Allosteric behaviour of 1:5 hybrids of mutant subunits of *Clostridium symbiosum* glutamate dehydrogenase differing in their amino acid specificity

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Hybrid hexamers were made by refolding mixtures of two mutant forms of clostridial glutamate dehydrogenase. Mutant Cys320Ser (C320S) has a similar activity to the wild-type enzyme, but is unreactive with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoate) (DTNB). The triple mutant Lys89Leu/Ala163Gly/Ser380Ala (K89L/A163G/S380A), active with norleucine but not glutamate, is inactivated by DTNB, since the amino acid residue at position 320 is a cysteine residue. The chosen ratio favoured 1:5 hybrids of the triple mutant and C320S. The renatured mixture was treated with DTNB and separated on an NAD⁺-agarose column to which only C320S subunits bind tightly. Fractions were monitored for glutamate and norleucine activity and for releasable thionitrobenzoate to establish subunit stoichiometry. A fraction highly enriched in the 1:5 hybrid was identified. Homohexamers (C320S with 40 mM glutamate and 1 mM NAD⁺ at pH 8.8, or K89L/A163G/S380A with 70 mM norleucine and 1 mM NAD⁺ at pH 8.5) showed allosteric activation; succinate activated C320S approx. 50-fold ($EC_{50} = 70$ mM, $h = 2.4$), and glutarate gave approx. 30-fold activation ($EC_{50} = 35$ mM, $h =$

2.3). For the triple mutant, corresponding values were 80 mM and 2.2 for succinate, and 75 mM and 1.7 for glutarate, but maximal activation was only about 2-fold. In the 1:5 hybrid, with only one norleucine-active subunit per hexamer, responses to glutarate and succinate were still co-operative, and activation was more extensive than in the triple mutant homohexamer. A single norleucine-active subunit can thus respond co-operatively to a substrate analogue at the other five inactive sites. On the other hand, similar hyperbolic dependence on the norleucine concentration for the hybrid and the triple mutant homohexamer reflected the inability of C320S subunits to bind norleucine. With glutamate at pH 8.8, an h value of 3.6 was obtained for the 1:5 hybrid, in contrast with an h value of 5.2 for the C320S homohexamer. The 'foreign' subunit evidently impedes inter-subunit communication to some extent.

Key words: allosteric mechanism, hybrid hexamers, norleucine dehydrogenase, site-directed mutagenesis.

INTRODUCTION

The glutamate dehydrogenase (GDH) family (EC 1.4.1.2-4), offers many examples of complex kinetic behaviour (e.g. [1–6]) and the bovine enzyme (EC 1.4.1.3) was invoked as an early example of putative allosteric regulation [7]. Detailed structural evidence to support this interpretation has been lacking until very recently, when a crystallographic structure became available for the bovine GDH homohexamer, revealing the auxiliary heterotropic GTP site, long postulated on the basis of kinetics and ligand binding studies [8]. The homologous enzyme from *Clostridium symbiosum* (EC 1.4.1.2), for which several high-resolution structures have been solved [9–11], lacks this site and the corresponding heterotropic regulatory behaviour. Nevertheless, this enzyme, also hexameric, still displays complex kinetic behaviour with respect to the variation of both NAD⁺ [6] and glutamate [12] concentrations. The solved structures have not yet provided an obvious clue to the basis of the inter-subunit communication that must underlie an allosteric mechanism for this enzyme. They have, however, permitted the design of various disabled mutant forms of the enzyme. Mutants in which the interfacial interactions are destabilized [13,14] provide evidence

that only the intact hexamer is catalytically active. This in itself suggests that events at the interfaces influence the catalytic machinery, since the structure shows that there are six spatially separated active sites [9–11]. Likewise, a mutant, Asp165His, in which the charge balance at the active site has been perturbed, is dimeric [15], providing evidence of information flow in the opposite direction, i.e. from the active sites to the subunit interfaces.

