Site and significance of chemically modifiable cysteine residues in glutamate dehydrogenase of *Clostridium symbiosum* and the use of protection studies to measure coenzyme binding

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Protein chemical studies of NAD⁺-dependent glutamate dehydrogenase (GDH; EC 1.4.1.2) from *Clostridium symbiosum* indicate only two cysteine residues/subunit, in good agreement with the gene sequence. Experiments with various thiol-modifying reagents reveal that in native clostridial GDH only one of these two cysteines is accessible for reaction. This residue does not react with iodoacetate, iodoacetamide, *N*-ethylmaleimide or *N*-phenylmaleimide, but reaction with either *p*-chloromercuribenzenesulphonate or 5,5'-dithiobis(2-nitrobenzoic acid) causes complete inactivation, preventable by NAD⁺ or NADH but not

by glutamate or 2-oxoglutarate. Protection studies with combinations of substrates show that glutamate enhances protection by NADH, whereas 2-oxoglutarate diminishes it. These studies were also used to determine a dissociation constant (0.69 mM) for the enzyme–NAD⁺ complex. Similar data for NADH indicated mildly cooperative binding with a Hill coefficient of 1.32. The significance of these results is discussed in the light of the high-resolution crystallographic structure for clostridial GDH and in relation to information for GDH from other sources.

INTRODUCTION

The glutamate dehydrogenases (GDHs; EC 1.4.1.2–4) have been intensively studied both because of their metabolic importance and because of their interesting allosteric properties [1–3]. Understanding at a molecular level, however, was held back for some years by the absence of a three-dimensional structure, despite determined efforts with crystals from vertebrate sources [4,5]. Our approach has been first to identify a GDH suitable for crystallography and then to carry out the necessary associated functional, chemical and genetic studies. Crystallization of the NAD⁺-dependent GDH (EC 1.4.1.2) from *Clostridium symbiosum* [6] has now led to determination of a high-resolution structure [7–9]. This GDH, like most others [1,2], is hexameric, and the subunit *M_r* is 49 295 [6,10]. The gene for this enzyme has also been cloned, sequenced and overexpressed in *Escherichia coli* [10]. Detailed kinetic studies are reported elsewhere [11,12]. A notable feature is the pH-dependent conformational equilibrium between active and inactive forms [13,14], reminiscent of the transition reported for the NADP⁺-dependent GDH (EC 1.4.1.4) of *Neurospora crassa* [3,15,16].

Chemical modification studies can make a useful contribution, by providing insight into the catalytic chemistry, by labelling active-site peptides which are likely to be in highly conserved regions of the sequence, and by providing a means of detecting substrate binding through protection studies. The only clear consensus emerging from such studies of GDHs from various sources is that one or more lysyl residues are involved in the active site [1,2,17,18]. In the case of GDH from *C. symbiosum*, indeed, a cluster of three essential lysine residues has been identified [19] and these are clearly implicated in substrate binding and catalysis [8,9,20]. Cysteine residues are another obvious target for modification for several reasons. Their relative in-

frequency and the availability of reasonably specific reagents provide an opportunity for clean, unambiguous modification and hence for labelling and counting enzyme subunits. The location of reactive thiol groups has also been of importance in the crystallographic context of locating binding sites for Hg atoms in heavy-metal derivatives. Finally, cysteine side-chains are frequently involved in enzyme catalysis, though not previously invoked in the GDH mechanism [18].

In this paper we report that, of only two cysteine residues per subunit in purified clostridial GDH, one can be modified by certain thiol reagents with loss of activity. Other reagents which fail to inactivate apparently do not react with the residue in question. Protection of the catalytic activity is used to study coenzyme binding.

EXPERIMENTAL

Materials

Thiol-modifying agents, including 5,5'-dithiobis(2-nitrobenzoate) (DTNB), *p*-chloromercuribenzenesulphonate (*p*CMBS), iodoacetic acid, iodoacetamide, *N*-ethylmaleimide and *N*-phenylmaleimide were from Sigma Chemical Co., Poole, Dorset, U.K. ¹⁴C-labelled iodoacetamide was from Radiochemicals, Amersham, Bucks., U.K. Monosodium glutamate of the purest grade was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. NAD⁺ (Grade 1) was obtained from Boehringer Corp., Lewes, East Sussex, U.K. Rathbone, Walkerburn, Scotland, U.K. supplied h.p.l.c.-grade acetonitrile and trifluoroacetic acid.

