PURIFICATION AND PARTIAL KINETIC AND PHYSICAL CHARACTERIZATION OF TWO NADP-SPECIFIC GLUTAMATE DEHYDROGENASE ISOENZYMES AND THEIR PROTEIN PRECURSORS, AND MEASUREMENT OF THE PATTERNS OF ACCUMULATION AND RATES OF DEGRADATION OF THEIR NONIDENTICAL SUBUNITS IN SYNCHRONIZED CELLS OF *CHLORELLA* CULTURED IN DIFFERENT CONCENTRATIONS OF AMMONIA

By

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LIST OF ABBREVIATIONS

NAD-GDH	Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase
NADP-GDH	Nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase
GS	Glutamine Synthetase
GOGAT	Glutamate Synthase
DON	6-diazo-5-oxo-L-norvaline
MSO	methionine sulfoximine
ATase	adenylyltransferase
AT_a	adenylylation site of ATase
AT_d	deadenylation site of ATase
PITC	phenyl isothiocyanate
mU	milliunits of enzyme activity
DTT	dithiothreitol
SDS	sodium dodecyl sulfate
FPLC	Pharmacia Fast Protein Liquid Chromatography System

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Two ammonium-inducible, chloroplast-localized, NADP-specific glutamate dehydrogenases were purified from Chlorella sorokiniana. They were homopolymers of either alpha or beta subunits with molecular weights of 55,500 and 53,000, respectively. Purification was achieved by gel filtration (Sephacryl S-300), fast protein liquid chromatography (Pharmacia, Mono Q column), and substrate affinity chromatography (Pharmacia, NADP-Sepharose 4B, Type 4). These isoenzymes were separated by their differential binding to the substrate affinity column. Peptide mapping of purified alpha and beta subunits showed them to have a high degree of sequence homology. Because the ammonia K_m of alpha holoenzyme was sensitive to changes in NADPH

concentration, its K_m ranged from 0.02 mM to 3.5 mM. With changes in NADPH concentration, the ammonia K_m of beta holoenzyme remained constant at 70 mM.

By use of SDS slab-gel electrophoresis and a Western blot/immunodetection procedure, patterns of accumulation of alpha and beta subunits (in their holoenzyme) were measured in cells cultured in media, containing different concentrations of ammonia. Pulse-chase experiments with [^{35}S]sulfate were performed to measure the rates of degradation of the two isoenzymes. When the culture medium contained 2 mM ammonia or lower, cells accumulated only the alpha holoenzyme. Above 2 mM ammonia, cells contained both isoenzymes; however, their patterns of accumulation and rates of degradation were very different. The physiological role of alpha and beta holoenzymes appears to be ammonia assimilation at low and high external ammonia concentrations, respectively.

From in vitro translation studies with total cellular poly(A)$^+$ RNA, isolated from cells engaged in synthesis of alpha or beta holoenzymes or both, it was concluded that alpha and beta subunits have protein precursor(s) of identical molecular weight (M_r= 58,500). When the putative protein-precursor(s) were incubated in vitro, with cell-free extracts from Chlorella cells, they were processed to proteins the size of alpha and beta subunits. From in vitro processing experiments, it was concluded that

either two different precursors of the same molecular weight are specifically processed to give alpha and beta subunits, or the specificty or type of processing-enzyme changes to give the two subunits from a single precursor-protein.

INTRODUCTION

In this laboratory, synchronous cultures of the eucaryotic microorganism Chlorella sorokiniana are being used as a model system to elucidate the regulatory mechanisms which control gene expression. Recent emphasis in research has been on the induction and inactivation of the ammonium inducible NADP-specific glutamate dehydrogenase.

Before this dissertation research, C. sorokiniana was reported (8,38,51,52,85,112,117,130,136,137) to contain only two isoenzymes of glutamate dehydrogenase. A tetrameric NAD-specific GDH, localized in the mitochondria, was present in cells cultured in medium containing either nitrate or ammonia as the sole nitrogen source (77). A hexameric NADP-specific form was present only in cells cultured in ammonia-containing medium (51,52,130). The two isoenzymes have been shown to be physically, chemically, kinetically, and antigenically distinct from each other (38,77,147). Furthermore, the two enzymes had distinct patterns of accumulation during the cell cycle and were regulated independently in different culture conditions (52). The NADP-GDH has been shown to be synthesized as a protein of higher molecular weight on cytosolic ribosomes

(99). The precursor of the NADP-GDH is presumably processed to the mature protein following transport into the chloroplast.

In contrast to many eucaryotic cells, which have high basal levels of inducible (adaptable) enzymes (4,110,135), the NADP-GDH activity was barely detectable in uninduced cells. The absence of significant NADP-GDH activity was found to be due to the inactivation of newly synthesized enzyme, and not due to a lack of its synthesis.

A model for the regulation of the NADP-GDH of Chlorella was proposed (113). The model proposed that the mRNA for the NADP-GDH is present in induced and uninduced cells. In the presence and absence of the inducer, the subunits are degraded following covalent modification by dimerization of subunits. When the inducer is present, however, there is a net accumulation of subunits which form the active holoenzyme (113). When the inducer is removed from previously induced cells, there is a rapid shift towards modification and degradation.

Recent studies (99) have shown the induction process to be much more complex than was proposed in the model. When Chlorella cells were transferred from nitrate-containing medium to ammonia-containing medium, a multi-step process of induction was initiated which resulted in the accumulation of NADP-GDH antigen and activity. During the first 120 min of induction, two subunits were detected which were designated beta

(M_r=55,500) and alpha (M_r=53,000) (101). The pattern of accumulation of the subunits could be varied by changing the culturing conditions. The pattern of induction of enzyme antigen in the light was different from the pattern of antigen accumulation seen when cells were induced in the dark in the absence of an exogenous carbon source and subsequent illumination of the culture. During the first 120 min of a normal induction period in the light, in 30 mM ammonia, the alpha and beta subunits were observed to have different patterns of accumulation. The alpha subunit began to accumulate after the addition of ammonia. However, after 120 min, the alpha subunit ceased to accumulate, but the beta subunit continued to accumulate at the same rate. After 180 min, most of the NADP-GDH antigen was present as the beta subunit. During the induction period, the holoenzyme was seen as a series of isoforms which changed in number and apparent molecular weight. After native polyacrylamide gel electrophoresis, two to three active isoforms were visible by activity staining. These isoforms had low relative mobilities with an apparent molecular weight range of 380,000 to 420,000. As induction continued, more forms became visible. By 100 min, seven active isoforms of the NADP-GDH could be detected. These isoforms had a range in apparent molecular weight from 280,000 to 420,000.

When poly(A)$^+$ mRNA was isolated from fully induced cells and translated <u>in vitro</u>, the immunoprecipitated

product was 58,500 d (99). This protein was 3,000 d larger than the beta subunit, and 5,500 d larger than the alpha subunit. It was proposed that the additional amino acid residues comprised the transit peptide for direction of the NADP-GDH to the chloroplast. It is not known whether the alpha and beta subunits are encoded by the same mRNA with the same precursor protein processed differentially, or whether at early times of induction there are two different mRNAs and precursor proteins of NADP-GDH.

The purpose of the dissertation research project was (a) to measure the rates of synthesis and degradation of the alpha and beta subunits; (b) to show whether each type of subunit is found in holoenzyme proteins, and if so, to examine the chemical, physical and kinetic properties of these holoenzymes; (c) to determine if a precursor-product relationship exists between the alpha and the beta subunits; and (d) to determine the physiological significance of having different accumulation patterns of alpha and beta subunits at different induction times, and different induction conditions.

The designation of alpha and beta in this dissertation is in accordance with standard procedure of naming proteins on a gel from top to bottom. The designation utilized by Prunkard (99) was based on the chronological order of appearance of the subunits when cells were induced with ammonia in the dark. Therefore, any comparison of this dissertation with the thesis of Prunkard (99) must account

for the difference in designation of the alpha and beta subunits. Hereafter, the protein with a molecular weight of 55,500 will be designated the alpha subunit and the protein with a molecular weight of 53,000 will be designated as the beta subunit.

LITERATURE REVIEW

In all biological systems, the assimilation of nitrogen is required for the synthesis of macromolecules essential for growth. In this survey of past and current literature, I will present an overview of the pathways involved in ammonia assimilation and examine the three key enzymes of ammonia assimilation (GS, GOGAT, and GDH) in more detail. The survey will focus on the enzymes of algae, fungi, and higher plants.

The Path of Ammonia Assimilation

Until the middle of the 1970s, it was generally accepted that the main route of ammonia assimilation into plants and microorganisms was via the GDH-catalyzed reductive amination of 2-oxoglutarate. The GDH had several characteristics which favored its potential role in ammonia assimilation; (a) it could catalyze the synthesis of glutamate *in vitro* with an equilibrium which favored synthesis, and (b) ^{15}N kinetic labeling studies of Folkes and Sims (31) with *Candida utilis*, and of Bassham and Kirk (9) with *Chlorella*, showed glutamate was the major initial product of ammonia assimilation *in vivo*. However, the GDH had certain characteristics which were unusual for an

enzyme which occupied a key position in a biosynthetic process; (a) the enzyme was readily reversible, and (b) in general, it had a low affinity for ammonia.

The studies by Folkes and Sims (31) showed glutamine was the only other major product from initial assimilation. The formation of glutamine from ammonia, by GS, resulted in the incorporation of ammonia-N into the amide-N of glutamine. The amide-N of glutamine was known to be transferred to carbamoyl phosphate and nucleic acids. Because there was no known way for the transfer of the amide-N of glutamine to the amino group of amino acids, the GS was not considered to be involved in the majority of ammonia assimilation

Until 1970, when Tempest, Meers and Brown (75,131,132,133) performed experiments with Klebsiella aerogenes which were maintained in an ammonia-limited chemostat, the role of the GDH remained the central theme for ammonia assimilation. The GDH had a K_m for ammonia of about 4 mM. Knowing this K_m for ammonia, it was anticipated that, if ammonia was maintained at 0.5 mM (which would be below the concentration for which the GDH would be most effective), the cell would increase the level of the GDH. By increasing the level of GDH, the ammonia would continue to be assimilated, although the process would be less efficient. When K. aerogenes was cultured in medium maintained at 0.5 mM ammonia, the activity of the GDH decreased to 3% of the activity found when this organism

was cultured in high concentrations of ammonia. Analysis of intermediates labeled by ^{15}N suggested the sequence of labeling was ammonia to glutamine to glutamate. These experiments led to the discovery of GOGAT. The GOGAT catalyzes the reductive transfer of the amide amino group of glutamine to the 2-oxo position of 2-oxoglutarate to yield two molecules of glutamate.

Since these original experiments were performed, other studies have continued to emphasize the role of the GS/GOGAT pathway in ammonia assimilation and to minimize the role of the GDH.

The presence of GS, GOGAT, and GDH in all bacteria provides them with two alternative routes for the assimilation of ammonia. In many bacteria, it has been possible to show that the GS/GOGAT pathway operates in some situations (e.g., ammonia limitation), while the GDH pathway operates under other conditions (e.g., ammonia excess) (70,138).

The GDH of green algae has characteristics which make it favorable for being involved in ammonia assimilation. Morris and Syrett (87) reported an NADP-GDH level three times higher than the calculated requirement for ammonia assimilation in Chlorella vulgaris. Brown et al. (14) have also reported a K_m for ammonia for a GDH of 0.3 mM to 0.5 mM. This K_m was much lower than reported in higher plants, and would allow assimilation of ammonia before intracellular toxic levels are reached. Tischner (134) has

reported the presence of two NADP-GDH isoenzymes in cells cultured in 5 mM ammonia which have K_m values for ammonia of 5.5 mM for one, and between 0.95 and 0.05 mM for the other (depending on the concentration of NADPH). In the marine plankton diatom Ditylum brightwellii (28), the NADP-GDH has a high K_m for ammonia (10 mM) but the internal level of ammonia was also high, in the range of 5-10 mM. Since the K_m for ammonia is close to the physiological concentration of ammonia in this organism, the NADP-GDH may be involved in ammonia assimilation.

Initial attempts to detect GOGAT in Chlorella were hindered by problems in solubilization of GOGAT activity. Once these problems were alleviated, high activities of GOGAT could be measured (62). Dougall (24) demonstrated the presence of the enzyme in carrot tissue culture. Lea and Mifflin (62) described the discovery of a ferredoxin-dependent GOGAT which was inactive with pyridine nucleotides. High levels of GS and GOGAT have been reported (5,10,111,129) in Chlorella. These enzymes are probably responsible for the assimilation of ammonia in green algae in most situations (81). In yeast and fungi, the level of GOGAT was found to be very low (11,14,54,106,133). In Neurospora, the assimilation of ammonia appears to be via the NADP-GDH and GS (50,61). In yeast (14,106) and Neurospora (25,50), the GOGAT appears to function only at low levels of ammonia, or when the NADP-GDH is absent or ineffective.

The ferredoxin-dependent GOGAT was discovered during studies on the assimilation of nitrogen in leaves. It was found that the nitrate reductase was present in the cytosol (20), while the next enzyme in the reduction of nitrate to ammonia, nitrite reductase, was present in the chloroplast fraction (29,79). Since the conversion of nitrate to ammonia results in the accumulation of ammonia in the chloroplast, it was proposed that the assimilation of ammonia into amino acids should also initially occur in the chloroplast. The GDH activity was found in the chloroplast fraction (35,63); however, the K_m of the enzyme was 5.8 mM. Since the level of ammonia required to uncouple photosynthesis in chloroplasts was 2 mM (36), it was considered to be unlikely that the GDH plays any role in ammonia assimilation. It was possible, however, that the *in vivo* K_m was lower than that which was determined *in vitro*. Another reason for doubting an important role of the GDH in ammonia assimilation was that the GS had also been detected at high levels in isolated chloroplasts from several plant tissues (44,55,89,90,91). Moreover, the GS had a low K_m for ammonia (in the range of 0.02 mM) (91).

Methionine sulfoximine (MSO) is an analogue of the glutamylphosphate-enzyme complex of the glutamine synthetase and is therefore a very potent inhibitor of this enzyme. Due to their structural similarities to glutamine, two other inhibitors, 6-diazo-5-oxo-L-norvaline (DON) and azaserine, are inhibitors of all glutamine-amide transfer

reactions. DON and azaserine both inhibit the GOGAT, while neither of them will inhibit the GDH. Therefore, by use of MSO and/or DON or azaserine, it was possible to specifically inhibit the GS/GOGAT pathway without affecting assimilation via GDH. When the effect of MSO was examined in blue-green algae, it was found that the assimilation of ammonia was blocked (126). When DON and azaserine were supplied to green algae, there was accumulation of 2-oxoglutarate and glutamine. These results indicated that glutamate was being synthesized via the glutamine-amide transfer reaction of the GOGAT and not via the reductive amination reaction catalyzed by the GDH.

Based on the following evidence of (a) the presence of the necessary enzymes, (b) the irreversibility of the reactions, (c) the low K_m for the substrate ammonia, (d) the incorporation pattern of the labeled nitrogen, (e) the use of specific inhibitors, it was concluded (104) that, for plants and algae (with the possible exception of fungi), the primary route of entry of ammonia is via the GS/GOGAT pathway. The GDH may be important under special circumstances and in conditions of ammonia excess.

Glutamate Dehydrogenase

Glutamate dehydrogenase catalyzes the reductive amination of 2-oxoglutarate by ammonia in a reversible reaction, utilizing either NADPH or NADH. The enzyme is widespread in living organisms and has been found in higher

plants (7,19,78,88,146), algal species (53,87), fungi (66,118), and bacteria (59,74,118). However, in certain species of Bacillus (118), and cyanobacteria (97), the GDH activity has been detected at very low levels or has been absent altogether.

The GDH from plants has been shown to have a high specificity for ammonia and 2-oxoglutarate (21,33,95). The GDH from Neurospora and Bacillus show less specificity and have low activities with several 2-oxo acids and amino acids (13).

Higher plants contain two isoenzymes of GDH: one a mitochondrial form which preferentially utilizes NAD, and the other a chloroplastic form, which preferentially utilizes NADP. Both isozymes utilize either coenzyme to a certain extent. The sub-cellular localization is well conserved among different organisms with the NAD-GDH activity being located in the mitochondria (26,61,95) and the NADP-GDH being located in the chloroplast (65,113). The mitochondrial enzyme showed less coenzyme specificity and used either NADH or NADPH in the aminating direction (52,95) but showed higher activity with NADH. In the deaminating direction, only NAD was used.

The chloroplastic GDH will also use both coenzymes but shows a preference for NADPH. The amount of coenzyme cross-reactivity varies with different organisms. In higher plants, the coenzyme specificity of the GDH is relatively low (63,65). The GDH with lowest coenzyme specificity had

been found in lettuce (63), where NADH and NADPH were used equally well. In algae, there was also a range in coenzyme specificity, from a ratio of 33:1 (NADPH:NADH) for Chlorella pyrenoidosa (130) to 75:1 (NADPH:NADH) for Caulerpa simpliciuscula (33). Fungi and bacteria show the highest coenzyme specificity, and often show absolute specificity for one coenzyme or the other (37,57,109).

The NAD-GDH exists as either a tetramer, with identical subunits of approximately 45,000 d (67,77,141) or as a hexamer with subunits of approximately 55,000 d (1,86) depending on the species. Several investigators (38,118,141) have shown the NAD-GDH and the NADP-GDH to have significantly different primary structures. The pH optima for the two isoenzymes appear to be similar (124) but the optima for the aminating and deaminating direction differ by 1.0-1.5 pH units, with the deaminating reaction optimum at approximately pH 9.0. In higher plants, algae, fungi, and bacteria (124), the K_m for ammonia ranges from 0.32 mM for Clostridium SB_4 (145) to 70 mM for pea epicotyl (21). Most of the K_m values determined are high compared to physiological concentrations. Recently, Tischner (134) and Pahlich and Gerlitz (94) have reported a K_m for GDH which is in the physiological range and is present in Chlorella cultured in low ammonia.

The GDH from pea root appears as only a single band on native polyacrylamide gel electrophoresis (95), but it was subsequently found that there are isoenzymes of different

mobility depending on the culture conditions (92,93). It was found that in seeds of pea there were seven isoenzymes of GDH which decreased in number during germination and generated a new isoenzyme pattern. In the plant, the shoots contain some isoenzymes seen in the seed, and some isoenzymes seen during early germination. The seed retains the seven isoenzymes and the roots maintain the isoenzyme pattern seen during germination. Similar results were reported by Hartmann et al. (42) for Medicago sativa. It was proposed that the isoenzymes which produced the pattern seen in seeds could be responsible for deamination reactions, and the isoenzymes which produced the pattern seen in roots and during germination could be involved in ammonia assimilation. When the GS/GOGAT pathway was discovered, this proposal was discounted. The different isoenzymes were proposed to be associated with cellular differentiation, intracellular compartmentation, or physiological function (63). Pahlich (93) suggested that the different activity bands seen on polyacrylamide gel electrophoresis were different conformers. It was postulated that two subunits which have the same amino acid composition can exist in two different tertiary conformations. The random distribution of these two conformers in a six subunit holoenzyme would give rise to the seven activity bands seen on polyacrylamide gel electrophoresis.

There are several reports of allosteric regulatory properties of GDH in higher plants (18,26,92), fungi (118), and bacteria (74,116). Most of these effects are minimal and are not believed to be a primary means of regulation of the enzyme activity. However, more studies need to be performed to determine the extent of allosteric regulation which may occur *in vivo*.

Talley et al. (130) have shown that the NADP-GDH of *Chlorella sorokiniana* is inducible at all times throughout the cell cycle. Turner et al. (137) have shown that the enzyme potential (i.e., initial rate of induction) of this enzyme increases continuously during the cell cycle, with a change in rate occurring at the time of DNA synthesis. The change in rate of enzyme potential could be blocked by the DNA synthesis inhibitor, 2′-deoxyadenosine. From these studies, it was concluded that the gene for this enzyme was continuously available for transcription during the cell cycle. It was further shown (136) that the mRNA for this enzyme was present in induced and uninduced mother cells at approximately the same level. Therefore, the regulation of induced accumulation of enzyme activity was proposed to be at the post-transcriptional level at certain stages of the cell cycle. Turnover studies showed that the GDH protein was synthesized in uninduced cells but that it was covalently modified and degraded, preventing its accumulation (136). During induction, the rate of covalent modification of the enzyme decreased so that active enzyme

accumulated, even though the enzyme was still being degraded during induction. When these cells were transferred from inducing conditions to non-inducing conditions (i.e., deinduction), enzyme activity was lost with a half-life of 5-10 min. The rate of turnover of the total enzyme antigen did not change; however, the subunits were converted to putative covalent dimers at the same rate as loss of enzyme activity (8).

The NADP-GDH from Candida utilis was shown to be structurally similar to the NADP-GDH of Chlorella but to have a different mechanism of regulation (46). This enzyme was observed to undergo reversible conformational transitions which affected its activity. It was also shown to be regulated by feedback inhibition (31). When the organism was starved for carbon, the NADP-GDH was rapidly inactivated with a concomitant loss of antigen. The enzyme normally had a low turnover rate; therefore, it was proposed that enzyme inactivation was by selective proteolytic degradation (46). In the same organism, the NAD-GDH was also inactivated by glutamate starvation (48). Its mechanism of inactivation was shown to occur by the reversible phosphorylation of enzyme subunits, resulting in a decrease in the specific activity of the enzyme (45,47). Uno et al. (139) found the regulation of an NAD-GDH of Saccharomyces cerevisiae to be regulated by phosphorylation during glutamate starvation by a mechanism similar to that reported for the enzyme of Candida.

Light has also been shown to be involved in the regulation of enzyme activity of NADP-GDH in Chlorella and Euglena (16,85,140). In Chlorella the NADP-GDH is induced by ammonia but it has been reported (85) that the induction is also dependent on light. In the presence of the inducer in the dark, there was no detectable enzyme activity. When the culture was illuminated, there was an immediate accumulation of enzyme activity without any lag. In the same series of experiments, the NAD-GDH, which was constitutively expressed in these cells, showed no alteration in accumulation of enzyme activity. Vankova et al. (140) have shown that the NADP-GDH of Chlorella can be induced in the dark when supplied with glucose as a carbon source. The enzyme which was induced in the dark did not have the same apparent molecular weight as the enzyme induced in the light when determined by non-denaturing polacrylamide gel electrophoresis.