A route to a more systematic exploration of inter-subunit communication has been provided by the successful refolding of the enzyme [16], allowing the construction of hybrid hexamers. Defined inter-subunit hybrids have been made by combining chemical modification and site-directed mutagenesis [17–20]. Using the Cys320Ser mutant (C320S) [21], which is unable to react with 5,5'-dithiobis(2-nitrobenzoate) (DTNB), as the source of the single active subunit in each hexamer, 5:1 hybrids were made both with wild-type (WT) GDH [20] and with the triple mutant Lys89Ser/Ala163Gly/Ser380Ala (K89L/A163G/S380A) [18], which contributes subunits that are active with L-methionine and L-norleucine and catalytically inactive towards glutamate [22,23], but are nevertheless still able to bind this amino acid [18]. In both cases, after treatment with DTNB

Abbreviations used: DTNB, 5,5'-dithiobis(2-nitrobenzoate); GDH, L-glutamate dehydrogenase (EC 1.4.1.2); TNB, thionitrobenzoate; WT, wild-type.

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[24,25], the hexamers have only one subunit (C320S) able to bind NAD^+ , and show classical Michaelis–Menten kinetics with regard to NAD^+ , unlike the C320S homohexamer [18] or WT GDH [6], which both show marked deviation from classical kinetics. This clearly proves that the non-classical kinetic behaviour in this case depends on communication between coenzyme-binding sites on the different subunits in a hexamer.

More recently, another 5:1 hybrid has been constructed [26], in which C320S again provides the only catalytically active subunit, but in which the other subunits are inactive by virtue of the replacement of a key catalytic residue rather than disabled binding of either substrate. The inactive partner in this case is the mutant Asp165Ser [27], lacking the catalytic aspartate residue [10]. This hybrid showed a characteristic allosteric response to NAD^+ unless the five Asp165Ser subunits were blocked by thionitrobenzoate (TNB) moieties [26], showing that the subunits mediating the allosteric behaviour need not themselves be catalytically active.

WT GDH also displays a markedly sigmoidal response to glutamate concentration at pH 9.0, with an h value of 5.4 [12], indicating strong positive co-operativity in glutamate binding. The C320S mutant shows very similar behaviour, with an h value of 5.2 [18]. The sigmoidal response of the DTNB-treated 5:1 (WT:C320S) hybrid to glutamate at high pH persisted ($h = 3.6$), since, though inactive, the chemically modified WT subunits can presumably still bind glutamate [20]. Similarly, the DTNB-treated hybrid hexamer, 5:1 (triple mutant:C320S) gave an h value of 3.0 for glutamate at high pH, again reflecting the fact that co-operativity in glutamate binding is not completely eliminated. The allosteric responses to the two substrates, glutamate and NAD^+ , thus appear to be independent, and the glutamate response, like the coenzyme response, does not require the mediating subunits to be catalytically active.

As a further test of this concept, we have now investigated in the present study the capacity of glutamate-active subunits to regulate the activity of a subunit with a different substrate activity within the same hexamer, namely the novel catalytic activity of the triple mutant, K89L/A163G/S380A, towards L-norleucine and L-methionine [22,23]. In the present study, we have attempted to investigate how a single L-norleucine-active subunit in a hybrid hexamer responds to five glutamate-active C320S partners. The present study documents the separate behaviour of the two pure mutants, providing baselines against which the behaviour of the hybrid is then compared.

MATERIALS AND METHODS

Materials

Grade II NAD^+ (free acid) was obtained from Boehringer Mannheim (Roche Diagnostics; Lewes, East Sussex, U.K.). Tris, L-norleucine, L-glutamate (monosodium salt), glutarate and NAD^+ -agarose were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Sepharose CL-6B and Sephadex G-25 (fine) were from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). Succinate (disodium salt) was from Fisons Scientific (Loughborough, Leics., U.K.). All other chemicals were of analytical reagent grade from BDH (Poole, Dorset, U.K.), Fisons Scientific, Merck (Darmstadt, Germany) or Riedel-deHäen (Seelze, Germany).