Enzyme preparation and assay

Clostridial GDH was purified by dye-ligand chromatography on Remazol Red–Sephacrose as described elsewhere [11,21]. The

Abbreviations used: GDH, glutamate dehydrogenase (EC 1.4.1.2,3,4); DTNB, 5,5'-dithiobis(2-nitrobenzoate), Ellman's reagent; *p*CMBS, *p*-chloromercuribenzenesulphonate.

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enzyme, stored as a suspension in 60 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ at 4 °C, was dialysed before use against several changes of 0.1 M potassium phosphate, pH 7.0, and then clarified by centrifugation. GDH activity was measured fluorimetrically with an instrument of the type described by Dalziel [22], calibrated by the method of Engel and Hornby [23]. Reaction mixtures (4 ml) contained 1 mM NAD^+ and 40 mM L-glutamate in 0.1 M potassium phosphate, pH 7.0, at 25 °C, and reaction was usually initiated by addition of 10 μl of enzyme solution. Protein concentrations were calculated from A_{280} on the basis of a molar absorption coefficient of $1.05 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ [21].

Amino acid analysis

Protein (0.08–0.33 mg) was hydrolysed with 6 M HCl at 110 °C for periods of 24, 48 and 72 h and amino acids were separated with a Locarte analyser. While tryptophan is completely destroyed by this treatment, loss of threonine and serine is slower and approximately linear with time. These amino acids were estimated by extrapolation to zero time.

Total cysteine content was determined in duplicate by oxidation to cysteic acid [24]. Samples (25–100 μl) of stock GDH solution (3.33 mg/ml) were lyophilized and treated with 75 μl of performic acid for 2 h at 0 °C. 2-Mercaptoethanol (10 μl) and distilled water (0.7 ml) were added and the samples were again lyophilized. The cysteic acid was quantified by amino acid analysis as described above.

Cysteine content was also estimated by incubating 0.5 mg/ml GDH with 0.5 mM DTNB at 25 °C in 0.1 M potassium phosphate buffer, pH 7, containing 6 M guanidinium chloride and 61 μM dithiothreitol. A_{412} values of the resulting thionitrobenzoate anion were monitored to constancy, the reference blank being a cuvette containing all components except the protein. The stoichiometry of the reaction under denaturing conditions was calculated by using a molar absorption coefficient of $13.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for the thionitrobenzoate anion [25]. Accessible cysteine content in native GDH was estimated similarly, except that the guanidinium chloride was omitted. Several incubations were carried out in each case in order to obtain a mean value of the thiol content.

Labelling with iodoacetamide

GDH solution (5 ml) at 25 mg/ml was dialysed against 5 litres of 0.1 M ammonium bicarbonate, pH 8.3, lyophilized, and then redissolved in the round-bottomed flask in 8 ml of 6 M guanidinium chloride/0.1 M Tris/HCl, pH 8.3/0.5 mM EDTA. Solid dithiothreitol (5 mg) was added and O_2 -free N_2 was bubbled through via a stopper. After incubating the stoppered O_2 -free solution in the dark for 2 h, 1.25 ml of 50 mM iodoacetamide in the same Tris/HCl buffer and 50 μCi of ^{14}C -labelled iodoacetamide in 0.25 ml were added. The solution was again bubbled with N_2 and further incubated for 1 h at 37 °C in the dark. After the addition of 10 μl of 2-mercaptoethanol, the solution was dialysed twice against 5 litres of 0.1 M ammonium bicarbonate, pH 8.3, and then lyophilized. Radioactive counts were monitored at all stages of the labelling procedure by standard scintillation counting; typically 20–200 μl samples were added to 10 ml of standard scintillation cocktail. Counting efficiency was approx. 83 %.