The induction of the NADP-GDH of Euglena requires light and is closely associated with the regreening of these cells even though this NADP-GDH is a cytosolic enzyme (16).

It can be seen from these few examples that many systems have been developed for the regulation of GDH activity. The NADP-GDH from Salmonella and the one from Neurospora have been cloned and the respective genes sequenced (58,83) and studies have been initiated on the structure of the controlling elements of these genes.

Glutamine Synthetase

Glutamine synthetase catalyzes the synthesis of glutamine from ammonia and glutamate by use of energy supplied from ATP. Like GDH, GS was found in higher plants (55,73,89,125), algae (101,121), and fungi (56,96,117).

When hydroxylamine was used in place of ammonia in the reaction catalyzed by GS, the GS was able to form glutamyl hydroxamate. Since the K_m and V_{max} for the reaction which utilized either ammonia or hydroxylamine were very similar, the production of the hydroxamate was used as a convenient method for measurement of enzyme activity (91). In addition to the biosynthetic reaction, which results in the formation of glutamine, GS was also shown to catalyze a transferase reaction which results in the exchange of hydroxylamine for ammonia on glutamine (91). This reaction required only catalytic amounts of ADP, a divalent metal, and phosphate or arsenate. The transferase assay gave activities which were several times higher than those obtained for the synthetase assay (91).

GS has been shown to be present in seeds, seedlings, shoots, roots, root nodules, and leaves (124). The activity of the shoot GS is higher than the root GS, except in plants which are very active in nitrogen assimilation in the roots (64).

Isolated chloroplasts have been shown to contain GS activity; however, not all of the activity is associated with the chloroplast (44,90). There was usually about 60%

of the total activity in the chloroplast (102). In maize, the distribution of GS was approximately 80% in the mesophyll cells, which also contain most of the nitrate and nitrite reductase, and a lower amount in the bundle sheath cells (102). Experiments performed on maize, by other workers (41), have found that the bundle sheath cells and the mesophyll cells contain approximately equal amounts of GS activity. Several workers have shown that chloroplasts contain a light-activated GS in sufficient amount to catalyze the reaction for ammonia assimilation (34,84). A high level of the enzyme was also found in the cytosol (15). In legume nodules, the activity of the GS was higher in the cytosol and may constitute up to 2% of total soluble protein (73). The high GS level is probably due to the role of the GS in assimilation of ammonia from nitrogen fixation. In cyanobacteria, the activity of the enzyme is higher in the heterocysts (23); again, this is the location of ammonia assimilation from nitrogen fixation.

Several laboratories have reported the presence of multiple forms of GS which can be separated by ion-exchange chromatography (10,29,72,129). In barley, the two forms, designated GS I and GS II, have similar molecular weights and similar apparent K_m values for ammonia and ATP; however, they have different stability characteristics, pH optima, and reactivity to thiol reagents (71). The GS I exhibits negative cooperativity with respect to glutamate but GS II does not (40). The GS I was localized in the

cytosol whereas the GS II was localized in the chloroplast. The difference between GS I and GS II does not seem to be just a difference in the modification of a single protein form (67).

The enzymes from higher plants (89,104), and lower eucaryotes (56,96), have very similar properties with molecular weights of 350,000-400,000 and are composed of eight identical subunits with molecular weights of 45,000-47,000. The GS from Chlorella pyrenoidosa is apparently different in that it has a molecular weight of 320,000 and is composed of six subunits with molecular weight of 53,000 each (101). The eucaryotic enzyme is significantly different from the procaryotic one which has a molecular weight of 590,000-600,000 and consists of twelve subunits each with a molecular weight of 50,000 (123).

The K_m values for substrates of the GS from different sources are similar (124). The K_m for ammonia was found to be very low and usually in the range from 10 to 20 micromolar. The K_m for glutamate was between 1-13 mM and 0.1-1.5 mM for ATP. The K_m for glutamate varied with the ATP and magnesium concentration (125). When magnesium concentration was decreased 8-fold, there was a 3-fold increase in the K_m for glutamate.

The reaction mechanism for the plant GS has not been extensively studied; however, the mammalian enzyme has been studied and shows many similarities to the plant enzyme.

The reaction seems to have an ordered sequence of addition of reactants, with Mg-ATP binding first, followed by glutamate. This complex reacts to form an enzyme bound glutamyl phosphate, followed by the binding of ammonia which displaces the phosphoryl group and allows the release of products (76).

Regulation of GS activity has been studied extensively in bacteria. The GS activity from Gram-negative bacteria was found to be under extremely tight control by repression/derepression, feedback inhibition, and covalent modification. The covalent modification has been shown to be controlled by an intricate cascade mechanism resulting in the adenylylation/deadenylylation of GS. In the proposed mechanism, GS is adenylylated by adenylyltransferase (ATase), which contains two active sites (103), one for adenylylation and one for deadenylylation (AT_a and AT_d, respectively). The adenylyation of the GS by the AT_a site results in the modification of a specific tyrosine residue on a subunit (115). Under physiological conditions, the specific activity of GS can vary, due to the different number of subunits which are adenylylated in the holoenzyme. Since the AT_a and AT_d act oppositely on the GS, these activities must also be regulated; otherwise, a futile cycle would be established with GS as the carrier. The ATase was found to be regulated by the nucleotidylylation of another protein, P_{II} (6), which was uridylylated by the single protein UR/UT. When P_{II} was

uridylylated (P_{IID}) at a specific tyrosine residue, it caused the activation of the AT_d site of the ATase, leading to the activation of the GS. The unmodified form of P_{II} (P_{IIA}) activated the AT_a site of ATase, which adenylylated the GS, and caused the inactivation of GS. Therefore, through reciprocal coupling, a closed type of bicyclic cascade system is generated, resulting in the fine control of the GS activity (122).

Besides the regulation of activity by nucleotidylation, there is also evidence for regulation of the level of the GS protein level by oxidative modification of a specific histidine residue in the active site (68). Following oxidation, the enzyme is proposed to be more susceptible to proteolytic degradation (69).

GS has also been studied in Neurospora and shows a much different type of regulation. In N. crassa the nitrogen source regulates the level and the de novo synthesis of GS (108). A change in the ammonia concentration, or in the type of nitrogen compound supplied, was found to alter the rate of accumulation of the GS by affecting its rate of synthesis (100). The change in rate of synthesis was found to be due to a change in the level of the GS mRNA (108). These studies have shown that the level of the GS was high when the organism was cultured on glutamate, and the level was low when ammonia or glutamine was present in the culture medium (61). Further studies have shown the presence of two different GS

subunits, designated alpha and beta (107). These studies have also indicated that the route of ammonia assimilation was different, depending on the concentration of ammonia (61). At high ammonia levels, assimilation was by the GDH and an octameric form of GS. The subunit present in the GS was the beta type, and its level was regulated by the type of nitrogen source supplied. When cells were cultured on glutamate, the beta subunit was at its highest level; it was at an intermediate level on ammonia, and lower on glutamine. When N. crassa was cultured in conditions of ammonia limitation, the alpha subunit was present and the holoenzyme was present as a tetramer. Under these conditions, ammonia assimilation occurred via the tetrameric GS and the GOGAT (61). The alpha and beta subunits were made from separate mRNAs, as determined by in vitro translation of size selected RNA (61).

The regulation of the GS has been studied in two different strains of Chlorella. In Chlorella pyrenoidosa a partially purified GS was found to be activated by thioredoxin (114). It was not shown however, which form of the GS was activated. In Chlorella kessleri two forms of GS were separated, and their levels were measured under different culture conditions (129). The GS I and GS II were both present in cells cultured autotrophically or heterotrophically. The GS I was always present at a higher level than the GS II and its level changed more than the GS II level under different conditions. Both forms were

present at higher levels when cultured in nitrate containing medium or in nitrogen free medium. Both forms were also higher in cells cultured heterotrophically than autotrophically.

Glutamate Synthase

Glutamate synthase is probably the least studied of the enzymes involved in ammonia assimilation. GOGAT was originally discovered in Aerobacter aerogenes by Tempest et al. (131) in 1970, and it was implicated in the assimilation of ammonia in conjunction with the glutamine synthetase. It was not until 1974 that GOGAT was discovered in green plants. Originally a pyridine nucleotide-dependent form was reported (24,32) but shortly afterwards a ferredoxin-dependent form was identified which has since been shown to be the form present in most plant species (62). The ferredoxin GOGAT has been found in roots (2,80,126), shoots (62,64,104,142), and seeds (82); the majority appears to be in mesophyll cells (102). Its presence in the mesophyll cells was expected, since this is the site of nitrate reduction to ammonia; however, its function in bundle sheath cells is unknown. The pyridine nucleotide-dependent form has not been reported in chlorophyll-containing cells of higher plants (62,104,126). The pyridine nucleotide-dependent activity has been found in roots (32,80,126) developing seeds (119) and root nodules (64,105).

Ferredoxin-dependent GOGAT has been localized in the chloroplast (41,102,142). The pyridine nucleotide GOGAT has been found in the cytosol of developing pea cotyledons (127) while the enzyme from tissue culture cells, and from roots has been found almost exclusively in the plastids (27).

The purified GOGAT from bacteria appears to be active as a dimer or tetramer. The dimer is composed of two nonidentical subunits with molecular weights of approximately 135,000 and 35,000 (82). The pyridine dependent GOGAT from root nodules is a single protein with a molecular weight of 235,000. The ferredoxin-dependent GOGAT from Vicia faba has a molecular weight of 150,000 with an undetermined subunit structure. The bacterial enzymes are iron-containing flavoproteins, but the enzyme from lupin root contains only FMN (12). The enzyme from V. faba does not appear to contain iron or flavin (143,145). The K_m values for higher plant GOGAT show a low affinity for 2-oxoglutarate (0.4-1.0 mM) and a higher affinity for glutamine (0.03-0.05 mM) (124).

There have been few studies on the regulation of the GOGAT in plants. One study was conducted in Neurospora crassa in which the levels of GDH, GS, and GOGAT were examined during different nitrogen shifts (61). Cultures in ammonia excess were found to have a low GOGAT activity, while cultures on glutamate or glutamine had almost no GOGAT activity. When cultures were shifted from ammonia

excess to ammonia limitation, there was a 25-fold increase in GOGAT activity. Cultures shifted from glutamate to ammonia limitation showed a 2-fold increases in activity, and cultures shifted from glutamine to ammonia limitation showed an eight-fold increase in enzyme activity. Cultures shifted from ammonia limitation to ammonia excess had a 20-fold decrease in the activity of GOGAT. The mechanism for change of enzyme activity was not addressed in this or any other study found.

EXPERIMENTAL PROCEDURES

Materials

NADP was obtained from P-L Biochemicals Inc.; electrophoresis reagents, Bio-Rad; $H_2{}^{35}SO_4$, New England Nuclear; [^{35}S]methionine, [^{125}I]Protein-A, and [^{32}P]ATP, Amersham; $H_3{}^{32}PO_4$, ICN; nitrocellulose and Nytran, Schleicher and Schuell; rabbit reticulocyte lysate, Green Hectares; oligo(dT)-cellulose, Collaborative Research; all other reagents were of the highest quality available from Fisher, Sigma Chem. Co., or Baker.

Culture Conditions

Chlorella sorokiniana was cultured autotrophically in a medium which was modified from that previously reported (52). The modified medium was composed of (in mM): KH_2PO_4, 18.4; K_2SO_4, 2; $MgCL_2$, 1.5; $CaCl_2$, 0.34; (in μM): $FeCl_3$, 71.6; EDTA, 72; H_3BO_3 38.8; $MnCl_2$, 10.1; $ZnCl_2$, 0.734; $CoCl_2$, 0.189; $CuCl_2$, 0.352; $NiCl_2$, 0.19; $SnCl_2$, 0.19; NH_4VO_3, 0.20.

The medium contained either 29 mM KNO_3 (nitrate medium) or 29 mM NH_4Cl (ammonium medium). The nitrate medium was adjusted to pH 6.8 and the ammonium medium was adjusted to pH 7.4. All media were filter sterilized; glassware was autoclaved using a self-contained

steam generator. Cells were cultured under light conditions to give a division number of four. Cells were synchronized in nitrate medium using 3 alternating light:dark periods of 9h:7h.

Enzyme Assays and Ammonia Determination

The activity of the NADP-GDH was measured by a spectrophotometric assay as described (51). All assays were perfomed in the deaminating direction, except where noted. The 0.5 ml reaction mixture contained 44 mM Tris, 200 mM L-glutamate, 1.02 mM NADP, pH 8.6 at 22°C. The aminating assay contained 44 mM Tris, 306 mM $(NH_4)_2SO_4$, 26 mM 2-oxoglutarate, 0.37 mM NADPH, pH 7.4. One unit of enzyme activity was defined as the amount of enzyme required to reduce, or to oxidize one micromole of coenzyme per min at 38.5°C.

Ammonia was measured by use of the procedure of Harwood and Kuhn (43). The data for a standard curve were prepared by diluting 30 mM ammonia medium with nitrogen-free medium. The standard curve was linear up to 3 μg nitrogen/ml. By this method, it was possible to detect less than 0.1 μg nitrogen/ml.

Polyacrylamide Gel Electrophoresis

Denaturing SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (60).
Non-denaturing polyacrylamide gel electrophoresis was

performed as described by Davis (22). Activity stains of non-denaturing gels were performed by use of the phenazine methosulfate/nitro blue tetrazolium coupled detection procedure, as described by Talley et al. (130).

Western blots were prepared from SDS- and non-denaturing polyacrylamide gels by use of the following procedure described by Wahl et al. (142) and modified by Prunkard (99). Chlorella cells, cultured at a cell density of 180 x 10^6 cells/ml, were harvested (5 ml), washed two times with 0.1 M Tris-HCl (pH 8.25), resuspended in 3 ml of the same buffer, and stored at -20°C until breakage. Cells were broken at 20,000 psi in a French pressure cell, and the homogenate was centrifuged at 8,000 x g for 2 min immediately prior to preparation for electrophoresis. The proteins from 30 µl of cell homogenate were separated electrophoretically as described above. After electrophoresis, the polyacrylamide gel was equilibrated in transfer buffer (24.8 mM Tris, 192 mM glycine, 20% methanol, pH 8.4) for 30 min and the proteins were electrophoretically transferred to nitrocellulose paper at 50 V (200 mA maximum) for 20 h. The excess binding sites on the nitrocellulose were blocked by incubation of the filter in ligand buffer (10 mM Tris, 3% BSA, 0.15 M NaCl, pH 7.4) for 1 h at 37°C in a plastic boiling bag (Sears, Seal-N-Save) by use of 0.5 ml of ligand buffer per cm^2 of nitrocellulose. The nitrocellulose was incubated with Protein-A purified anti-NADP-GDH IgG (147) (1 mg/ml, 5

l/lane diluted in ligand buffer to a final volume of 0.2 ml/cm^2 of nitrocellulose) for 2 h at 37°C. Following incubation with the antibody, the nitrocellulose was washed for 1 h in ligand buffer, and incubated for 2 h at 37°C with 0.25 μCi/lane of ^{125}I-Protein-A, in a volume of 0.2 ml/cm^2 of nitrocellulose, diluted in ligand buffer. The nitrocellulose paper was then washed for 2 h at 37°C in 10 mM Tris, 0.15 M NaCl (pH 7.4), dried, and exposed to Kodak X-Omat AR film for 36 h at -70°C.

Immunoprecipitation of NADP-GDH

NADP-GDH was immunoprecipitated from *in vivo* or *in vitro* labeled proteins by use of a modified indirect immunoprecipitation procedure (147). All buffers, antibody solutions, and homogenates were centrifuged at 15,000 x *g* for 15 min before use. All incubations were performed at 4°C in 1.5 ml microcentrifuge tubes. Cell homogenates (0.2 ml) or *in vitro* translation products from rabbit reticulocyte lysate (0.05 ml) were diluted with an equal volume of immunoprecipitation buffer (10 mM Tris, 150 mM NaCl, 2% Triton X-100, 5 mg/ml BSA, pH 7.2) and centrifuged at 15,000 x *g* for 15 min. After centrifugation, the supernatant was removed and placed into a clean tube containing 2 μl of Protein-A purified anti-NADP-GDH IgG (1 mg/ml). After incubation at 4°C for 30 min, 0.012 ml of sheep anti-rabbit IgG (5 mg/ml) was added and incubated at 4°C for an additional 30 min. The mixture was then

layered onto 0.2 ml of immunoprecipitation buffer containing 20% sucrose and centrifuged at 15,000 x g for 15 min. The supernatant and sucrose were removed, and the pellet was resuspended in 0.2 ml of immunoprecipitation buffer without BSA. The immunoprecipitate was resuspended and centrifuged at 15,000 x g for 10 min through 0.2 ml of 20% sucrose. The immunoprecipite was resuspended and centrifuged through 20% sucrose two additional times. After the final wash, the immunoprecipitate was dissolved in 0.02 ml of Laemmli incubation buffer (60) and subjected to electrophoresis on a 10% polyacrylamide-SDS gel. After electrophoresis, the separated proteins were electrophoretically transferred to a sheet of Nytran membrane. The membrane was dried and placed directly against Kodak X-Omat AR film for autoradiography. For quantitation, the autoradiograms were scanned on an LKB laser scanning densitometer with a 0.05 mm slit width. The areas were automatically computed by a Hewlett Packard integrator.

Purification of Alpha and Beta NADP-GDH Holoenzymes

To purify the holoenzyme composed of alpha subunits, a homogenate was prepared from cells induced for 3 h in 1 mM ammonia. The holoenzyme, composed of beta subunits, was obtained from cells induced for 6 h in 30 mM ammonia. After gel filtration chromatography on Sephacryl S-300 (in 25 mM Tris, 2 mM DTT, pH 7.4), the NADP-GDH activity was applied

to a Mono Q column (Pharmacia) and eluted with a gradient of 0.15 to 0.35 M NaCl in 10 mM Tris, (pH 7.4). Each fraction that contained more than 5% of the total enzyme activity was combined and applied to a 25 ml NADP-Sepharose (Pharmacia, Type 3) column equilibrated with 25 mM sodium phosphate, (pH 6.5). After washing with 10 bed-volumes of 25 mM sodium phosphate (pH 6.5), the enzyme was eluted with 2 mM NADP. The alpha subunit holoenzyme was separated from the beta subunit holoenzyme by use of a 25 ml NADP-Sepharose (Pharmacia, Type 4) column. The sample was applied in 25 mM sodium phosphate buffer (pH 6.5), and washed with 50 ml of the same buffer. The bound enzyme (alpha subunit holoenzyme) was eluted with 1 M NaCl in 25 mM sodium phosphate buffer. As a final purification step, the enzyme from the Type 4 NADP-Sepharose affinity column was applied to an anti-NADP-GDH affinity column. The column was washed with 50 ml of 10 mM sodium phosphate, 15 mM NaCl, pH 7.2. The enzyme was eluted with 0.1 M sodium acetate, pH 2.5.

The Type 4 NADP-Sepharose affinity resin was synthesized rather than obtained commercially. The affinity resin was synthesized in a manner to produce NADP linked to Sepharose through the nicotinamide-ribose moiety and a six carbon linker (equivalent to Pharmacia, Type 4). The resin was prepared according to the method of Wilchek and Lamed (144) as follows. A saturated solution of adipic acid dihydrazide was prepared by stirring 30 g of the adipic acid

dihydrazide in 200 ml of water. The solution was placed at 4°C for 2 h and then filtered through a Whatman GF/C filter. The filtrate (50 ml) was added to 15 g of washed Sepharose 4B CL and shaken at 4°C for 16 h. The NADP was subjected to conditions for cleavage with periodate by preparing 30 ml of 10 mM NADP and adding sodium periodate to a final concentration of 10 mM periodate. The solution was incubated in the dark for 1 h at 4°C. The periodate-cleaved NADP was diluted with 45 ml of water, followed by the addition of 75 ml of 0.1 M sodium acetate (pH 5.0). The entire solution of periodate-cleaved NADP was added to the Sepharose-adipate which had been washed and resuspended in 100 ml of 0.1 M sodium acetate (pH 5.0). The mixture was incubated in the dark at 4° C for 5 h and then washed with 4 L of water and 4 L of 0.2 M NaCl. The column was stable in the NaCl solution for several months.

Radioisotope Labeling Conditions

Pulse-chase experiments with [^{35}S]sulfate were performed with synchronized Chlorella daughter cells. Cells were harvested by centrifugation, washed once in ice-cold low-sulfate (0.2 mM sulfate) ammonium medium, and resuspended to a culture turbidity of 5.5 A_{640} with pre-equilibrated (38.5°C, 2% CO_2-air), radioactive low-sulfate (0.2 mM sulfate, 0.667 Ci/mmol [^{35}S]sulfate), ammonium medium. To initiate the chase period, cells were harvested and washed by centrifugation in ammonium

medium without radioactive sulfate. Cells were resuspended to the desired turbidity in pre-equilibrated ammonium medium (38.5°C, 2% CO_2-air).

Pulse-chase experiments with [^{32}P]phosphate were performed as described above, except the sulfate was maintained at its normal concentration (2 mM) while phosphate was decreased to 0.25 mM and Tris was added to 30 mM as a buffer. The specific activity of the [^{32}P]phosphate in the medium was 0.33 Ci/mmol. Synchronized daughter cells, cultured in nitrate medium, were labeled for 90 min after the addition of ammonia. The cells were then harvested, washed, and resuspended in ammonium medium without radioactive phosphate. At frequent intervals, samples were harvested and prepared for immunoprecipitation as described previously. In a parallel culture, cells were induced for 3 h in non-radioactive ammonium medium. At the end of the induction period, cells were harvested, washed, and resuspended in nitrogen-free medium, containing [^{32}P]phosphate to start the deinduction period. Samples were harvested and prepared for immunoprecipitation as described above. All immunoprecipitates were subjected to electrophoresis in SDS-polyacrylamide gels, followed by electrophoretic transfer of the proteins to Nytran and autoradiography.