Enzyme preparation

Transformants of *Escherichia coli* Q100 harbouring plasmids carrying the genes for either mutant C320S [21] or K89L/A163G/S380A [22,23] of clostridial GDH were separately incu-

bated at 37 °C in Luria–Bertani medium supplemented with 100 $\mu\text{g}/\text{ml}$ 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 at 27000 g for 30 min at 4 °C. The mutant enzymes were purified to homogeneity with a dye-affinity column of Remazol Brilliant Red GG-Sepharose CL-6B [6]. The purified enzymes, stored in 60 %-saturated ammonium sulphate at 4 °C, were desalted on a Sephadex G-25 column before use. The concentration of enzyme was determined spectrophotometrically at 280 nm by using a value of the absorption coefficient of $1.05 \text{ l} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ determined for the WT enzyme [6]. The specific activity of the purified triple mutant was 1.25 units/mg using L-norleucine as substrate in 0.1 M Tris/HCl (pH 8.0). The C320S mutant had a specific activity of 24.8 units/mg with L-glutamate as substrate in 0.1 M potassium phosphate buffer (pH 7.0). Under the same conditions, the WT enzyme had a specific activity of 23 units/mg.

Enzyme assays

The activities of both mutants were measured spectrophotometrically at 25 °C (Uvikon 941 Plus; Kontron Instruments, Neufahrn, Germany) by recording the increase in A_{340} due to the production of NADH. The C320S mutant was assayed at pH 7.0 in 0.1 M potassium phosphate buffer containing 1 mM NAD^+ and 40 mM L-glutamate, and the K89L/A163G/S380A mutant was assayed at pH 8.0 in 0.1 M Tris/HCl containing 1 mM NAD^+ and 100 mM L-norleucine.

Construction of inter-subunit hybrids

The construction of hybrids was initiated with 6 mg of the triple mutant protein and 30 mg of C320S mutant protein. To unfold and separate the subunits [28], the two proteins, each at 1.5 mg/ml protein concentration in 0.1 M potassium phosphate buffer (pH 7.0), were incubated separately at 25 °C in 4.5 M urea for 60 min (triple mutant) or 4 M urea for 30 min (C320S). The two denatured enzymes (residual activities < 1 %) were mixed at a 1:5 (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 36 h for refolding and reactivation as described previously [16]. Controls were run for both the mutants to check the effect of incubation without urea treatment, and later of mixing and diluting the two mutants. The renatured mixture (600 ml) was concentrated and separated from NAD^+ by ion-exchange chromatography on a DEAE-Sephadex A-50 column (1 cm $\times$ 5 cm) pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) at a flow rate of 1.2 ml/min. The enzyme was eluted with 1 M NaCl in 0.1 M potassium phosphate buffer (pH 7.0), and 18 mg of protein was obtained.

The triple mutant, like the WT enzyme [24,25], is inactivated by DTNB, which blocks the coenzyme-binding sites. Accordingly, like WT [20], the triple mutant is unable to bind to an NAD^+ -agarose column after DTNB treatment. By contrast, the C320S mutant, which is unable to react with DTNB, binds to NAD^+ -agarose even after treatment with this reagent. The refolded enzyme was therefore treated in two batches (9 mg of each protein) with 10 mM DTNB at pH 7.0 and 25 °C. The residual activity of the triple mutant (assayed with norleucine) in the sample was less than 2 % after 1 h. Unreacted DTNB and other small molecules were removed by gel filtration on a Sephadex G-25 column (equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.0). After separation, 6 mg of protein was obtained in two batches and the side fractions were pooled separately. A 1 ml portion of this DTNB-modified enzyme

(3 mg/ml) was loaded on to the NAD^+ -agarose column (1 cm $\times$ 7 cm) and eluted with a linear gradient of 0–0.5 M NaCl in 10 mM potassium phosphate buffer (pH 7.0). A_{280} was measured on 1 ml fractions. Partial concentrations of triple mutant and C320S subunits incorporated into renatured samples were determined as described previously [20]. Enzyme activities towards glutamate and norleucine were determined after removal of TNB from modified subunits with 5 mM 2-mercaptoethanol. As the cross-specificity between the two GDH mutants is negligible, assays with these two substrates give an accurate estimate of the content of C320S and triple mutant subunits respectively.

