Coregulation of Oxidized Nicotinamide Adenine Dinucleotide (Phosphate) Transhydrogenase and Glutamate Dehydrogenase Activities in Enteric Bacteria During Nitrogen Limitation

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The relationship between oxidized nicotinamide adenine dinucleotide (phosphate) [NAD(P)+] transhydrogenase (EC 1.6.1.1) and NAD(P)+ glutamate dehydrogenase in several enteric bacteria which differ slightly in their regulation of nitrogen metabolism was studied. Escherichia coli strain K-12 was grown on glucose and various concentrations of NH4Cl as the sole nitrogen source. In the range of 0.5 to 20 mM NH4Cl, the energy-independent transhydrogenase increased two- to threefold. Comparable changes occurred in NAD(P)+-linked glutamate dehydrogenase. NH4Cl concentrations of 20 to 60 mM resulted in relatively constant specific activities for both enzymes. Higher exogenous NH4Cl, however, led to a decline in both activities. Isocitrate dehydrogenase, another potential source of cellular NADPH, was insensitive to NH4Cl limitation. Similar studies in the presence of glutamate and different exogenous NH4Cl concentrations again showed concerted effects on both enzymes. Growth on glutamate as the sole nitrogen source led to severe repression of both transhydrogenase and glutamate dehydrogenase. In Salmonella typhimurium, both enzymes were unaffected by limiting NH4Cl or growth on glutamate as the sole nitrogen source. Both were, however, repressed by growth on aspartate, a potential source of cellular glutamate. Coordinate changes in glutamate dehydrogenase and transhydrogenase were also evident in Klebsiella aerogenes, particularly under conditions in which glutamate dehydrogenase was regulated inversely to glutamine synthetase. Coordinate changes in glutamate dehydrogenase and transhydrogenase in enteric bacteria are discussed in terms of the possible involvement of the latter enzyme as a direct source of NADPH in the ammonia assimilation system.

NAD(P)+ transhydrogenase (EC 1.6.1.1) catalyzes the reversible transfer of reducing equivalents between the pyridine nucleotides. The reaction can be identified in both bacterial and mammalian systems. In enteric bacteria, the reaction in the direction of NAD(P)+ reduction requires the input of energy which can be provided either from ATP or respiration.

The precise physiological role of the NAD(P)+ transhydrogenase is not clear (18). In Escherichia coli, energy-linked transhydrogenase has often been associated with the supply of NADPH for the biosynthesis of amino acids since the presence of the latter in the growth medium repressed the level of the enzyme in the cells (9). The involvement in NADPH production for general biosynthesis is, however, not borne out by recent studies designed to probe cellular sources of NADPH (4). A role for the enzyme in branched-chain amino acid transport has also been suggested (5). Evidence was presented that leucyl-tRNA functions as a regulator of the enzyme. These data, however, do not reconcile with the repressive abilities of other amino acids, indicating the possibility of other regulatory mechanisms. The recent isolation of a mutant lacking transhydrogenase activity has as yet provided little information concerning the role of the enzyme. Such mutants grow normally under growth conditions so far tested (6, 22), but a phenotype of slow growth in the absence of the hexose monophosphate shunt has been indicated (7). In the insertion mutation pnt::Tn5, slow growth occurs on glucose but not on succinate or Casamino Acids. Because of the indicated relationship between transhydrogenase and glutamate dehydrogenase in mitochondria (18), work was initiated in E. coli to probe this situation and to try to correlate any observed relationship with the known amino acid repres-
sion of the transhydrogenase. An interrelation-
ship between the two systems in E. coli may
well indicate a role for transhydrogenase in ni-
trogen metabolism, in particular, the assimila-
tion of ammonia. We have also extended these
studies to other enteric bacteria, in particular
Klebsiella aerogenes, in which ammonia assimila-
tion appears to be under the control of a
regulatory gene close to or within the structural
gene for glutamine synthetase (11), and Salmo-
nella typhimurium, in which glutamate dehy-
drogenase is subject to different regulation (20).