The labelled, lyophilized protein (1.3 mg) was hydrolysed in 6 M HCl at 110 °C for 24 h. Amino acids were analysed as described previously, but 80 μl fractions were collected and 100 μl was withdrawn from each for scintillation counting.

Tryptic digestion and peptide purification

^{14}C -labelled, lyophilized protein was dissolved to a concentration of approx. 5 mg/ml in 5 ml of 0.1 M ammonium bicarbonate, pH 8.3, containing 0.1 mM CaCl_2 . Trypsin solution (10 mg/ml; 50 μl) was added and the solution was incubated at 37 °C for 1 h. After a second addition of trypsin, the sample was further incubated for 1 h, and the reaction was then terminated by lyophilizing the protein solution. Peptides were separated and purified by reverse-phase h.p.l.c. on a C18 column eluted with gradients of acetonitrile running into methanol/1 % phosphoric acid.

Inactivation by thiol-modifying agents

GDH solutions (0.09–0.2 mg/ml) in 0.1 M potassium phosphate, pH 7.0, at 25 °C were separately incubated with iodoacetic acid, iodoacetamide, *N*-ethylmaleimide, *N*-phenylmaleimide (all 5 mM), *p*CMBS (10–200 μM) and DTNB (1 mM). In a separate experiment, the concentration of DTNB was varied over the range 0–1 mM. GDH activity was monitored in each case by assaying 10 μl samples periodically as described earlier. Reagent solutions at the appropriate concentrations were prepared gravimetrically except for DTNB, the concentration of which was calculated from A_{323} by using a molar absorption coefficient of $16.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [25].

In a series of double incubations in the same buffer, GDH at 0.4 mg/ml was incubated separately with iodoacetic acid, iodoacetamide, *N*-ethylmaleimide, *N*-phenylmaleimide (all 5 mM) and DTNB (1 mM) and assayed periodically as above. Except for the sample initially treated with DTNB, all other samples were further treated with 1 mM DTNB under the same conditions as given previously. A solution of GDH not previously treated with any of the reagents was included in this set as a control. The final protein concentration in the second round of incubations was 0.36 mg/ml. Modification by DTNB was followed by monitoring the increase in A_{412} with a Cary 219 recording spectrophotometer (Varian Instruments, Palo Alto, CA, U.S.A.) and cuvettes of 1 cm pathlength. GDH activity was followed as previously described.

Protection experiments

GDH was incubated with either 10 μM *p*CMBS or 1 mM DTNB in 0.1 M potassium phosphate, pH 8.0, at 25 °C. Rates of inactivation were compared with and without various additions of substrates singly or in combination. In view of the contribution of high substrate concentrations to the ionic strength, control incubations of GDH with 1 mM DTNB were set up containing 0–100 mM NaCl. Enzyme activity was monitored as described earlier.

Determination of dissociation constants for NAD^+ and NADH

In a series of incubations of 0.2 mg/ml GDH with 1 mM DTNB as above, the concentration of NAD^+ and NADH was varied over the ranges 0.1–13 mM and 2–1000 μM respectively. Samples were assayed as before. Rate constants for the inactivating reaction were evaluated by using the Enzfitter program of Dr. R. J. Leatherbarrow (Elsevier-Biosoft).

RESULTS

Cysteine content and cysteine-containing peptides

Performic acid oxidation gave a value of 1.78 ± 0.07 cysteic acid residues/enzyme subunit (mean of four determinations). This is in reasonable agreement with a mean value of 1.97 mol of

Table 1 Amino acid sequences of two radioactive and two non-radioactive tryptic peptides purified from clostridial GDH treated with [¹⁴C]iodoacetamide: alignment with homologous sequences of *E. coli* GDH

All four peptides were purified by h.p.l.c. from a tryptic digest of clostridial GDH alkylated with [¹⁴C]iodoacetamide and were analysed by automated solid-phase sequencing. Peptides 1 and 2 were the only radioactive peptides present in significant amounts. The sequences for peptides 2 and 3 were clearly incomplete. That for peptide 4 shows a complete peptide with a normal tryptic cleavage point. Peptide 1 also appeared to be complete, despite the anomalous cleavage. Asterisks denote identical residues in the alignment of clostridial sequence (CS) with *E. coli* sequence (EC) from [26,27]. Subsequent information [10,19] indicates that the level of identity is slightly higher than indicated below: the first amino acid in peptide 4 should be H, not V, and the missing two unassigned residues are LG. Also in peptide 3 the sequence SSS should be SSL.