RESULTS AND DISCUSSION

Separation of hybrid hexamer mixture

On elution from the DEAE-Sephadex A50 column, and prior to separation on NAD^+ -agarose, the specific activity for each individual enzyme in the refolded mixture of hybrid hexamers was measured. The specific activities were 20.6 units/mg with glutamate (83% of the original 24.8 units/mg for pure C320S) and 0.21 unit/mg with norleucine (16.5% of the original 1.25 units/mg for pure triple mutant). This suggested that the refolded subunits are present in the chosen ratio of 1:5 (triple mutant:C320S), i.e. that refolding yields are the same for both mutants.

Figure 1 compares the gradient elution profiles of C320S and of DTNB-treated samples of triple mutant and the hybrid mixture, all run on the NAD^+ -agarose column. Without DTNB treatment, WT GDH (results not shown), the native C320S mutant and the triple mutant (results not shown) gave virtually identical elution profiles, reflecting tight binding to the column via intact, unblocked, coenzyme-binding sites. The DTNB-treated triple mutant, on the other hand, eluted much earlier (Figure 1) as it is not retarded by specific binding to the coenzyme ligand. For the DTNB-modified hybrid mixture, however, the position of the main protein peak differed from that for either of

Figure 1 Elution profiles of GDH mutants during affinity chromatography on an NAD^+ -agarose column

DTNB-treated triple mutant (1), native C320S mutant (2) and DTNB-treated refolding mixture containing triple mutant and C320S subunits in the ratio 1:5 (3) were loaded on to the NAD^+ -agarose column connected to FPLC. The column was equilibrated with 10 mM potassium phosphate buffer (pH 7.0) and the enzymes were eluted with a linear gradient of NaCl (0–0.5 M) (4) in the same buffer at a flow rate of 0.25 ml/min. Proteins were detected by A_{280} .

Figure 2 Analysis of the fractions of the DTNB-treated 1:5 (triple mutant:C320S) hybrid after elution from the NAD^+ -agarose column

The activity of DTNB-treated 1:5 (triple mutant:C320S) hybrid fractions towards glutamate ($\circ$; Glu) and norleucine ($\bullet$; Nle) were measured after 2-mercaptoethanol treatment of the fractions and the ratio of the two activities (C320S:triple mutant) ($\square$) was determined. A_{280} ($\blacktriangle$) was also measured.

the pure parent proteins, although a very small peak around fractions 8 and 9 indicated a minor proportion of triple mutant homohexamer. The slightly advanced elution volume of the main peak, relative to that for pure C320S GDH, suggests that the refolded mixture contained inter-subunit hybrids binding less tightly to the column, because not all their subunits could bind NAD^+ .

The ratio of activity towards glutamate (C320S) and norleucine (triple mutant), indicating the relative amounts of the two mutants, was determined for each fraction in the elution of the hybrid mixture (Figure 2). The increase in ratio of activity (C320S:triple mutant) with the fraction number indicated that different hybrids in the eluting fractions interact differently with NAD^+ -agarose. For the peak fraction 28, the specific activities for the triple mutant and C320S (related to total protein concentration) were calculated in order to find out how much activity is contributed individually by the two types of subunits. For the triple mutant subunits it was 0.22 unit/mg (17% of the original activity) with norleucine as the substrate, and for C320S subunits 21.6 units/mg (87% of the original activity) with glutamate as the substrate. These two independent values are both close to the predicted percentages for a 1:5 hybrid (16.7% and 83.3%). Correspondingly, the ratio of activities (triple mutant:C320S) was 98.6 for fraction 28, as compared with a theoretical value of 99 calculated for 1:5 (triple mutant:C320S) hybrids from the specific activities of triple mutant for norleucine and C320S for glutamate. In the neighbouring fractions, the triple mutant and C320S specific activities were 0.28 unit/mg and 19 units/mg respectively (fraction 27), and 0.16 unit/mg and 23.2 units/mg respectively (fraction 29). These activity values suggest that fraction 28 contained predominantly 1:5 hybrid hexamers, whereas the neighbouring fractions were contaminated with 2:4 and 0:6 species.

