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ARTICLE

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Alteration of the quaternary structure of glutamate dehydrogenase from *Clostridium symbiosum* by a single mutation distant from the subunit interfaces

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Abstract X-ray crystallographic studies have previously shown that glutamate dehydrogenase from *Clostridium symbiosum* is a homohexamer. Mutation of the active-site aspartate-165 to histidine causes an alteration in the structural properties of the enzyme. The mutant enzyme, D165H exists predominantly as a single species of lower molecular mass than the wild-type enzyme as indicated by gel filtration and sedimentation velocity analysis. The latter technique gives an $s_{20,w}$ value for D165H of (6.07 ± 0.01)S which compares with (11.08 ± 0.01)S for the wild-type, indicative of alteration of the homohexameric quaternary structure of the native enzyme to a dimeric form, a result confirmed by sedimentation equilibrium experiments. Further support for this is provided by chemical modification by Ellman's reagent of cysteine-144 in the mutant, a residue which is buried at the dimer-dimer interface in the wild-type enzyme and is normally inaccessible to modification. The results suggest a possible structural route for communication between the active sites and subunit interfaces which may be important for relaying signals between subunits in allosteric regulation of the enzyme.

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Introduction

Glutamate dehydrogenases (GDH; E.C. 1.4.1.2–4) are all homohexameric enzymes with the exception of the NAD^+-dependent GDHs of fungi which are tetrameric (Smith et al. 1975; Britton et al. 1992). The homohexameric GDHs have subunits (with M_r ranging from 47 000 to 55 000) which each comprise a coenzyme binding and a substrate binding domain. The crystal structure of the apoenzyme from *Clostridium symbiosum* displays a large cleft between these two domains. Determination of X-ray structures of wild-type (Stillman et al. 1993) and mutant enzymes (Dr M. Waugh, personal communication) co-crystallized with either L-glutamate or NAD^+ respectively has revealed that GDH subunits display extensive conformational flexibility and suggests that in the conformation adopted during the hydride ion transfer step in the enzyme's catalytic cycle the cleft is completely closed. The conformational change between the catalytically inactive open forms and the catalytically active closed forms of the enzyme can be approximated to a rigid body motion in which the coenzyme binding domain undergoes a 25° hinge closure relative to the substrate binding domain. The movement of the two domains relative to each other is achieved through changes in torsion angles in the hinge region (which comprises amino acids 200–379 and 391–434).

At this point then, through the use of high-resolution crystallography and site-directed mutagenesis we are beginning to shed light on structure/function relationships in GDH. However, aside from catalysing a metabolically fundamental reaction, GDHs have been the subject of intensive study, owing to their interesting allosteric regulation by homotropic and or heterotropic effectors (Goldin and Frieden 1971; Smith et al. 1975). Wang and Engel (1995)

have recently shown that the 'model GDH' from *C. symbiosum* used in our protein engineering program, in addition to displaying negative cooperativity with NAD^+ (as for the bovine enzyme (Dalziel and Engel 1968; Syed et al. 1991) also exhibits strong positive cooperativity with the substrate, L-glutamate, with a Hill coefficient of 6.0.

This paper describes a structural change induced by substitution of aspartate-165 a residue in the L-glutamate binding site, with His, and the implications this has for our current understanding of the structural basis for allostery in GDH. This mutant was originally created in an attempt to test the hypothesis that Asp-165 functions as a general base in catalysis (Stillman et al. 1993) by replacement with an alternative base.

Experimental

Site-directed mutagenesis of GDH

The same strategy for recombinant DNA manipulations was used as described for the construction of D165S-*gdh* (Dean et al. 1994). This strategy involved mutagenesis according to Kunkel et al. (1987) of an M13mp19-*gdh* construct, which was then subcloned into the expression vector ptac-85 (Marsh 1986) and finally subcloned back into M13mp19. The last step facilitated sequencing of the entire *gdh* gene (Sanger et al. 1977) and verified that no undesired secondary mutation had been introduced during DNA manipulations. The oligonucleotide used in mutagenesis was 5′-TCCTGCAGGT**CAC**CTTGGTGTAG-3′, where the codon shown in boldface (coding for histidine) replaced the wild-type GAC codon (coding for aspartate). The host strain *E. coli* TG1 (*Δlac-proAB supE thi hsdΔ5/F′ traD36 proA + B + lacI^{q}Z ΔM15*), containing a functional *E. coli gdh* gene, was used throughout DNA manipulation and mutant protein expression, minimising selection for wild-type revertants during cell culture. Note that the enzyme purification procedure used readily removes the $NADP^+$-dependent GDH of the *E. coli* host (Dean et al. 1994).