CS	FCQAF	Peptide 1
	* * * * *	
EC	FCQAL	
	146 150	
CS	VAIIMPCATQNDVDL.....	Peptide 2
	* * * * * * * *	
EC	VDIALPCATQNELDV.....	
	315 329	
CS	YADEPEFVQTVEEVLSSSGPV††	Peptide 3
	* * * * *	
EC	DPNQTEFAQAVREVMTTLWPF	
	18 38	
CS	VIGPDIDVPAGD?VGAR	Peptide 4
	* * * * * * *	
EC	HLGADTDVPAGDIGVGR	
	157 174	

Table 2 Amino acid composition of GDH

The cysteine content was obtained as cysteic acid after performic acid oxidation and also by reaction with DTNB. The contents of the other amino acids, averaged from two experiments, were estimated by standard amino acid analysis with detection by ninhydrin reaction. The values in parentheses, given for comparison, are the values deduced from the recently published gene sequence [10].

Amino acid residue	Residues per 49000- <i>M_r</i> protein
Cysteine	2 (2)
Aspartic acid	45 (44 = 24 Asp + 20 Asn)
Glutamic acid	52 (50 = 34 Glu + 16 Gln)
Glycine	49 (48)
Alanine	44 (43)
Methionine	13 (17)
Leucine	28 (27)
Tyrosine	18 (21)
Phenylalanine	19 (19)
Histidine	8 (6)
Lysine	28 (27)
Arginine	18 (18)
Proline	15 (20)
Threonine	18 (17)
Serine	20 (20)
Valine	42 (45)
Isoleucine	21 (20)

thiol/mol of subunit determined from the release of thionitrobenzoate upon reaction of the enzyme with DTNB in 6 M guanidinium chloride (mean of four determinations). On the other hand, in the absence of the denaturant, only 0.79 mol of thionitrobenzoate/mol was released (mean of three determinations). It appears, therefore, that clostridial GDH contains only

two cysteine residues/subunit, of which only one is accessible to DTNB in the native state. There are no disulphide bridges.

The stoichiometry was confirmed by labelling the denatured reduced protein with [¹⁴C]iodoacetamide. In a standard amino acid separation performed on the acid hydrolysate, the single peak of radioactivity, corresponding precisely in elution position with the carboxymethylcysteine standard (the amide group is lost during acid hydrolysis), contained 84 % of the counts loaded. The stoichiometry of labelling indicated 2.0 residues modified/subunit.

After digestion of the radioactively labelled protein with trypsin, peptides were separated by h.p.l.c. Only two peaks of radioactivity were seen, which is in keeping with the amino acid analysis. The small amounts recovered after further purification only allowed partial sequencing (Table 1), but in both cases the sequence obtained included the labelled cysteine residue. In the case of the shorter of the two sequences obtained, it seems likely that the termination reflects anomalous, chymotrypsin-like cleavage rather than incomplete sequencing. Two other peptides were easily obtained in a pure state during purification, and these were sent for sequencing also. Alignment of the four fragments of sequence with the sequence of *E. coli* NADP⁺-dependent GDH (EC 1.4.1.4) [26,27] reveals levels of identity ranging from approx. 30 % to approx. 80 % (Table 1). This evidence, recently extended and confirmed by much fuller sequencing of tryptic and CNBr-cleaved peptides of the unmodified protein [28] and by DNA sequencing of the entire gene [10], provided the first clear indication that this NADP⁺-dependent GDH belongs to the same enzyme family as the well-studied NADP⁺-dependent and dual-specificity GDHs.

