Intersubunit Communication in Hybrid Hexamers of K89L/A163G/S380A and C320S Mutants of Glutamate Dehydrogenase from *Clostridium symbiosum*†

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ABSTRACT: The triple mutant K89L/A163G/S380A (inactive with glutamate but active with L-Nle and L-Met) and C320S (fully active with glutamate, entirely inactive with L-Nle and L-Met, and also lacking reactive cysteine) mutant of glutamate dehydrogenase (EC 1.4.1.2) of *Clostridium symbiosum* could be completely denatured by urea with the loss of structure and activity. The mutants denatured by urea could be reassociated to give stable hexamers with recovery of activity of ∼67% by dilution in 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM NAD^+. The native, urea-denatured, and renatured states of mutant enzymes were characterized by size exclusion chromatography on FPLC and native PAGE. Intersubunit hybrid hexamers containing five subunits of triple mutant and one subunit of C320S mutant were constructed by *in vitro* subunit hybridization followed by affinity chromatography. Kinetic analysis showed that a 5:1 hybrid hexamer, with only one C320S subunit able to bind NAD^+ after DTNB modification, shows classical Michaelis−Menten kinetics with regard to NAD^+. This contrasts with the apparent negative co-operativity shown by pure C320S hexamers and suggests that the interaction in NAD^+ binding among subunits is eliminated in the hybrid. After removal of thionitrobenzoate, however, all of the subunits in the hybrid are able to bind NAD^+. In this state the hybrid enzyme showed slight deviation from classical behavior with regard to NAD^+, indicating reintroduction of some level of allosteric interaction. The hybrid hexamer also showed much reduced co-operativity with glutamate at pH 8.8, with a Hill coefficient of 3 for DTNB-treated hybrid (as compared to 5.2 for the pure C320S mutant) and 2.2 for the untreated hybrid. The fact that co-operativity in glutamate binding is not entirely eliminated correlates with evidence that the triple mutant subunits, though inactive toward glutamate, can nevertheless still bind this amino acid.

Hexameric glutamate dehydrogenases (GDHs,[1] EC 1.4.1.2−4) from various sources are often allosteric enzymes (*1*−*5*). Specifically, *Clostridium symbiosum* glutamate dehydrogenase (EC 1.4.1.2), for which both the cloned, overexpressed gene (*6*) and a high-resolution structure (*7, 8*) are available, displays (i) apparent negative interaction in kinetics with NAD^+ (*9*) and (ii) strong positive interaction with glutamate at high pH (*10*). Unlike aspartate transcarbamoylase, with independent regulatory and catalytic subunits (*11*), clostridial GDH has only one type of subunit (*12*). This implies that the allosteric effects in GDH are mediated by interactions among six identical subunits. Site-directed mutagenesis opens the door for investigation of this proposed allostery, either by constructing dimeric (*13*) or trimeric GDHs or, alternatively, by examining the behavior of defined intersubunit hybrids in which some of the subunits are functionally disabled with respect to coenzyme or substrate binding or catalysis.

In one such study intersubunit hybrids were constructed using wild-type (WT) clostridial GDH and a mutant form C320S (*14*; S. Aghajanian and P. C. Engel unpublished experiments). Cys320 is close to the coenzyme binding site (*7*), and the reaction of its −SH group with DTNB inactivates the WT enzyme (*15*). Replacement with serine gives a fully active enzyme, unaffected by DTNB (*16*). The use of C320S as a neutral marker mutation offers the possibility of counting subunits of different types in hybrids. Besides Cys320, there is only one other Cys residue, at position 144, and that is inaccessible to DTNB (*15*). It was demonstrated that a hybrid containing five subunits of WT inactivated by DTNB and one subunit from C320S (which cannot be modified by DTNB) shows classical Michaelis−Menten kinetics with NAD^+, suggesting that the nonlinear kinetic behavior of the native enzyme is the result of interaction between the six identical NAD^+ binding sites in the hexamer (*14*). To study this further, we have now constructed a different hybrid involving two mutants, *viz.* C320S (*16*) and a triple mutant, K89L/A163G/S380A (*17*). The two residues Lys89 and Ser380 are directly involved in the recognition of the γ-COO^- of the side chain of the substrate glutamate, and Ala163 is one of the residues that lines the binding pocket for this side chain (*7, 8*). The triple mutant K89L/A163G/S380A was constructed with a view to shifting the substrate specificity away from the preference for glutamate and toward acceptance of alternative monocarboxylic amino acid

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[1] Abbreviations: GDH, L-glutamate dehydrogenase (EC 1.4.1.2); IPTG, isopropyl β-thiogalactopyranoside; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB^-, thionitrobenzoate, FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; DEAE, diethylaminoethyl.

