The crystal structure of glutamate dehydrogenase from *Clostridium symbiosum* at 0.6 nm resolution

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The structure of glutamate dehydrogenase from *Clostridium symbiosum* has been solved by single-crystal X-ray-diffraction studies at 0.6 nm resolution by using a combination of isomorphous replacement and molecular averaging. The electron-density map reveals that this glutamate dehydrogenase is a hexameric oligomer, arranged in 32 symmetry, of cylindrical appearance and dimensions, of length 10.8 nm and radius 4.4 nm. From an analysis of this map each subunit appears to contain some 55% α -helix and is organized into two distinct globular domains separated by a deep cleft. The subunits associate using the domain closest to the 32-symmetry point, making intimate contacts around the threefold and twofold interfaces. The second domain shows structural homology to the NAD-binding domain of other dehydrogenases, and difference Fourier analysis has shown that the NAD is bound in both a structurally equivalent position and a similar conformation to that observed for those related enzymes.

INTRODUCTION

Glutamate dehydrogenases (GDHs) (EC 1.4.1.2–4), as a class, catalyse the oxidative deamination of L-glutamate to 2-oxoglutarate and ammonia (Frieden, 1963). The reversible reaction occurs at the expense of the reduction of one molecule of NAD(P)⁺:

GDHs fall into two metabolic categories, being involved in either ammonia assimilation (generally NADP⁺-dependent) or glutamate catabolism (generally NAD⁺-dependent), with GDHs in vertebrates being an exception to this in that they can utilize both coenzymes with similar efficiency (Smith *et al.*, 1975). The enzyme has been isolated from very diverse sources and has been the subject of extensive investigation by a wide variety of techniques (see Goldin & Frieden, 1971, and references therein). Commonly found properties of GDHs include reversible polymerization and activation–inactivation phenomena, which are both pH-dependent and also affected by the presence of small ligands (Gore, 1981). The vertebrate GDHs, in particular, have also been shown to exhibit complex allosteric properties, being exquisitely sensitive to the concentration of purine nucleotides. However, it has proved impossible to crystallize animal GDHs in a form suitable for X-ray analysis (Birktoft *et al.*, 1979a,b), and so attention has shifted to the enzyme from bacterial sources. This work has been encouraged by amino acid sequence studies on both bacterial and fungal GDHs, which have detected significant homology across the family of GDHs from widely different species (Kinnaird & Fincham, 1983; Wootton, 1974; McPherson & Wootton, 1983). Thus it was hoped that the properties of the mammalian enzyme

could be modelled from the structure of a related bacterial GDH.

A further major point of interest in the study of GDH would be the discovery of the exact nature of the relationship between its mode of nucleotide binding and that of the related dehydrogenases. For the latter there has been great interest in the nature of the structural motif that binds the NAD molecule. Here, a structural arrangement of six strands of parallel β -sheet, with a common strand order, flanked by α -helices, has been seen in the structure of many of the dehydrogenases so far elucidated: lactate dehydrogenase (Adams *et al.*, 1970), malate dehydrogenase (Hill *et al.*, 1972), alcohol dehydrogenase (Eklund *et al.*, 1976) and glyceraldehyde-3-phosphate dehydrogenase (Buehner *et al.*, 1974). Although sequence comparisons (Wootton, 1974; Rossmann, 1975) have indicated the presence of such a fold in GDH, confirmation of its existence could only come from a complete three-dimensional structure determination, since the observed sequence homology is low. The crystallization of an NAD⁺-dependent GDH (EC 1.4.1.3) from *Clostridium symbiosum* has recently been reported (Rice *et al.*, 1985), and the present paper details the solution of the structure of this enzyme to 0.6 nm resolution by X-ray diffraction and a preliminary analysis of the results.

EXPERIMENTAL

X-ray data collection and isomorphous phasing

Crystals of GDH grown from 35% -satd. (NH₄)₂SO₄ (BDH Chemicals, Poole, Dorset, U.K.) solutions are in the monoclinic space group C2, with three subunits (a half-molecule) in the asymmetric unit. For convenience, the cell dimensions have been redefined compared with those published earlier (Rice *et al.*, 1985) to give

Abbreviation used: GDH, glutamate dehydrogenase.