Amino acid composition

The amino acid composition of clostridial GDH, with the exception of tryptophan, is compared in Table 2 with the prediction from the recently published gene sequence [10]. With the exception of methionine, proline and valine, which are

Figure 1 Time courses of inactivation of GDH by DTNB and pCMBS

The enzyme was incubated with the thiol-modifying reagent in 0.1 M potassium phosphate, pH 7.0, at 25 °C. The concentration of DTNB was varied over the range 0–1 mM whereas data for only one concentration of pCMBS (10 μM) are shown. Samples (10 μl) were periodically withdrawn from each incubation for assays of enzyme activity. See text for details of assay. ●, No DTNB or pCMBS; ▽, 0.1 mM DTNB; ×, 0.2 mM DTNB; △, 0.8 mM DTNB; ▲, 1 mM DTNB; ○, 10 μM pCMBS. Pseudo-first-order plots of the decline of GDH activity are shown.

Table 3 Protection of GDH by its substrates and coenzymes against inactivation by DTNB and pCMBS and protection in the presence of substrates and coenzymes of the reaction

The Table shows the relative values of inactivation rate constants, obtained from the slopes of plots such as Figures 1 and 2, expressed as percentages of the values in the absence of protecting ligands, i.e. 0.068 min^{-1} with 1 mM DTNB and 0.165 min^{-1} with $10 \mu\text{M}$ pCMBS.

Substrate added		Percentage of rate constant in absence of added substrate
DTNB (1 mM)	None	100
	NAD ⁺ (0.5 mM)	70
	NADH (0.5 mM)	6
	Glutamate (40 mM)	100
	NADH (0.5 mM) + glutamate (40 mM)	0
	2-Oxoglutarate (39 mM)	100
	NH ₄ Cl (100 mM)	77
pCMBS (10 μM)	None	100
	NADH (200 μM)	11
	NADH (200 μM) + 2-Oxoglutarate (40 mM)	60

Figure 2 Protection by NAD⁺ against inactivation of GDH by 1 mM DTNB

Conditions were as described in Figure 1 except that the DTNB concentration was fixed at 1 mM and NAD⁺ at various concentrations was added to the incubations: Δ , no added NAD⁺; $\bullet$, 0.1 mM; $\blacktriangle$, 0.5 mM; $\circ$, 1 mM; ∇ , 2 mM; $\blacktriangledown$, 4 mM; $\times$, 8 mM; $+$, 13 mM.

significantly underestimated, and histidine which is overestimated, the values agree to within 1 or 2 residues.

Reagent specificity of inactivation by thiol modification

Of the thiol modifiers investigated, only the charged aromatic reagents, DTNB and pCMBS, inactivated the enzyme. DTNB (1 mM) caused 95% inactivation after 20 min, whereas with pCMBS as little as $10 \mu\text{M}$ gave complete inactivation over the same period. With different concentrations of the mercurial (10 and $200 \mu\text{M}$), complete inactivation was obtained after 11 and 0.5 min respectively. By contrast, iodoacetate, iodoacetamide, *N*-

Figure 3 The use of protection against inactivation by DTNB to study the binding of coenzymes to GDH

Double reciprocal plots of $1/(k_{\text{max}} - k_{\text{coenzyme}})$ against $1/[\text{coenzyme}]$ are derived from data of the type shown in Figure 2 for NAD⁺ (Figure 3a) and NADH (Figure 3b). In the case of a linear plot, the intercept on the horizontal axis gives the dissociation constant for the protecting coenzyme. The concentration of NADH was varied from 2–1000 μM and the data for the lowest concentrations are not shown in Figure 3b. In view of the non-linearity, the full data-set for NADH is presented as a Hill plot in Figure 4.

ethylmaleimide and *N*-phenylmaleimide, all present at 5 mM, were entirely without effect on activity. Figure 1 shows linear pseudo-first-order plots of $\log [\text{activity remaining (\%)}]$ versus time for the inactivation of GDH by DTNB at different concentrations. The plot for $10 \mu\text{M}$ pCMBS, also shown in Figure 1, yields a pseudo-first-order rate constant of 0.165 min^{-1} , 2.5-fold

Figure 4 Hill plot of the binding of NADH to GDH as revealed by protection against inactivation by DTNB

The protection data for the full range of NADH concentrations (see legend to Figure 3) are displayed as a Hill plot to highlight the apparent binding cooperativity. $\bar{Y}$, used to calculate the ordinate variable, is the fractional saturation of the enzyme with NADH.

higher than the rate constant (0.066 min^{-1}) for 1 mM DTNB, the highest concentration used.