Enzyme preparation

E. coli TG1 transformants separately harbouring wild-type and D165H expression constructs were cultured for 16 hr in LB broth supplemented with 100 μg ml^{-1} ampicillin and 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside). The cells were then harvested by centrifugation, sonicated, and the resulting extract clarified by centrifugation at 27 000 g for 30 min. D165H and wild-type enzymes were purified to homogeneity (as judged by 12% SDS-PAGE gels stained with Coomassie Brilliant Blue R-250) on Remazol Brilliant Red GG Sepharose CL-6B according to Syed et al. (1991). Purified enzymes were stored in 70% ammonium sulphate at 4 °C. They were dialysed before use against three changes of 0.1 M potassium phosphate, pH 7.0 and clarified by centrifugation.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to Garfin (1990) using the discontinuous system of Laemmli (1970) which utilises Tris-glycine buffering and includes stacking gels to increase band resolution. For native-PAGE 7.5% cross-linked stacking and running gels were used. Samples were prepared in low-conductivity buffer (60 mM Tris-Cl, pH 6.8) and were loaded between the higher conductivity electrode (25 mM Tris, 192 mM glycine, pH 8.3) and stacking gel (125 mM Tris-Cl, pH 6.8). The resolving gel was buffered with 375 mM Tris-Cl, pH 8.8.

For SDS-PAGE 7.5% stacking and 10–12% running gels were used. 5% 2-mercaptoethanol (in sample buffer) and SDS (1% in sample buffer and 0.1% in all other buffers) were included for SDS-PAGE. Electrophoresis was performed using 8×12 cm minigels (Hoefer, Newcastle under Lyme, UK) at 20 mA constant current (per gel) for native-PAGE and approx. 50 mA constant current (per gel) for SDS-PAGE. Gels were stained for 20 min in a solution of 0.1% Coomassie Brilliant Blue R-250 (w/v) (Sigma), 40% methanol (v/v) and 10% acetic acid (v/v). Gels were destained with 40% methanol (v/v) and 10% acetic acid (v/v) for approx. 2 hrs with frequent changes of destaining solution.

Gel filtration

Gel filtration was performed on a Superose-6 pre-packed column (Pharmacia, UK) using a Pharmacia FPLC system. The buffer used was 50 mM potassium phosphate, 150 mM NaCl. Purified wild-type and D165H enzymes were applied separately to the Superose 6 column equilibrated with 0.1 M potassium phosphate, 0.15 M NaCl (the same buffer was also used during running). 200 μl samples of 0.28 mg ml^{-1} protein were injected onto the column. The flow rate used was 0.5 ml min^{-1}. The eluted protein was monitored by an absorbance detector set at 280 nm.

Analytical ultracentrifugation

For the ultracentrifuge experiments the Beckman Optima XL-A (Beckman, Spinco Division) analytical ultracentrifuge equipped with scanning absorption optics was used. The sedimentation velocity experiment was carried out at 25,000 rev./min and 20 °C whereas the sedimentation equilibrium experiment was performed at 7,500 rev./min at the same temperature using 6-channel KEL-F centrepieces. The sedimentation coefficient as well as the sedimentation equilibrium data were evaluated from the absorption traces of the Optima XL-A, scanning at 280 nm. The sedimentation coefficient was evaluated at least three times to min-

imize the errors resulting from the graphical evaluation. The sedimentation coefficient in the buffer (0.1 M potassium phosphate), $s_{T,b}$, was corrected to that at 20 °C in water, $s_{20,w}$, using the following formula (Tanford 1961):

$$s_{20,w} = \left(\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{T,b}}\right)\left(\frac{\eta_{T,b}}{\eta_{20,w}}\right)s_{T,b}$$

where $\bar{v}$ = partial specific volume of the polymer, ρ = density, η = viscosity and s = sedimentation coefficient with the indices w = water, b = buffer and T = temperature. The partial specific volume of the protein was calculated from the amino acid composition (Teller et al. 1992) of the biopolymer, giving $\bar{v} = 0.736$ ml/g. The molar mass of the monomer was calculated from the amino acid sequence to be 49,315 g/mol. A loading concentration of 1.5 mg/ml was used for sedimentation velocity and we make the assumption $s_{20,w} \approx s^0_{20,w}$.