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stract at the ASBC meeting in New Orleans,
1980.)

MATERIALS AND METHODS

Bacterial strains. E. coli strain K-12 and strain
W6 (a proline-requiring auxotroph derived from
ATCC 9637 and obtained from S. Luria) were used in
these studies. S. typhimurium strain TA1650 (dhyA1,
hisJ5601) was a gift from S. Kustu, University of
California, Davis. The parental strain of K. aerogenes
CG567 (nadB1 rha-l) was a gift from B. Magasanik,
Department of Biology, Massachusetts Institute of
Technology. Bacillus subtilis strain VB217 (phe trp)
was obtained from Robert Bondaryk, Department of
Metabolic Regulation, Boston Biomedical Research
Institute.

Growth conditions. Cells were grown in 500 ml of
a modified M9 minimal salts medium (15) in 2-liter
shake flasks, using 30 mM glucose as the carbon
source. NH₄Cl was omitted from the minimal salts
medium and added at the required level. Amino acids
were added at the levels designated in the experi-
ments described in the text. E. coli, B. subtilis, and S. typhi-
murium were grown at 37°C, whereas K. aerogenes
was cultured at 30°C. E. coli strain W6, when used,
was grown on a medium supplemented with 50 µg of
L-proline per ml. K. aerogenes received growth sup-
plements of niacinamide and L-rlamnosine. All cells
were harvested at middle- to late-exponential phase
(as determined by the optical density of cultures at
650 nm) and washed twice in 50 mM Tris-sulfate
buffer (pH 7.8) containing 1 mM EDTA and 1 mM
dithiothreitol. In the case of limiting ammonia levels,
cells were harvested before expiration of growth due
to usage of NH₄Cl, as predetermined from the extent
of growth in a separate experiment. Cells were usually
frozen in an 80% glycerol stock.

Preparation of enzymatic fractions. Respira-
tory particles (enriched for inner membrane proteins)
in each case were prepared after breakage of cells in a
French pressure cell at 20,000 lb/in² in 50 mM Tris-
sulfate buffer (pH 7.8) containing 1 mM EDTA and 1
mM dithiothreitol (9). After removal of cell debris by
centrifugation at 26,000 × g for 15 min, the respiratory
particles were obtained by ultracentrifugation at
180,000 × g for 90 min. The resulting membrane
fractones were resuspended in 50 mM Tris-sulfate
buffer (pH 7.8) containing 1 mM dithiothreitol and
10% (wt/vol) glycerol. Approximately 96% of the glu-
tamate dehydrogenase could be demonstrated in the
high-speed supernatant, along with glutamine synthet-
tase and isocitrate dehydrogenase. NAD(P)⁺
transhydrogenase and NADH-Fe(CN)₆³⁻ reductase
were predominantly in the membrane. In most cases
measurements were obtained from at least three separate
experiments. Normally, in each experiment activities
were measured in duplicate.

Enzyme assays. Energy-independent NAD(P)⁺
transhydrogenase was measured as the reduction of
acetylpyridine NAD⁺ by NADPH as described previ-
ously (8). Glutamate dehydrogenase was measured by
following the oxidation of NADPH as described (19).
Glutamine synthetase in E. coli was measured by a
γ-glutamyl transferase assay (21), and units are
described as nanomoles of γ-glutamylhydroxamate
formed per minute per milligram of protein. In K.
aerogenes, because of the different isoactivity point at
pH 7.55, γ-glutamyl transferase assays were performed
described for this organism (1). Isocitrate dehydro-
genate was monitored by the reduction of NAD(P)⁺
during the oxidation of isocitrate to α-ketoglutarate
(16). NADH-Fe(CN)₆³⁻ reductase activity was mea-
sured in a 3-ml assay, containing final concentrations
of 187 mM potassium phosphate buffer (pH 8.0), 1
mM potassium ferricyanide, and 0.2 mM NADH,
which was used to initiate the reaction. Enzyme assays
in E. coli, B. subtilis, and S. typhimurium were per-
formed at 37°C, whereas those in K. aerogenes were
performed at 30°C. Protein determination was done
by the method of Lowry, using bovine serum albumin
as the standard. In all cases, transhydrogenase and
 glutamate dehydrogenase activities were measured on
the same day they were prepared. γ-Glutamyl trans-
ferase activities were measured on the next day (within
15 h) after storage on ice overnight.