In the case of modification by DTNB it is possible to monitor the time courses of inactivation and of thiol modification conveniently on the same sample, by taking advantage of the increase in A_{412} upon release of anionic thionitrobenzoate. Such experiments yielded identical values of the rate constants for the two processes, consistent with the view that modification of a single thiol group/subunit is directly responsible for inactivation.

Protection by substrates

Partial protection (Table 3) against 1 mM DTNB was given by 0.5 mM NAD^+ and, much more effectively, by 0.5 mM NADH (inactivation rate constants decreased to 70% and 6% respectively). A mixture of NADH and glutamate (40 mM) gave complete protection, whereas glutamate or 2-oxoglutarate without coenzyme offered no significant protection. Limited protection was obtained, however, with 100 mM NH_4Cl , and this was apparently not due merely to the high ionic strength as 100 mM NaCl did not significantly affect the rate of inactivation.

In the case of inactivation by pCMBS, fewer combinations of protecting agents were explored, but in a striking result (Table 3) 2-oxoglutarate greatly diminished the considerable degree of protection afforded by NADH alone.

Concentration dependence of protection by coenzymes

In order to obtain estimates of the dissociation constants for

Table 4 Effects of DTNB on clostridial GDH with and without incubation with other thiol-modification reagents

Clostridial GDH was incubated, as described in the Experimental section, with the reagents listed in the left-hand column. Activity was monitored over 30 min. In the case of DTNB, A_{412} was also monitored. After 30 min, 1 mM DTNB was added to all samples other than the one already treated with this reagent, and activity and A_{412} were monitored over a further 30 min period. Increases in A_{412} values in the right-hand column are expressed as a percentage of the increase seen in the sample treated with DTNB alone over the first 30 min.

Reagent in first incubation	Activity remaining after 30 min (%)	Activity remaining after second incubation (with DTNB) (%)	Increase in A_{412} (% of change in first incubation with DTNB)
Iodoacetic acid (5 mM)	98.2	4.0	100
Iodoacetamide (5 mM)	97.8	3.2	100
N-Ethylmaleimide (5 mM)	99.6	4.2	100
N-Phenylmaleimide (5 mM)	100.5	2.4	100
DTNB (1 mM)	5.25	—	—
None (control)	100	100	100

enzyme-coenzyme binary complexes, GDH was incubated with DTNB in the presence of NAD^+ and NADH at various concentrations. Pseudo-first-order plots for the inactivation of the enzyme in the presence of NAD^+ (Figure 2) show nearly complete protection by 13 mM NAD^+ . The dissociation constant (K_d) for either coenzyme should be obtainable from a plot of $1/[\text{E.coenzyme}]$ against $1/[\text{coenzyme}]$, where $[\text{E.coenzyme}]$ represents the concentration of the binary complex. In the present case, the difference between slopes of pseudo-first-order plots in the absence (k_{max}) and presence (k_{NAD^+} or k_{NADH}) of coenzyme is proportional to $[\text{E.coenzyme}]$, provided that saturation with either coenzyme would give complete protection. Plots of $1/(k_{\text{max}} - k_{\text{NAD}^+})$ against $1/[\text{NAD}^+]$ and $1/(k_{\text{max}} - k_{\text{NADH}})$ against $1/[\text{NADH}]$ are shown in Figures 3(a) and 3(b) respectively. The extrapolated intercepts of these plots on the horizontal axis should give, in either case, the reciprocal of the dissociation constant. (This analysis rests on the assumption of a simple second-order reaction between the reagent and the target thiol group. This assumption remains to be tested and it is possible that modification involves prior formation of a non-covalent enzyme-DTNB complex.) A K_d value of $0.69 \pm 0.05 \text{ mM}$ for NAD^+ is obtained from Figure 3(a). In contrast with the linear plot for NAD^+ , however, the corresponding plot for NADH (Figure 3b) cannot be convincingly fitted by a straight line. Rather it is a smooth curve, concave upwards, suggestive of positive cooperativity. Accordingly, a Hill plot of the same data (Figure 4) shows a linear middle section with a slope of 1.32. Although it might be argued that in this experiment the similarity of the enzyme concentration ($4 \mu\text{M}$) to the lowest NADH concentration entails a risk of systematic error, the curvature is most clearly evident for high NADH concentrations.