The partial concentrations of triple mutant and C320S subunits in a fraction can also be estimated spectrophotometrically by exploiting the fact that only the triple mutant subunits react with DTNB (compare with [20]). Estimation of the released TNB anion showed that the partial concentration of triple mutant in fraction 28 was 0.12 mg/ml out of a total of 0.83 mg/ml. This

Figure 3 Effect of succinate on the sigmoidal dependence upon glutamate concentration of the rates of the C320S mutant GDH

Initial rates of reaction were measured at a fixed concentration of NAD^+ (1 mM) and over a range of glutamate (Glu) concentrations (0–300 mM) in the presence of 0 (●), 10 (■), 50 (▲) and 100 mM (▼) succinate in 0.1 M Tris/HCl (pH 8.8). U, units.

Figure 4 Effect of succinate and glutarate on the sigmoidal dependence of the reaction rate of C320S mutant

Activity of the C320S mutant was measured at fixed concentrations of NAD^+ (1 mM) and L-glutamate (40 mM) and varying concentrations of succinate (●) or glutarate (○) in 0.1 M Tris/HCl (pH 8.8). U, units.

value, approx. 14.5% of the total, is again close to the theoretical expectation (16.7%). On the same basis, the percentages of triple mutant in fractions 27 and 29 were 21% and 6.5%. This independent method of estimation therefore also suggests a preponderance of 1:5 hybrid species in fraction 28. Contamin-

Figure 5 Effect of succinate and glutarate on the dependence of the reaction rate of triple mutant

Activity of the triple mutant was measured at fixed concentrations of NAD^+ (1 mM) and L-norleucine (70 mM) with varying concentrations of succinate (●) or glutarate (○) in 0.1 M Tris/HCl (pH 8.5). U, units.

Figure 6 Effect of succinate and glutarate on the reaction rate of 1:5 (triple mutant:C320S) hybrids with norleucine

Activity of the triple mutant subunit was measured at fixed concentrations of NAD^+ (1 mM) and L-norleucine (70 mM) with varying concentrations of succinate (●) or glutarate (○) in 0.1 M Tris/HCl (pH 8.5). U, units.

ation by hybrids of differing composition (e.g. 0:6 and 2:4) must either be slight or else maintain the overall 1:5 ratio for the fraction as a whole.

Effect of succinate and glutarate on C320S

In order to provide a basis for assessing the behaviour of the hybrid hexamer, it was essential to define the relevant properties

Figure 7 Effect of norleucine on native triple mutant and 1:5 (triple mutant:C320S) hybrid

Activity was measured with the native triple mutant (A) and the 1:5 (triple mutant:C320S) hybrid (B) at a fixed concentration of NAD^+ (1 mM) with varying concentrations of L-norleucine in 0.1 M Tris/HCl (pH 8.5). U, units.

of the parent molecules, i.e. the C320S and triple mutant homohexamers. As with WT GDH [12], the sigmoidal dependence upon glutamate concentration of the rate of glutamate oxidation catalysed by pure C320S mutant GDH was gradually removed by succinate (Figure 3). The h value decreased from 5.2 to 4.2, 2.5 and finally 1.3 as the succinate concentration increased from 0 to 10, 50 and then 100 mM. At a fixed concentration of 40 mM glutamate and 1 mM NAD^+ , varying concentrations of succinate (0–75 mM) gave a large activation with a sigmoidal dependency, with EC_{50} at 70 mM and an h value of 2.4 (Figure 4). There was a similar sigmoidal response to glutarate, with EC_{50} at 35 mM (Figure 4) and an h value of 2.2.