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substrates. The prediction has been borne out by the actual experimental result (*17*). The triple mutant folds correctly and binds NAD^+, but, unlike WT, it has no activity toward glutamate. However, it can use methionine and norleucine, compounds that give barely measurable activity as substrates with WT GDH or the C320S mutant.

The strategy adopted for constructing the hybrid hexamers is renaturation of a mixture of the two mutants, separately unfolded by urea. We have shown elsewhere (*18*) that, after denaturation in 4 M urea to yield unfolded monomers, the WT enzyme can be successfully refolded. Optimal conditions for unfolding the mutant hexamers to give stable fragments and for refolding to form native structures have now also been identified. Following such a study, hybrid hexamers (5:1) of triple mutant and C320S have been constructed and investigated to assess the involvement of intersubunit communication in coenzyme and substrate binding.

MATERIALS AND METHODS

Materials. Grade II NAD^+ (free acid), grade II NADH (disodium salt), and 2-oxoglutarate (disodium salt) were obtained from Boehringer Mannheim (East Sussex, U.K.). Tris[hydroxymethyl]aminomethane (Tris), L-norleucine, 2-oxocaproic acid, L-glutamate (monosodium salt), NAD^+-agarose (NAD^+ immobilized through the N-6 atom of the adenine ring to cross-linked 4% beaded agarose), and molecular mass marker proteins for size exclusion chromatography were purchased from Sigma Chemical Co. (Dorset, U.K.). Sepharose CL-6B, Sephadex G-25 (fine), and FPLC prepacked column Superose 6 HR 10/30 were from Pharmacia Biotech. Ltd. (St. Albans, U.K.). All other chemicals were of analytical reagent grade from BDH Chemicals Ltd. (Warwickshire, U.K.) or Fisons Scientific Ltd. (Leicester, U.K.).

Enzyme Preparation. Transformants of *Escherichia coli* Q100 harboring plasmids carrying the genes for either mutant C320S (*16*) or K89L/A163G/S380A (*17*) of clostridial GDH were separately incubated at 37 °C in LB medium supplemented with 100 μg/mL ampicillin and 0.5 mM isopropyl β-thiogalactopyranoside (IPTG). The cells were harvested after 16 h, suspended in 0.1 M potassium phosphate buffer (pH 7.0), sonicated, and centrifuged for 30 min at 27000*g* and 4 °C. The mutant enzymes were purified to homogeneity, using a dye-affinity column of Remazol Brilliant Red GG−Sepharose CL-6B (*9*). Fractions containing purified enzyme were pooled and precipitated with ammonium sulfate. The pure enzyme stored in 60% saturated ammonium sulfate at 4 °C was dialyzed before use against 0.1 M potassium phosphate buffer (pH 7.0) with several changes. The concentration of enzyme was determined spectrophotometrically at 280 nm by using a value of the absorption coefficient of 1.05 L g^{-1} cm^{-1} determined for the WT enzyme (*9*). The specific activity of the purified triple mutant was 1.3 units/mg using L-norleucine as substrate in 0.1 M potassium phosphate buffer (pH 8.0), and the C320S mutant had a specific activity of 25.4 units/mg with L-glutamate as substrate in 0.1 M potassium phosphate buffer (pH 7.0) (as compared with a figure of 23 units/mg for WT enzyme).

Native PAGE was carried out on the Bio-Rad mini gel system using 7.5% gel according to the method of Garfin (*19*). The gels were stained with Coomassie Brilliant Blue R-250.

Enzyme Assays. The activities of both mutants were measured spectrophotometrically at 25 °C (Uvikon 941 Plus, Kontron Instruments) by recording the increase in absorbance at 340 nm due to the production of NADH. The C320S mutant of GDH was assayed at pH 7.0 in 0.1 M potassium phosphate buffer containing 1 mM NAD^+ and 40 mM L-glutamate, and the activity of the triple mutant was assayed at pH 8.0 in 0.1 M potassium phosphate buffer containing 1 mM NAD^+ and 100 mM L-norleucine.

Denaturation of GDH Mutants by Urea. The enzymes were incubated at 25 °C in 0.1 M potassium phosphate buffer (pH 7.0) with different concentrations of urea, and the residual activities of samples were measured by taking 5−20 μL aliquots into 1 mL of assay solution. The effect of various added ligands on the course of inactivation was investigated.

Reactivation after Urea Treatment. The procedure for reactivation of the GDH mutants after urea denaturation was similar to that described previously for WT GDH (*18*). After denaturing for specified time periods, the enzymes were diluted 25-fold into 0.1 M potassium phosphate buffer (pH 7.0) containing NAD^+ and other ligands. Samples (10−50 μL) were withdrawn at intervals for activity assays.