Double incubations

As shown above, although DTNB and pCMBS inactivate clostridial GDH, several other standard thiol-modification re-

agents do not. This could mean either that the relevant thiol group is not accessible to these reagents, or else that they modify the same cysteine residue without affecting activity. In order to resolve this question, double incubation experiments were carried out, in which the enzyme was first treated over 30 min with one of the apparently ineffective reagents and then, in a second incubation, exposed to DTNB for 30 min. As before, the four reagents in question gave little or no loss of activity. The result of the second inactivation was unequivocal (Table 4): in all four cases the loss of activity and release of thionitrobenzoate upon secondary treatment with DTNB were similar to the values for a sample treated only with DTNB.

DISCUSSION

The agreement in Table 2 between the experimental amino acid analysis and the prediction from the DNA sequence [10], taken together with the crystallographic findings [8,9], gives some confidence that the sequence deduced from the gene indeed represents the sequence of the protein as produced *in vivo* and purified, i.e. that there are not significant post-translational modifications to be taken into account, as, for example, in the case of the GDH of *Sulfolobus* [29]. Tryptophan is missing from the analysis, but of all the amino acids this one is most easily identified unambiguously in an electron-density map, and the high-resolution structure [8] leaves no room for doubt that the five tryptophan residues specified by the gene sequence, including the C-terminal residue, are all present in the purified protein.

The analysis confirms that clostridial GDH has the unusually low number of two cysteine residues, both present in the free sulphhydryl form. The results of cysteine modification are in marked contrast with those for ox-liver GDH, which is under some circumstances activated by modification with mercurial compounds [30–32]. The residue involved in ox-liver GDH is Cys-319 [31], which is not generally conserved and is missing in clostridial GDH [10]. The NAD⁺-dependent GDH of *Neurospora crassa*, on the other hand, has a single reactive thiol group/subunit, and although iodoacetate and iodoacetamide do not react with this group, *N*-ethylmaleimide, *p*-hydroxymercuribenzoate and DTNB all partially inactivate the enzyme [33]. The NADP⁺-dependent GDH of the same organism is inactivated by *N*-ethylmaleimide [34], but this appears to be due to modification of a residue other than cysteine, since prior carboxymethylation of all the cysteines neither inactivates nor prevents subsequent inactivation by *N*-ethylmaleimide.

Against this background, it appears that, out of only two cysteines in the 450-residue [10] clostridial GDH polypeptide chain, one is sufficiently buried to be inaccessible to the six varied thiol reagents employed in this study. The other one, however, is modified by the negatively charged aromatic reagents DTNB and *p*CMBS, in both cases with total loss of activity. (With *p*CMBS, a decline to unmeasurably low levels of activity was directly demonstrated; with DTNB, the persistence of a small percentage of activity presumably reflects the equilibrium nature of thiol–disulphide exchange and the presence at equilibrium of a small but finite fraction of unreacted enzyme.) The modification reaction can be prevented, or at least moderated, by various substrates or substrate combinations. This suggests either that the cysteine in question is part of the active site or else that it is sufficiently close that its modification by a bulky reagent causes total steric hindrance of the catalytic reaction. The fact that this cysteine is present in a similar sequence in *E. coli* (Table 1) initially suggested that it might be an essential residue in the strict sense. It now appears, however, from a wider comparison [10] of the growing list of published GDH sequences that, even though