Cyanogen Bromide Peptide Mapping of Alpha and Beta Subunits of NADP-GDH

The alpha and beta subunits were characterized by mapping of the cyanogen bromide fragments by the procedure

of Chang et al. (17). The purified alpha or beta subunits, from the antibody affinity columns, were each dialyzed against 4 L of deionized water overnight and then lyophilized to dryness. The subunits (approximately 2 nmol) were dissolved in 0.05 ml of 70% formic acid, and 0.015 ml of CNBr (0.189 M) was added. After overnight incubation at room temperature, 1 ml of water was added, and the solution was lyophilized in an acid-washed vial. The peptides were dissolved in 0.04 ml of 50% pyridine (freshly distilled), and derivatized by the addition of 5 µl of pure PITC. The vial was flushed with nitrogen and incubated at 54°C for 10 min. The excess PITC was removed by 3 extractions with heptane:ethyl acetate (2:1 v/v). The derivatized peptides were dried *in vacuo* and redissolved in 70% ethanol before use.

The derivatized peptides were separated on a C_{18} reverse phase column (Pharmacia PepRPC) with 0.035 M sodium acetate (pH 5.5) and acetonitrile. The derivatized peptides were detected by their absorbance at 260 nm. For each separation, 20 pmol of derivatized peptide was used.

Isolation and In Vitro Translation of Poly(A)$^+$ RNA

To increase the yield and the purity of the poly(A)$^+$ RNA from *Chlorella*, several modifications were made from the procedure previously used in this laboratory (136). Cells (1 g fresh weight) were harvested by

centrifugation at 8,000 x g for 5 min, resuspended in 10 ml of 100 mM Tris,pH 8.5, 400 mM LiCl, 10 mM EGTA, 5 mM EDTA, 100 U/ml heparin, and broken by passage through a French pressure cell at 20,000 psi. The homogenate was treated with 0.05 volumes of 0.2 M EDTA (pH 8.0) and 0.1 volumes of 20% SDS for 10 min at room temperature. The homogenate was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and twice with chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated by addition of an equal volume of 2x LiCl-urea buffer (4M LiCl, 4M urea, 2 mM EDTA) at 0°C overnight. The RNA was pelleted and washed with 1x LiCl-urea buffer, resuspended in 10 mM Tris, 1mM EDTA (pH 8.0), and precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate. The pellet was washed with 70% ethanol and dissolved in water to give 10-15 A_{260}/ml. The RNA in 10 ml was adjusted to 0.1% SDS with 20% SDS stock and heated at 65°C for 5 min. After quick-chilling in an ice-ethanol bath, the solution was adjusted to 0.5 M NaCl with 5 M NaCl stock. The RNA solution was heated briefly at 65°C (30 s) to redissolve the SDS and immediately applied to a 1 ml column of oligo(dT)-cellulose. The RNA solution was applied to the column and the eluate was reapplied. The column was then washed with 20 ml of 10 mM Tris, 1 mM EDTA, 0.5 M NaCl, pH 8.0. The poly(A)$^+$ RNA was eluted with ten 0.5 ml aliquots of 10 mM Tris, 1 mM EDTA, pH 8.0, at 65°C. The poly(A)$^+$ RNA eluted in the second through sixth fractions. The poly(A)$^+$ RNA was precipitated overnight at

-20°C with 0.3 M sodium acetate and 2.5 volumes of ethanol.The RNA was pelleted, washed with 70% ethanol, and redissolved in 10 mM Tris, 1 mM EDTA, pH 8.0. The final precipitation was made in 2.5 M ammonium acetate and 2.5 volumes ethanol overnight at -20°C. By this procedure, typically 30 µg of poly(A)$^+$ RNA could be obtained from 1.0 g fresh weight of Chlorella cells.

The poly(A)$^+$ RNA was translated in a rabbit reticulocyte lysate system as described by Pelham and Jackson (98), modified to contain 2 mM of each amino acid except methionine. The radioactive amino acid used in the in vitro translation system was [^{35}S]methionine.

In Vitro Processing of NADP-GDH Precursor Protein(s)

The total translation products (0.05 ml), produced after in vitro translation for 60 min, were added to 0.05 ml of the freshly prepared cell-free extract. A cell-free extract was prepared from 1.5 g fresh weight of C. sorokiniana cells, induced for either 80 min or 180 min in 30 mM ammonia. Cells were broken in 3 ml of 250 mM Tris, pH 7.5, 25 mM KCl, 25 mM $MgCl_2$, 2 mM dithiothreitol and centrifuged for 3 h at 100,000 x g. After 10 min and 30 min of incubation, the mixture of in vitro translation products and cell-free extract was diluted with immunoprecipitation buffer and the alpha and beta subunits of NADP-GDH were immunoprecipitated as described.

Intracellular Localization of Alpha Holoenzyme NADP-GDH

To determine where the alpha holoenzyme exists in the cell, C. sorokiniana cells were cultured for 80 min in 2 mM ammonium medium and stained for NADP-GDH activity as described by Prunkard (99). Chlorella cells were cultured to an A_{640} of 5.0, washed by centrifugation into 0.1 M Tris (pH 8.25), and frozen and thawed twice. The frozen-thawed cells were washed in 0.1 M Tris (pH 8.25), pelleted by centrifugation, and the pellet was resuspended in 1.0 ml of NADP-GDH assay mixture which also contained 0.3 mM nitroblue tetrazolium and 0.15 mM phenazine methosulphate. The cells were incubated at 37°C for 30 min, washed by centrifugation, and examined microscopically for the cellular location of the formazan blue precipitate.

Separation of NADP-GDH Holoenzyme-Size and Free Subunit-Size Proteins in Cell Homogenates by Gel-Filtration Column Chromatography

To determine whether all of the NADP-GDH antigen was present in holoenzyme size molecules, cell homogenates were fractionated by gel filtration chromatography. A 2.5 x 65 cm column was packed with Sephacryl S-300 and run at 1 ml/min. The column was calibrated with NADP-GDH catalytic activity (>100,000 d fraction) and A_{280} of bovine serum albumin (<100,000 d fraction). A 1 ml sample prepared from 0.5 g fresh weight of Chlorella cells was applied to the column and 1 ml fractions were collected. After

chromatography the appropriate fractions were combined and concentrated to 0.5 ml by ultrafiltration on a YM 10 membrane (Amicon). From these two concentrated preparations, 0.03 ml aliquots were applied to a 10% SDS polyacrylamide gel and the NADP-GDH antigen was detected by Western blot/immunodetection. The fractionation procedure described was performed on Chlorella homogenates prepared from cells induced for 80 min and 180 min with ammonium-containing medium. The NADP-GDH activity was present only in the high molecular fraction as determined by the spectrophotometric assay.

RESULTS

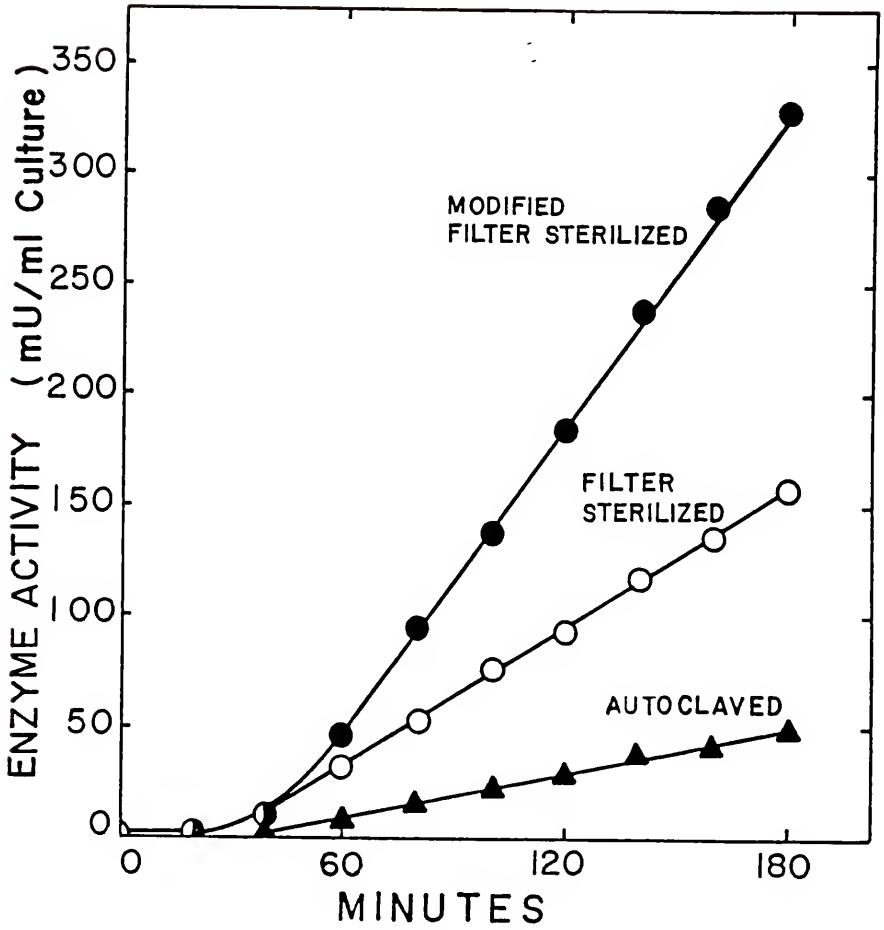
Modifications of Culture Medium and Effect on Growth Rate and Induction of NADP-GDH activity

Much of the previous research on the regulation of gene expression of the NADP-GDH from C. sorokiniana was performed at Virginia Polytechnic Institute and State University. When the cells of this same organism were cultured at the University of Florida, they were observed to be yellowish-green instead of a dark green color, to accumulate unusually large numbers of starch granules within their chloroplasts, to have a low growth rate (i.e., less than 50% of optimal), and to clump into large aggregates during growth in suspension culture. These characteristics of the organism were shown to be caused by two factors. First, the low growth rate of the cells was in part due to the absence of trace micronutrients in the Florida deionized water that were present in the Virginia deionized water. Second, the university steam supply used to autoclave media and glassware at the University of Florida, contained additives (e.g., polyamines, detergents, acrylates, etc.) that inhibited growth rate of the cells and also induced cell aggregation.

To determine what factors were causing the poor growth characteristics of the cells at the University of Florida, culture media were prepared with water from Virginia Polytechnic Institute and State University. A comparison was made of the growth characteristics of the cells in water obtained from the latter university and water which had been deionized an additional cycle through a mixed bed ion-exchange resin. The cells cultured in water from Virginia, which had been deionized one additional time, did not grow as well as cells which were cultured in the untreated water. This observation led to the proposal that micronutrients were present in the water from Virginia which were not present in the water at the University of Florida. The soil in Florida has been found to be deficient in several micronutrients. Two of these (nickel and tin) had not been added to the culture media which was developed by Sorokin (120) and used for culture of Chlorella. In addition to these two micronutrients, Arnon and Wessel (3) have reported a requirement for vanadium in one of the dark reactions associated with photosynthesis. These three micronutrients (Ni, Sn, and V) were added and a change was made from Sorokin medium (120) to the media developed by Sueoka (128) which has a lower concentration of EDTA. This change to the modified medium was made to allow for the optimal use of the micronutrients and to allow a higher concentration of unchelated micronutrients in the medium.

After making the changes indicated above, the cells grew approximately 2-times faster. However, cells would

Fig. 1 Comparison of C. sorokiniana NADP-GDH catalytic activity in extracts prepared from synchronized cells cultured in two different media sterilized by autoclave steam or by filtration. Autoclaved medium (▲); filter sterilized medium (○); modified, filter sterilized medium (●).


ENZYME ACTIVITY (mU/ml Culture)
350
300
250
200
150
100
50
0
0
60
120
180
MINUTES
MODIFIED
FILTER STERILIZED
FILTER
STERILIZED
AUTOCLAVED

frequently aggregate in culture. A comparison of filter sterilized medium and autoclaved medium revealed a toxic effect of the University of Florida steam on Chlorella. This toxicity was postulated to be due to polyamines, detergents, acrylates, and other compounds used in maintenance of the steam lines.

The higher growth rate of the organism resulted in a higher rate of induction of the NADP-GDH activity. When cells were cultured in the original medium, which had been sterilized by autoclaving with university steam, the NADP-GDH reached 50 mU/ml of culture in 3 h (Fig. 1). When cells were cultured in original medium, which had been filter sterilized, and when cells were cultured in filter sterilized modified medium, as described above, the NADP-GDH activity reached 150 mU/ml and 320 mU/ml in 3 h, respectively. Since the NADP-GDH was induced 6-fold faster in modified filter sterilized medium than autoclaved medium, and only a 2-fold increase in growth rate was observed, there was approximately a 3-fold change in the differential rate of synthesis of the NADP-GDH.

Turnover of Total NADP-GDH Antigen in Original and Modified Media

The rate of synthesis and degradation of total NADP-GDH antigen during induction and deinduction periods was compared in original and modified medium. The experiment was performed by use of the same pulse-chase procedure as performed previously in this laboratory (8).

Fig. 2 Pattern of induction and inactivation of C. sorokiniana NADP-GDH activity during a pulse-chase experiment. Cell extracts were prepared from synchronized cells cultured in either modified filter-sterilized medium (●), or in original filter-sterilized medium (○). Uninduced cells were transferred to ammonium medium and labeled for 90 min with radioactive sulfate. The labeling period was followed by a chase period of 90 min, in medium containing non-radioactive sulfate and ammonia. Cells were then transfered to nitrogen-free medium for an additional 60 min chase period.

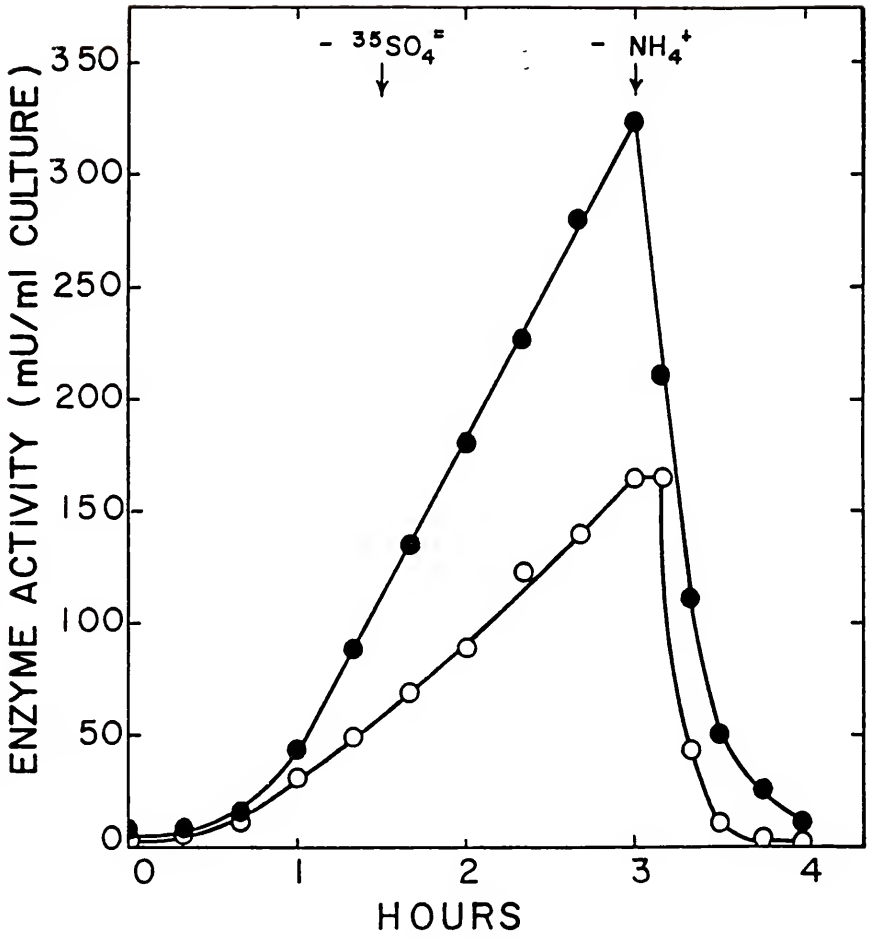
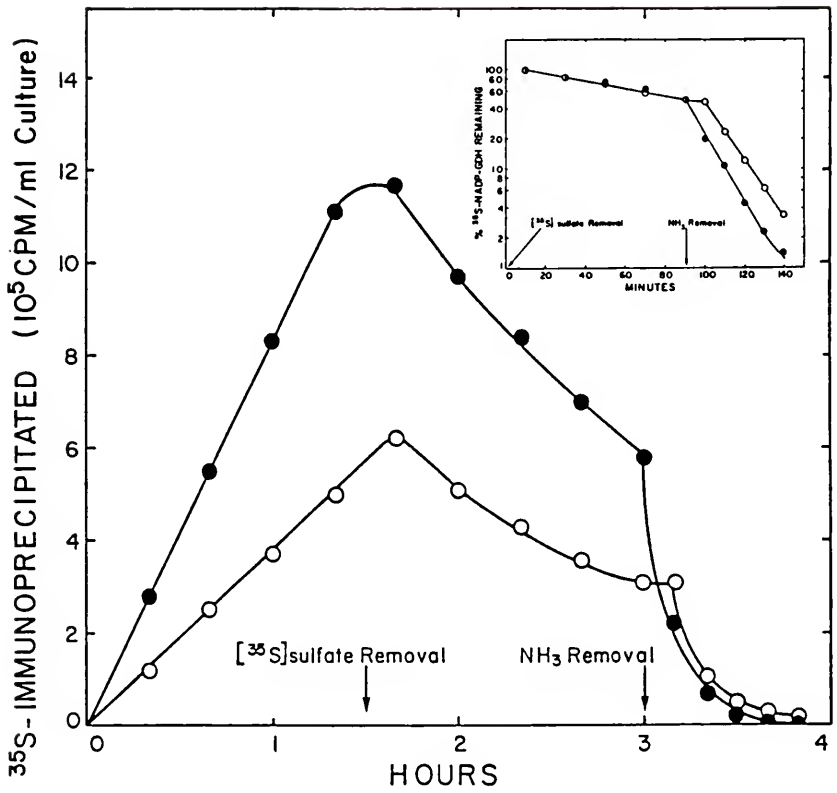

- $^{35}SO_4^{=}$
- NH_4^+
ENZYME ACTIVITY (mU/ml CULTURE)
350
300
250
200
150
100
50
0
0
1
2
3
4
HOURS

Fig. 3 Total radioactive NADP-GDH recovered by immunoprecipitation from cells labeled with radioactive sulfate in a pulse-chase experiment. C. sorokiniana was synchronized and labeled in either filter-sterilized modified medium (●), or filter-sterized original medium (○) as described in Figure 2. Extracts were prepared from labeled cells and total NADP-GDH antigen was immunoprecipitated. The radioactivity in the immunoprecipitates was determined by liquid scintillation counting. The inset shows a semi-log plot of the loss of radioactivity from total immunoprecipitated NADP-GDH from the two cultures during the chase and deinduction periods. The zero time on the inset corresponds to 90 min on the main graph.


^{35}S- IMMUNOPRECIPITATED (10^5CPM/ml Culture)
HOURS
[^{35}S]sulfate Removal
NH_3 Removal
% ^{35}S-NADP-GDH REMAINING
MINUTES

To adapt the cells for this experiment, cells were cultured for one week in either original or modified medium. Cultures were maintained at a turbidity (A_{640}) below 5.5 by dilution every 12 h to an A_{640} of 0.5. After adaption of the cells, the cultures were synchronized in their respective media.

Cells cultured in the modified medium showed a greater rate of accumulation of NADP-GDH activity than cells cultured in original medium (Fig. 2). This difference was reflected in the higher rate of incorporation of [^{35}S]sulfate into total NADP-GDH antigen in cells cultured in modified medium than cells cultured in original medium (Fig. 3). During induction of enzyme activity, the half-life of the NADP-GDH antigen (measured during the chase period) was approximately 80 min in cells cultured in both the modified and original media. During the deinduction period, the NADP-GDH from cells cultured in both types of media had a half-life of 9 min for antigen and activity.

In Vitro Inactivation of NADP-GDH

In earlier studies (8), it was observed that the inactivation of the NADP-GDH, during a deinduction period, led to the formation of a protein which was twice the size of a NADP-GDH subunit. This putative dimer was not dissociable by boiling in SDS with 2-mercaptoethanol or dithiothreitol. Therefore, it was proposed that the bond

Fig. 4 Effect of $CaCl_2$ concentration on the rate of *in vitro* inactivation of the NADP-GDH. A cell extract was prepared from *C. sorokiniana* cells, cultured for 36 h in ammonium medium, by breakage in a French pressure cell at 20,000 psi, followed by centrifugation at 8,000 x g for 3 min. Each reaction contained 2 mM ATP and $CaCl_2$ at the concentrations indicated. The amount of NADP-GDH activity was measured spectrophotometrically after 3 min of inactivation at 38.5°C.