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Table 1. Data-processing statistics

$$R_m = \Sigma_{hkl} \Sigma_{i=1}^p |I_i - \bar{I}| / \Sigma_{hkl} n \bar{I}$$

Data set	Total no. of measurements	No. of independent reflexions	R_m (%)	Mean fractional isomorphous difference (%)
Native	4613	3805	3.0	—
Ethyl mercury phosphate	7281	5216	3.9	16.6
Dimercury acetate	9583	6685	6.4	19.0
NAD ⁺	3735	3507	7.6	14.2

parameters $a = 14.71$ nm, $b = 15.13$ nm and $c = 9.45$ nm, $\beta = 132.75$. Data for the native crystals and two potential heavy-atom derivatives, produced by equilibrating crystals in a solution containing either 0.5 mM-ethyl mercury phosphate or 0.5 mM-dimercury acetate, were collected to 0.6 nm resolution by using Ni-filtered Cu K_α -radiation on the modified Hilger and Watts multicounter 5 circle X-ray diffractometer in the Laboratory of Molecular Biophysics, University of Oxford, Oxford, U.K. In order to shorten the data-collection time only a single equivalent of data, plus scaling overlaps, was collected for the native enzyme and two equivalents for the heavy-atom derivatives to provide estimates of the anomalous scattering. Pertinent data-processing statistics are provided in Table 1.

Since the asymmetric unit of the cell was known to contain three subunits, it was expected that the difference Patterson function would be solved by $3n$ sites and hence could potentially be quite complex. In practice, the solution of the Patterson function was straightforward owing to the apparent presence in this enzyme of a single reactive thiol group forming a covalent derivative with either of the mercury compounds. Both derivatives produced the same solution to the Patterson function in which a clear threefold relationship between the mercury-binding sites was immediately obvious. Only small differences in heavy-atom parameters were found for the two derivatives, which were refined by using the F_{HLE} method (Dodson & Vijayan, 1971). Both derivatives were used in the calculation of the protein phases

by using the method of Blow & Crick (1959), incorporating contributions from anomalous scattering (North, 1965). The mean figure of merit for the phases to 0.6 nm was 0.62, and a list of heavy-atom parameters is given in Table 2.

Molecular averaging

An analysis of the resultant isomorphous map provided good evidence for the structural similarity of the three subunits in the asymmetric unit, which was further confirmed by using the Crowther (1972) fast-rotation function on the strongest 1450 data from 1.0 nm to 0.6 nm. This yielded a peak corresponding to a threefold rotation with a peak height equal to 82% of the origin. The isomorphous map was then averaged without a molecular boundary, about the non-crystallographic three fold axis, by using the SKEWPLANES procedure of Bricogne (1976), implemented to run on the VAX 11/780 at the M.R.C. Laboratory of Molecular Biology, Cambridge, U.K. Inspection of the averaged map allowed the determination of the molecular boundary, and subsequently the averaging process was repeated to include this envelope. The averaged map was then back-transformed to provide structure factors and phases, which were then recombined with the isomorphous phase information to produce a new map. This whole procedure was repeated for a total of ten cycles, after which the mean figure of merit for the phase set was 0.81. The final electron-density map was produced in the molecular frame and traced on to clear plastic sheets.

Table 2. Heavy-atom parameters

$$R_{F_{HLE}} = \frac{\Sigma |F_{HLE}| - |F_{Hcalc.}|}{\Sigma |F_{HLE}|}$$

F_{HLE} is the lower estimate of the heavy-atom structure factor F_H . E is the root-mean-square lack of closure for all reflexions from 'best' phases.

Derivative	Site	Relative occupancy	X	Y	Z	$R_{F_{HLE}}$	Root-mean-square $F_{Hcalc.}/E$
Ethyl mercury phosphate	1	0.2500	0.1729	0*	0.3279	44.1	2.3
	2	0.2065	0.1996	-0.2382	0.8231		
	3	0.2362	0.1993	0.1388	0.9154		
Dimercury acetate	1	0.2677	0.1721	0*	0.3255	46.8	1.9
	2	0.2046	0.2013	-0.2364	0.8283		
	3	0.2362	0.1960	0.1386	0.9078		

* Not refined.