the neighbouring residues Pro-319 and Ala-321 (peptide 2) are totally conserved so far, both the cysteine residues shown in Table 1 can be replaced by other residues, the cysteine in peptide 1 by serine or threonine and that in peptide 2 by serine or alanine. Although these are conservative changes, there is clearly no absolute requirement for a thiol group at either position. Results previously reported for similar experiments with other bacterial GDHs are remarkably dissimilar from those for the clostridial enzyme. Thus, although GDH from *Salmonella* sp. was inactivated by *p*-hydroxymercuribenzoate [35], even with 10 mM reagent (1000 times higher than the concentration of *p*CMBS used in the present study), loss of activity was only 90% after 40 min. More surprisingly, the activity of *E. coli* GDH was unaffected by either 1 mM *p*-hydroxymercuribenzoate or 2 mM DTNB even though one thiol group/subunit appeared to react with DTNB [36]. *E. coli* GDH has four cysteine residues/subunit [26,27] and presumably its reactive thiol is not the homologue of the reactive residue in clostridial GDH. The sequence similarity highlighted in Table 1 was nevertheless important in assisting the solution of the crystal structure. Ethyl mercury phosphate was used to obtain a satisfactory heavy-metal derivative in order to solve the phasing problem [37], and two sites of additional electron density, one strong and one weak, were seen. In the absence, at that stage, of a full sequence for the clostridial enzyme, landmarks in the structure were needed to aid in tracing the polypeptide backbone. Similarity in primary sequence between the GDHs of *C. symbiosum* and *E. coli* suggested that the proteins were likely also to have similar folded structures. Furthermore it pinpointed where in the folded structure the two mercury atoms might bind. This facilitated fitting of the medium-resolution electron density to amino acid sequence, with consensus sequence for other members of the GDH family being employed wherever authentic clostridial sequence was not yet known [7].

The crystallographic solution now in turn explains the basis of the chemical results reported here. One of the two cysteine residues, Cys-320 [10] (see peptide 2, Table 1), corresponding to the stronger of the two mercury-binding sites [37], is in a loop joining the secondary structure elements β_k and α_{13} [8]. It is in the active-site cleft, on the surface of Domain 2 (which binds the coenzyme [8]), and it is obvious from the location of this residue that, even though it is not directly involved in binding, a bulky substituent would prevent coenzyme docking. Conversely this also explains why specifically the coenzyme(s) should protect activity completely, whereas glutamate and 2-oxoglutarate in the absence of coenzyme give no protection. This situation is similar to that of the lactate dehydrogenases [38] where the much-studied conserved essential thiol group was ultimately revealed by crystallography not to be a central part of the catalytic machinery.

The other cysteine residue (peptide 1, Table 1), Cys-144 [10], is in helix α_7 , in the substrate-binding domain [8], but is remote from the active site and somewhat less accessible from the surrounding solution than Cys-320, in keeping with the observed results. In view of the possibility of clean modification of Cys-320, this residue clearly has potential as a labelling site, especially if less bulky substituents can be introduced. The protection studies shown in Table 3 and Figure 2 also provide a useful addition to the pyridoxal 5'-phosphate system [19] as a simple method of monitoring the binding of active-site ligands. The results with combinations of substrates suggest that there is mutual enhancement of binding of NADH and glutamate in an abortive complex. This has been observed for GDH from other species. Interestingly, however, the data for protection against *p*CMBS appear to indicate mutual antagonism in binding

between the correct productive partners NADH and 2-oxo-glutarate. Logically, an altered mode of NADH binding that no longer screened Cys-320 would provide an alternative explanation, but structurally this appears implausible.

The value of 0.69 mM for the dissociation constant of the GDH–NAD⁺ binary complex obtained from Figure 3(a) is more than two orders of magnitude larger than the value of 2.7 μ M obtained from rapid-reaction kinetic analysis of the formation of the ternary complex [12]. It seems clear that the protection studies measure binding of NAD⁺ at the correct coenzyme site. Whether, however, they measure binding to catalytically competent subunits or perhaps to a subset that are temporarily in an inactive conformation is less certain.

The protection data for NADH, on the other hand, cannot be interpreted in terms of a single dissociation constant, and are suggestive of weakly cooperative binding of the coenzyme. This again poses a puzzle, because such cooperativity has not so far been detected in steady-state studies [11], and it is the rate behaviour as a function of NAD⁺ concentration, rather than NADH, that shows obvious departure from typical Michaelis–Menten behaviour. Discovering the basis of the mismatch between the binding studies reported here and the available kinetic information may be the key to a fuller understanding of the GDH mechanism.

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