Chromatographic Analysis of Urea-Treated and Reactivated Triple Mutant. To estimate apparent M_r values, size exclusion chromatography was carried out on a Pharmacia FPLC system with a Superose 6 HR 10/30 prepacked column equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl. For molecular mass calibration, thyroglobulin (669 000), apoferritin (443 000), β-amylase (200 000), alcohol dehydrogenase (150 000), and albumin (66 000) were used. One hundred microliters of each (1 mg/mL) was loaded separately. The flow rate was 0.5 mL/min.

Construction of Intersubunit Hybrids. The mutants K89L/A163G/S380A and C320S, each at 1.5 mg/ml protein concentration, were denatured separately at 25 °C, by 4.5 and 4 M urea for 60 and 30 min, respectively. The two denatured enzymes were mixed in 5:1 (triple mutant:C320S) volume ratio, diluted 25-fold into 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM NAD^+, and incubated at 25 °C for 24 h. Controls for both the mutants without urea treatment and with and without mixing the two after dilution were run in parallel. The renatured mixture was concentrated and separated from NAD^+ by ion-exchange chromatography on a DEAE-Sephadex A-50 column. The enzyme was eluted with 1 M NaCl in 0.1 M potassium phosphate buffer (pH 7.0).

Affinity Chromatography of Refolded Enzyme Samples on NAD^+-Agarose. It has been shown (*14*, *20*) that DTNB-modified enzyme fails to bind to NAD^+-agarose as a consequence of steric blocking of the coenzyme binding site by a TNB^- moiety. The triple mutant, like the WT enzyme, is inactivated by DTNB, and NAD^+ is able to protect against this inactivation (*17*). Accordingly, DTNB-modified triple mutant was not retarded on an NAD^+-agarose column. By contrast, the C320S mutant shows no effect upon DTNB treatment and, therefore, does bind to the NAD^+-agarose column after such treatment. Refolded samples were treated with 2 mM DTNB at pH 7.0 and 25 °C. The residual activity of triple mutant in the sample was <1% after 30 min. Gel filtration on a column of Sephadex G-25 (equilibrated and eluted with 25 mM potassium phosphate buffer, pH 7.0) was used to remove unreacted DTNB and other small molecules. In separate experiments 200 μL each (1 mg/mL) of DTNB-

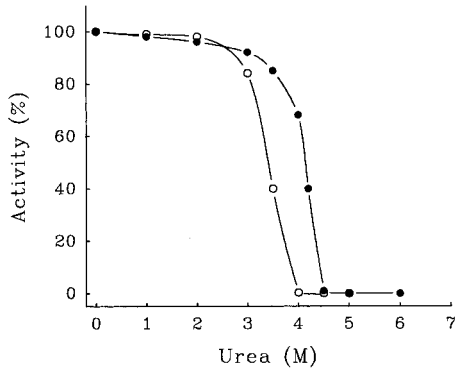

FIGURE 1: Denaturation of K89L/A163G/S380A and C320S mutant GDHs by urea. The enzymes, 0.5 mg/ml of K89L/A163G/S380A (●) or 0.028 mg/mL of C320S (○) in 0.1 M potassium phosphate buffer (pH 7.0), were treated with various concentrations of urea for 60 and 30 min, respectively, at 25 °C. The residual activities were determined by taking aliquots (10−20 μL) in 1 mL of assay mixture as described under Materials and Methods.

modified triple mutant, native C320S, and refolded hybrid mixture was loaded onto the NAD^+-agarose column and eluted using a linear gradient of 0−1 M NaCl in 25 mM potassium phosphate buffer (pH 7.0). One milliliter fractions were collected, and protein concentration was measured. The enzyme activities of fractions toward glutamate and norleucine were determined after treatment with 5 mM β-mercaptoethanol, which removes the TNB^- from modified subunits.

Determination of Concentrations of the Two Mutant Subunit Types in Refolded Sample. The concentrations of triple mutant (x) and C320S (y) subunits incorporated into renatured samples were calculated from absorption spectra as described elsewhere (S. Aghajanian, and P. C. Engel, unpublished experiments) using the equations

$$x = A_{336}/\epsilon_{336}^{\text{mod-TM}}$$

$$y = (A_{280} - x\epsilon_{280}^{\text{mod-TM}})/\epsilon_{280}^{\text{C320S}}$$

where A_{336} and A_{280} are the absorbance values of the sample at 336 and 280 nm (corrected for buffer contribution and at 336 nm also for the very small contribution of the unmodified protein) and $\epsilon_{336}^{\text{mod-TM}}$, $\epsilon_{280}^{\text{mod-TM}}$, and $\epsilon_{280}^{\text{C320S}}$ are the absorption coefficients for modified triple mutant and C320S enzymes at 336 and 280 nm, respectively. The absorption coefficient for the C320S mutant at 280 nm was assumed to be identical to that for the WT enzyme ($\epsilon_{280}^{\text{C320S}} = 1.05$ L g^{-1} cm^{-1}) (*9*). Likewise, the absorption coefficients for DTNB-modified triple mutant at 280 and 336 nm, which contain a contribution from the bound TNB^- moiety, were taken to be equal to those for modified WT enzyme ($\epsilon_{280}^{\text{mod-TM}} = 1.155$ L g^{-1} cm^{-1} and $\epsilon_{336}^{\text{mod-TM}} = 0.16$ L g^{-1} cm^{-1}) (S. Aghajanian and P. C. Engel, unpublished experiments).