Sedimentation equilibrium experiments were performed with GDH loading concentrations of 1.5 and 0.5 mg/ml. For the evaluation of the sedimentation equilibrium data the MSTARA program was used (Harding et al. 1992; Cölfen and Harding 1997). This provides amongst other information an estimate of the molar mass independent of the behaviour of any expected model. As an additional independent check, the equilibrium data were evaluated by fitting them to a model of a single ideal sedimenting species using the routine provided by Beckman (McRorie and Volker 1993). This latter routine performs a linear regression analysis *over a selected dataset* within the equilibrium distribution: for an ideal single system this leads to no overestimate or underestimate for the whole equilibrium distribution between cell meniscus and base.

The buffer density was determined to be 1.00792 g/ml at 20 °C using a precision density meter (Anton paar DMA 02C) according to the method of Kratky et al. (1973). For the determination of the buffer viscosity at 20 °C, an automatic viscometer (Schott Geräte AVS 310) was used. The viscosity of the buffer was determined to be 1.0065. For the density as well as for the viscosity 10 consistent readings were taken to minimize the experimental error.

Modification with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)

The stoichiometry of modification by Ellman's reagent (Ellman 1959) was determined by monitoring the increase in A_{412} at 25 °C in incubations of 0.2 mg ml^{-1} enzyme with 1 mM DTNB in 0.1 M potassium phosphate buffer, pH 7.0.

Enzyme assays

Rates were measured spectrophotometrically by recording A_{340} in standard two-substrate and three-substrate assays at 25 °C. Activity in the two-substrate reaction was determined with 1 mM NAD^+ and 40 mM L-glutamate in 0.1 M potassium phosphate buffer, pH 7.0. Reaction mixtures in three-substrate assays contained 0.1 mM NADH, 50 mM ammonium chloride, 5 mM 2-oxoglutarate and 0.1 M potassium phosphate, pH 7.0.

Results

SDS-PAGE of crude extracts and purified wild-type and D165H prepared from transformants showed comparable expression levels when cultured in LB broth with 100 μg ml^{-1} ampicillin and 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside); (Fig. 1a, lanes 1 and 2). The mutant enzyme exhibited identical mobility to wild-type, and both enzymes were of high purity (Fig. 1a, lanes 3 and 4). It is noteworthy that the mutant enzyme was satisfactorily purified by the highly selective dye-ligand procedure routinely used for the wild-type enzyme. This chromatographic behaviour has proved in general to be a good indicator of a well-folded mutant.

The activity of wild-type and D165H was determined in standard two-substrate and three-substrate assays. For the forward reaction the activity with 1 mM NAD^+ and 40 mM L-glutamate, 0.1 M potassium phosphate buffer, pH 7.0 was 21.4 U mg^{-1} for wild-type enzyme. The activity of the mutant enzyme was judged to be not more than 6×10^{-4} U mg^{-1} from assays monitoring A_{340} for 10 min. For the reverse reaction assays in 0.1 mM NADH, 50 mM ammonium chloride, 5 mM 2-oxoglutarate and 0.1 M potassium phosphate, pH 7.0 gave activities of 4.9×10^{-3} (D165H) and 108 U mg^{-1} (wild-type). Thus activity in both directions is at least 2×10^4 fold less than that of the wild-type enzyme.

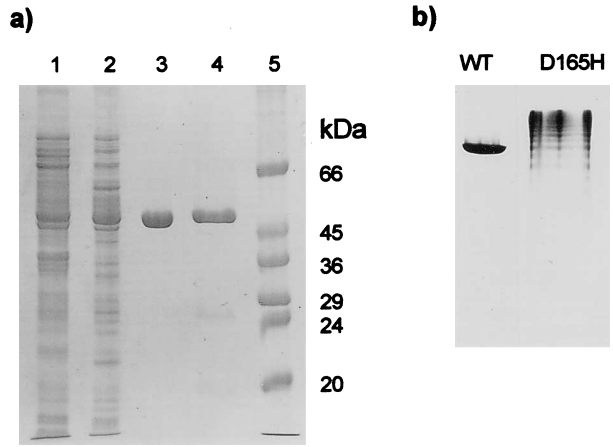

Fig. 1a, b PAGE of wild-type and D165H GDH enzymes and crude extracts. **a** SDS/PAGE on a 12% gel. Lane 1, 10 μl of a 100×dilution of wild-type crude extract; 2, 10 μl of a 100×dilution of D165H mutant crude extract; 3, 10 μl of 0.9 mg ml^{-1} purified wild-type enzyme; 4, 10 μl of 1.0 mg ml^{-1} purified D165H mutant enzyme; 5 molecular mass markers. **b** Non-denaturing PAGE (7.5% gel) of purified wild-type and D165H-GDH. Samples contained 10 μl of 1.0 mg ml^{-1} wild-type or D165H protein. Gels were run and stained as described in Experimental