Fig. 1. Portion of the electron-density map for GDH

The phases derived from the combination of isomorphous replacement and molecular averaging were used to compute a Fourier map at 0.6 nm resolution. This map was averaged about the non-crystallographic threefold axis and plotted in the molecular frame. Sections 15/54 to 19/54 of this map are shown. The long rod of density dominating the view is a segment of helix approx. 4.0 nm in length.

RESULTS AND DISCUSSION

Interpretation of the electron-density map

The electron-density map, derived by a combination of isomorphous replacement and molecular averaging, was of exceptional quality, as can be seen from Fig. 1. This map was readily interpretable in terms of a hexameric molecule of cylindrical appearance and of overall dimensions length 10.8 nm and radius 4.4 nm, with the threefold axis running along the length of the cylinder. With the exception of only a small section of the chain (probably less than 5%), the electron density could be unambiguously assigned to the individual subunits. In order to visualize the overall organization of the molecular structure, two models were constructed, one following the detailed shape traced by the electron density of a single subunit and the other representing the approximate molecular surface of the hexamer as defined by the boundary of the electron density. The first of these is shown in Fig. 2, and a clear definition of the molecular structure can be seen. Particularly well defined are the α -helical segments of secondary structure, which are characterized by long rods of electron density at this resolution. In all a minimum of 16 helical segments varying in length from 1.3 to 4.0 nm can be defined, constituting some 240 residues of the protein. On the basis of a molecular-mass estimate of 49000 Da for this GDH (Rice *et al.*, 1985) there must be approximately 445 amino acid residues in the polypeptide chain, and thus residues in helical conformation account for at least 55% of the protein structure. Furthermore, in many places strong connectivity could be seen between elements of secondary structure. One such region, which lies on the

Fig. 2. Model of a single GDH subunit

From the electron density attributable to a single subunit of GDH a plywood model was constructed. At this resolution α -helical segments of the protein appear as long rods, and in all some 16 helical segments can be located on the model. Two views are depicted, showing (a) the complete subunit in which the division of the molecule into two domains separated by a deep cleft can be seen, and (b) a region of the molecule where several connected α -helices are visible. The helix on the left is thought to be one of the chain termini because of the abrupt end to the electron density and the lack of a piece of unconnected structure nearby.

surface of the molecule, is shown in Fig. 2(b). Elements of connected helical structure are very evident in this view and it can be seen that the electron density terminates abruptly at the end of one of the helices. Inspection of the structure behind this particular helix shows it to be composed of a tightly packed bundle of connected helices, and therefore the passage of the chain through this region is precluded. Since no other unconnected electron density can be found close to this helix, we presume this to be either one of the chain termini or, alternatively, the end of the ordered region of the polypeptide chain that either follows or precedes the *N*- or the *C*-terminus respectively.

Domain structure and function

The model of the subunit presented in Fig. 2(a) shows that the polypeptide chain of GDH is folded into two distinct globular domains (A and B), separated by a deep cleft, with domain A being some 50% larger than domain B. Studies of other enzymes have shown that enzyme active sites are commonly situated in such clefts in the molecular surface, and thus the presence of this structural feature in GDH strongly suggests that the active site lies between these two domains. The domain interface is dominated by three α -helices, which are 3.0, 4.0 and 4.5 nm in length. Together these form the base of the cleft and provide a structure on to which the two domains are assembled.

An analysis of the electron density showed that the inter-subunit contacts made by these two domains were substantially different. The upper domain in Fig. 2(a), referred to below as domain A, lies closest to the 32-symmetry point and makes extensive contacts with its

symmetry-related equivalents across both the molecular threefold and twofold axes. Some 20% of its exposed surface is buried in the interaction across the twofold interface between the trimers and 40% is buried in the interaction between the threefold related subunits. Domain B, on the other hand, makes no contacts across the twofold axis and very little contact across the threefold axis. Thus the organization of the molecule into its 32 symmetry appears to be a property of only one of the two domains. A picture of the model showing the overall shape of the GDH hexamer is illustrated in Fig. 3. The cylindrical appearance of the molecule, the deep cleft between the two domains of a subunit and the close contact between the domains nearest to the 32-symmetry point are very apparent.