RESULTS AND DISCUSSION

Figure 1 shows urea denaturation profiles for the triple mutant (K89L/A163G/S380A) and C320S mutant enzymes of clostridial GDH. The mutant C320S was denatured completely by 4 M urea in 30 min, but the triple mutant retained 68% of its activity even after 60 min at this concentration of urea. Complete denaturation of the triple mutant was achieved by treatment with 4.5 M urea for 60 min. Native PAGE analysis also showed that the triple mutant was largely stable up to 4 M urea treatment (Figure 2A). The C320S mutant was stable only up to 3 M urea and lost its native structure completely in 4 M urea after 30 min of incubation (Figure 2B), in keeping with the activity result.

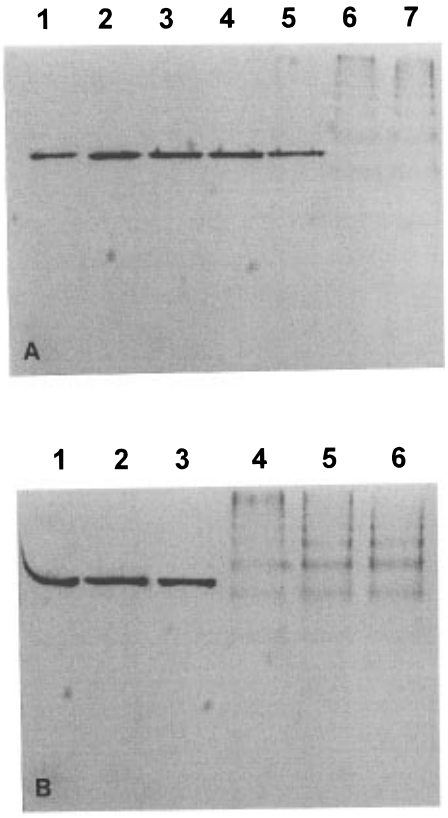

FIGURE 2: Native PAGE (7.5%) of K89L/A163G/S380A (0.5 mg/mL) and C320S (0.03 mg/mL) mutants of GDH samples treated with different concentrations of urea and stained for protein with Coomassie Brilliant Blue. (A) Lanes represent (1) native WT-GDH (2) native K89L/A163G/S380A mutant, and (3−7) K89L/A163G/S380A after 60 min of incubation with 2 (lane 3), 3 (lane 4), 4 (lane 5), 5 (lane 6), or 6 M urea (lane 7). (B) Lanes represent (1) native C320S and (2−6) C320S after 30 min of incubation with 2 (lane 2), 3 (lane 3), 4 (lane 4), 4.5 (lane 5), or 5 M urea (lane 6).

Structural effects of urea treatment on the triple mutant were also analyzed by FPLC. Samples of native enzyme, treated with 4.5 M urea for 15 and 60 min in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C, were diluted 5-fold in 50 mM potassium phosphate buffer (pH 7.0) containing 150 mM NaCl and loaded onto the FPLC prepacked column of Superose 6 HR 10/30 equilibrated with the same buffer. In the untreated native state the triple mutant appeared as a single sharp peak at a position corresponding to M_r of 300 000 (Figure 3a). After 15 min of incubation with 4.5 M urea, the enzyme eluted from the column in two peaks (Figure 3b), the first at the native hexamer position (M_r = 300 000) and the second at an intermediate position between those predicted for folded monomer and dimer, corresponding to an apparent subunit M_r of 80 000−90 000. Finally, after 60 min of incubation (residual activity <1%), the hexamer peak was completely replaced by a sharp peak corresponding to the monomer (Figure 3c). A broad shoulder associated with the hexamer peak indicated the formation of aggregates of high molecular weight, which were also seen in the nondenaturing PAGE (Figure 2A). These results are very similar to previous observations for the WT enzyme (*20*).

