RESEARCH COMMUNICATION

The catalytic role of aspartate in the active site of glutamate dehydrogenase

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A putative catalytic aspartyl residue, Asp-165, in the active site of clostridial glutamate dehydrogenase has been replaced with serine by site-directed mutagenesis. The mutant enzyme is efficiently overexpressed in *Escherichia coli* as a soluble protein and can be successfully purified by the dye-ligand chromatographic procedure normally employed for the wild-type enzyme. By several criteria, including circular dichroism spectrum, sulphhydryl reactivity with Ellman's reagent, crystallization and mobility in non-denaturing electrophoresis, the enzyme appears to be correctly folded. NAD⁺ protects the D165S mutant against

modification by Ellman's reagent, suggesting unimpaired binding of coenzyme. In standard assays the specific activity is decreased 10³-fold in the reductive amination reaction and 10⁵-fold for oxidative deamination. Kinetic studies show that apparent K_m values for NADH and 2-oxoglutarate are almost unchanged. The large reduction in the reaction rate coincides with a weakening of the affinity for ammonium ion ($K_m > 300$ mM, compared with 60 mM for the wild-type). The data are entirely consistent with the direct involvement of D165 in catalysis rather than in the binding of coenzyme or 2-oxoglutarate.

INTRODUCTION

Glutamate dehydrogenases (GDH; EC 1.4.1.2–4) catalyse the reversible oxidative deamination of L-glutamate, with NAD(P)H as cofactor. Although iminoglutarate has long been considered a likely intermediate [1], non-enzymic formation of this compound is too slow to account for observed rates of catalysis [2]. In the presence of the enzyme, however, small quantities of L-glutamate are produced from 2-oxoglutarate and ammonia by reduction with sodium borohydride, suggesting that iminoglutarate is formed and bound in the catalytic pocket [3].

In a proposal [4] for the catalytic mechanism of the enzyme, based on the pH-dependence of inhibition by substrate analogues, the proposed iminoglutarate intermediate is attacked, by a water molecule polarized by its binding to a lysine residue. A group of pK 4.5, possibly an aspartyl carboxyl group, has been postulated to act as a base deprotonating the α -amino group. No direct evidence for the involvement of a carboxyl group was available, however.

The recently solved crystallographic structure for a glutamate complex of clostridial GDH [5] not only shows electron density for the bound water molecule between the ϵ -amino group of Lys-125 and the α -carbon of glutamate, but also identifies the carboxyl group of Asp-165 as being ideally placed to receive a proton from the α -amino group. This has permitted the formulation of a detailed mechanism in which the aspartate plays a crucial role in accepting and donating protons throughout the catalytic cycle [5].

In this paper we have tested the role of Asp-165 by site-directed mutagenesis. Substitution by serine was chosen to remove the negative charge and proton acceptor/donor capacity of the aspartate carboxyl whilst minimizing secondary structural changes.

EXPERIMENTAL

DNA methods and site-directed mutagenesis of GDH

Manipulations of recombinant DNA were performed using standard protocols [6,7]. The *gdh* gene of *Clostridium symbiosum* was subcloned into M13mp19 from pGS516 [8] using the *Bam*HI and *Sal*I restriction sites. DNA fragments were extracted from 1% agarose gels with the GeneClean II kit (BIO 101 Inc.).

Oligonucleotide-directed mismatch mutagenesis was performed by selection against template strands containing uracil [9,10]. In the mutagenic oligonucleotide, 5'-TCCTGC-AGGTAGCCTTGGTGTAG-3', the underlined serine codon (AGC) replaced the wild-type aspartate codon (GAC). Confirmation that the mutation of Asp-165 to Ser had been successful was obtained by single-stranded DNA sequencing [11] of the mutant gene inserted into M13mp19. The D165Sgdh gene was subcloned into the expression vector ptac-85 [12] and overexpressed in *Escherichia coli* TG1. Wild-type *gdh* contamination was avoided by repeated subculturing of the TG1 ptac-D165Sgdh. The possibility of undesired secondary mutations was excluded by sequencing the entire *gdh* gene following subcloning back into M13mp19. The host strain *E. coli* TG1 (Δ lac-proAB supE thi hsdΔ5/ F' traD36 proA+B+ lacI^qZΔM15), which contains a functional *E. coli* *gdh* gene, was used throughout DNA manipulation and expression. This minimizes the selective advantage conferred by reversion to wild-type in the mutant *gdh* gene.

Enzyme preparation

Wild-type GDH was purified to homogeneity from *E. coli* TG1pGS516 cell paste on Remazol Brilliant Red GG Sepharose CL-6B [13]. D165S-GDH was partially purified from *E. coli* TG1 ptac-D165Sgdh on a fresh column of the same type and further

Abbreviations used: GDH, glutamate dehydrogenase; IPTG, isopropyl β -D-thiogalactopyranoside; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); RF, replicative form.

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purified by gel filtration on Sephacryl S300-HR. Storage, dialysis and clarification of the enzymes and determination of concentration were according to Syed et al. [13]. Column materials were washed with 2 column volumes of 6 M urea/2 M NaCl, followed by 10 column volumes of distilled water, prior to equilibration with buffer. Cuvettes were also washed with 5 M NaOH to avoid contamination with wild-type enzyme.

