Regulation of Synthesis and Reversible Inactivation *in vivo* of Dual Coenzyme-specific Glutamate Dehydrogenase in *Bacteroides fragilis*

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Regulation of the dual coenzyme-specific glutamate dehydrogenase (GDH; EC 1.4.1.3) was studied in the anaerobic bacterium *Bacteroides fragilis*. Cells grown at a low concentration of ammonia had a specific activity for the enzyme 10-fold higher than that for cells grown with excess ammonia. Immunochemical determination with a GDH-specific antiserum showed that the content of immuno-precipitated protein was about 8% of the total protein in the former cells and was 4% in the latter cells. When cells grown on 50 mM-NH₄Cl were transferred to a fresh medium containing 0.5 mM-NH₄Cl, an increase in the molecular activity of the enzyme occurred, and synthesis of immuno-reactive protein started. Rapid inactivation of the GDH occurred when cells grown on 1 mM-NH₄Cl were exposed to 50 mM-NH₄Cl. However, the amount of immuno-precipitated protein was not decreased. The inactivation was specifically induced by ammonia and was reversed by transferring the cells to an ammonia-limited medium even in the presence of chloramphenicol. These findings suggest that the synthesis of the GDH is stimulated under low ammonia conditions and that the enzyme activity is controlled by means of a reversible activation/inactivation mechanism which is regulated by ammonia. However, no phosphorylation of GDH was observed before and after exposure of cells to high concentrations of ammonia.

INTRODUCTION

Bacteroides fragilis is a strictly anaerobic bacterium predominating in the human intestinal tract (Finegold *et al.*, 1974; Moore & Holdeman, 1974) and frequently found in clinical isolates (Finegold, 1977). In *B. fragilis*, glutamate dehydrogenase (GDH) provides the principal means of incorporation of ammonia into glutamate even under ammonia-limited conditions (Yamamoto *et al.*, 1984). This anaerobe contains two electrophoretically distinct GDHs: one of them has dual specificity for pyridine nucleotides, and the other is NAD⁺-specific. The dual coenzyme-specific GDH (EC 1.4.1.3) has been purified and characterized (Yamamoto *et al.*, 1987). This enzyme shows relatively high affinity for ammonia (K_m about 1 mM) in the NADPH-dependent amination. Enzyme activities with NAD(P)H and NAD(P)⁺ are not controlled by amino acids, dicarboxylic acids or adenine nucleotides. Higher NADPH-dependent activity is found in cells grown in a medium containing a low concentration of ammonia. The NAD⁺-specific enzyme is induced, to a small extent, only under conditions of excess ammonia. *B. fragilis* cannot use amino acids as a nitrogen source (Varel & Bryant, 1974) and the cellular levels of the GDHs are not affected by the presence of glutamate in the growth medium (Yamamoto *et al.*, 1984). Some properties of the dual-specificity enzyme purified from *Bacteroides thetaiotaomicron* have also been investigated by Glass & Hylemon (1980). The presence of a similar enzyme has been demonstrated in the other species of the DNA homology group in the

Abbreviation: GDH, glutamate dehydrogenase.

genus *Bacteroides* (Glass & Hylemon, 1980; Shah & Williams, 1982). However, the mechanism of its regulation is not understood.

In this study the regulatory properties of the dual coenzyme-specific GDH in *B. fragilis* were investigated using a specific antiserum against the purified enzyme.

METHODS

Organism and culture. *Bacteroides fragilis* ATCC 23745 was grown anaerobically in the defined medium described previously (Yamamoto *et al.*, 1984). The NH_4Cl concentrations were as indicated. Cultures incubated for 18 h were washed with sterile 50 mM-Tris/HCl buffer pH 7.4, then the cells were transferred to a fresh medium. When cultures were exposed to ammonia shock, NH_4Cl solutions were added to 18 h cultures originally containing 1 mM- NH_4Cl to give a final concentration of 50 mM.

Preparation of crude extracts. Cells were harvested by centrifugation at 12000 *g* for 10 min and disrupted with a sonicator (Tomy model 150P) at 20 kHz and 74 W for 5 min in 4 vols of 50 mM-Tris/HCl pH 7.6. The supernatants, following centrifugation at 15000 *g* for 15 min, were used as crude extracts.