Effect of succinate and glutarate on triple mutant

Even though the triple mutant GDH is inactive with the dicarboxylate amino acid glutamate as a substrate, it retains an allosteric response, albeit a weak one, to dicarboxylate ligands. Thus, at a fixed substrate concentration of 70 mM norleucine, there was a sigmoidal increase in activity with varying succin-

Figure 8 Effect of glutamate on 1:5 (triple mutant:C320S) hybrid

Activity of the 1:5 (triple mutant:C320S) hybrid was measured after treatment with 5 mM 2-mercaptoethanol at a fixed concentration of NAD^+ (1 mM) with varying concentration of L-glutamate in 0.1 M Tris/HCl (pH 8.8). U, units.

ate concentrations, maximally about 1.8-fold, with EC_{50} at 80 mM (Figure 5) and an h value of 2.2. The response to glutarate (Figure 5) was less co-operative, with EC_{50} of 75 mM and an h value of 1.7, but somewhat more pronounced (maximally just over 2-fold).

Independent evidence of a conformational effect of dicarboxylate ligands on the triple mutant GDH comes also from a remarkable stabilization against unfolding in urea. When treated with 4.5 M urea for 60 min, the triple mutant was completely inactivated, but in the presence of 100 mM succinate, under the same conditions, it retained over 70 % of its activity after 60 min.

Investigation of the effects of substrates and other ligands on the hybrid hexamer

Hybrids (1:5; triple mutant:C320S), prepared as described above, were used for these experiments. With norleucine as the substrate, 1:5 hybrids, containing only one subunit per hexamer able to use this substrate, still gave sigmoidal responses to succinate and glutarate (Figure 6). h values of 2.4 and 2.6 respectively, higher than those for the pure triple mutant (Figure 5), indicated increased co-operativity among the hybrid subunits. Pure C320S hexamers showed higher co-operativity with these ligands (Figure 4), and the five C320S subunits in the hybrid thus contribute to a higher overall response, even though it is the norleucine catalytic activity of the single non-C320S subunit which is being used to sense this global effect.

The 1:5 hybrid (triple mutant:C320S) gave a similar pattern with varying norleucine concentrations (Figure 7B) to that shown by the native triple mutant (Figure 7A). This shows that the five C320S subunits per hexamer have little influence on the single triple mutant subunit with regard to the reaction with norleucine. In the presence of 100 mM succinate (results not shown), the hybrids gave an almost linear dependence on norleucine concentration, whereas the graph for the native triple mutant showed some hyperbolic character, indicative of approach towards

saturation (note that the low solubility of norleucine limits the achievable concentration).

The C320S mutant showed a sigmoidal curve of activity at higher concentrations of glutamate at pH 8.8, with an h value of 5.2 (Figure 3), indicating strong positive co-operativity in glutamate binding to the homogeneous hexamer. After the removal of blocking TNB groups with 2-mercaptoethanol, 1:5 hybrids (i.e. with all subunits active) showed reduced co-operativity in the glutamate dependence of the rate, with an h value of only 3.6 (Figure 8). Thus the presence of a 'foreign' subunit in the hybrid hexamer apparently impedes communication between the subunits.

Overall the results described in the present study show unambiguously that, just as coenzyme-binding information is transmitted between subunits of the hexamer, information about the binding of dicarboxylate effectors, which may be seen as glutamate surrogates, may also independently be efficiently transmitted to a norleucine-active subunit from five subunits that do not use norleucine as a substrate. These allosteric characteristics do not require all the subunits to be catalytically competent even though it is through the catalytic reaction that the co-operativity is manifest.