The effects of ligands on the urea denaturation of both mutants were studied. In most cases the concentrations of

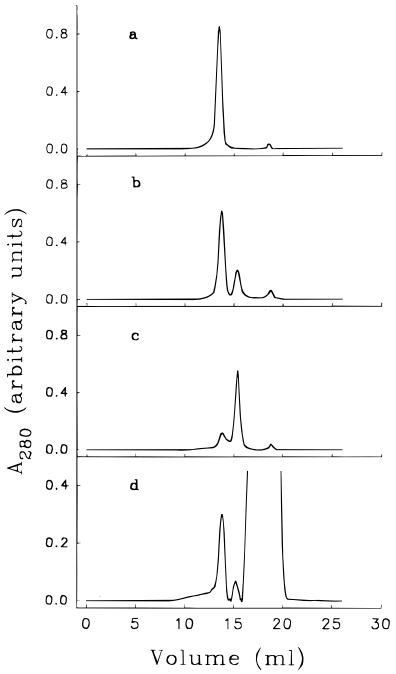

FIGURE 3: Elution profiles of native, urea-treated, and refolded samples of the triple mutant on Superose 6 HR column. Native triple mutant (0.3 mg/mL) in 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl was loaded on a FPLC column of Superose 6 HR (a). Fifty microliters of enzyme samples (1.5 mg/mL) after 15 (b) and 60 min (c) of incubation with 4.5 M urea at 25 °C were withdrawn, diluted 5-fold with elution buffer, and loaded onto the column. The sample of enzyme (1.5 mg/mL) after 60 min of treatment with 4.5 M urea was diluted 10-fold into 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM NAD^+ and was loaded onto the column after 20 h of incubation at 25 °C (d). In each case 200 μL sample was loaded. Note that in view of the greater dilution of sample (d) 2-fold higher sensitivity was used for detection at 280 nm.

ligands were chosen to give a near-maximal effect. In the case of norleucine, however, the low solubility makes it impossible to make such a statement. Data in Table 1 show that neither of the two forms of coenzyme, NAD^+ and NADH, was able to protect C320S effectively against denaturation, but this mutant was almost completely resistant to 4 M urea in the presence of 2-oxoglutarate or in the combined presence of NADH and glutamate. This is similar to more detailed published results for the WT enzyme that show only moderate protection by the coenzymes and much more potent stabilization of the native structure by 2-oxoglutarate (*20*). Surprisingly, although glutamate and 2-oxoglutarate are not substrates for the triple mutant, they provided higher protection against urea denaturation than norleucine or 2-oxocaproic acid at the same concentration (50 mM) (Table 1). Of all the ligands individually, NADH gave the best protection of the triple mutant and was considerably more effective in this regard with the triple mutant than with C320S. Activity staining analysis of the triple mutant treated with urea in the presence of ligands by native PAGE also showed that with glutamate and 2-oxoglutarate the native protein band is more intense than with norleucine or 2-oxocaproic acid (data not shown). These results show that although the substrate specificity of the triple mutant is altered, glutamate and 2-oxoglutarate still bind, but unproductively. Bearing this out, a kinetic study of the triple mutant with norleucine as substrate showed that glutamate acts as a weak inhibitor competitive with norleucine (K_i = 125 mM) (data not shown).

Table 1: Effect of Ligands on Denaturation of Triple Mutant and C320S by Urea[a]

ligand(s)	activity (%) triple mutant	C320S
no ligand	1.0(±0.1)	0.3 (±0.1)
NAD^+ (2 mM)	22 (±0.5)	1.0 (±0.1)
NADH (2 mM)	50 (±1)	5.0 (±0.3)
norleucine (50 mM)	20 (±0.5)	
2-oxocaproic acid (50 mM)	3.0 (±0.2)	
glutamate (50 mM)	35 (±1)	55 (±1)
2-oxoglutarate (50 mM)	30 (±1)	100 (±1)
NAD^+ (2 mM) + 2-oxocaproic acid (50 mM)	26 (±1)	
NAD^+ (2 mM) + 2-oxoglutarate (50 mM)	38 (±1)	
NADH (2 mM) + norleucine (50 mM)	65 (±0.5)	
NADH (2 mM) + glutamate (50 mM)	55 (±1)	90 (±1)

[a] The triple mutant (0.6 mg/mL) and C320S (0.03 mg/mL) were incubated at 25 °C with indicated ligands followed by treatment with 4.5 and 4.0 M urea, respectively. Aliquots (5−20 μL) were withdrawn after 1 h from triple mutant and after 30 min from C320S incubation mixtures and assayed for residual activity. The complete experiment was carried out at least twice with each set of incubation conditions run in duplicate. The reported values, which are rounded to two significant figures, are thus the means of activities measured for four or more separate incubations. The error levels given in parentheses indicate the approximate observed range of the experimental measurements. The blank entries in the C320S column indicate that these measurements were not made.