Assay of enzyme activities. All enzyme activities were assayed at 30 °C. For assaying GDH activities, the decrease in the absorption of NAD(P)H at 340 nm was monitored with a recorder-equipped spectrophotometer (Jasco model Uvidec-340). The reaction mixture contained 0.1 M-Tris-HCl pH 8.0, 0.15 mM-NADPH, 40 mM- NH_4Cl and 5 mM-2-oxoglutarate. NADH-dependent activity was assayed at pH 7.4.

One unit of enzyme activity was defined as the amount of enzyme that catalysed the oxidation of 1 μmol NAD(P)H min^{-1} .

Protein was assayed by the Lowry method, using bovine serum albumin as standard.

PAGE. Crude extracts were subjected to electrophoresis by the method of Davis (1964). After electrophoresis, the gels were stained for enzyme activity. The staining solution consisted of 50 mM-Tris/HCl pH 8.6, 1 mM-NADP⁺, 10 mM-glutamate, 1 mM-phenazine methosulphate (Sigma) and 0.2% triphenyltetrazolium chloride.

Preparation of antiserum. The purified enzyme (Yamamoto *et al.*, 1987) containing hexameric and dodecameric enzymes (homogeneous by SDS-PAGE) was used to immunize a male rabbit. Approximately 90 μg of the GDH protein mixed with Freund's complete adjuvant was injected subcutaneously into the back of the rabbit. This was repeated several times at intervals of 7–10 d. Blood was collected every week after the 3rd injection until the cross reactivity in the serum became low. The blood was allowed to clot and was then centrifuged at 8000 *g* for 5 min. The supernatant obtained was used as the antiserum.

Immunological techniques. The isolation of immunoglobulins and other immunological methods were according to Johnstone & Thorpe (1982).

Antibody reaction was tested using the Ouchterlony double immunodiffusion method in a 1% (w/v) agarose gel containing 10 mM-potassium phosphate pH 7.8 and 0.15 M-NaCl.

Immuno-electrophoresis was done in 12 ml 1.2% agarose with a 30 mM-barbital buffer pH 8.3 as the electrolyte, on glass plates (10 × 6 cm) at 4 °C in a water-cooled electrophoresis chamber (Bio-Rad model 1415). About 5 μl of extract (approximately 100 μg protein) in each well (3 mm diameter) was electrophoresed at 2.5 mA per 6 cm of gel width for about 4 h. The antiserum was added to troughs made on the gels and incubated at 4 °C.

Rocket immuno-electrophoresis was done in 24 ml 1% agarose containing the barbital buffer and 5% (v/v) of the anti-GDH antiserum, on glass plates (10 × 12 cm) in the cooled electrophoresis chamber. The purified enzyme was used as a standard for assaying immuno-reactive protein. After electrophoresis at 2 mA per 12 cm of gel width overnight, the gels were soaked in saline for 2 d and given a change of saline after the first day. After washing with distilled water and drying the gels, protein was stained with Coomassie blue G-250 in 3.4% (w/v) perchloric acid.

³²P-labelling. Cells grown for 18 h in 1 litre 1 mM- NH_4Cl medium containing 1 mCi (37 MBq) [³²P]orthophosphoric acid (ICN Radiochemicals) were harvested before and after ammonia shock treatment. The final concentration of phosphate was 0.26 mM after inoculation, and about 20% of the radioactivity was recovered in harvested cells. Cell-free extracts (0.5 ml), prepared as above followed by ultracentrifugation at 80000 *g* for 1.5 h, were mixed with purified anti-GDH antibody (2 mg ml^{-1}). After 1 h incubation at room temperature, mixtures were centrifuged and pellets were washed twice with 50 mM-Tris/HCl-containing saline (pH 7.8) and suspended in 50 μl 50 mM-Tris/HCl pH 7.8. Purified GDH and the precipitates containing 750–1650 c.p.m. were subjected to SDS-PAGE (Laemmli, 1970). Gels were stained for protein, dried, and then used for autoradiography. Detection of radioactivity was done by exposing an X-ray film (Kodak X-Omat AR) for 9 d at –75 °C. Cerenkov radiation from ³²P was counted as radioactivity by a liquid scintillation counter (Beckman LS-230).

Calculation of GDH molecular activity. Values were estimated using the assumptions that the molecular mass of the GDH is 300000 (Yamamoto *et al.*, 1987) and that all material cross-reacting with the anti-GDH antiserum was the GDH protein. The molecular activity was defined as mol substrate reacted min^{-1} (mol enzyme)^{–1}.