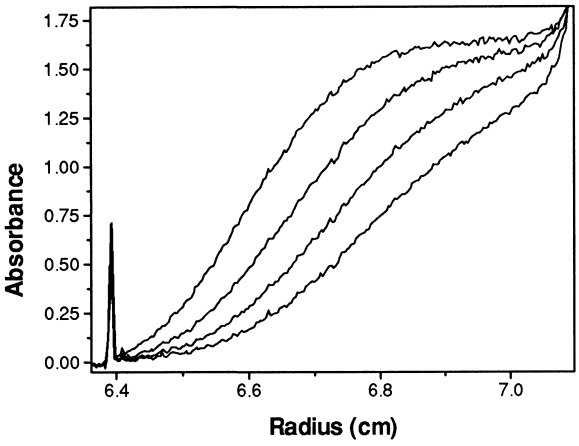

Fig. 2 Sedimentation velocity boundary (recorded using UV absorption optics in the XL-A ultracentrifuge at 280 nm) for D165H 1.5 mg/ml in 0.1 M potassium phosphate buffer at 20 °C and 25 000 rev./min. The time interval between the 4 scans shown above is 40 min. {Many more scans were recorded at intervening time intervals, but for the clarity of the figure only 4 scans are shown}

Two tests used routinely in the laboratory, native-PAGE and the stoichiometry of cysteine modification with DTNB, were employed to check the overall structural integrity of the mutant enzyme. In contrast to the results on SDS-PAGE, under non-denaturing conditions purified wild-type and D165H enzymes show markedly different electrophoresis patterns (Fig. 1b). Wild-type protein migrates as a single, well-defined band and this is indicative of hexamer formation, whereas the mutant profile consists of many diffuse bands and shows extensive smearing. Since the purified mutant protein stains as one major band with Coomassie Brilliant Blue R-250 under denaturing conditions, the numerous bands produced with the same stain under non-denaturing conditions all derive from D165H-GDH. A Laemmli Tris-Cl/glycine buffering system was used for the native gel shown in Fig. 1b. To test the possibility that the mutant enzyme is unstable in these buffer and pH conditions non-denaturing PAGE was also performed with 0.1 M sodium phosphate (pH 7.0) in both gel and running buffer rather than Tris-Cl/glycine. Under these conditions the resulting electrophoresis pattern is very similar (not shown).

DTNB modifies only one of the two cysteine residues per wild-type CsGDH subunit (Cys-320; Syed et al. 1994, Wang and Engel 1994). DTNB treatment of mutant enzme resulted in the release of 1.73 mol of thionitrobenzoate/mol protein subunit after 30 min as indicated by the change in A_{412}. This shows that both Cys-320 and Cys-144 are accessible to modification in this mutant. A clear deviation from the single exponential curve normally obtained for the timecourse of DTNB modification of wild-type enzyme was also observed for D165H (not shown). The new form of the curve suggests that the kinetics are complicated by the modification of more than one group, and the timecourse may reflect two pseudo-first-order rate constants for D165H rather than only for the wild-type-enzyme.

To characterise the mutant protein further Superose 6 gel filtration was performed. Both purified proteins eluted from the column as single symmetrical peaks showing that under these conditions mutant and wild-type exist predominantly (>99%) as single molecular mass species. The R_f values for wild-type enzyme and D165H were 2.15 and 2.42 respectively, indicating that the apparent relative molecular mass of D165H is lower than that of wild-type enzyme. Taking this together with identical mobilities for both enzymes on SDS/PAGE it can be seen that the overall conformation/shape and/or quaternary structure of the mutant protein is significantly altered relative to wild-type.

The quaternary structure of D165H was further investigated using analytical ultracentrifugation. A sedimentation velocity experiment (see Fig. 2) shows a quite well defined boundary, indicating that under the conditions used D165H is a single sedimenting species. Furthermore, it can be seen that the preparation is free of major impurities.