Table 2: Effect of Ligands on Reactivation of Triple Mutant Denatured by Urea[a]

ligand(s)	activity (%)
buffer	8.0 (±0.5)
NAD^+ (2 mM)	67 (±1)
2-oxocaproic acid (50 mM)	7.0 (±0.5)
NAD+ (2 mM) + 2-oxocaproic acid (50 mM)	30 (±1)
NAD+ (2 mM) + NaCl (50 mM)	69 (±1)
NADH (2 mM)	24 (±1)
norleucine (50 mM)	3.0 (±0.5)
norleucine (100 mM)	4.0 (±0.5)
NADH (2 mM) + norleucine (50 mM)	28 (±1)

[a] The enzyme (1.5 mg/mL) samples after treatment with 4.5 M urea for 60 min were diluted 25−fold in 0.1 M phosphate buffer (pH 7.0) containing the indicated ligand(s), and the activities of samples were determined after 20 h of incubation at 25 °C. The complete experiment was carried out at least twice with each set of incubation conditions run in duplicate. The reported values, which are rounded to two significant figures, are thus the means of activities measured for four or more separate incubations. The error levels given in parentheses indicate the approximate observed range of the experimental measurements.

To make intersubunit hybrids, it is essential that the mutants are able to refold correctly to give active hexamers. The triple mutant, after denaturing with 4.5 M urea, was diluted 25-fold into 0.1 M potassium phosphate buffer (pH 7.0) containing different ligands (Table 2). For the triple mutant, as for WT GDH (18), NAD^+, with or without NaCl, gave the highest levels of reactivation (69% and 67%, respectively). The substrates norleucine and 2-oxocaproic acid failed to promote the reactivation of triple mutant. 2-Oxocaproic acid rather retarded the rate of reactivation by NAD^+. With the C320S mutant also, 66% of enzyme activity could be recovered on reactivation in the presence of NAD^+, which was therefore adopted as the sole ligand to be added in hybridization experiments.

Figure 3d shows the FPLC elution profile of triple mutant reactivated in the presence of 2 mM NAD^+ after 20 h of incubation. The sample renatured without NAD^+ gave a main peak in the monomer position and a very small peak

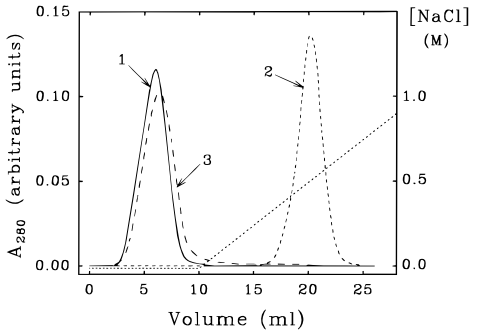

FIGURE 4: Elution profiles of affinity chromatography on NAD^+-agarose column. The DTNB-treated samples were (1) triple mutant, (2) C320S, and (3) refolded mixture in the ratio 5:1 (triple mutant: C320S). Chromatographic details were as described under Materials and Methods. Note that the C320S sample, though treated with DTNB, remains unmodified.

at the native hexamer position, very similar to that seen in Figure 3c. By contrast, the enzyme sample renatured with 2 mM NAD^+ showed a sharp peak at the hexamer and a very small peak at the monomer position, again showing that the mutant monomers are able to refold in the presence of NAD^+ to give the native hexameric structure in high yield. All of these results indicate the potential for constructing intersubunit hybrids of the two mutant enzyme forms.

From the standpoint of dissecting the basis, nature, and extent of interaction between binding sites on different subunits within a hexamer, the most useful initial construct is a 5:1 hybrid in which one "active" subunit of a hexamer is surrounded by five partners that are disabled in some way. In a preceding paper we have used this approach with blockage of NAD^+ binding as the functional defect (*14*; S. Aghajanian P. C. Engel, unpublished experiments). Our present objective was to extend this approach to discover the importance of amino acid binding to the six subunits in mediating the transfer of information. Hybrid hexamers of triple mutant and C320S were made as described under Materials and Methods. The refolded mixture of hybrid hexamers (5:1 triple mutant:C320S) was treated with DTNB, loaded onto an NAD^+-agarose column, and eluted with a gradient of NaCl. Figure 4 shows the elution profiles of DTNB-treated samples of triple mutant, C320S, and the hybrid mixture. Binding to the NAD^+-agarose column depends on an intact, unblocked coenzyme binding site, and WT GDH, the C320S mutant, and the triple mutant all gave virtually identical elution profiles, reflecting their tight binding to the column. Accordingly, in the separation shown in Figure 4, only the C320S mutant, which remains unmodified by DTNB, bound tightly to the column (Figure 4, peak 2), whereas the DTNB-modified samples, *viz.* triple mutant (Figure 4, peak 1) and hybrid hexamer (Figure 4, peak 3), both gave early elution peaks. The shape of the elution profile, and the position of the protein peak for the DTNB-modified hybrid mixture differed, however, from those for the DTNB-modified triple mutant. This indication of slight retardation suggested that the refolded mixture of the two mutants contains intersubunit hybrids. For this sample, 1 mL fractions were collected and analyzed for protein concentration and enzyme activities after β-mercaptoethanol treatment. The ratio of activity toward glutamate (C320S) and norleucine (triple mutant), indicating the relative amounts of the two mutants, was determined for each fraction (Figure 5). Increase in the activity ratio with the fraction number indicated that different hybrids in the eluting fractions interact differently with NAD^+-agarose. The theoretical value for