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REFERENCES

- Frieden, C. (1959) Glutamic dehydrogenase: II Effect of various nucleotides on association-dissociation and kinetic properties. *J. Biol. Chem.* **234**, 815–819
- Tomkins, G., Yielding, K. L., Talal, N. and Curran, J. (1963) Protein structure and biological regulation. *Cold Spring Harbor Symp. Quant. Biol.* **28**, 461–470
- Engel, P. C. and Dalziel, K. (1969) Kinetic studies of glutamate dehydrogenase with glutamate and norvaline as substrates: coenzyme activation and negative homotropic interactions in allosteric enzymes. *Biochem. J.* **115**, 621–631
- West, D. J., Tuveson, R. W., Barratt, R. W. and Fincham, J. R. S. (1967) Allosteric effects in nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Neurospora*. *J. Biol. Chem.* **242**, 2134–2138
- Lé John, H. B., Stevenson, R. M. and Meuser, R. (1970) Multivalent regulation of glutamic dehydrogenases from fungi: effects of adenylates, guanylates and acyl coenzyme A derivatives. *J. Biol. Chem.* **245**, 5569–5576
- Syed, S. E.-H., Engel, P. C. and Parker, D. M. (1991) Functional studies of a glutamate dehydrogenase with known three-dimensional structure: steady-state kinetics of the forward and reverse reactions catalysed by the NAD⁺-dependent glutamate dehydrogenase of *Clostridium symbiosum*. *Biochim. Biophys. Acta* **1115**, 123–130
- Monod, J., Changeux, J. P. and Jacob, F. (1963) Allosteric proteins and cellular control systems. *J. Mol. Biol.* **6**, 306–329
- Peterson, P. E. and Smith, T. J. (1999) The structure of bovine glutamate dehydrogenase provides insights into the mechanism of allostery. *Structure* (London) **7**, 769–782
- Baker, P. J., Britton, K. L., Engel, P. C., Farrants, G. W., Lilley, K. S., Rice, D. W. and Stillman, T. J. (1992) Subunit assembly and active site location in the structure of glutamate dehydrogenase. *Proteins: Struct. Funct. Genet.* **12**, 75–86
- Stillman, T. J., Baker, P. J., Britton, K. L. and Rice, D. W. (1993) Conformational flexibility in glutamate dehydrogenase: role of water in substrate recognition and catalysis. *J. Mol. Biol.* **234**, 1131–1139
- Stillman, T. J., Migueis, A. M. B., Wang, X.-G., Baker, P. J., Britton, K. L., Engel, P. C. and Rice, D. W. (1999) Insights into the mechanism of domain closure and substrate specificity of glutamate dehydrogenase from *Clostridium symbiosum*. *J. Mol. Biol.* **285**, 875–885
- Wang, X.-G. and Engel, P. C. (1995) Positive cooperativity with Hill coefficients of up to 6 in the glutamate concentration dependence of steady-state reaction rates measured with clostridial glutamate dehydrogenase and the mutant A163G at high pH. *Biochemistry* **34**, 11417–11422
- Pasquo, A., Britton, K. L., Stillman, T. J., Rice, D. W., Cölfen, H., Harding, S. E., Scandurra, R. and Engel, P. C. (1996) Construction of a dimeric form of glutamate dehydrogenase from *Clostridium symbiosum* by site-directed mutagenesis. *Biochim. Biophys. Acta* **1297**, 149–158
- Millevoi, S., Pasquo, A., Chiaraluce, R., Consalvi, V., Giangiacomo, L., Britton, K. L., Stillman, T. J., Rice, D. W. and Engel, P. C. (1998) A monomeric mutant of *Clostridium symbiosum* glutamate dehydrogenase: comparison with a structured monomeric intermediate obtained during refolding. *Protein Sci.* **7**, 966–974