The corrected sedimentation coefficient $s_{20,w}$ for D165H is 6.07 (±0.01)S. For reasonably globular proteins this corresponds to a molar mass of ≈100 000 g/mol. Given a subunit M_r calculated from the amino acid sequence of the mutant of 49 315 this therefore corresponds to a dimeric mutant enzyme (M_r 98 630): this $s_{20,w}$ value compares with a value of (11.08 ± 0.01)S for the wild-type hexameric enzyme and a value of 5.3 S for the deliberately engineered dimer obtained earlier (Pasquo et al. 1996). The $s_{20,w}$ values appear to reasonably fit the classical $s_{20,w} \propto M^{2/3}$ relationship for globular structures, with the dimer more spheroidal: (its $s_{20,w}$ value is higher than the ~5.5 S predicted if both dimer and hexamer were spherical). In the absence of other hydrodynamic data one cannot say much more at the present stage about the conformation of the dimer, other than that it must be reasonably spheroidal and of low aspect ratio.

Sedimentation equilibrium experiments yielded linear plots of ln A (A = absorbance) vs. r^2 typical for an ideal ~monodisperse species. Together with the information from sedimentation velocity that D165H is a single species it is possible to fit the equilibrium concentration profiles to a model for a single ideal species using the routine IDEAL1 (McRorie and Voelker 1993). The apparent weight average molar masses $M_{w,app.}$ obtained at loading concentrations of 0.5 mg/ml and 1.0 mg/ml were (90 000 ± 1000) and 103 000 ± 1000) respectively. The low error reflects the precision, not the accuracy of the determination, since only a selected part of the concentration distributions are analysed. The data were also analysed using the routine MSTARA (Cölfen and Harding 1997), which considers the entire solute distribution in the ultracentrifuge cell, using appropriate extrapolations into the region near the cell base that is beyond optical registration. These values, of (92 500 ± 5000) and (100 000 ± 5000) at respective loading concentrations of 0.5 and 1.0 mg/ml, although of lower precision (because of the extrapolation) confirm the values from IDEAL1.

Discussion

In the initial characterisation of the D165H mutant nondenaturing electrophoresis indicated that replacement of Asp-165 by histidine affects the structural properties of the enzyme, pointing to experiments to discover the nature of the structural perturbation. Gel filtration showed that the mutant exists as a single species of molecular mass lower than wild-type enzyme at a protein concentration of 0.28 mg ml^{-1} in 0.1 M potassium phosphate buffer (pH 7.0), with 0.15 M NaCl. Sedimentation velocity experiments on 0.5 and 1.5 mg ml^{-1} D165H protein performed in 0.1 M potassium phosphate buffer, pH 7.0, again indicated a single species with a molecular mass consistent with a dimeric quaternary structure. Sedimentation equilibrium experiments have confirmed that the D165H mutant exists predominantly as a dimer in 0.1 M potassium phosphate at 20 °C, although there is evidence for some possible further dissociation to monomers at the lower protein concentration used.

DTNB modification of 0.2 mg ml^{-1} enzyme in 0.1 M potassium phosphate buffer, pH 7.0 implies that both cysteines in the mutant (Cys-144 and 320) are now accessible to modification by this reagent (unlike the native wild-type enzyme in which only Cys-320 is modified (Syed et al. 1994)). Accessibility of Cys-144 could easily arise through a local structural perturbation in the hexameric protein in the vicinity of this residue. However, given the close proximity of Cys-144 to the dimer-dimer subunit interface (Fig. 3), the observed stoichiometry is fully consistent with the D165H mutant existing predominantly as a dimer under these conditions. In the mutant F187D, a dimer deliberately created by destabilising the subunit interfaces, both cysteines are also accessible (Pasquo et al. 1996). Taken together, the different techniques are all consistent with a dimeric structure of the mutant occurring over a range of enzyme concentrations in 0.1 M potassium phosphate buffer. The gel filtration experiment also suggests that the quaternary structure is unaffected by the addition of 0.15 M NaCl.

A possible reason for failure of the hexamer to assemble fully following mutation of Asp to His may be the close proximity of the histidine side chain to the side chains of Arg-93 and Lys-125. In the structure of the wild-type enzyme with glutamate bound (Stillman et al. 1993) Arg-93 makes two hydrogen bonds with one of the carboxyl oxygen atoms of Asp-165 of length 3.1 and 3.2 Å. Lys-125 also makes a 3.0 Å hydrogen bond with the other carboxyl oxygen of Asp-165. Modelling a histidyl side chain at position 165 showed that in both of the two most appropriate orientations to accommodate this side chain there are distances as low as 2.5 Å between an imidazole nitrogen atom and a nitrogen atom from either Arg-93 or Lys-125. Given a van der Waals radius of 1.55 Å for nitrogen, this indicates a steric clash, and suggests at least some perturbation in the mutant compared with the native structure. In addition, replacement of Asp-165 by histidine could prevent adequate solvation of charge on Lys-125 and Arg-93, giving rise to electrostatic repulsion between these two residues, resulting in active-site perturbation. Further structural rearrangements could then be transmitted to the dimer-dimer subunit interface which lies close to the catalytic pocket.