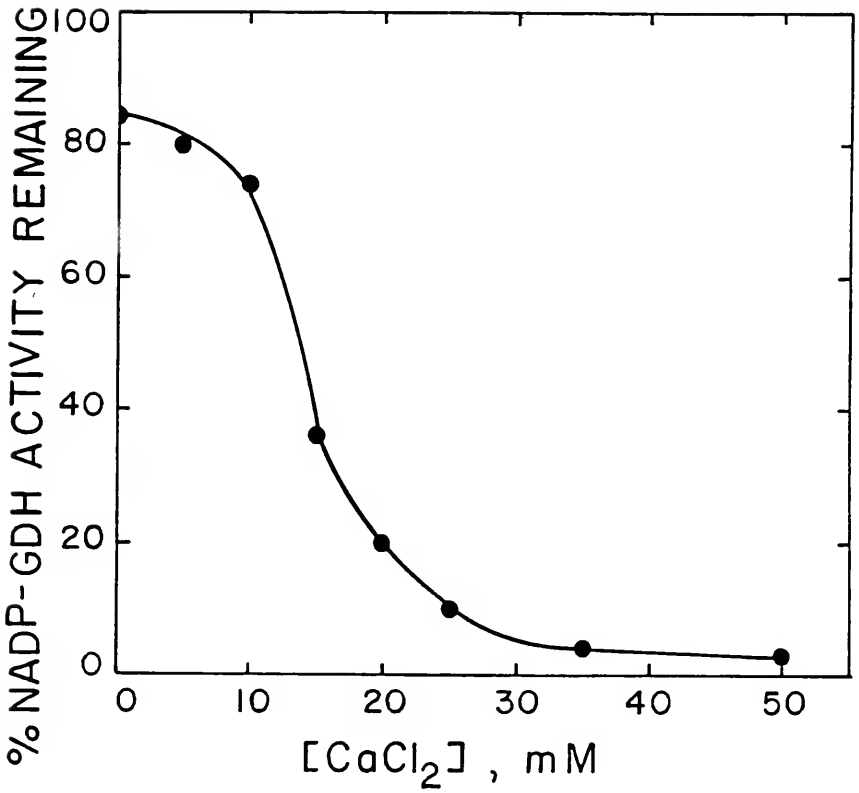

% NADP-GDH ACTIVITY REMAINING
100
80
60
40
20
0
0
10
20
30
40
50
[CaCl2] , mM

Fig. 5 Effect of ATP concentration on the rate of *in vitro* inactivation of the NADP-GDH. Cell extracts were prepared as described in Figure 4. Each reaction mixture contained 50 mM $CaCl_2$ and ATP at the concentraion indicated. The amount of NADP-GDH catalytic activity was determined after 3 min of inactivation at 38.5° C.

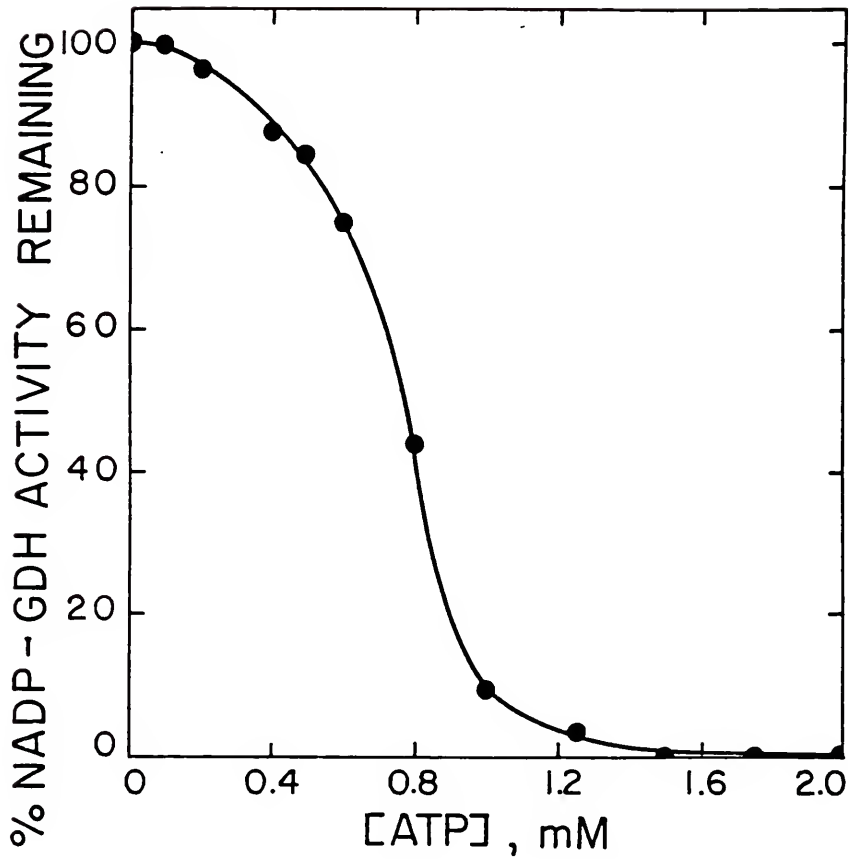
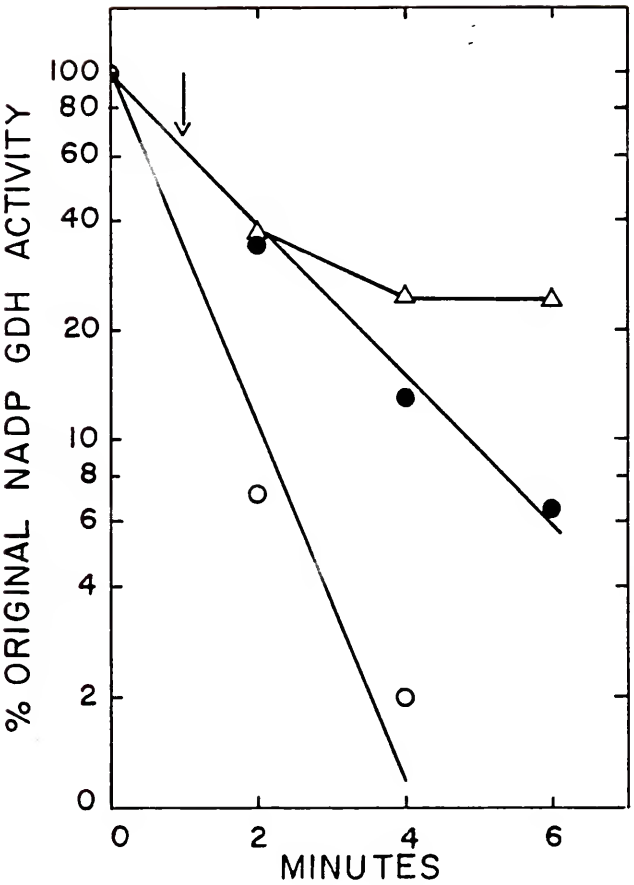

% NADP-GDH ACTIVITY REMAINING
100
80
60
40
20
0
0
0.4
0.8
1.2
1.6
2.0
[ATP] , mM

Fig. 6 Cessation of *in vitro* inactivation of NADP-GDH after the addition of EGTA. Cell extracts were prepared as described in Figure 4 with 20 mM $CaCl_2$ and either 2.5 mM ATP (●), or 5 mM (○) ATP. At the time designated by the arrow, EGTA was added to the 2.5 mM ATP reaction to bring the reaction to 20 mM EGTA (△). The same amount of buffer was added to the control culture (●).


% ORIGINAL NADP GDH ACTIVITY
100
80
60
40
20
10
8
6
4
2
0
0
2
4
6
MINUTES

formed between subunits was an isopeptide bond. The formation of an isopeptide bond in proteins is catalyzed by a class of enzymes referred to as transglutaminases (30). All of these enzymes (except one) have been reported to require calcium as a cofactor. Therefore, experiments were performed to test for the inactivation of the NADP-GDH with calcium as a possible requirement.

To keep proteins in the cellular compartment in which they might function, and to prevent dilution of cellular proteins, the initial in vitro inactivation experiments used fully induced cells which were permeabilized by two cycles of freeze-thaw. A first-order decay of NADP-GDH activity was obtained when calcium and ATP were added to the permeabilized cells. Subsequently, it was found that NADP-GDH inactivation could occur in cell homogenates, and was still dependent on the addition of calcium (Fig. 4) and ATP (Fig. 5). A maximal rate of inactivation was obtained at pH 6.5.

Addition of cycloheximide or ammonia prevented inactivation in vivo (50). In contrast, the in vitro inactivation was unaffected by the addition of either of these compounds. The addition of glutamine and lysine had no effect on the inactivation of the NADP-GDH in vitro. Magnesium or manganese would not substitute for calcium. The addition of EGTA caused a cessation in the inactivation of the NADP-GDH, presumably by sequestering the calcium (Fig. 6).

Fig. 7 Loss of NADP-GDH catalytic activity in the ATP/$CaCl_2$ *in vitro* inactivation system. *C. sorokiniana* cells were cultured for 12 h in radioactive sulfate medium and cell extracts were prepared as described in Figure 4. The reaction mixture contained 50 mM $CaCl_2$ and 0.6 mM ATP. Use of these concentrations resulted in a half-life of NADP-GDH activity of approximately 5 min. Samples were taken at zero time and at the time indicated by the arrow, for immunoprecipitation and analysis by SDS polyacrylamide tube-gel electrophoresis.

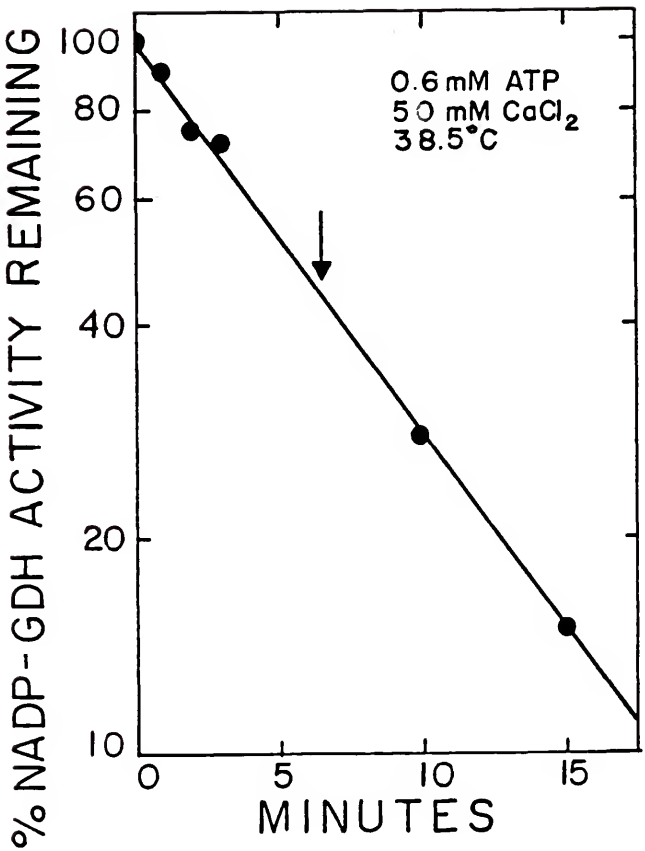
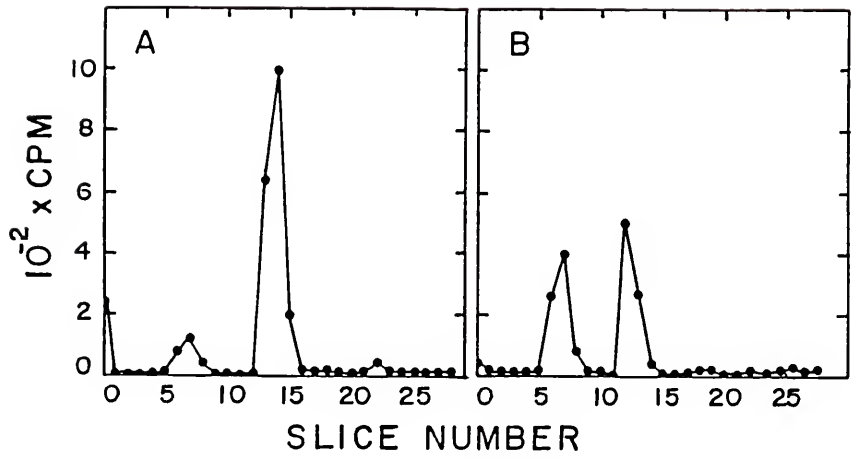

% NADP-GDH ACTIVITY REMAINING
100
80
60
40
20
10
0
5
10
15
MINUTES
0.6 mM ATP
5.0 mM CaCl2
38.5°C

Fig. 8 SDS polyacrylamide tube-gel electrophoresis of total NADP-GDH antigen immunoprecipitated from samples during *in vitro* inactivation. Radioactive cell extract was prepared as described in Figure 7. Samples were removed after zero (A) or 6 min (B) of *in vitro* inactivation, as shown in Figure 7. After SDS polyacrylamide tube-gel electrophoresis of the immunoprecipitated NADP-GDH, the gels were sliced into 2 mm sections and the radioactivity in each slice determined by liquid scintillation counting.


A
B
10^{-2} x CPM
0
2
4
6
8
10
0 5 10 15 20 25
0 5 10 15 20 25
SLICE NUMBER

Studies on in vitro inactivation of NADP-GDH were also performed with extracts prepared from cells in which 50% of the NADP-GDH activity had decayed in vivo after removal of ammonia from the culture medium. When calcium and ATP were tested again, it was found that the addition of calcium was no longer necessary, and the concentration of ATP required was 75% lower.

To determine whether the in vitro inactivation of NADP-GDH activity was accompanied by formation of putative dimer, homogenates from ^{35}S-labeled cells were used for in vitro inactivation in the calcium/ATP dependent system (Fig. 7). Inactivation was allowed to proceed for 6 min at which time a 45% loss of enzyme activity was observed. Samples were removed at 0 and 6 min, inactivation was stopped with EGTA at 4°C, and the total NADP-GDH antigen was immunoprecipitated with specific antibodies. The immunoprecipitates were subjected to electrophoresis in 10% polyacrylamide SDS tube-gels, the gels were sliced, and the radioactivity in each slice measured by liquid scintillation counting. As shown in Figure 8A, the zero time sample shows predominant label in a protein, corresponding to the size of the NADP-GDH subunit. Additionally, a small amount of radioactivity was observed in a protein with the molecular weight of the putative dimer. After 6 min of in vitro inactivation, there was a loss of label from the NADP-GDH subunit and an increase in the label in the putative dimer (Fig. 8B). This observation

Fig. 9 Comparison of the amount of NADP-GDH activity and antigen during *in vitro* inactivation, using cell extracts prepared from cells cultured in modified medium. The reaction mixture which contained cell extracts, prepared as described in Figure 4, 1 mM ATP, and 50 mM $CaCl_2$, caused a loss of enzyme activity and antigen with a half-life of 5 min at 38.5°C Samples were taken every 5 min to analyze for NADP-GDH activity (○), and antigen (●). The antigen was determined by densitometric scans of autoradiograms made from an SDS polyacrylamide slab-gel which was analyzed by Western blot/immunodetection. The inset shows the region of the gel displaying the NADP-GDH antigen. Lane 1, 0 min; 2, 5 min; 3, 10 min; 4, 15 min; 5, 20 min.

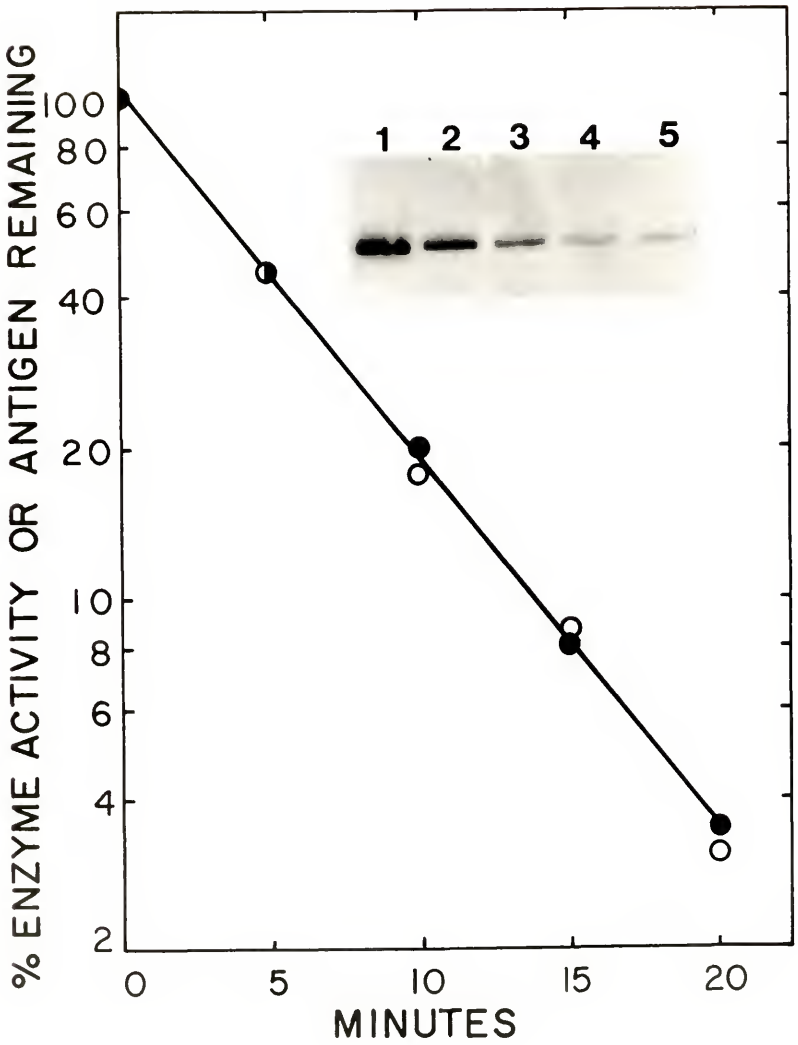
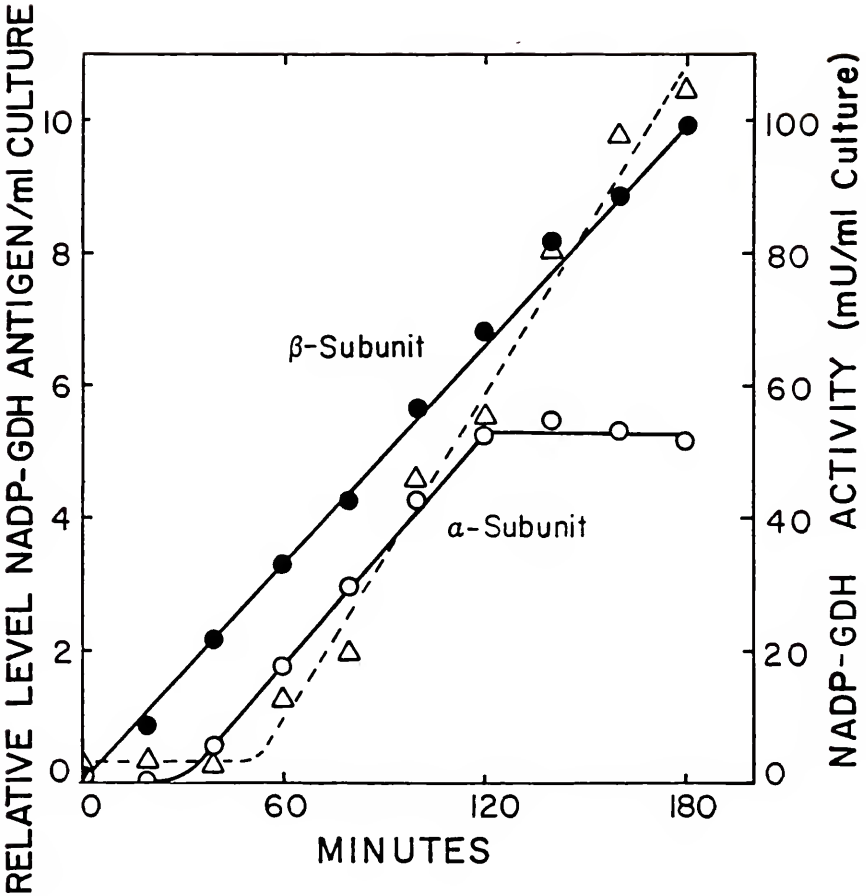

% ENZYME ACTIVITY OR ANTIGEN REMAINING
100
80
60
40
20
10
8
6
4
2
0
5
10
15
20
MINUTES
1
2
3
4
5

Fig. 10 Patterns of induced accumulation of the alpha and beta subunit antigens, and total catalytic activity, of NADP-GDH during induction in 30 mM ammonium medium. The autoradiogram from Prunkard
(see Fig. 19 ref 99) was scanned on a laser densitometer to determine the relative amounts of each subunit per ml of culture during synchronous growth of daughter cells of C. sorokiniana. Beta subunit (●), alpha subunit (○), catalytic activity (△).


RELATIVE LEVEL NADP-GDH ANTIGEN/ml CULTURE
NADP-GDH ACTIVITY (mU/ml Culture)
MINUTES
β-Subunit
α-Subunit
0
2
4
6
8
10
0
20
40
60
80
100
0
60
120
180

suggested that the calcium/ATP dependent *in vitro* inactivation of the NADP-GDH might be proceeding via dimerization, as proposed during *in vivo* inactivation.

When the *in vitro* inactivation system was tested with homogenates prepared from cells cultured on the modified medium, the requirements for inactivation of the NADP-GDH were the same as with homogenates from cells cultured in the original medium. However, during the calcium/ATP dependent loss of NADP-GDH activity, there was a coincident loss of total NADP-GDH antigen without the accumulation of the putative dimer (Fig. 9). By use of the Western blot/immunodetection procedure, it was not possible to detect the putative dimer before or during *in vitro* inactivation.

Accumulation and Turnover of Alpha and Beta Subunits of NADP-GDH

By use of a Western blot procedure, it was shown by Prunkard (99) that the total NADP-GDH antigen was composed of two different molecular weight antigens which were designated as subunits of the NADP-GDH. During induction of NADP-GDH activity in cells in the light, the pattern of accumulation of the alpha and beta subunits was examined.

In the experiments performed by Prunkard, a constant amount of protein from each time point was loaded onto a polyacrylamide slab-gel. Therefore, the results were described as the amount of alpha and beta subunit relative to total cellular protein. It was concluded that,

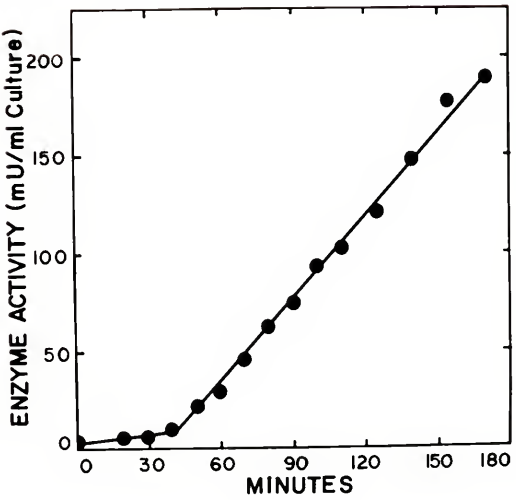
qualitatively, the alpha and beta subunits both accumulate early during induction with 30 mM ammonia. The alpha subunit appeared to decrease in concentration as induction proceeded beyond 80 min and was only a minor fraction (less than 10%) of total NADP-GDH antigen by 3 h. When I scanned this autoradiogram (see Fig. 19, ref. 99) with a laser densitometer and expressed the data on a per ml of culture basis, the pattern shown in Figure 10 was obtained. It was found that the beta subunit began to accumulate from the time of addition of ammonia. The alpha subunit, however, began to accumulate after a 30 min lag and increased for only an additional 90 min, and then ceased to accumulate.