Chemicals. Essentially ammonia-free L-glutamine
and L-histidine were obtained from Calbiochem. All
other reagents and NADs were purchased from Sigma
Chemical Co., except for potassium ferricyanide,
which was obtained from Fisher Scientific.

RESULTS

In Fig. 1, the effects of ammonia limitation on four
activities are shown: glutamine synthetase,
glutamate dehydrogenase, transhydrogenase,
and NADH dehydrogenase [NADH-Fe(CN)₆³⁻
reductase]. In the range of exogenous NH₄Cl
(0.5 to 20 mM), both transhydrogenase and glu-
tamate dehydrogenase increased in a similar
fashion. As expected, glutamine synthetase was
high at low concentrations of NH₄Cl (0 to 1 mM)
and decreased in the range of 1 to 20 mM, at
which point it reached a relatively constant level
of 100 to 150 nmol min⁻¹ mg of protein⁻¹. This
was consistent with the idea that glutamine syn-
thetase is the main route of ammonia assimila-
tion at low NH₄⁺ levels (20). In contrast to the
increasing transhydrogenase activity, NADH
dehydrogenase activity decreased to a constant
level of 600 nmol min⁻¹ mg of protein⁻¹. Analysis
of the ratio of the two activities, transhydrogen-
ase to glutamate dehydrogenase, indicates that
FIG. 1. Effect of exogenous NH₄Cl on various activities in E. coli K-12. Cells were grown on minimal salts medium containing glucose (30 mM) and various concentrations of NH₄Cl (0.5 to 150 mM). Activities were measured as described in the text. (a) Glutamine synthetase (●) and glutamate dehydrogenase (○) measured as nanomoles per minute per milligram of protein; (b) NADH-Fe(CN)₆⁻ reductase (□) and NAD(P)⁺ transhydrogenase (○) measured as nanomoles per minute per milligram of protein.

it remained relatively constant over the range of NH₄Cl concentrations used (0.6 to 0.8).

Growth on glutamate alone led to repression of both glutamate dehydrogenase and transhydrogenase with the specific activities decreasing to 35 to 90 nmol min⁻¹ mg of protein⁻¹, respectively (Fig. 2). Addition of increasing concentrations of NH₄Cl to the medium in the presence of glutamate led to concertedly higher specific activities for both enzymes, and at 20 mM NH₄Cl, both were comparable to that observed in the absence of added glutamate in the medium. Above this concentration of NH₄Cl, however, glutamate dehydrogenase was still repressed (Fig. 2a), an effect which probably related to the direct involvement of NH₄⁺ in this enzyme reaction, leading to the synthesis of glutamate. Transhydrogenase appeared to be relatively insensitive to NH₄Cl in the range of 20 to 150 mM and in the presence of glutamate. NADH dehydrogenase activity (Fig. 2b) varied very little throughout the whole range of NH₄Cl concent-