Table 1. *Specific activities and immuno-reactive protein content of GDH in extracts from B. fragilis grown at different concentrations of NH₄Cl*

Cells were harvested after 18 h growth in defined medium. Specific activities are expressed as units (mg protein)⁻¹, amount of immuno-reactive protein as µg (mg protein)⁻¹, and molecular activities as min⁻¹. Results are the mean values of two determinations for each extract, which differed from each other by no more than 10%.

NH ₄ Cl concn (mM)	OD ₆₅₀	GDH		
		Specific activity	Amount of immuno-reactive protein	Molecular activity
1	0.385	5.11	82.2	18700
5	0.714	3.17	56.2	16900
10	1.293	1.55	42.1	11000
50	2.160	0.458	44.5	3090
100	2.095	0.482	37.8	3840

RESULTS

Regulation of GDH synthesis

B. fragilis was grown in media containing NH₄Cl at different concentrations, and the specific activity of GDH and the amount of protein immuno-precipitated with the antiserum against the enzyme were determined in crude extracts (Table 1). The specific activity was highest in 1 mM-NH₄Cl-grown cells. Cells grown on 50 mM- or 100 mM-NH₄Cl showed a specific activity one-tenth of that for 1 mM-NH₄Cl-grown cells. The content of immuno-reactive protein was also the highest, 8% of the total protein, in 1 mM-NH₄Cl-grown cells, while it was about 4% for cells grown in 10 mM-, 50 mM- and 100 mM-NH₄Cl. The molecular activity of the GDH was 18700 min⁻¹ in the 1 mM-NH₄Cl-grown cells. This value was six times that for 50 mM- or 100 mM-NH₄Cl-grown cells. These findings suggest that in *B. fragilis* the synthesis of GDH is stimulated by less than 10 mM-ammonia and that the enzyme has active and inactive forms depending on the ammonia concentration in the growth medium.

To confirm these observations, cells grown in a 50 mM-NH₄Cl medium were transferred to a fresh medium containing 0.5 mM-NH₄Cl, and the enzyme activity and the immuno-reactive protein were determined in cell-free extracts (Fig. 1). After 3 h incubation, the specific activity increased by about 50%, although the amount of immuno-reactive protein did not increase, indicating that the enzyme was activated during the incubation of cells in the ammonia-limited medium. After 9 h incubation, the specific activity and the amount of immuno-reactive protein increased about 4- and 1.5-fold, respectively. The molecular activity was thus raised from 2820 min⁻¹ to 6660 min⁻¹. After 24 h incubation with 50 µg chloramphenicol ml⁻¹, a 50% increase in the specific activity was observed, whereas a small drop (10–20%) in the amount of immuno-reactive protein and a decrease in the OD₆₅₀ of the culture by 20% were also observed; the molecular activity was 5220 min⁻¹ in this culture. These results indicate that synthesis of GDH is induced under ammonia-limited conditions, and activation of the enzyme occurs post-translationally also under ammonia-limited conditions.

In vivo inactivation and reactivation

When growth in a 1 mM-NH₄Cl medium reached stationary phase, a culture was subjected to ammonia shock by the addition of NH₄Cl to a final concentration of 50 mM. The activity of GDH was assayed in crude extracts from shocked and unshocked cultures (Fig. 2). The NADPH-linked activity of GDH decreased by 80% within 15 min of ammonia shock. The molecular activity of the enzyme decreased from 19270 min⁻¹ to 3700 min⁻¹. The NADH-dependent activity of the enzyme also decreased to the same extent (data not shown). The decrease in antigen content was accompanied by the growth of cells after ammonia shock. Thus, the total amount of the immuno-precipitated protein which was estimated in terms of GDH content × cell amount (OD₆₅₀) was almost constant for at least 2 h after ammonia shock. The

Fig. 1. Induction and activation of the dual coenzyme-specific GDH in *B. fragilis* under low ammonia conditions. Cells from an 18 h culture in 50 mM-NH₄Cl were transferred to the defined medium containing 0.5 mM-NH₄Cl (open symbols) and also to that containing 0.5 mM-NH₄Cl plus chloramphenicol (50 μg ml⁻¹, filled symbols). Enzyme activity and immuno-reactive protein (protein precipitated with anti-GDH antiserum) were assayed in crude extracts prepared from the cells by sonication. ○, ●, Specific activity of GDH; ▽, ▼, immuno-reactive protein; □, ■, molecular activity of GDH; △, ▲, OD₆₅₀. Results are the mean values of two determinations for each sample, which differed from each other by no more than 10%.