Since the alpha subunit remained at a constant level per ml of culture after 120 min, this subunit represented a smaller percentage of both total cellular protein and total NADP-GDH antigen as induction continued. The constant level of alpha subunit from 120 min to 180 min could have been due to the absence of synthesis of a stable form of the NADP-GDH antigen. Alternatively, the constant level could represent a steady state between synthesis and degradation of the alpha subunit.

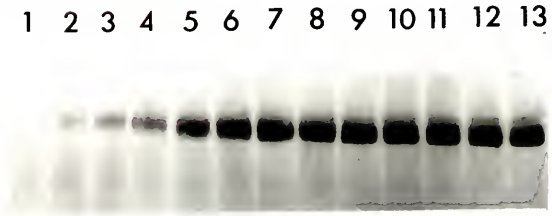
To measure the relative rates of degradation of the alpha and beta subunits during the induction and deinduction periods, a pulse-chase experiment was designed similar to the one for measurement of the turnover of total NADP-GDH antigen. In this experiment, the total NADP-GDH antigen was fractionated by SDS slab-gel electrophoresis

Fig. 11 Patterns of induced accumulation of NADP-GDH activity and antigens during growth of synchronized daughter cells of C. sorokiniana in 30 mM ammonium medium. Panel A, accumulation of NADP-GDH catalytic activity. Panel B, native polyacrylamide slab-gel analyzed by Western blot/immunodetection of NADP-GDH during induction of enzyme activity. Lane 1, 30 min; 2, 40 min; 3, 50 min; 4, 60 min; 5, 70 min; 6, 80 min; 7, 90 min; 8, 100 min; 9, 110 min; 10, 125 min; 11, 140 min; 12, 155 min; 13, 170 min. Panel C, SDS polyacrylamide slab-gel analyzed by Western blot/immunodetection of NADP-GDH during induction of enzyme activity. Lanes 1-12 from this panel correspond to lanes 1-12 from Panel B.

A


ENZYME ACTIVITY (mU/ml Culture)
200
150
100
50
0
0
30
60
90
120
150
180
MINUTES

B


1 2 3 4 5 6 7 8 9 10 11 12 13

C

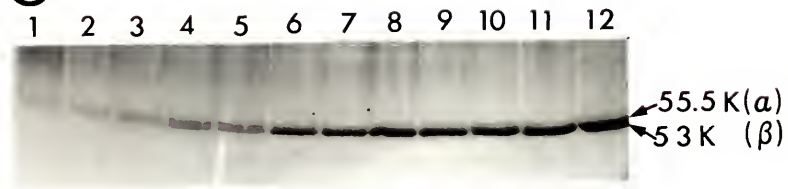

1 2 3 4 5 6 7 8 9 10 11 12
55.5 K(α)
53 K (β)

Fig. 12 Pattern of induced accumulation of NADP-GDH activity in synchronized daughter cells of C. sorokiniana during growth in 30 mM ammonium medium. The inset shows the region of a native polyacrylamide slab-gel developed with an activity stain for NADP-GDH (150). Lane 1, 60 min; 2, 80 min; 3, 100 min; 4, 120 min; 5, 130 min; 6, 140 min; 7, 150 min; 8, 165 min, 9, 180 min after illumination, and addition of ammonia, to the culture.

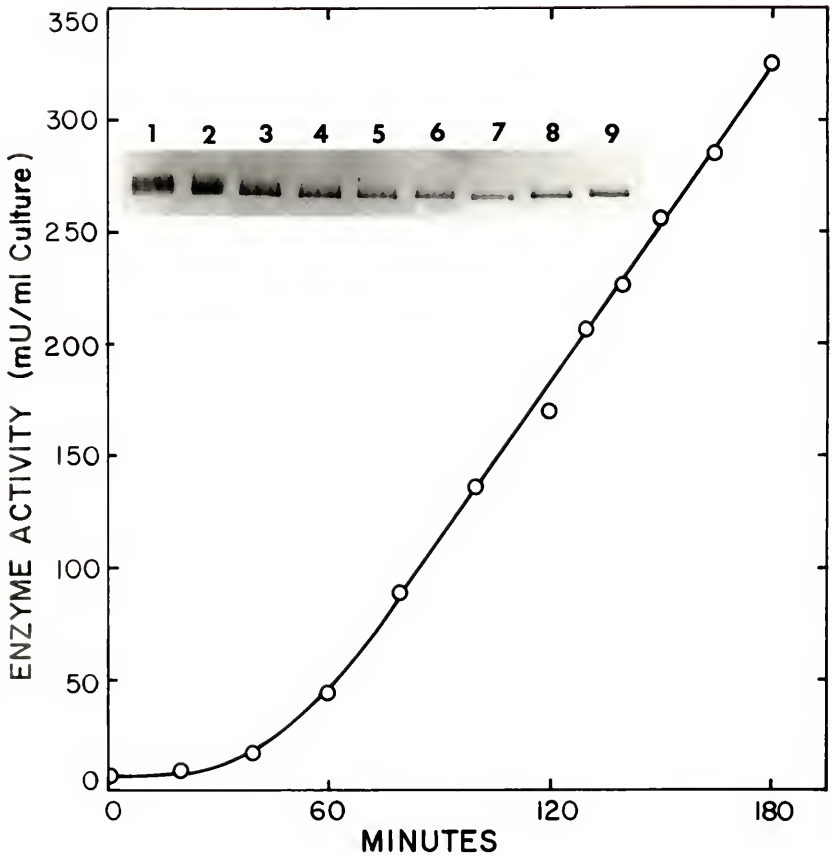
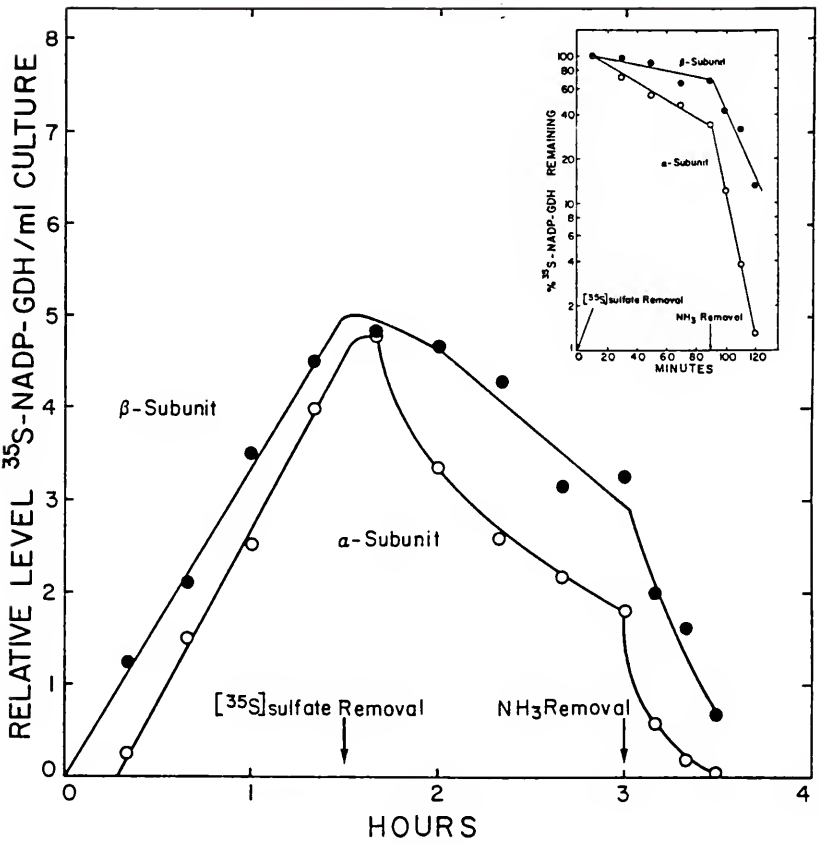

350
300
250
200
150
100
50
0
ENZYME ACTIVITY (mU/ml Culture)
1 2 3 4 5 6 7 8 9
0
60
120
180
MINUTES

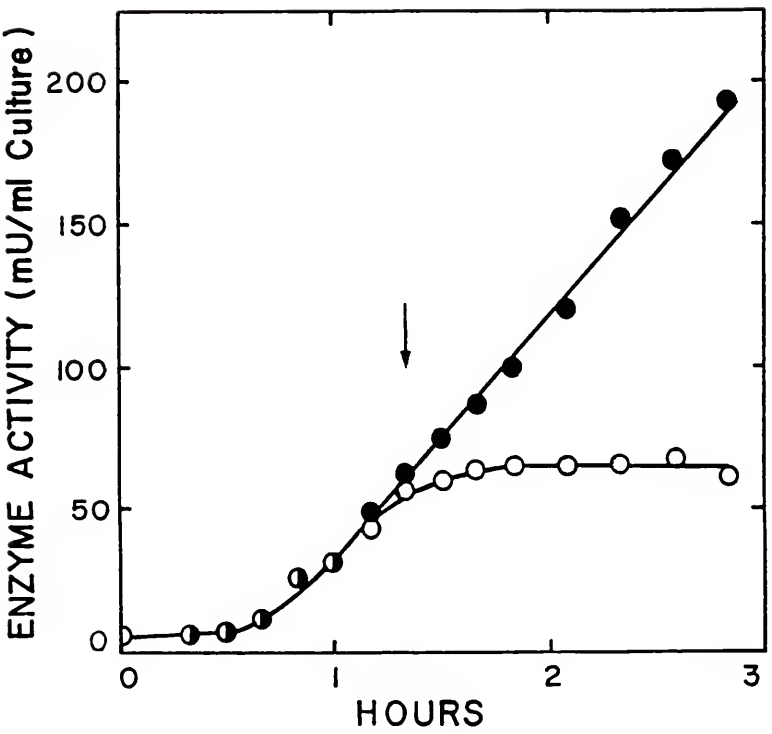
Fig. 13 Patterns of labeling of NADP-GDH subunits during induction and inactivation of NADP-GDH in synchronized daughter cells of C. sorokiniana. Cells were labeled with radioactive sulfate for 90 min during the induction period in 30 mM ammonia, then transferred to non-radioactive ammonium medium for a 90 min chase period. After 3 h of induction of enzyme activity, the cells were transferred to nitrogen-free medium to initiate the inactivation of the NADP-GDH. Total NADP-GDH antigen was immunoprecipitated during induction (pulse and chase periods) and deinduction. The immunoprecipitates were fractionated by SDS polyacrylamide gel electrophoresis, and an autoradiogram was prepared. The autoradiogram was scanned to determine the amount of each subunit present. The inset shows a semi-log plot of the radioactivity from the NADP-GDH subunits during the chase and deinduction periods. The zero time on the inset corresponds to 90 min on the main graph. Alpha subunit (○), beta subunit (●).


RELATIVE LEVEL ^{35}S-NADP-GDH/ml CULTURE
HOURS
β-Subunit
α-Subunit
[^{35}S]sulfate Removal
NH$_3$Removal
% ^{35}S-NADP-GDH REMAINING
MINUTES

into alpha and beta subunits, and the amount of radioactivity in each subunit was determined after autoradiography. As a preliminary control experiment, a light induction experiment was performed similar to the one performed by Prunkard (99). The patterns of accumulation of NADP-GDH activity, and alpha and beta subunits, were the same as observed by Prunkard (99); however, the resolution of the alpha and beta subunits was not as good as obtained by Prunkard (Fig. 11). In a similar experiment, the pattern of the NADP-GDH holoenzyme isoforms was examined by activity stain following native polyacrylamide gel electrophoresis (Fig.12). After 60 min of induction, the NADP-GDH holoenzyme existed as seven different isoforms which decreased in number, and apparent molecular weight as the induction continued.

Total NADP-GDH antigen was immunoprecipitated from homogenates prepared from ^{35}S-labeled cells cultured in the modified medium. The NADP-GDH antigen from modified medium was seen as two radioactive bands, with molecular weights of 55,500 and 53,000. The molecular weights of these antigens correspond to the alpha and beta subunits, respectively. During induction (Fig. 13), the beta subunit was labeled first, and ^{35}S incorporation into this subunit appeared to occur immediately after the addition of ammonia. The alpha subunit became labeled after a 20 min lag; however, by 80 min, the amount of ^{35}S in the alpha subunit was approximately equal to that in the beta

Fig. 14 Pattern of induced accumulation of NADP-GDH activity, and the effect of addition of the protein synthesis inhibitor, cycloheximide, during accumulation of NADP-GDH activity in synchronized daughter cells of C. sorokiniana cultured in 30 mM ammonium medium. Two cultures were labeled with radioactive sulfate for 80 min. At the time indicated by the arrow (80 min), the cells from one culture were transferred to 30 mM ammonium medium without radioactive sulfate to initiate the chase period. Cells from the parallel culture were transferred to ammonium medium without radioactive sulfate, but which contained 0.025 mg/ml cycloheximide. Control culture (●); culture treated with cycloheximide at initiation of chase period (○).


ENZYME ACTIVITY (mU/ml Culture)
200
150
100
50
0
0
1
2
3
HOURS

subunit. For the first 80 min of induction, the increase in NADP-GDH catalytic activity had a pattern coincident with labeling of the alpha subunit. During chase and deinduction periods, the loss of radioactivity from the two subunits occurred at different rates. The half-lives of the alpha and beta subunits during the induction and deinduction periods were 50 min and 150 min, and 5.0 and 13.5 min, respectively.

To determine whether processing of one subunit to the other could occur in a sequential manner, pulse-chase experiments were performed with [^{35}S]sulfate. One culture was induced under normal conditions for a pulse-chase experiment, and a parallel culture was maintained which had cycloheximide added at the beginning of the chase period to prevent any further protein synthesis. In addition, it was observed by Prunkard (99) that NADP-GDH antigen was synthesized at a low rate in the dark. When cells cultured for 3 h in the dark with ammonia were illuminated, the NADP-GDH activity accumulated without any lag. For this experiment, the ammonia was added at the same time that the culture was illuminated and label was added. A comparison of the induction pattern of NADP-GDH activity from normal and cycloheximide treated cultures is shown in Figure 14. An induction lag of approximately 40 min was observed in both cultures. The enzyme activity then began to accumulate in a linear manner. At 80 min, the cells from one culture were washed and resuspended in non-radioactive medium.

Fig. 15 Pattern of incorporation of ^{35}S into total NADP-GDH antigen and the effect of addition of cycloheximide on the loss of ^{35}S during a pulse-chase experiment in 30 mM ammonium medium. NADP-GDH was immunoprecipitated from cell homogenates prepared from C. sorokiniana labeled as described in Figure 14. The amount of radioactivity in the immunoprecipitated NADP-GDH was determined by liquid scintillation counting. Control culture (○); culture treated with cycloheximide at initiation of chase period (●).

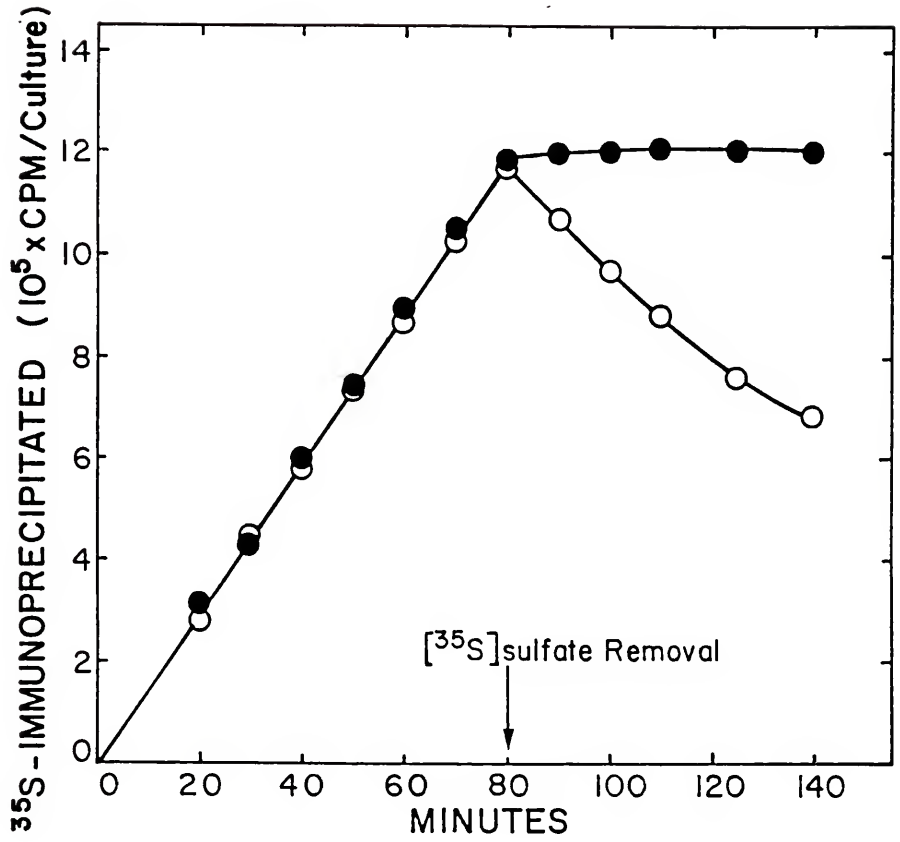
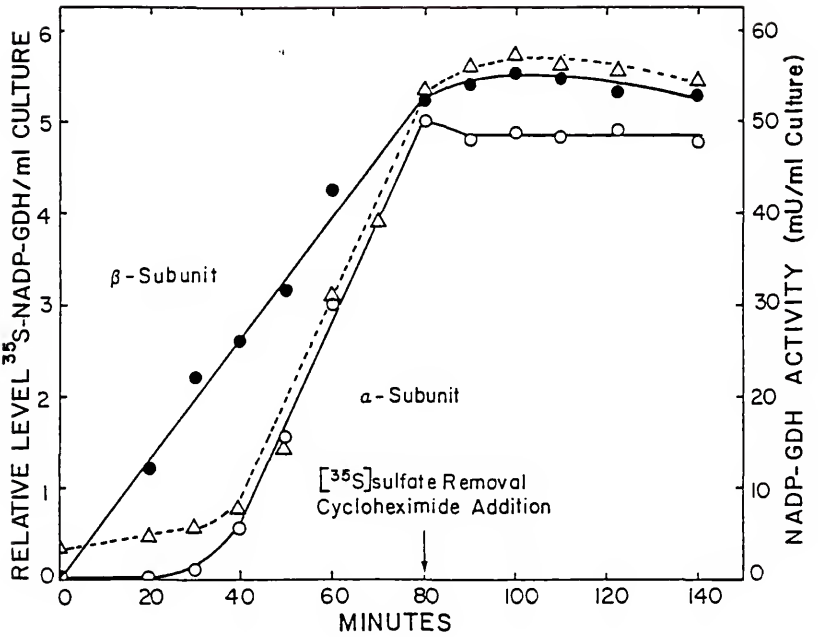

^{35}S-IMMUNOPRECIPITATED (10^5 x CPM/Culture)
14
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
MINUTES
[^{35}S]sulfate Removal

Fig. 16 Patterns of incorporation of ^{35}S into alpha and beta subunits and the effect of addition of cycloheximide on the loss of ^{35}S during a pulse-chase experiment in 30 mM ammonium medium. NADP-GDH was immunoprecipitated from cell homogenates prepared from C. sorokiniana labeled as described in Figure 14. The immunoprecipitated NADP-GDH was fractionated by SDS polyacrylamide slab-gel electrophoresis. The gel was used to prepare an autoradiogram which was scanned to determine the amount of radioactivity in each subunit. Relative amount of alpha subunit (○), beta subunit (●), and actual catalytic activity (△).

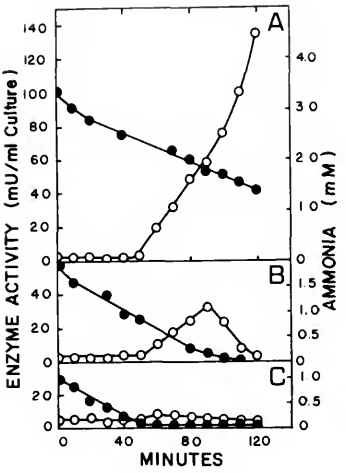

RELATIVE LEVEL ^{35}S-NADP-GDH/ml CULTURE
NADP-GDH ACTIVITY (mU/ml Culture)
MINUTES
β-Subunit
α-Subunit
[^{35}S]sulfate Removal
Cycloheximide Addition
0
20
40
60
80
100
120
140
1
2
3
4
5
6
10
30
50

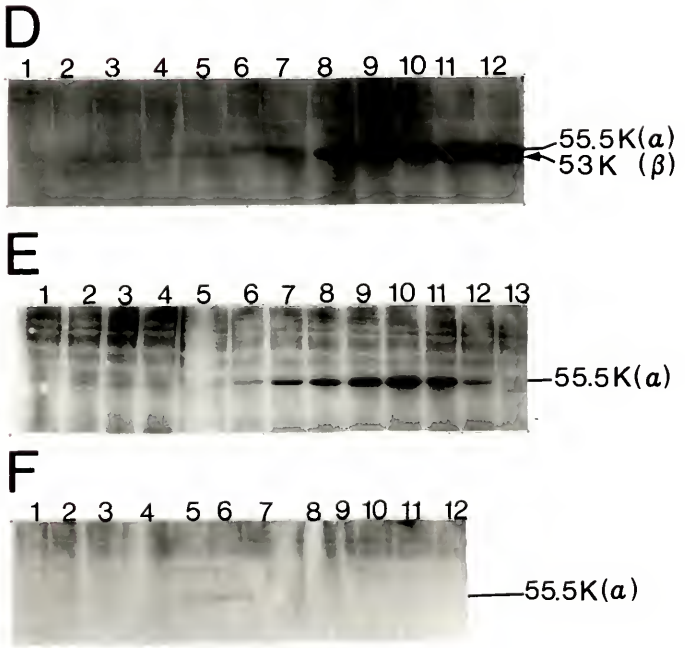
These cells continued to show normal, linear induction of NADP-GDH activity. In the other culture, cells were washed and resuspended in non-radioactive sulfate medium with cycloheximide, to stop protein synthesis. In this culture, enzyme activity ceased to accumulate and remained at a constant level.