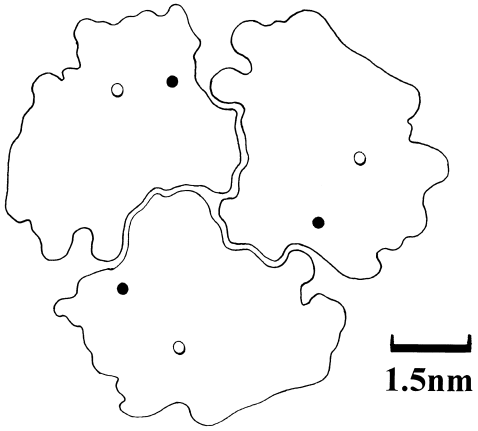

Fig. 3 Schematic diagram showing the proximity of Cys-144 to the dimer-dimer interface. The side chain SH group of Cys-144 (*filled circles*) which becomes accessible to modification by DTNB in the D165H mutant is shown in each subunit lying close to the dimer-dimer interface. The side chain SH group of the other cysteine residue which is accessible to DTNB modification in both wild-type and D165H (Cys-320) is shown near the centre of each subunit (*open circles*)

The replacement of Asp-165 for histidine causes a substantial reduction in catalytic activity (at least 10^4-fold), and it is possible that the residual activity may in fact represent a very small amount of wild-type enzyme introduced by misreading errors during translation as seen for other mutants of *Cs*GDH (Wang et al. 1995). Given the important role of Asp-165 in catalysis (Dean et al. 1994; Dean 1996) the large observed decrease in activity upon mutation to histidine is not surprising. Unfortunately, in view of this fact no further light can be shed on the question of whether GDH can display activity in dimeric, rather than hexameric form.

As pointed out earlier, despite a detailed description of the conformational changes required for catalysis to occur, very little is known about the structural basis for allosteric regulation. Clearly for homotropic allosteric regulation to occur the substrate binding event must be relayed to other binding sites via the subunit interfaces. However, examination of X-ray structures of GDH has not yet revealed the molecular mechanism by which conformational changes are transmitted between the active sites of subunits across the dimer-dimer or trimer-trimer interfaces.

The outcome of mutation of Asp-165 to histidine is a quaternary structural change. Although this is clearly a more radical change than the normal allosteric transition in a wild-type hexamer, nevertheless the change must be mediated at the dimer-dimer interfaces in response to an event within the binding cleft. In addition to the recent discovery that clostridial GDH exhibits strong positive cooperativity with L-glutamate Wang and Engel (1995) also ob-

served that mutation of an alanine residue (A163) in the glutamate binding site causes a significant reduction in the level of cooperativity (Hill coefficient of 2.6). This highlights a possible region of the active site that is sensitive to conformational signals from the other subunits in the hexamer. It is interesting to note that aspartate-165 lies on the same β-strand as alanine-163 (Baker et al. 1992). This raises the possibility that a major route for inter-subunit active-site communication may be transmission of a structural change in residues on the β-strand (β-e) in the active site (created upon L-glutamate binding), through to the interfaces between subunits which make up a trimer (the dimer-dimer interface).

The midpoint for the plot of velocity as a function of ligand (in this case substrate) concentration ($S_{0.5}$) at 150 mM glutamate for wild-type enzyme at pH 9.0 (Wang and Engel 1995) suggests that the ligand dissociation constant may be high, with a correspondingly low free energy change of binding. This would imply that the free energy barrier between active and inactive states is very low, suggesting that the conformational change at the subunit interfaces required to trigger this allosteric transition may be very subtle. This emphasises the importance of the protein engineering approach for establishing links between the ligand binding site and the subunit interfaces in systems such as clostridial GDH, as demonstrated in the present study.

It is possible that determination of the X-ray structure of this mutant may yield further clues to the nature of the relay system linking the L-glutamate binding sites and subunit interfaces in the protein and so shed light on the basis of allosteric interaction in GDH.

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