Fig. 2. Effect of ammonia shock on GDH activity in *B. fragilis* growing in medium containing 1 mM-NH₄Cl. NH₄Cl (to a final concentration of 50 mM) was added to the culture in the stationary phase of growth at the time indicated with an arrow (solid lines). Another culture without the shock treatment (dashed lines) was grown in parallel. Samples were taken to prepare crude extracts from the cells. △, ▲, OD₆₅₀; ○, ●, specific activity of GDH; ▽, ▼, immuno-reactive protein; □, ■, total immuno-reactive protein (OD₆₅₀ × amount of immuno-reactive protein). The results obtained after ammonia shock are represented by filled symbols. Results are the mean values of two determinations for each sample, which differed from each other by no more than 10%.

Table 2. *Effects of salts on GDH activity in B. fragilis*

Each salt indicated was added to a 20 h culture initially containing 1 mM-NH₄Cl, and the cells were harvested after incubation for 15 min. The specific activity of GDH was measured in cell-free extracts. Results are the mean values of two determinations for each extract, which differed from each other by no more than 10%.

Salt	Concn (mM)	GDH specific activity	
		Units (mg protein) ⁻¹	%
No addition		1.97	100
NH ₄ Cl	5	1.00	51
	15	0.643	33
	30	0.661	34
	50	0.643	33
	80	0.533	27
NaCl	50	1.86	94
KCl	50	2.13	108
CH ₃ NH ₂ .HCl*	50	2.04	104
NH ₂ OH.HCl*	50	1.86	94

* These salts were neutralized with NaOH before addition to the culture.

double immunodiffusion test showed a single fused band without a spur between crude extracts from shocked and unshocked cells, and immunoelectrophoresis also showed a single precipitin band in each extract. The relative mobility of the enzyme in each crude extract was the same when examined by PAGE. These observations indicate that the decrease in enzyme activity following the addition of ammonium chloride was not due to degradation of the enzyme, and that the enzyme is probably converted to an inactive form.

Preparations of GDH, precipitated with a GDH-specific antibody from high-speed supernatants of ammonia-shocked and -unshocked cells grown in [³²P]phosphate-containing medium, were analysed by SDS-PAGE. The amounts of phosphorus and GDH protein applied to the gel were about 1 nmol and 10 µg, calculated from the radioactivity and density of stained protein respectively. However, no radioactivity was detected in GDH protein by autoradiography. The antibody-antigen complexes from shocked and unshocked cells were identical in SDS-PAGE, with no degradation product of GDH visible after ammonia shock.

The kind of compound that would trigger the inactivation of GDH was examined by adding salts or ammonia analogues to 18 h cultures originally containing 1 mM-NH₄Cl (Table 2). NH₄Cl at 5 mM decreased the activity by 50%, and almost maximum inactivation occurred at 15 mM. The other salts, 50 mM-KCl and 50 mM-NaCl, showed no effect. This suggests that ammonia or the ammonium ion is the effector inducing inactivation, and not the chloride ion. No changes in enzyme activity were observed after the addition of the ammonia analogues monomethylamine and hydroxylamine (Table 2). When cells were suspended in medium lacking glucose or in Tris/HCl buffer (50 mM, pH 7.4), incubated anaerobically at 37 °C for 1.5 h, and then exposed to ammonia shock, decreases of 80% in the specific activity of the enzyme were observed (data not shown). The inactivation of GDH may therefore be energy-independent in *B. fragilis*.

To test whether the inactivation of GDH was reversible, cells exposed to ammonia shock were washed and transferred to two fresh media, one containing 0.5 mM-NH₄Cl and the other containing 0.5 mM-NH₄Cl plus 50 µg chloramphenicol ml⁻¹. By removing the high concentration of ammonia, the enzyme activity recovered to about 90% of that observed before the ammonia shock (Fig. 3). No increase in the content of immuno-reactive protein was observed during 7 h incubation in the presence or absence of chloramphenicol after the transfer.