The NADP-GDH was immunoprecipitated from cell homogenates from the untreated and the cycloheximide treated culture. Figure 15 shows the radioactivity present in the immunoprecipitated NADP-GDH. Both cultures showed a linear incorporation of the label into protein during the pulse period. During the chase period, the NADP-GDH from the untreated culture was degraded with a half-life of 80 min. The NADP-GDH from the cycloheximide treated culture showed no loss of ^{35}S during this period.

Cell extracts from the treated culture were subjected to immunoprecipitation, SDS slab-gel electrophoresis, and autoradiography. The amount of NADP-GDH antigen was determined from the autoradiograms (Fig. 16). The incorporation of ^{35}S into the beta subunit was seen to begin at the time of addition of ammonia. However, incorporation of radioactivity into the alpha subunit was observed only after a 30 min lag. By 80 min, the alpha and beta subunits contained nearly equal amounts of ^{35}S. A comparison of the patterns suggests that the initial increase of NADP-GDH activity was due primarily to the alpha subunit. Moreover, there was no apparent processing of one subunit to the other.

Fig. 17 Patterns of NADP-GDH activity, uptake of ammonia, and accumulation of alpha or beta subunits during induction of enzyme activity in synchronized daughter cells of C. sorokiniana in three different concentrations of ammonia. Synchronous cells were transferred to medium containing either (A), 1 mM; (B), 2 mM; or (C), 3.4 mM ammonia. At frequent intervals during induction, samples were removed for measurement of NADP-GDH catalytic activity (○), and the medium was analyzed for the amount of ammonia remaining (●). The NADP-GDH antigen was determined by analysis of an SDS polyacrylamide slab-gel by the Western blot/immunodetection procedure. Panel D, antigen induced in cells cultures in 3.4 mM ammonium medium. Lane 1, 0 min; 2, 10 min; 3, 20 min; 4, 30 min; 5, 40 min; 6, 50 min; 7, 60 min; 8, 70 min; 9, 80 min; 10, 90 min; 11, 100 min; 12, 110 min after addition of ammonia. Panel E, antigen induced in cells cultured in 2.0 mM ammonium medium. Lanes 1 through 12 are as indicated in Panel D; Lane 13, 120 min. Panel F, antigen induced in cells cultured in 1.0 mM ammonium medium. Lanes 1 through 12 are as indicated in Panel D.


A
B
C
ENZYME ACTIVITY (mU/ml Culture)
AMMONIA (mM)
MINUTES
0
40
80
120


D
1 2 3 4 5 6 7 8 9 10 11 12
55.5K(α)
53K (β)
E
1 2 3 4 5 6 7 8 9 10 11 12 13
55.5K(α)
F
1 2 3 4 5 6 7 8 9 10 11 12
55.5K(α)

It has been shown (46) that in Candida utilis the phosphorylation of the NAD-GDH causes inactivation of the enzyme and appears to be required for its subsequent degradation. A similar system was proposed for the regulation of the NADP-GDH of Saccharomyces cerevisiae (139). To determine whether the NADP-GDH activity of Chlorella might be regulated by phosphorylation of the alpha or beta subunits, and whether the alpha and the beta subunits might show different patterns of labeling by phosphate, an in vivo labeling experiment was performed with [^{32}P]phosphate. Cells were labeled during an induction period as in a pulse-chase experiment, and during a deinduction period when the NADP-GDH was undergoing rapid inactivation. During the pulse, chase, and inactivation periods, there was no detectable incorporation of ^{32}P into the NADP-GDH subunits that were immunoprecipitated. However, there was incorporation of ^{32}P into other proteins as indicated by the high amount of TCA-precipitable radioactivity.

Effect of Ammonia Concentrations on the Accumulation of Alpha and Beta Subunits

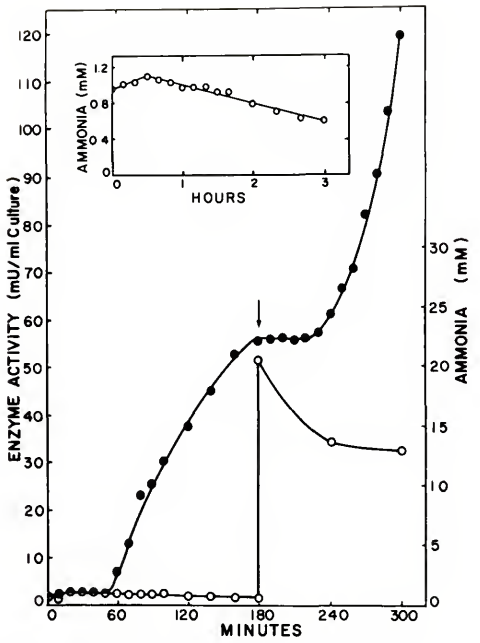
To test the effect of different ammonia concentrations on the induction of the alpha and beta subunits of the NADP-GDH, cells synchronized in nitrate medium were induced with either 1 mM, 2 mM, or 3.4 mM ammonia. Cells were

harvested periodically and assayed for NADP-GDH activity. The concentration of ammonia in the medium was measured. The proteins from cell homogenates were fractionated by SDS polyacrylamide slab-gel electrophoresis for Western blot/[^{125}I]Protein-A immunodetection analysis.

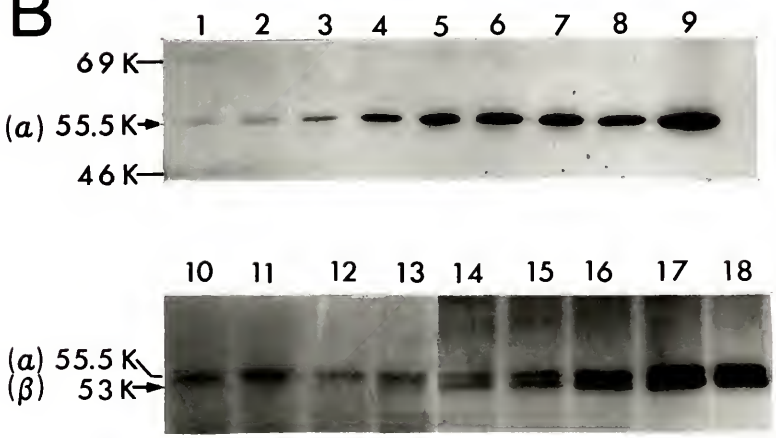
During the first 120 min of induction, the ammonia concentration decreased from 3.4 mM to 1.4 mM (Fig. 17A). After a 50 min lag, the NADP-GDH activity accumulated linearly until 90 min. The rate of accumulation of the NADP-GDH activity began to increase after 90 min. In the culture started with 2 mM ammonia, the ammonia concentration decreased to 0.15 mM in 90 min. In this culture, the NADP-GDH activity accumulated after a 50 min lag period; however, the enzyme activity accumulated at a lower rate than in the culture with 3 mM ammonia (Fig. 17B). When the ammonia concentration dropped to below 0.15 mM, a net decrease in enzyme activity was observed. The cells cultured in 1 mM ammonia utilized almost all of the ammonia by 55 min, which was before the end of the induction lag. Thus, in this culture, only a slight increase was seen in the enzyme activity (Fig. 17C). The ammonia concentration in the medium of all cultures decreased by 1 mM before NADP-GDH activity began to accumulate. To eliminate any variation in ammonia uptake due to a different number of cells per ml of culture, all cultures were started with synchronized cells at a cell concentration of 140 x 10^6 cells per ml, and the cells were not diluted during the subsequent induction period.

Fig. 18 Patterns of NADP-GDH activity and antigen, in a culture of synchronized daughter cells of C. sorokiniana, during induction in low ammonium medium, maintained between 1.0 mM and 0.65 mM ammonia. After 3 h in low ammonia, the culture was adjusted to 20 mM ammonia and induction was continued for 2 additional hours. Panel A, NADP-GDH activity (●) and the concentration of ammonia in the medium (○). The ammonium concentration was maintained by the addition of 0.2 ml of a solution of 0.54 M ammonium chloride every two min to a 3 L culture (0.018 mmol/min/L). The inset is an expanded scale which shows the change in concentration of ammonia in the culture medium during the first 3 h of induction. Panel B, NADP-GDH antigen visualized by Western blot/immunodetection of an SDS polyacrylamide slab-gel. Lane 1, 60 min; 2, 70 min; 3, 80 min; 4, 90 min; 5, 100 min; 6, 120 min; 7, 140 min; 8, 160 min; 9, 180 min; 10, 190 min; 11, 200 min; 12, 210 min; 13, 220 min; 14, 230 min; 15, 240 min; 16, 250 min; 17, 260 min; 18, 280 min. The autoradiogram for lanes 1-9 was exposed for 60 h, and the autoradiogram for lanes 10-18 was exposed for 18 h, to obtain proper exposure of all bands.

A


ENZYME ACTIVITY (mU/ml Culture)
AMMONIA (mM)
MINUTES
AMMONIA (mM)
HOURS

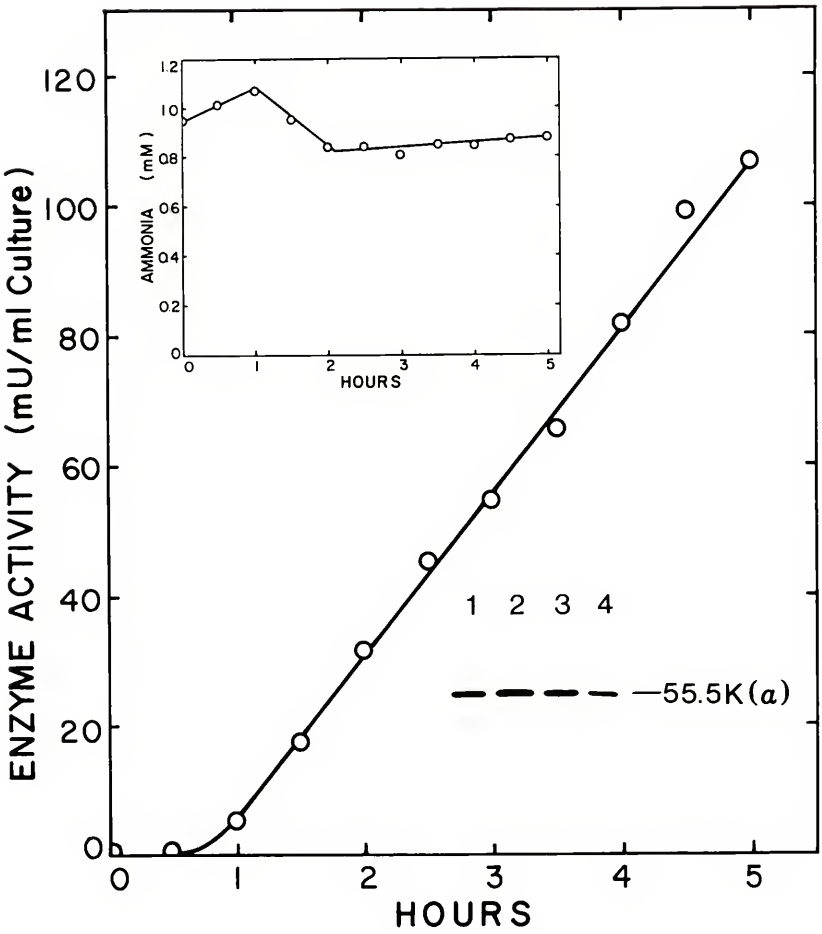
B


1 2 3 4 5 6 7 8 9
69 K
(a) 55.5 K
46 K
10 11 12 13 14 15 16 17 18
(a) 55.5 K
(β) 53 K

In cells induced with 3.4 mM ammonia, only the alpha subunit accumulated for the first 70 min after the addition of ammonia; thereafter, the beta subunit also began to accumulate (Fig. 17D). The appearance of the beta subunit was coincident with the time at which an acceleration in the accumulation of NADP-GDH activity was observed. Only the alpha subunit was seen in cells cultured in 2 mM ammonia. The appearance of the alpha subunit coincided with the appearance of NADP-GDH activity and its disappearance coincided with the loss of enzyme activity during deinduction (Fig. 17E). In 1 mM ammonia, the appearance and disappearance of the alpha subunit coincide with the accumulation and loss of enzyme activity, respectively (Fig. 17F).

To determine whether the holoenzyme composed of the alpha subunit could be made to accumulate continuously during induction, without the accumulation of the beta subunit, cells were induced in 1 mM ammonia. An attempt was made to maintain a constant concentration of ammonia by the frequent addition of ammonia to match the pre-determined rate of its uptake (18 μmol/min). However, during the first 3 h, the concentration of ammonia in the medium actually decreased from 1.0 mM to 0.65 mM. The rate of induction of NADP-GDH activity in cells in this culture was significantly less than the rate of induction of NADP-GDH activity in cells in 30 mM ammonia (Fig. 12). The pattern

Fig. 19 Induction of NADP-GDH activity and antigen in synchronized daughter cells of C. sorokiniana by a concentration of ammonia maintained between 1.0 mM and 0.85 mM ammonia in the culture medium. In this experiment, the ammonia concentration was held at a value closer to 1 mM. This experiment is essentially a duplicate of the experiment described in Figure 18 except that after 40 min the rate of addition of ammonia was increased to 0.0182 mmol/min/L) by increasing the volume of ammonium chloride stock added to 0.202 ml every 2 min to a 3 L culture. The ammonia concentration measured in the culture medium is shown in the inset (○). The antigen was detected by Western blot/immunodetection analysis of an SDS polyacrylamide slab-gel. Lane 1, 1 h; 2, 2 h; 3, 3 h; 4, 4 h.


ENZYME ACTIVITY (mU/ml Culture)
120
100
80
60
40
20
0
0
1
2
3
4
5
HOURS
AMMONIA (mM)
1.2
1.0
0.8
0.6
0.4
0.2
0
HOURS
1 2 3 4
—55.5K(a)

of accumulation of enzyme activity was non-linear in 1 mM ammonia. This pattern might have been due to either the low concentration of ammonia, the failure to hold the ammonia concentration constant, or the decrease in effective light intensity due to increased culture turbidity during synchronous growth.

After 3 h in 1 mM ammonia, the culture was adjusted to 20 mM ammonia. This addition caused a 50 min cessation of accumulation of NADP-GDH activity (Fig. 18A); thereafter, the activity began to accumulate at a rate comparable to the rate observed in cells induced initially with the 30 mM ammonia.

During the 3 h induction period in 1 mM ammonia, the only form of the enzyme which accumulated was the alpha subunit (Fig. 18B). Following the addition of 20 mM ammonia to the medium, the alpha subunit remained at a constant level, and the beta subunit began to increase 50 min later.

To determine whether the non-linear accumulation of NADP-GDH activity was due to the failure to hold the ammonia concentration constant, the induction experiment was repeated to maintain the ammonia concentration more closely to 1 mM ammonia. In this experiment, the NADP-GDH activity increased in a linear manner and only the alpha subunit was detected for the duration of the 5 h experiment (Fig. 19).

Purification, Physical and Kinetic Characterization of Alpha and Beta Holoenzymes of NADP-GDH

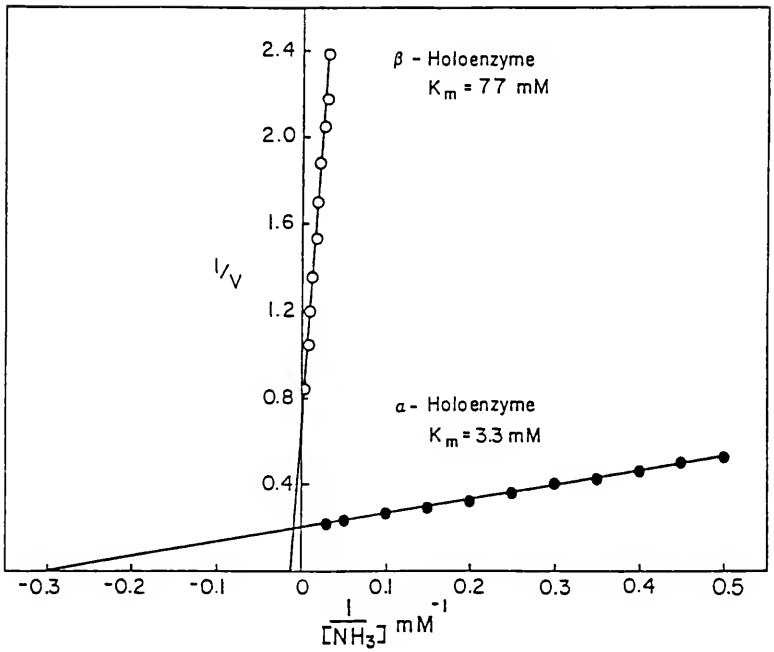
To determine whether both the alpha and beta subunits were in holoenzyme size proteins or whether one was present as free subunits, a cell homogenate from 80 min of induction, which contained both alpha and beta subunits, was fractionated by gel filtration on a Sephacryl S-300 column. The eluate from the Sephacryl S-300 column was collected in two fractions, one fraction of high molecular weight proteins (greater than 100,000 d), and one fraction of smaller proteins (less than 100,000 d). If both subunits were present in holoenzyme-size molecules, they should both be in the high molecular weight fraction. If alpha or beta was not in a holoenzyme size protein, one subunit should be in the high molecular weight fraction, and the other subunit should be in the low molecular weight fraction. The presence of both sububits in the low molecular weight fraction might indicate a slow rate of assembly of the subunits into holoenzyme. A Western blot of an SDS polyacrylamide gel of both fractions showed that both subunits were in the high molecular weight fraction. Moreover, all of the NADP-GDH activity was associated with this fraction. Neither subunit-type could be detected in the low molecular weight fraction.

Beginning with homogenates from cells induced for 3 h in 1 mM ammonia, or cells induced for 6 h in 30 mM ammonium medium, it was possible to purify alpha holoenzyme or beta

holoenzyme to homogeneity, respectively. The purification procedure included gel filtration chromatography, FPLC anion-exchange column chromatography on a Mono Q column, and chromatography on substrate affinity columns.

Because of its ability to bind differentially the alpha and beta holoenzymes, the NADP-Sepharose (Pharmacia, type 4) column was useful in fractionation of the NADP-GDH subunits. A preparation (15.5 U) of NADP-GDH, from 3 h induction in 30 mM ammonium medium, which contained 90% alpha and 10% beta, was partially purified by fractionation on a Mono Q anion exchange column. When this preparation was applied to an NADP-Sepharose (Pharmacia, type 4) affinity column, only 20% (3.07 U) of the enzyme activity would bind. To determine whether the low retention of NADP-GDH activity was due to limited binding capacity of the column, 5.5 U of enzyme in the eluate was re-applied to the same column after the enzyme preparation was stored at 4°C overnight. The number of enzyme units, applied the second time, was approximately twice the number that bound to the column the first time. If the column could bind only 5 units of enzyme activity, it was predicted that approximately 50% of the enzyme activity would bind. Once again it was observed that 20% (1.1 U) of the total enzyme activity bound to the column, whereas 80% (4.4 U) of the enzyme did not bind. The NADP-GDH which bound to the first column was re-applied to a second column and all of this enzyme bound the second time. The holoenzyme which bound to

Fig. 20 Lineweaver-Burk plot of initial velocities (μmol/min) measured at varied concentrations of ammonia for holoenzymes composed of either alpha or beta subunits. Enzyme was obtained from cells induced for 80 min in 2 mM ammonia (alpha subunit holoenzyme), or 6 h in 30 mM ammonia (beta subunit holoenzyme). Each point represents the average of two spectrophotometric assays. The concentration of 2-oxoglutarate and NADPH were 26 mM and 0.37 mM, respectively. Slope and intercept values were determined by linear regression analysis.