- Dean, J. L. E., Cölfen, H., Harding, S. E., Rice, D. W. and Engel, P. C. (1997) Alteration of the quaternary structure of glutamate dehydrogenase from *Clostridium symbiosum* by a single mutation distant from the subunit interfaces. *Eur. Biophys. J.* **25**, 417–422
- Aghajanian, S. and Engel, P. C. (1997) Reactivation of *Clostridium symbiosum* glutamate dehydrogenase from subunits denatured by urea. *Biochem. J.* **326**, 649–655
- Aghajanian, S. A., Wang, X.-G. and Engel, P. C. (1996) *In vitro* construction of inter-subunit hybrids in *Clostridium symbiosum* glutamate dehydrogenase. *Biochem. Soc. Trans.* **24**, 136S
- Goyal, A., Aghajanian, S., Hayden, B. M., Wang, X.-G. and Engel, P. C. (1997) Intersubunit communication in hybrid hexamers of K89L/A163G/S380A and C320S mutants of glutamate dehydrogenase from *Clostridium symbiosum*. *Biochemistry* **36**, 15000–15005
- Goyal, A., Aghajanian, S., Hayden, B. M., Wang, X.-G. and Engel, P. C. (1998) Construction and investigation of co-operativity in hybrids of norleucine- and glutamate-active subunits of clostridial glutamate dehydrogenase. *Biochem. Soc. Trans.* **26**, S27
- Aghajanian, S. and Engel, P. C. (1998) Use of protein engineering to explore subunit interactions in an allosteric enzyme: construction of inter-subunit hybrids in *Clostridium symbiosum* glutamate dehydrogenase. *Protein Eng.* **11**, 569–575
- Wang, X.-G. and Engel, P. C. (1994) Identification of the reactive cysteine in clostridial glutamate dehydrogenase by site-directed mutagenesis and proof that this residue is not strictly essential. *Protein Eng.* **7**, 1013–1016
- Wang, X.-G. (1996) Alteration in substrate specificity in glutamate dehydrogenase by site-directed mutagenesis. PhD Thesis, University of Sheffield, Sheffield
- Wang, X.-G., Britton, K. L., Stillman, T. J., Rice, D. W. and Engel, P. C. (2001) Novel enzyme specificity: engineering of a monocarboxylic amino acid dehydrogenase with activity towards L-methionine by site-directed mutagenesis of a clostridial glutamate dehydrogenase gene. *Eur. J. Biochem.* **268**, 5791–5799
- Syed, S. E.-H., Hornby, D. P., Brown, P. E., Fitton, J. E. and Engel, P. C. (1994) Site and significance of chemically modifiable cysteine residues in glutamate dehydrogenase of *Clostridium symbiosum* and the use of protection studies to measure coenzyme binding. *Biochem. J.* **298**, 107–113
- Basso, L. A. and Engel, P. C. (1994) Initial formation of a non-covalent enzyme-reagent complex during the inactivation of clostridial glutamate dehydrogenase by Ellman's reagent: determination of the enzyme's dissociation constant for the binary complex with NAD⁺ from protection studies. *Biochim. Biophys. Acta* **1209**, 222–226
- Hayden, B. M. and Engel, P. C. (2001) Construction, separation and properties of hybrid hexamers of glutamate dehydrogenase in which five of the six subunits are contributed by the catalytically inert D165S. *Eur. J. Biochem.* **268**, 1173–1180
- Dean, J. L. E., Wang, X.-G., Teller, J. K., Waugh, M. L., Britton, K. L., Baker, P. J., Stillman, T. J., Martin, S. R., Rice, D. W. and Engel, P. C. (1994) The catalytic role of aspartate in the active site of glutamate dehydrogenase. *Biochem. J.* **301**, 13–16
- Aghajanian, S. A., Martin, S. R. and Engel, P. C. (1995) Urea-induced inactivation and denaturation of clostridial glutamate dehydrogenase: the absence of stable dimeric or trimeric intermediates. *Biochem. J.* **311**, 905–910

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