FIG. 2. Effect of exogenous NH₄Cl on cellular activities in E. coli K-12 in the presence of added glutamate. Cells were grown on a minimal salts medium containing glucose (30 mM) and various concentrations of NH₄Cl (0 to 150 mM) in the presence of L-glutamate (20 mM). Activities were measured as described in the text. (a) Glutamine synthetase (●) and glutamate dehydrogenase (○), measured as nanomoles per minute per milligram of protein; (b) NADH-Fe(CN)₆⁻ reductase (□) and NAD(P)⁺ transhydrogenase (○), measured as nanomoles per minute per milligram of protein. The activity scales for glutamate dehydrogenase and glutamine synthetase are the same.
the same range of NH₄Cl concentrations, NADH dehydrogenase decreased from 2,330 to 1,120 nmol min⁻¹ mg of protein⁻¹, and the transhydrogenase increased from 1,220 to 2,650 nmol min⁻¹ mg of protein⁻¹. The activities of these latter two enzymes in strain W6 (a proline auxotroph of ATCC 96377) were consistently higher than those in strain K-12. This has previously been noted, and this strain has been used systematically for studies on the isolation of transhydrogenase (12).

Growth of E. coli on ammonia in the presence of L-leucine resulted in this amino acid being identified as the most repressive towards transhydrogenase (5). With excess ammonia in the presence of 10 mM leucine (Table 1), transhydrogenase activity was repressed to approximately a quarter of the optimal activity as observed with corresponding concentrations of NH₄Cl in the absence of leucine (Fig. 1b). This was in accordance with the reported repression of this amino acid. The glutamate dehydrogenase activity with excess ammonia and with leucine present was, however, comparable to that observed in the absence of leucine. Growth on limiting ammonia in the presence of leucine leads to activation of glutamate dehydrogenase under conditions in which transhydrogenase is still repressed (Table 1). This suggests that leucine has other effects on nitrogen metabolism which are as yet unidentified and are the subject of further investigation. In this context, it should be noted that in mitochondria, L-leucine exerts an allosteric control on glutamate dehydrogenase, and there is evidence for presence of specific leucine-glutamate dehydrogenase complexes (14).

Not all nitrogen compounds when used as the sole source of cellular nitrogen led to the highly repressive effects observed with glutamate (Table 1). Growth of E. coli K-12 on glucose and L-alanine or adenosine resulted in transhydrogenase specific activities of 667 and 683 nmol min⁻¹ mg of protein⁻¹, respectively. These were comparable to that detectable at optimal concentrations of exogenous NH₄Cl. Growth on glucose and glutamine as the sole nitrogen source resulted in a two- to threefold reduction in transhydrogenase.

In S. typhimurium the regulation of ammonia metabolism has been shown to be slightly different than that in E. coli, in that glutamate de-

<table>
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<tr>
<th>Organism</th>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>NADP transhydrogenase</th>
<th>Glutamate dehydrogenase</th>
<th>Glutamine synthetase</th>
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<td><strong>K. aerogenes</strong></td>
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<td>NH₄Cl</td>
<td>&lt;13</td>
<td>&lt;9</td>
<td>62</td>
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*Glucose was present at 30 mM.

Glutamine (prepared fresh before use) was at 0.2%. Histidine and glutamate were at 0.4%. NH₄Cl was at 50 mM (excess) and 0.5 mM (limiting). When used in combination with histidine it was 15 mM.

Assays in K. aerogenes were performed at 30°C. In other organisms, they were performed at 37°C.

Not determined.
hydrogenase was unresponsive to growth on limiting ammonia or glutamate as sole nitrogen source (3). It was, however, still sensitive to amino acids and could be effectively repressed by aspartate (3), a good source of cellular glutamate. Transhydrogenase was coregulated in a similar manner and further signifies a relationship between the two enzymes.