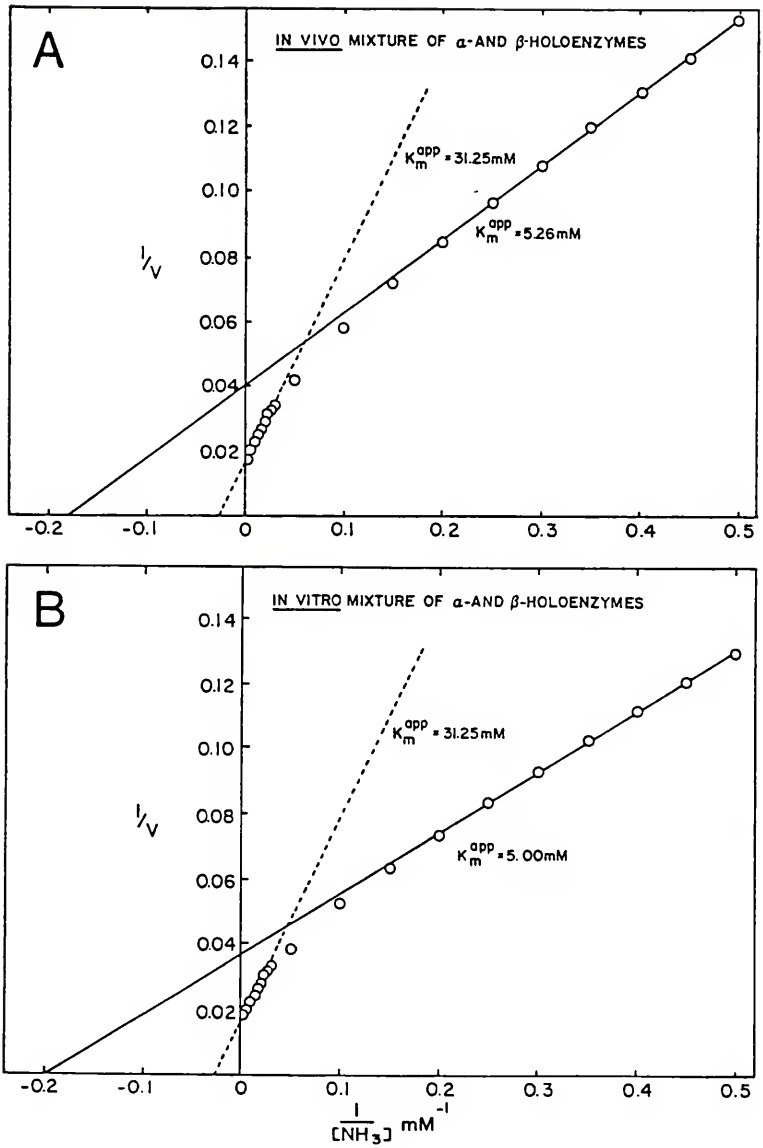

β - Holoenzyme
K_m = 7.7 mM
α - Holoenzyme
K_m = 3.3 mM
1/V
2.4
2.0
1.6
1.2
0.8
0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
$\frac{1}{[NH_3]}$ mM^{-1}

the column was found to be composed of only alpha subunits. A portion of the NADP-GDH which did not bind to the column was dialyzed against 0.01 M sodium phosphate (pH 6.5) and acid-washed Norit-A to remove any nucleotide which might be bound to the enzyme. When the dialyzed NADP-GDH was applied to the affinity column, all of the enzyme activity and antigen was bound. Therefore, it was possible to use this column to purify holoenzymes containing either alpha subunits or beta subunits from a mixture of holoenzymes containing the two subunit types. In addition, based on the data presented, it was proposed that a binding site exists on beta holoenzyme which when occupied, would not allow the beta holoenzyme to bind to the column. If the site was unoccupied, then the beta holoenzyme could bind to the column. The ratio of bound/unbound enzyme activity might be a result of the dissociation constant of the site for a specific factor, possibly NADPH.

In this study, the patterns of accumulation of alpha and beta subunits of the NADP-GDH have been shown to be regulated differently in low or high concentrations of ammonia. It seemed possible that the two subunits could be assembled into holoenzymes with kinetic characteristics better suited to the cell under culture conditions of low or high concentrations of ammonia.

The NADP-GDH from cells cultured in 1 mM ammonia for 3 h, or 30 mM ammonia for 6 h, was used to determine the kinetic characteristics of holoenzymes composed of the

Fig. 21 Lineweaver-Burk plot of initial velocities (μmol/min) measured at varied concentrations of ammonia, using NADP-GDH from a cell extract of C. sorokiniana which contained equal amounts of alpha and beta holoenzymes, and an in vitro mixture of cell extracts to obtain equal amounts of alpha and beta subunits in holoenzymes. The experiment was performed as described in Figure 20. Panel A, initial velocities measured from a cell extract which contained equal amounts of alpha and beta subunits in vivo. Panel B, initial velocities measured from an in vitro mixture of cell extracts to produce a combined extract with equal amounts of the alpha and beta subunits as determined by Western blot/immunodetection analysis.


A
IN VIVO MIXTURE OF α-AND β-HOLOENZYMES
K_m^{app} = 31.25mM
K_m^{app} = 5.26mM
1/V
0.14
0.12
0.10
0.08
0.06
0.04
0.02
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
B
IN VITRO MIXTURE OF α-AND β-HOLOENZYMES
K_m^{app} = 31.25mM
K_m^{app} = 5.00mM
1/V
$\frac{1}{[NH_3]}$ mM^{-1}

alpha or beta subunits, respectively. As shown in Figure 20, the K_m for the beta subunit holoenzyme of NADP-GDH was 77 mM. This K_m value is similar to the K_m for ammonia determined for the NADP-GDH which was purified and characterized previously from cells cultured in 30 mM ammonium medium (38). The K_m values for glutamate and NADP, for the beta holoenzyme NADP-GDH, were 32.3 mM and 0.031 mM, respectively. The K_m for NADPH for the beta holoenzyme was 0.14 mM. As shown in Figure 20, the K_m of the alpha holoenzyme NADP-GDH was approximately 3.3 mM. The K_m values for glutamate and NADP, for this form of the enzyme, were 38.2 mM and 0.040 mM, respectively. The K_m for NADPH for the alpha holoenzyme NADP-GDH was 0.10 mM.

When the K_m for ammonia was determined for a crude enzyme preparation from cells, having equal amounts of alpha and beta subunits, the resulting double reciprocal plot was non-linear (Fig. 21A). The observed non-linear double reciprocal plot could be due to the presence of two NADP-GDH holoenzymes composed of homopolymers of either alpha or beta subunits, each with different K_m values for ammonia (116). Alternatively, the NADP-GDH holoenzymes could be heteropolymers, consisting of a mixture of alpha and beta subunits, with each type of subunit acting independently in the holoenzyme.

An equal mixture of alpha and beta holoenzymes was prepared from different cell homogenates to test whether the *in vitro* mixture of holoenzymes would produce the same

Fig. 22 Eadie-Hofstee plot of initial velocities (μmol/min) measured at varied concentrations of ammonia using two different cell extracts to obtain different ratios of alpha and beta subunits in holoenzymes of NADP-GDH. Each line is generated from the enzyme activity from a combination of extracts which contained either alpha or beta holoenzyme. The cell extracts were combined to produce different ratios of alpha and beta holoenzymes. The homogenates were allowed to remain at 4°C overnight before being assayed. 100 alpha subunit (□), 80 alpha subunit:20 beta subunit (▲), 50 alpha subunit:50 beta subunit (△), 20 alpha subunit:80 beta subunit (●), 100 beta subunit (○). The inset shows the theoretical pattern generated if one assumes two isoenzymes with different K_m and V_{max} values. The values used in the calculations for the alpha holoenzyme were 3.5 mM and 160 U/mg for K_m and V_{max}, respectively. The values used for the contribution of the beta holoenzyme in the calculations, were 70 mM and 480 U/mg, for K_m and V_{max}, respectively. Line 1, 100 alpha subunit; line 2, 80 alpha subunit:20 beta subunit; line 3, 50 alpha subunit:50 beta subunit; line 4, 20 alpha subunit:80 beta subunit; line 5, 100 beta subunit.

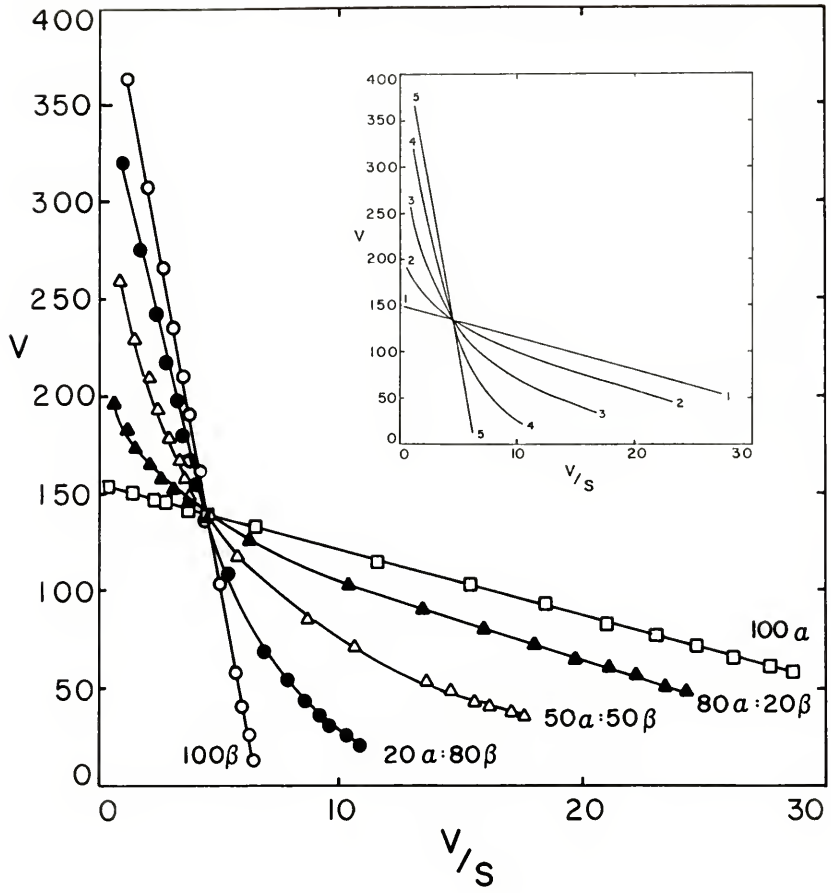
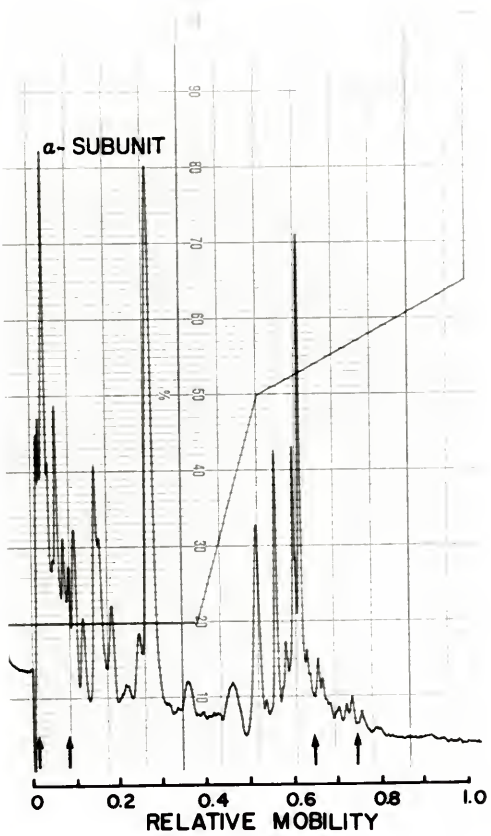
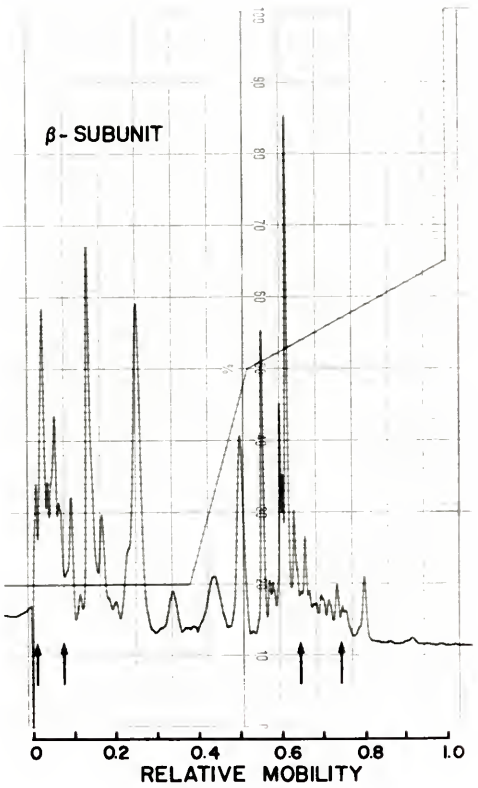

400
350
300
250
200
150
100
50
0
V
0
10
20
30
V/S
100β
20 a:80β
50a:50β
80a:20β
100 a
1
2
3
4
5

TABLE I
AMMONIA K_m VALUES FOR NADP-GDH
ALPHA AND BETA HOLOENZYMES AT VARIOUS
NADPH CONCENTRATIONS

Subunit Type	Relative Specific Activity	Concentration of NADPH, mM		
		0.03	0.10	0.37
		K_m for Ammonia, mM		
Alpha	1.0	0.02	1.32	3.98
Beta	3.0	75	72	77

Fig. 23 Comparison of PITC peptide derivatives, obtained from CNBr cleavage of purified alpha and beta subunits, separated by FPLC reverse phase chromatography. The peptide derivatives were separated on a Pharmacia PepRPC column using a gradient of 0.035 M sodium acetate, pH 5.5 (buffer A) and acetonitrile (buffer B). Peptides were detected at 260 nm and 20 pmol of deivatized peptide were used in each chromatographic run. The arrows indicate the absence or presence of peptide derivatives not seen in both chromatograms.


α- SUBUNIT
%
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY


β- SUBUNIT
%
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

kinetic plot for ammonia as was seen for cell extracts which contained equal amounts of both holoenzymes. The double-reciprocal plot, with varying concentrations of ammonia that was obtained using this extract (Fig. 21B), was very similar to the kinetic plot for the *in vivo* mixture of alpha and beta holoenzymes (Fig. 21A).

To determine whether the K_m values for the NADP-GDH holoenzymes composed of either alpha or beta subunits, are maintained in a population of holoenzymes composed of both the alpha and beta subunits, 5 different ratios of alpha and beta holoenzymes were prepared from cell homogenates. To allow for possible subunit exchange among holoenzyme species, the mixtures of holoenzymes were allowed to equilibrate at 4°C overnight. To allow for accurate determination of the K_m values for ammonia, the initial velocities were measured over an ammonium concentration range of 2 mM to 300 mM. The results are shown in the Eadie-Hofstee plot in Figure 22. The values determined for the K_m and V_{max} of the holoenzyme(s) composed of either alpha or beta subunits were used in an equation (114) which mathmatically describes the total velocity of an enzyme preparation composed of two isoenzymes with different K_m and V_{max} values for the same substrate. As can be seen in the inset in Figure 22, when these experimentally determined values for K_m and V_{max} were used in the theoretical model, there was very close correlation between the experimental data and the theoretical calculations.

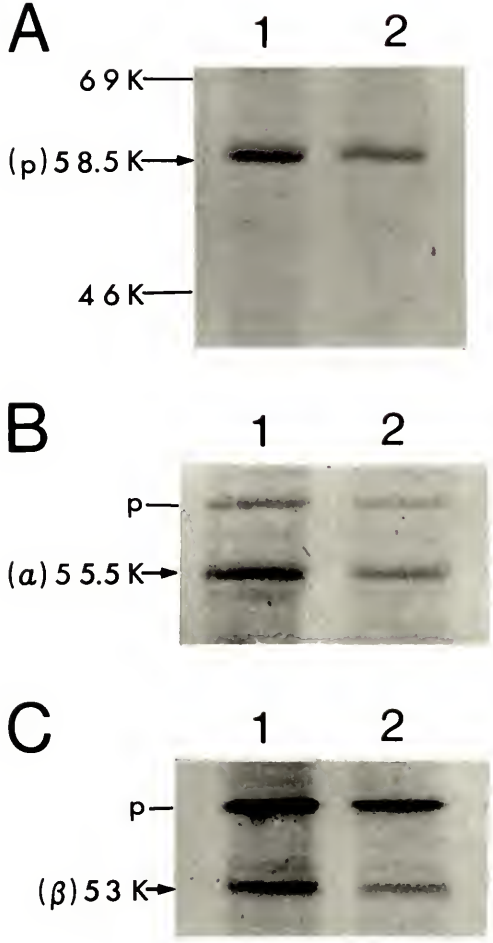
It has been reported recently by Tischner (134) that Chlorella contains two isoenzymes of NADP-GDH which show different allosteric properties with NADPH. To determine whether the concentration of NADPH might affect the K_m for ammonia for alpha or beta holoenzymes of NADP-GDH, the concentration of NADPH was decreased from 0.37 mM to 0.03 mM during measurement of the K_m for ammonia. As shown in Table 1, the K_m for ammonia remained constant for the beta holoenzyme; however, the K_m for ammonia for the alpha holoenzyme decreased from 3.5 mM to 0.02 mM ammonia.

To compare the peptide maps of the alpha and beta subunits, the individual purified proteins were treated with cyanogen bromide to cleave the protein chain at methionine residues (39). The resulting peptides were then derivatized with PITC and separated by FPLC reverse phase chromatography. As shown in Figure 23, the peptide maps of the alpha and beta subunits are very similar, with 40 peptide derivatives in common and 4 peptide derivatives that are different.

In Vitro Processing of NADP-GDH Subunit Precursor(s)

Because the alpha and beta subunits were shown to have similar molecular weights, peptide maps, and antigenic properties, the question can be asked as to whether these two subunits are formed by differential processing of a common precursor protein or formed by specific processing of structurally-related but different precursor proteins.

Fig. 24 *In vitro* processing of NADP-GDH precursor protein(s) produced by translation *in vitro* from poly(A)$^+$ RNA isolated from cells induced for 80 min and 180 min in 30 mM ammonium medium, and *in vitro* processing of precursor-protein(s) to alpha or beta subunits. The poly(A)$^+$ RNA was translated in a rabbit reticulocyte translation system (100) using [^{35}S]methionine to label the translation products. NADP-GDH antigen was immunoprecipitated and fractionated by SDS polyacrylamide slab-gel electrophoresis. The fractionated proteins were transferred to nitrocellulose and used for autoradiography. Panel A, NADP-GDH immunoprecipitated from an *in vitro* mixture programmed with poly(A)$^+$ RNA from cells induced for 80 min (lane 1) or 180 min (lane 2) of induction. Panel B, *in vitro* translation products synthesized from RNA as in Panel A, lane 1, and processed with 100,000 x *g* extract prepared from freshly broken cells induced for 80 min in 30 mm ammonium medium. NADP-GDH antigen was immunoprecipitated after processing was allowed to proceed for 10 min (lane 1) and 30 min (lane 2). Panel C, *in vitro* translation products synthesized from poly(A)$^+$ RNA as in Panel A, lane 2, and processed with 100,000 x *g* extract prepared from freshly broken cells induced for 180 min in 30 mM ammonium medium. The NADP-GDH antigen was immunoprecipitated after processing was allowed to proceed for 10 min (lane 1) and 30 min (lane 2).


A
1
2
69K
(p)58.5K
46K
B
1
2
p
(α)55.5K
C
1
2
p
(β)53K

The rationale of the next series of experiments was (a) to isolate poly(A)$^+$ RNA from cells engaged in synthesis of the alpha subunit, beta subunit, or both; (b) to translate these different poly(A)$^+$ RNA preparations *in vitro* to determine the size of the subunit precursor protein(s) synthesized, and (c) to attempt to process the precursor protein(s) with extracts prepared from cells in which either the alpha or beta subunit holoenzymes were accumulating.

The first experimental approach was to compare the size of the NADP-GDH translated *in vitro* from poly(A)$^+$ RNA isolated from cells after 80 min and 180 min of induction. If the alpha and beta subunits are synthesized from two different mRNAs, the cells harvested at these two times were predicted to have different amounts of the two types of mRNA, according to the different rates of accumulation of the alpha and beta subunits during these times of induction. When these poly(A)$^+$ RNAs were translated *in vitro*, in a rabbit reticulocyte lysate translation system, the protein(s) immunoprecipitated with anti-NADP-GDH antibody had a molecular weight of 58,500 (Fig. 24A). This protein was the same size as the precursor which was synthesized from poly(A)$^+$ RNA isolated from fully induced cells cultured in 30 mM ammonium medium for 6-12 h (99). There was little, if any, difference in the size of the immunoprecipitated NADP-GDH from the two RNA preparations.

The second experimental approach, used to answer the question, took advantage of the observation that the 58,500

d precursor protein can be processed in vitro by Chlorella homogenates (99). To test for the existence of two protein precursors of the alpha and beta subunits of NADP-GDH, poly(A)+ RNA, isolated from cells induced for 80 min and 180 min, were processed with extracts from 80 min and 180 min of induction, respectively.

As shown in Figure 24B, when the 58,500 d precursor protein(s) produced from 80 min poly(A)+ RNA was processed with homogenate from freshly broken 80 min cells, the primary product formed was the alpha subunit. However, a small amount of the beta subunit was also formed. When the 58,500 d precursor protein(s) from 180 min poly(A)+ RNA was processed by homogenate from 180 min (Fig. 24C), a large amount of the beta subunit and a small amount of the alpha subunit was formed.

Localization of the Alpha Subunit NADP-GDH Holoenzyme

Since the alpha and beta size proteins could be produced in vitro from proteins synthesized in vitro, it was postulated that both subunits were localized in the chloroplast. Prunkard (99) has shown by two different methods that the NADP-GDH is present in the chloroplast in cells cultured 24 h in 30 mM ammonium medium. In this dissertation research, it was found that cells cultured in 2 mM ammonia for 80 min had NADP-GDH activity localized only in the chloroplast. Therefore, it appears that both of the alpha and beta holoenzymes are localized in the chloroplast.

DISCUSSION

Results reported previously (113) and in this dissertation indicate the presence of two antigens for the NADP-GDH from Chlorella. The previous method for analysis of radioactive enzyme antigen was by slicing tube gels into 2 mm sections and measuring the radioactivity by liquid scintillation counting. Therefore, since the molecular weights and migration rates of the antigens were similar, it should not be surprising that only one radioactive peak was observed in previous studies. By use of Western blots and autoradiography, it was possible to show that total NADP-GDH antigen is composed of two antigen subunits (designated alpha and beta).

Prunkard (99) did not determine whether the two subunit-size antigens seen by Western blot/immunodetection, were actually in holoenzyme-size proteins. By gel-filtration chromatography and Western blot/immunodetection, it was possible to show that both antigens fractionated with the high molecular weight proteins, and presumably in the NADP-GDH holoenzymes. The absence of free subunits suggests that the subunits are rapidly assembled into the holoenzyme.

The NADP-GDH alpha and beta holoenzymes were purified from extracts prepared from cells cultured in low or high

concentrations of ammonia, respectively. Their purification was accomplished by gel-filtration chromatography, ion-exchange chromatography on a Mono Q column, and substrate affinity chromatography. The use of the Mono Q column and the Fast Protein Liquid Chromatography system resulted in high resolution and the rapid purification of the NADP-GDH. To purify and characterize the holoenzymes composed of only one type of subunit, it was necessary to utilize an NADP-Sepharose affinity column (Pharmacia, Type 4) which was able to fractionate the two types of holoenzyme. The alpha holoenzyme was able to bind to the Type 4 NADP-Sepharose column, whereas, the beta holoenzyme was unable to bind unless dialyzed against charcoal.