DISCUSSION

In order to investigate the regulatory mechanisms for the dual coenzyme-specific GDH of *B. fragilis*, immunochemical methods were used with a GDH-specific antiserum. The synthesis of

Fig. 3. Reversibility of the inactivation of the dual coenzyme-specific GDH. A stationary-phase culture grown with 1 mM- NH_4Cl was shocked by the addition of 50 mM- NH_4Cl at the time indicated by the arrow, and then washed with a sterile buffer. The washed cells were transferred to a defined medium containing 0.5 mM- NH_4Cl (open symbols) or to another medium containing 0.5 mM- NH_4Cl and chloramphenicol (50 $\mu\text{g ml}^{-1}$, filled symbols). Symbols are the same as in Fig. 1. Results are the mean values of two determinations for each sample, which differed from each other by no more than 10%.

GDH was regulated in response to the concentration of extracellular ammonia. Immuno-reactive protein was about 4% of the total protein in cells grown on more than 10 mM- NH_4Cl , while it was twice that level at lower concentrations. Moreover, the synthesis of the enzyme protein was induced when the cells grown with 50 mM- NH_4Cl were transferred to a low ammonia medium. It is likely that a large amount of the enzyme is synthesized to incorporate ammonia under ammonia-limited conditions. Such regulation of the synthesis of GDH appears to be unique to *B. fragilis*. The anaerobic rumen bacterium *Selenomonas ruminantium* also has a dual coenzyme-specific GDH, the activity of which decreased while growing on limited ammonia (Smith *et al.*, 1980). In many bacteria the NADP^+ -specific GDH which functions in glutamate synthesis is induced by ammonia and repressed by glutamate or other amino acids (Smith *et al.*, 1975).

GDH in cells was inactivated within 15 min by ammonia shock and GDH activity was recovered without synthesis of GDH protein by removing high concentrations of ammonia from the growth medium. This indicates the presence of another regulatory mechanism for GDH in *B. fragilis*, namely a reversible inactivation caused by changes in the extracellular ammonia concentration. Ammonia shock is known to cause the inactivation of glutamine synthetase in *Escherichia coli* due to the adenylation/deadenylation control mechanism (Stadtman & Ginsburg, 1974). The energy-dependent inactivation of some enzymes has also been found in micro-organisms (Switzer, 1977). Other mechanisms for inactivation of microbial enzymes have also been found. Degradation inactivation of NADP^+ -dependent GDH occurs in *Pseudomonas aeruginosa* (Smits *et al.*, 1984), *Candida utilis* (Hemmings, 1978a) and *Saccharomyces cerevisiae* (Mazon & Hemmings, 1979; Bogonez *et al.*, 1985). Protein phosphorylation is also known to be a regulatory mechanism for NAD^+ -dependent GDH in *C. utilis* (Hemmings, 1978b, 1981) and *S. cerevisiae* (Hemmings, 1980), and for isocitrate dehydrogenase in *E. coli* (Garnak & Reeves, 1979) and *Salmonella typhimurium* (Wang & Koshland, 1981). The inactivation of the *B. fragilis* GDH is unlikely to be due to its degradation, since a single protein with the same mobility was detected immunoelectrophoretically in each extract from shocked and unshocked cells and the amount of immuno-precipitated protein was not decreased by ammonia shock. Phosphorylation or adenylation of the enzyme was also excluded as the cause of the inactivation or activation mechanism, because no phosphorylated forms of the enzyme were detected autoradiographically in either shocked or unshocked cells grown with ^{32}P -phosphate.

The values obtained for the molecular activity of the GDH fell in a range of about 3000 min⁻¹ to 19000 min⁻¹. The activity seemed to alter in relation to the concentration of extracellular ammonia. The *B. fragilis* GDH, composed of six identical subunits (Yamamoto *et al.*, 1987), may have multimolecular forms, in which subunits are modified by an unidentified compound in response to a rise in the ammonia concentration, similar to the adenylylated forms of glutamine synthetase in *E. coli* (Stadtman & Ginsburg, 1974).

Inactivation of the GDH was reversible in whole cells. However, we have never succeeded in inactivating and activating the GDH in crude extracts even under anaerobic conditions. An active form of the GDH was recently purified 37-fold from crude extracts (Yamamoto *et al.*, 1987) and 18-fold from high-speed supernatants of disrupted cells, in which GDH comprised 16% of soluble protein (unpublished results). These extents of purification of GDH activity were higher than those expected on the basis of GDH protein contents. This may have resulted from the removal of an inhibitory compound from the enzyme fraction. However, GDH activity was not increased by dialysis of each extract from shocked and unshocked cells. Studies are continuing in order to identify the modification mechanism of the enzyme.

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