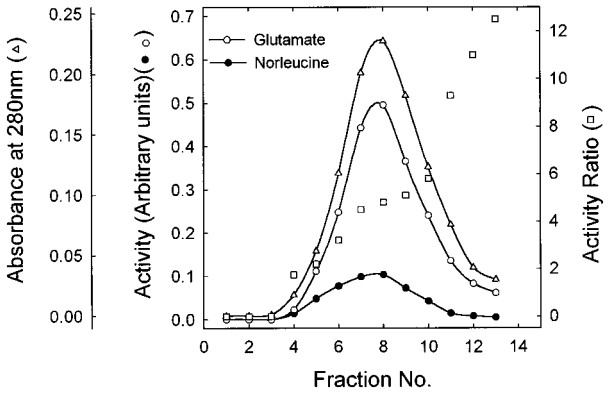

FIGURE 5: Analysis of the fractions of DTNB-modified 5:1 sample after elution from the NAD^+-agarose column. A_{280} (△); activities toward glutamate (○) and norleucine (●) measured after β-mercaptoethanol treatment of the fractions; and the ratio of the two activities (glutamate/norleucine) (□). Note that unmodified C320S hexamer elutes from fraction 16 to 24, outside the range of fractions assayed in this experiment.

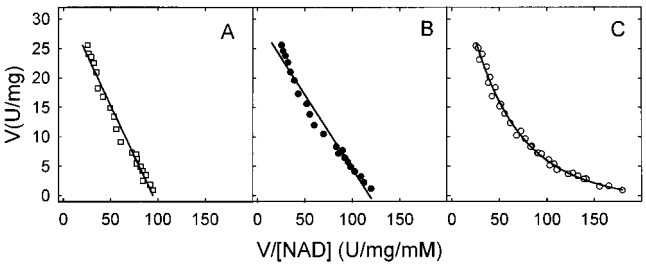

FIGURE 6: Eadie−Hofstee plots of activity with NAD^+ concentration for the DTNB-modified hybrid with five triple-mutant subunits and 1 C320S subunit before (A) and after (B) β-mercaptoethanol treatment and native C320S (C). The activity was assayed using 40 mM glutamate in 0.1M potassium phosphate buffer (pH 7.0) at 25 °C. The protein concentrations of 5:1 hybrids and C320S mutant in assay mixture were 2.3 and 0.35 μg/mL, respectively. The linear regression analysis of the data in (A) and (B) gave best-fit straight lines with $R = 0.99$ and 0.98, respectively.

the ratio of the activity for 5:1 (triple mutant:C320S) hybrids was 3.8 based on specific activities of C320S for glutamate and triple mutant for norleucine. The experimentally found value, 3.2 for fraction 6, was close to the theoretical value, showing that this fraction is rich in 5:1 hybrid hexamer species. The partial concentrations of triple mutant and C320S subunits in a fraction were estimated spectrophotometrically as described under Materials and Methods. Thus, for example, assuming only two species of hybrids, *viz.* 6:0 and 5:1 (triple mutant:C320S), are present in a particular fraction and in equal amount, the ratio of triple mutant subunits to C320S should be 11:1. On comparing the subunit ratios of triple mutant to C320S of each fraction with the theoretical value of 11:1, it was found that fraction 6 has the ratio of 11.8:1, showing that 6:0 and 5:1 hybrid species are present in approximately 1:1 ratio.

The WT GDH shows marked deviation from classical Michaelis−Menten kinetics with varied NAD^+ concentration at pH 7.0 (*9*). The effect of NAD^+ on C320S and on the 5:1 hybrid hexamer was investigated at pH 7.0 using glutamate as substrate. Eadie−Hofstee plots of kinetics with NAD^+ for the C320S mutant showed nonlinear kinetic behavior (Figure 6C). In the case of DTNB-modified 5:1 hybrids, NAD^+ cannot bind to the five triple mutant subunits in a hybrid hexamer. Classical kinetic behavior was observed (Figure 6A; the linear regression analysis of the data gave the best-fit straight line with $R = 0.99$), confirming that no NAD^+ binding interaction between subunits is possible. After