Alpha and beta holoenzymes, purified as described, were cleaved with cyanogen bromide to determine the extent of homology between the two types of subunits. It was anticipated that 16 possible peptides would result from the cleavage of a subunit with 15 methionine residues. The presence of 40 peptide derivatives (Fig. 23) indicates the formation of secondary products from the reaction with PITC. The most common of these secondary derivatives are formed from serine, threonine, and lysine which could form a total of 8 possible secondary products. Of the 40 peptide derivatives obtained from each subunit, there were 36 peptide derivatives in common and 4 peptide derivatives which were different,indicating a high degree of amino acid sequence homology between the alpha and beta subunits.

The data presented in this dissertation indicate that accumulation of the alpha and beta subunits of the NADP-GDH is differentially regulated in response to different concentrations of ammonia in the culture medium. When cells were induced in 30 mM ammonium medium, the beta subunit began to accumulate from the time of addition of ammonia. However, when cells were induced in 2 mM ammonium medium or lower, the beta subunit did not accumulate. Therefore, it appears the cell can sense the concentration of ammonia in the medium and regulate which form of the NADP-GDH that is to accumulate.

The GS and GOGAT have been proposed to be involved in the assimilation of ammonia in most situations; however, these activities are decreased in the presence of high concentrations of external ammonia. If the cell was in an environment with ammonia available, the cell would be at an advantage if it could utilize the ammonia for growth. In this dissertation, it has been shown that when the external concentration of ammonia approaches 1 mM the NADP-GDH activity is induced. In this situation, the NADP-GDH would be expected to have a low K_m for ammonia for most efficient utilization of the ammonia. However, if the cell was in an environment with a high external concentration of ammonia, it would not likely require a NADP-GDH with a low K_m for ammonia. A NADP-GDH with a high K_m might be advantageous to prevent the over-accumulation of nitrogen metabolites and utilization of reducing power.

The NADP-GDH from cell extracts, which contained only an alpha or beta holoenzyme, was utilized to test for a difference in kinetic parameters of the holoenzymes. The K_m and V_{max} values were determined for holoenzymes composed of all alpha or all beta subunits (Fig. 20). When these values were used in an equation which describes the velocity for two independent enzymes catalyzing the same reaction, it was found that the theoretical data matched the experimental data for each ratio of alpha and beta holoenzymes (Fig. 22). In these calculations, a K_m of approximately 3.5 mM ammonia was used for the alpha holoenzyme, and a K_m of approximately 70 mM was used for the beta holoenzyme. In addition, the V_{max} of the beta holoenzyme was 480 U/mg and the V_{max} of the alpha holoenzyme was 160 U/mg.

Although the alpha holoenzyme had a K_m of 3.5 mM for ammonia, it would still seem unlikely that this form of the enzyme would be involved in ammonia assimilation. The Chlorella GS has been reported to have a K_m for ammonia of 0.05 mM (129) However, the activity of the GS has been shown to decrease rapidly during induction of Chlorella NADP-GDH activity by ammonia (96). If the cell encounters sufficient ammonia (approximately 1 mM) to induce the NADP-GDH, the cell might inactivate the GS and assimilate ammonia by the NADP-GDH. It was reported by Tischner (134), that a Chlorella NADP-GDH isoenzyme can bind ammonia more tightly at low concentrations of NADPH. In this laboratory,

the effect of NADPH on the K_m for ammonia was tested for the alpha and beta holoenzymes (Table 1). It can be seen that decreasing the concentration of NADPH had no affect on the K_m for ammonia for the beta holoenzyme. However, the results indicate that, as the NADPH concentration is decreased, the affinity of the alpha holoenzyme increases for ammonia. This increased affinity would allow the NADP-GDH to bind ammonia more efficiently when the reducing power of the cell is low.

The autoradiogram from Prunkard (see Fig. 19, ref 99) was scanned, and the levels of the alpha and beta subunits were expressed on a per ml of culture basis (Fig. 10). Since the accumulation of enzyme activity was coincident with the accumulation of the alpha subunit, it appeared that the beta holoenzyme had little, if any catalytic activity during the first 120 min of the induction period. The alpha holoenzyme appeared to be responsible for the accumulation of all catalytic activity during the first 120 min of induction. However, it appeared that after 120 min, the beta subunit holoenzyme contributed most of the catalytic activity. Although the level of the alpha subunit remained constant after 120 min, and the level of the beta subunit continued to increase, it is possible that the alpha subunit holoenzyme could be increasing in catalytic activity due to some form of covalent modification.

A pulse-chase experiment was performed to determine how the rates of synthesis and degradation might affect the

patterns of accumulation of the alpha and beta subunits. As shown in Figure 13, during the first 80 min of the induction period, the alpha subunit had a 3-fold higher rate of degradation than the beta subunit. During this time, the rate of labeling of the alpha subunit was 1.67-fold higher than the rate of labeling of the beta subunit (Fig. 13, and 16). Therefore, the rate of synthesis of the alpha subunit could be as much as 4- to 5-fold higher than the rate of synthesis of the beta subunit.

If the patterns of labeling and degradation of the subunits (Fig. 13) are compared to the patterns of accumulation of the subunits (Fig. 10), it can be seen that the alpha subunit was being synthesized and degraded even after its level reached a plateau. This steady-state could be the result of a decreased rate of synthesis of the alpha subunit after 120 min.

An experiment was performed to determine whether there might be conversion of one NADP-GDH subunit to another. As can be seen in Figure 16, there was no apparent conversion of one subunit into the other during the chase period in the presence of cycloheximide. Also note, however, that the addition of cycloheximide at the beginning of the chase period stopped normal degradation of the alpha and beta subunits. Therefore, it is possible that cycloheximide might have inhibited the conversion of the alpha subunit to the beta subunit. However, if cycloheximide only inhibited protein synthesis, it can be concluded that one subunit is not the precursor of the other.

Israel et al. (51) showed, in an earlier study performed *in vivo*, that the addition of cycloheximide, at the time of removal of ammonia from the culture, blocked the loss of NADP-GDH activity. In contrast, it was shown that once the loss of NADP-GDH activity began, during a deinduction period, the addition of cycloheximide no longer blocked the loss in enzyme activity. The observations made by Israel et al. (51), and the results of the present study, suggest that a metabolite (e.g., an amino acid) utilized during protein synthesis, might protect the NADP-GDH from degradation. When the metabolite is utilized for protein synthesis and it is not replaced, due to the absence of an external source of nitrogen (e.g., ammonia), the NADP-GDH is no longer protected and is degraded. Therefore, it can be proposed that the addition of cycloheximide, at the time of ammonia removal, blocks protein synthesis and thereby prevents the utilization of the metabolite. Since the metabolite is not utilized, its level would remain sufficiently high to protect the NADP-GDH from inactivation.

In previous studies (8), it was shown that the half-life of total NADP-GDH antigen did not change after ammonia was removed from the culture medium. Moreover, a loss of subunit antigen was observed to occur with a concurrent and reciprocal increase in putative dimer. The combination of loss of subunit antigen and the accumulation

of putative dimer antigen gave a half-life of 80 min. The half-life for total NADP-GDH antigen during deinduction was identical to the half-life observed during the induction period.

In contrast, in the present dissertation research project, the half-life of total NADP-GDH antigen was observed to change from an 80 min half-life during the induction period to a 9 min half-life during the deinduction period in cells cultured in both original and modified media (Fig. 3). Thus, the loss in enzyme antigen paralleled the loss in enzyme activity during the deinduction period.

An *in vitro* system was developed to study the inactivation of the NADP-GDH in more detail, and to obtain a system to study the individual processes (i.e., dimerization and degradation) that might be involved in the inactivation and degradation of the NADP-GDH *in vivo*. Previous attempts to obtain an *in vitro* inactivation system in this laboratory were unsuccessful (51). After cells were broken, it was observed that the inactivation of the NADP-GDH ceased, and the process could not be restarted. Israel et al. (51) attempted to reconstitute the inactivation system by the addition of various compounds such as NADP, NADPH, and nucleotides.

In previous research (113), it was reported that a putative dimer is formed during inactivation of the NADP-GDH. Since the bond between the subunits did not have the characteristics of a disulfide bond, it was postulated

(113) that the bond might be an isopeptide bond. A class of enzymes which catalyze the formation of isopeptide bonds are referred to as transglutaminases (30). All transglutaminases studied so far require calcium as a cofactor. Since calcium might be required for the dimerization process, a test was made of the effect of calcium on the inactivation and dimerization of NADP-GDH *in vitro*.

The addition of calcium alone, to cells permeabilized by freeze-thaw, had no effect on NADP-GDH activity. It seemed possible that the dimerization reaction might be coupled to protein degradation. Therefore, since ATP is required for intracellular protein turnover in other organisms (49), it was added along with the calcium. Alternatively, calcium and/or ATP might be required to put the NADP-GDH into a conformation which makes the enzyme susceptible to inactivation or degradation. The addition of calcium and ATP to permeabilized cells resulted in a very rapid loss of NADP-GDH activity (Fig. 6).

It is unclear why there is a lag before the cessation of inactivation upon addition of EGTA to the *in vitro* inactivation system. The lag might be due to the time required for the chelation of the calcium, or it may be due to the time required for release of the calcium from whatever protein might be binding it. Alternatively, it might be an indication of a multi-step process in which calcium is required for the initiation of inactivation or

degradation. During the lag period,the activated components might be utilized.

The ubiquitinylation system which has been elucidated by Hershko and Ciechanover (49) could lead to formation of a molecule with the same molecular weight as a dimer of NADP-GDH subunits by addition of 6 molecules of ubiquitin (M_r=9,000) to a single NADP-GDH subunit. Also, since the ubiquitinylation pathway for protein degradation utilizes the free amino-terminus of the protein to be degraded (30), and the NADP-GDH from fully induced cells has a blocked amino terminus (38), the calcium and/or ATP might be required for removal of the amino-terminal blocking group and subsequent ubiquitinylation of the enzyme. Therefore, it is possible the NADP-GDH may be inactivated and marked for degradation by a ubiquitin-dependent pathway.

The loss of NADP-GDH activity and production of putative dimer in the *in vitro* inactivation system, prepared from cells cultured in original autoclaved medium, appeared to be the same as seen during the deinduction period *in vivo*. However, when cells cultured in modified filter-sterilized medium were deinduced, the dimer was not seen by Western blot/immunodetection analysis, and the loss of enzyme antigen was coincident with the loss in enzyme activity (Fig. 9). It is possible the Western blot/immunodetection procedure was unable to detect the dimer. However, the loss in NADP-GDH activity and radioactive antigen occurred concurrently *in vivo*, during a

pulse-chase experiment; and no accumulation of radioactive dimer could be detected (Fig. 13). Therefore, the pattern of dimer formation *in vitro* is still consistent with the observations *in vivo*. It is possible that the dimer is formed as a part of the inactivation process, but the degradation of the dimer might require a metalloprotease which was only partially active in conditions used previously for culturing the organism. Alternatively, a metal ion may be required for the activation of a protease which then degrades the dimer. By the addition of nickel, tin, and vanadium to the culture medium, a protease might be made fully active and remove the putative dimer before it is able to accumulate to a detectable level. The toxic components in the university steam supply might also have inhibited a protease, or might have altered normal metabolism of the cell.

The cessation of accumulation of the alpha subunit, after 120 min in 30 mM ammonia, might have been due to the accumulation of a metabolite of ammonia which prevents the accumulation of the alpha subunit. If such a repressing metabolite is not allowed to accumulate, the accumulation of the alpha subunit might not be repressed. Therefore, induction of the NADP-GDH in cells, cultured in a low concentration of ammonia, might allow for the continuous accumulation of the alpha subunit. It was predicted that, since the beta subunit appeared first, at presumably a low intracellular concentration of ammonia or a metabolite of

ammonia, its accumulation might be continuous in cells induced in low ammonium medium. When cells were cultured in medium, containing 2 mM ammonia, the alpha accumulated as predicted; however, the beta subunit did not accumulate (Fig. 17). This unexpected lack of accumulation of the beta subunit, in 2 mM ammonium medium, suggests that a threshold level of ammonia, or an ammonia metabolite, is required for the accumulation of the beta subunit of the NADP-GDH. Therefore, it was proposed that maintenance of a low concentration of ammonia in the culture medium might allow for the continuous induction of only the alpha subunit. In other words, induction of the NADP-GDH in cells cultured in 1 mM ammonia might be sufficient to supply nitrogen for cell growth but insufficient to permit excess accumulation of ammonia, or ammonia metabolites. When the ammonia level was held constant near 1 mM, the NADP-GDH activity accumulated in a linear manner for 5 h, and the alpha holoenzyme was the only form of the enzyme present in these cells (Fig. 19). This linear accumulation of activity occurred even though the effective light intensity per cell decreased due to an increase in culture turbidity. When the culture was adjusted to high ammonia (20 mM), after 3 h in 1 mM ammonia, there was a cessation of accumulation of the alpha subunit followed by the accumulation of the beta subunit (Fig. 18). The observed pattern of accumulation of only the alpha subunit in 1 mM ammonia and a cessation of the accumulation of this subunit in high ammonia (20 mM)

medium are consistent with the hypothesis that a metabolite of ammonia,accumulates in cells cultured in high ammonium medium and suppresses accumulation of the alpha subunit.

It was shown in previous studies (99) that, when poly(A)$^+$ RNA from fully induced cells was translated in an in vitro translation system, the NADP-GDH antigen that was synthesized had a molecular weight of 58,500. This antigenic species was proposed to be a precursor protein with a transit peptide at its amino terminus involved in transport of the precursor into the chloroplast. When poly(A)$^+$ RNA was isolated from cells cultured in 30 mM ammonia for 80 min or 180 min, and this RNA was translated in vitro, NADP-GDH antigen(s) were synthesized with a molecular weight of 58,500 d (Fig. 24A). When the 58,500 d precursor-protein(s) were processed in vitro with fresh cell extracts from the same harvest times (i.e., 80 or 180 min), it was observed that the precursor-protein(s) produced from 80 min poly(A)$^+$ RNA was processed primarily to a protein the size of the alpha subunit and the precursor-protein(s) produced from 180 min poly(A)$^+$ RNA was processed primarily to a protein the size of the beta subunit (Fig. 24A,B). These results are consistent with the hypothesis that non-identical precursor-protein(s) are synthesized in vitro, from poly(A)$^+$ RNA isolated at different induction times, which have similar, or identical molecular weights. An alternative explanation would be that only one precursor-protein is present and the specificity

of the processing enzyme changes, or different processing enzymes exist at different times during the induction period. It is also possible that a single precursor-protein is modified such that a processing enzyme can only cleave the transit peptide at one point to form the alpha subunit, or at two points to form the beta subunit. This type of differential processing could require modification of an amino acid near the peptide bond that is cleaved.

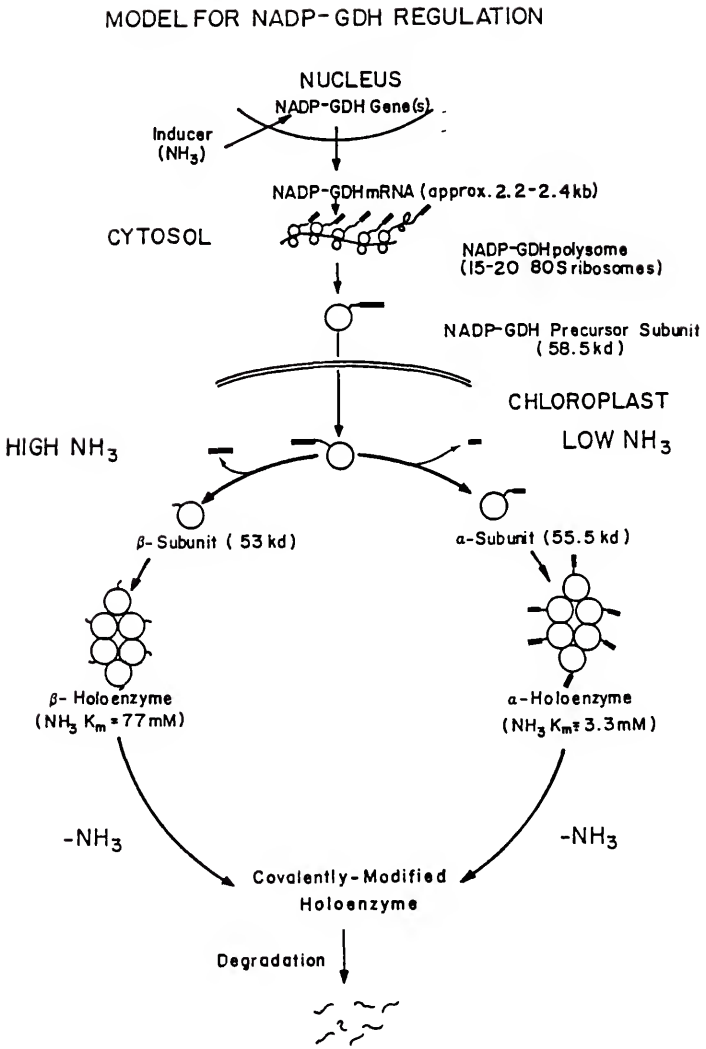
In summary, the NADP-GDH has been shown in this dissertation, to be synthesized on cycloheximide sensitive ribosomes; therefore, the NADP-GDH gene is probably in the nucleus. It is not known at this time whether there are two NADP-GDH genes coding for two different mRNAs, one gene coding for two different mRNAs, or one gene coding for one mRNA. If there is only one NADP-GDH mRNA, the precursor NADP-GDH produced might be differentially processed at different times during induction in previously uninduced cells, and in high versus low ammonium medium. When cells were cultured in high-ammonium medium, the alpha subunit accumulated after a 30 min lag. It increased linearly for 90 min and then ceased to accumulate. The alpha subunit was synthesized 4-5 times faster than the beta subunit during induction in 30 mM ammonium medium. During induction of enzyme activity in cells cultured in 30 mM ammonium medium, the alpha and beta subunits were degraded with half-lives of 50 min, and 150 min, respectively. After removal of ammonia from the culture medium, the half-lives for alpha

and beta subunits were 5.5 min, and 13.5 min, respectively. Since the alpha subunit reached a constant level after 120 min of induction, the rate of synthesis of this subunit probably decreased, such that a steady-state between synthesis and degradation was obtained. The decrease in the rate of synthesis might have been due to the accumulation of an ammonium metabolite which could affect transcription, translation, or processing of a precursor, such that less alpha subunit was produced. The low affinity of the beta holoenzyme for ammonia (K_m=70 mM) suggests that this holoenzyme is involved in ammonia assimilation only at high external concentrations of ammonia. The high affinity of the alpha holoenzyme for ammonia (K_m=0.02 to 3.5 mM) and its negative allosteric regulation by NADPH suggest that this holoenzyme is involved in ammonia assimilation at low concentrations of ammonia and when the intracellular concentration of NADPH is low. There are several possible reasons for the synthesis of two NADP-GDH isoenzymes in Chlorella. When the cells were in an environment with low external concentrations of ammonia (less than 2 mM), the alpha subunit of the NADP-GDH was observed to accumulate. In this environment, the level of GS activity in another species of Chlorella has been observed to decrease. Therefore, the low K_m form of the NADP-GDH might be involved in ammonia assimilation to compensate for the low activity of GS. The allosteric regulation by NADPH might be a mechanism whereby the cell cannot assimilate ammonia to

an extent sufficient to deplete the reducing equivalents of the cell. Similarly, when the cells were in an environment with high external concentrations of ammonia the beta subunit was observed to accumulate, resulting in the assembly of a holoenzyme with a low affinity for ammonia. It is proposed that the NADP-GDH with a low affinity for ammonia is induced to conserve reducing power of the cell rather than being involved in detoxification. If the cell could assimilate all of the ammonia to which it was exposed, it would utilize a large amount of reducing power that is also needed for ATP production, and subsequently for carbon assimilation.

The model shown in Figure 25 was proposed to summarize the data presented in this dissertation. The NADP-GDH is coded for by nuclear gene(s) inducible by ammonia in the light. The mRNA(s) is/are translated on cycloheximide sensitive ribosomes to produce precursor-protein(s) of 58,500 d. In cells cultured in medium, containing low (less than 2 mM) ammonia, the precursor-protein(s) is/are processed within the chloroplast to form only the alpha subunit, and the alpha holoenzyme accumulates. The precursor proteins(s) is/are processed within the chloroplast to form either alpha or beta subunits, depending on the concentration of ammonia in the culture medium. The subunits are assembled into homopolymers of alpha or beta subunits. In cells cultured in medium, containing less than 2 mM ammonia, only the alpha

Fig. 25 Model for the regulation of processing of NADP-GDH precursor-protein(s) to produce the alpha and beta holoenzymes, during induction of NADP-GDH activity in high or low ammonia.


MODEL FOR NADP-GDH REGULATION
NUCLEUS
NADP-GDH Gene(s)
Inducer
(NH_3)
NADP-GDH mRNA (approx. 2.2-2.4 kb)
CYTOSOL
NADP-GDH polysome
(15-20 80S ribosomes)
NADP-GDH Precursor Subunit
(58.5 kd)
CHLOROPLAST
HIGH NH_3
LOW NH_3
β-Subunit (53 kd)
α-Subunit (55.5 kd)
β-Holoenzyme
(NH_3 K_m = 77 mM)
α-Holoenzyme
(NH_3 K_m = 3.3 mM)
-NH_3
-NH_3
Covalently-Modified
Holoenzyme
Degradation

holoenzyme accumulates, whereas in medium with greater than this ammonia concentration, the cells accumulate only the alpha holoenzyme continuously. The alpha and beta holoenzymes have different K_m values for ammonia. (Although not shown in this model, the subunits may be assembled to form heteropolymers with the alpha and beta subunits contributing their own individual kinetic characteristics.) Upon removal of ammonia the subunits are covalently modified and degraded.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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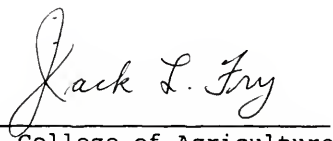

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