The lower domain in Fig. 2(a), referred to below as domain B, consists of five α -helices arranged in groups of two and three. The closest approach of the two groups of helices is some 2.0 nm, a distance that is too far to allow any molecular contact, and between these two helical groups diffuse density in the map, representing an intervening molecular structure, could be discerned. This distance is exactly that required in a structure that consists of two groups of helices separated by a β -sheet, and such a sheet would indeed appear as diffuse density at this resolution. Thus a tentative assignment to a region of β -sheet has been made for this density. If we assume that the β -sheet observed here is parallel, then the domain has a structure that is highly reminiscent of the nucleotide-binding domain of the dehydrogenases, although a high-resolution structure would be necessary to confirm the exact nature of this relationship. Taking this to be the case, the approximate location of the

Fig. 3. Quaternary structure of GDH

The overall organization of the GDH hexamer is illustrated in (a) by a molecular model built to represent the approximate molecular surface of the enzyme as defined by the boundary of the electron density. (b) is a schematic diagram in the same orientation that highlights the relative arrangement of the two domains of each subunit with respect to the assembled hexamer. The NAD moiety is coloured white in the model and shown as a hatched region in the schematic. The subunits are seen to come close together, making contacts around the 32-symmetry point that are primarily provided by only one of the two domains. The closest approach of the NAD-binding sites is some 5 nm.

NAD-binding site can be found from the position and orientation of the helices, since this site occurs in an equivalent position across the dehydrogenase family of enzymes having this structure. Thus the nicotinamide ring would lie deep into the cleft, on the upper surface of domain B, facing domain A.

NAD binding and location of the active site

Following the tentative assignment of domain B to the NAD-binding function, crystals of GDH were equilibrated in 10 mM-NAD⁺ for 18 h. These crystals were then transferred to the 5 circle diffractometer, and a single equivalent of data was collected to 0.6 nm resolution (see Table 1). After data reduction a difference Fourier was calculated between the data for the native and NAD⁺-soaked crystals, and the resultant map clearly showed a single large peak of electron density, associated with each subunit. Furthermore, the peak position corresponded exactly to that predicted for NAD binding on the basis of comparisons with the related dehydrogenases, and this density has been assigned to the bound NAD. The difference map also indicated that the binding of NAD had not been accompanied by any large-scale conformational changes of the enzyme.

This map was transferred to the Evans and Sutherland PS300 graphics, and an attempt was made to fit the NAD electron density, by using the model building program FRODO (Jones, 1978), with co-ordinates of NAD taken from substrate binding to lactate dehydrogenase (Grau *et al.*, 1981) and glyceraldehyde-3-phosphate dehydrogenase (Moras *et al.*, 1975). The NAD conformations in these two enzymes are very similar, with the exception of the nicotinamide ring, which is anti or syn to the ribose ring in lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase respectively. It was found that an excellent fit to the electron density could be achieved simply by translating either of these NAD moieties into position, and it was not necessary to rotate about any of the free bonds to alter the NAD torsion angles. Since the density can be fitted with the NAD with the nicotinamide ring in either the syn or anti conformation, the definite location of the active site remains unresolved. This arises from an inability to decide which is the B face of the

nicotinamide ring from which the proton is transferred or added during the catalytic cycle (You, 1985). However, the general picture that has emerged from the study of related dehydrogenases indicates that the active site is located in a spatially equivalent position with respect to the NAD-binding site. In order to preserve the A- or B-type stereospecificity of the reaction, the nicotinamide ring is maintained in the appropriate conformation so as to orient the correct face of the nicotinamide towards the active site (You, 1985). Although structural studies so far include three related A-type dehydrogenases, namely lactate dehydrogenase, alcohol dehydrogenase and malate dehydrogenase, the structure of only a single B-type enzyme, glyceraldehyde-3-phosphate dehydrogenase, is known, and, although this fits the pattern of preserving the active site by altering the NAD conformation, we must necessarily be cautious of extending this analysis to include GDH. Nevertheless, the fit of the NAD from glyceraldehyde-3-phosphate dehydrogenase, shown in Fig. 4, to the density from GDH is marginally better, and this would be consistent with the B-type stereospecificity of this enzyme. This would then predict a binding site for the second substrate in GDH deep into the cleft and surrounded by a cluster of helices, as shown in Fig. 5.