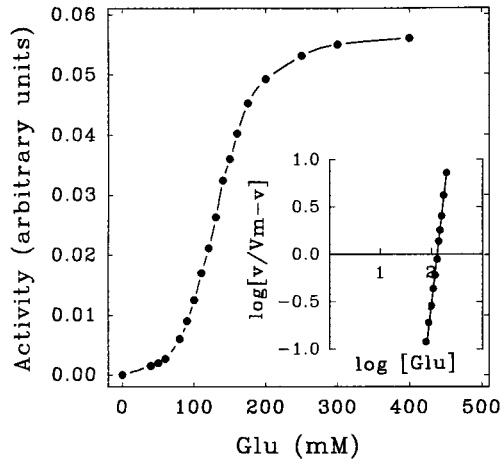

FIGURE 7: Effect of glutamate concentration on pure C320S at pH 8.8. Activity of the enzyme was measured using 1 mM NAD^+ in 0.1 M Tris-HCl buffer (pH 8.8). (Inset) Hill plot of the same data.

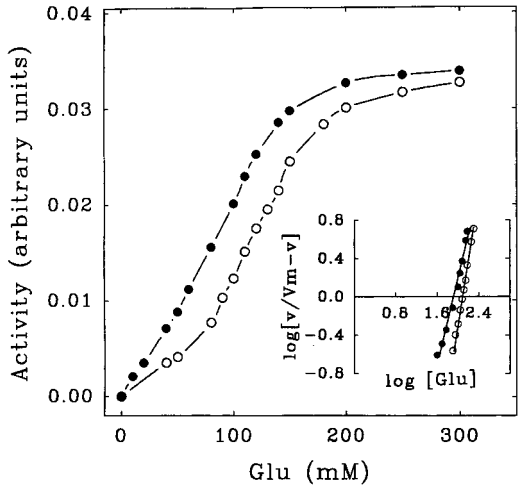

FIGURE 8: Effect of glutamate concentration on DTNB-modified 5:1 hybrids at pH 8.8 after (●) and before (○) β-mercaptoethanol treatment. Activity was measured using 1 mM NAD^+ in 0.1 M Tris-HCl buffer (pH 8.8). (Inset) Hill plot of the same data.

treatment with β-mercaptoethanol, the triple-mutant subunits are fully active with norleucine and are now able to bind NAD^+. The result here, showing only a slight deviation from linear kinetics (Figure 6B; the best straight line by transformation of the data gave a value of $R = 0.98$), was thus less predictable. Since all six subunits in this construct can bind NAD^+, more indication of allosteric interaction might have been anticipated. However, five of the six subunits cannot form a productive ternary complex with glutamate, and the result suggests that it is mainly interaction between fully liganded subunits, i.e. ternary complexes, that causes the observed kinetic behavior in WT GDH and the C320S mutant.

The WT GDH shows a sigmoidal curve of activity at higher concentrations of glutamate at pH 9.0, with a Hill coefficient (n) of 5.4 (*10*), indicative of strong positive co-operativity in glutamate binding. The mutant C320S shows very similar behavior, with a Hill coefficient of 5.2 (Figure 7). DTNB-modified 5:1 (triple mutant:C320S) hybrids (i.e. with only one active subunit) showed a reduced co-operative effect for glutamate binding, with a value of $n = 3.0$ at pH 8.8 (Figure 8, open circles and inset), showing that the subunit interaction is reduced but not abolished altogether. After the removal of thionitrobenzoate by the addition of β-mercaptoethanol to 5:1 hybrids, a similar behavior was observed, although the Hill coefficient was somewhat lower at 2.2 (Figure 8, solid circles and inset). The activity at intermediate glutamate concentrations was also somewhat higher, which may be attributed to a conformational response of the C320S subunit upon thionitrobenzoate release from the triple-mutant subunits. Thus, the communication between the subunits of two types in the 5:1 hybrid, either β-mercaptoethanol-treated or untreated, is reduced but not abolished, when compared to the interaction among the six C320S subunits of a homogeneous hexamer.

The initial assumption underlying these experiments was that the 5:1 hybrid construct might display classical Michaelis−Menten kinetics with glutamate since five of six subunits are impaired in their glutamate binding. Our results show that, even though the triple mutant shows no catalytic activity with glutamate, these subunits are still able to bind glutamate, as judged by both its effect on unfolding and by the competitive inhibition of L-norleucine oxidation. It seems that this residual binding is sufficient to support the co-operative interaction. It is too early at this stage to speculate on whether the observed Hill coefficient of 3 represents maximal co-operativity for a trimer, uncoupled in some way from its partner, or whether it reflects diminished co-operativity throughout the hexamer.

In a general sense, the results demonstrate the feasibility of the hybridization approach to dissecting the basis of allosteric interaction in GDH and lay foundations for further studies of this type with GDH mutants.

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