In _K. aerogenes_, the enzymes involved in the formation of glutamate are more strictly controlled by a regulatory gene close to or within the structural gene for glutamine synthetase (glnA) (11). Therefore, we determined the level of transhydrogenase activity in cells of _K. aerogenes_ grown on limiting and excess ammonia. In addition, we studied other growth conditions which have been shown to affect glutamine synthetase and glutamate dehydrogenase activities within the cell. The results of the experiments are outlined in Table 1. Again, decreases of both glutamate dehydrogenase and transhydrogenase occurred under conditions of limiting NH₄Cl (0.5 mM). The low levels of transhydrogenase and glutamate dehydrogenase coincided with the expected high glutamine synthetase activity (13). When glutamine was used as the sole nitrogen source in the presence of glucose, high levels of glutamine synthetase were produced, with corresponding repressed levels of glutamate dehydrogenase and transhydrogenase. Incorporation of excess NH₄Cl into the medium should have restored high levels of glutamate dehydrogenase and transhydrogenase and repressed glutamine synthetase. This was the case, as shown in Table 1. Growth on a medium containing l-histidine as a poor carbon source and ammonia and glutamine as a nitrogen source has been demonstrated to lead to severe repression of both glutamate and glutamine synthetase (2). Transhydrogenase once again was repressed under such conditions. Growth on glucose with l-histidine as the sole nitrogen source also resulted in repression of glutamate and dehydrogenase and transhydrogenase, under conditions in which glutamine synthetase and histidase are highly activated. As in the case of _E. coli_, growth on glutamate as the sole nitrogen source led to severe repression of transhydrogenase and glutamate dehydrogenase, with a correspondingly high level of glutamine synthetase (Table 1).

Growth of _E. coli_ K-12 on media containing l-histidine, glutamine, and NH₄Cl also led to severe repression of NAD(P)⁺ transhydrogenase and glutamate dehydrogenase, but not glutamine synthetase. This effect was similar to that observed in _K. aerogenes_. Activities were 15, 19, and 460 nmol min⁻¹ mg of protein⁻¹, respectively. In this case, however, glutamine, rather than l-histidine, was probably functioning as the carbon source, which may account for the high level of glutamine synthetase. Because of the strong correlations between the two enzymes in the family Enterobacteriaceae, it was of interest to test for the transhydrogenase in _B. subtilis_, in which NAD(P)⁺ glutamate dehydrogenase is essentially absent. Transhydrogenase was virtually absent, further strengthening the correlation between the two enzymes (Table 1). There is, however, some evidence for an NAD-dependent glutamate dehydrogenase in this organism (10) when it is grown under certain growth conditions. The role of this enzyme in nitrogen metabolism is not yet clearly defined.

**DISCUSSION**

The evidence presented would strongly suggest that in the three enteric bacteria studied, NAD(P)⁺ transhydrogenase and NAD(P)⁺ glutamate dehydrogenase are both coregulated under similar growth conditions and depend upon the nitrogen source. The organisms differ in their abilities to (i) derepress the histidine-utilizing enzymes and (ii) to repress glutamate dehydrogenase during nitrogen-limiting growth, as a result of the degree of control by a regulatory gene close to or within the structural gene for glutamine synthetase. Coordinate changes in transhydrogenase could indicate that the enzyme is involved in the supply of NADPH, specifically for glutamate dehydrogenase when it functions in the synthesis of glutamate. These observations, in turn, would explain the inability of investigators to demonstrate a role for transhydrogenase in the general production of NADPH in _E. coli_ as being due to the fact that it serves a specific role related to NH₃ assimilation. This view is strengthened by the demonstration of an interrelationship between the two enzymes in other enteric bacteria, but in particular _K. aerogenes_, and the corresponding low activities of the enzymes in _B. subtilis_. Several mutants are available in _K. aerogenes_ in which the regulation of nitrogen metabolism is affected. Analyses of such mutants are presently being pursued to understand further the possible role of NAD(P)⁺ transhydrogenase in ammonia assimilation. The initial data in wild-type cells of _K. aerogenes_ bode well for a positive outcome of such experiments. The inverse relationship between NADH-Fe(CN)₆³⁻ reductase activity and transhydrogenase activity at limiting NH₄Cl in _E. coli_ again exemplifies the interrelationship of these two systems. Further studies are necessary to understand the physiological control of this system. In particular, the potential regula-
tion of transhydrogenase by NADP+ and NADPH pools in the cell is under investigation.

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LITERATURE CITED