Relationship to other GDHs

The importance of this work on *C. symbiosum* GDH depends, to some extent at least, on the degree to which this particular structure can be related to the work on the GDH from other sources, and particularly the mammalian GDHs. Although the sequence-homology studies on the *Escherichia coli* and *Neurospora crassa* NADP⁺-dependent GDHs indicate strong homologies with the bovine enzyme and thereby imply the existence of strong structural relationships, the absence of a known sequence for the *C. symbiosum* enzyme as yet precludes such a definite conclusion.

A wide variety of models for the quaternary structure of bovine GDH, which is also hexameric, have been proposed on the basis of both low-angle X-ray scattering

Fig. 4. Interpretation of the NAD difference map

The density attributed to the bound NAD in the NAD difference map was fitted simply by translating the co-ordinates of NAD⁺ taken from glyceraldehyde-3-phosphate dehydrogenase (Moras *et al.*, 1975). The good agreement between the co-ordinates and the electron density is taken as indicating a very closely related conformation for bound NAD in these two B-type dehydrogenases.

Fig. 5. Location of the GDH active site

A view of the cleft between the two domains in GDH is shown including the bound NAD moiety (darker colour). The probable location of the active site deduced from the fitted position of the nicotinamide ring and a knowledge of the stereospecificity of the hydrogen-transfer reaction is shown as a white dot.

Fig. 6. Comparison of bovine liver and clostridial GDHs

An electron micrograph of bovine liver GDH looking down the molecular threefold axis of symmetry (b) [reprinted with permission from A. M. Fiskin *et al.* (Biochemistry 10, 2396–2408) copyright (1971) American Chemical Society] is compared with the model representing the molecular surface of the *C. symbiosum* enzyme (a). A close relationship between these two structures is very evident.

(Sund *et al.*, 1969) and electron-microscopic studies (see Fiskin *et al.*, 1971, and references cited therein). These imply that the GDH hexamer is organized in 32 symmetry and that the enzyme subunit is divided into two domains, one of which is larger than the other. These observations are fully consistent with our X-ray model of the *C. symbiosum* enzyme. Furthermore these models also predict the presence of a cavity at the centre of the molecular assembly, in excellent agreement with our structure, which has a cavity of about 1.0–1.5 nm diameter centred on the 32-symmetry point. This structural similarity is further indicated by comparisons of the electron micrographs for bovine GDH and our X-ray structure. In Fig. 6 an electron micrograph, presumed to be down the three-fold axis of bovine GDH, is compared with the view down the molecular threefold axis on the surface model of the *C. symbiosum* enzyme. The overall impression from these two photographs is one of considerable structural similarity, in terms of both size and appearance, and the description of the structure by Fiskin *et al.* (1971) as having a propeller shape with three broad blades is clearly defined in the X-ray structure. The only differences indicated by the electron-microscopy work are based on investigations of the distribution of mass in bovine GDH from electron images of tilted specimens (Fiskin *et al.*, 1975). These suggest a particle very similar to ours in terms of size, but having a slightly more isometric appearance, being wider at the mid-plane separating the two trimers. It is our conclusion then that the excellent agreement between the models in terms of the domain structure, the symmetrical organization of the subunits and the overall appearance of the electron-microscopy pictures points to a strongly related structure. However, we must await the arrival of information on the amino acid sequence of *C. symbiosum* GDH to confirm that the residues involved in tight packing around the 32-symmetry point are conserved in the bovine liver enzyme.

It is our belief that the structural work on this bacterial GDH should provide a firm framework around which many of the molecular properties of this much-studied enzyme may be explained. The division of

this molecule into two distinct domains and the separation of the function of self-assembly and NAD binding to these has already added much to our understanding of the overall relationship between structure and function in GDH. Furthermore, the preliminary indications of the similar mode of NAD⁺ binding between this enzyme and the other dehydrogenases should provide a useful source of information on the evolutionary relationship between members of this class of enzymes.

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