Polyacrylamide-gel electrophoresis

Native and SDS/PAGE were carried out in general according to Garfin [14], and gels were stained with Coomassie Brilliant Blue R-250. Purified enzyme samples, however, were loaded on SDS/PAGE gels immediately after addition of SDS loading buffer without heating. This avoids cleavage of GDH, which may occur with higher temperatures and longer incubation times (Dr. S. Aghajanian, personal communication).

Enzyme assays

Activity in the two-substrate reaction was measured with a recording fluorimeter [13] at 25 °C. Reaction mixtures (4 ml) contained 1 mM NAD⁺ and 40 mM L-glutamate in 0.1 M potassium phosphate, pH 7.0, and 2 nM or 3 μ M wild-type or mutant GDH subunits respectively.

The three-substrate reaction was measured spectrophotometrically (Kontron Uvikon 941 Plus) at 340 nm and 25 °C. Standard reaction mixtures (1 cm light path; 1 ml) contained 0.1 mM NADH, 5 mM 2-oxoglutarate and 50 mM NH₄Cl in 0.1 M potassium phosphate, pH 7.0. Final wild-type and mutant GDH subunit concentrations were 2 nM and 0.1 μ M respectively.

To determine kinetic parameters, rates were measured with 2 mM 2-oxoglutarate and 50 mM NH₄Cl over a range of NADH concentrations (1–200 μ M). Similarly, the 2-oxoglutarate concentration was varied (50 μ M–2 mM) with 0.1 mM NADH and 50 mM NH₄Cl.

Michaelis parameters were calculated by non-linear regression [15] with the program Enzpack (Biosoft Ltd., Cambridge, U.K.).

Inactivation with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)

The stoichiometry of inactivation by DTNB was determined by monitoring the increase in A_{412} at 25 °C [16] in incubations of 0.2 mg·ml⁻¹ enzyme with 1 mM DTNB in 0.1 M potassium phosphate (pH 7.0). Protection by coenzyme was determined by adding 0–15 mM NAD⁺.

N-terminal amino acid sequence determination

A 5 μ l sample of purified D165S mutant protein was separated by SDS/PAGE and electroblotted on to a Problot membrane (Applied Biosystems Inc.) [17]. The 49 kDa band was excised and loaded with a blot cartridge. The N-terminal 10 residues were sequenced (Applied Biosystems 476A Sequencer).

Crystallization of D165S mutant GDH

Crystallization trials were carried out by the hanging drop method of vapour diffusion over a range of conditions suitable for native GDH and its substrate complexes [18]. These included the addition of potassium tetracyanoplatinate, tartrate and NAD⁺.

Materials

Reagents for molecular biology were of the highest grade commercially obtainable. Bacterial strain *E. coli* TG1 and most reagents for mutagenesis were obtained from Boehringer Mannheim U.K. M13mp19 replicative form (RF) DNA was supplied by Pharmacia Biotech Ltd. (St. Albans, U.K.). Single-stranded DNA sequencing was carried out with the 7-deazadGTP reagent kit and Sequenase Version 2.0 DNA polymerase. T4 DNA polymerase was from Epicentre Technologies. Sepharose CL-6B and Sephacryl S300-HR were purchased from Pharmacia Biotech Ltd. Grade II NAD⁺ (free acid), grade II NADH (disodium salt), 98% NADP⁺ (disodium salt) and 2-oxoglutarate were supplied by Boehringer Mannheim. Other chemicals were supplied by Fisons or BDH. Oligonucleotides were synthesized using an Applied Biosystems 392 Oligonucleotide Synthesizer by the phosphoramidite method.

RESULTS

SDS/PAGE of crude extracts from TG1 cells transformed with ptac-D165S or pGS516 (wild-type) and grown overnight in Luria-Bertani broth with 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) shows comparable overexpression of the two proteins (Figure 1a, lanes 1 and 2). D165S also has identical mobility to wild-type GDH (Figure 1a, lanes 3 and 4). Mutant enzyme was purified to near-homogeneity (Figure 1a, lane 4) and a 1 litre cell suspension of a 16 h growth at 37 °C yielded approx. 30 mg of pure D165S protein after the two-step purification. This compares with about 80 mg for the wild-type enzyme after a single step on Remazol Red Sepharose CL-6B. The endogenous NADP(H)-dependent GDH activity of the *E. coli* host was completely washed from the Remazol Red column with loading buffer before elution of wild-type GDH or D165S with NaCl.

The polypeptide sequence of the first 10 N-terminal residues of

Figure 1 PAGE of wild-type and D165S enzymes and crude extracts

(a) SDS/PAGE on 12% gels. Lane 1, 10 μ l of a 100 $\times$ dilution of wild-type crude extract TG1pGS516; 2, 10 μ l of a 100 $\times$ dilution of D165S mutant crude extract TG1 ptac-D165S; 3, 10 μ l of 0.4 mg·ml⁻¹ wild-type enzyme purified on Red Remazol Sepharose CL-6B; 4, 10 μ l of 0.6 mg·ml⁻¹ D165S enzyme purified by Red Remazol and Sephacryl S300-HR chromatography; 5, molecular mass markers. Samples were treated as described in the Experimental section. (b) Non-denaturing PAGE (7.5% gels) of purified wild-type and D165S-GDH. Samples contained 10 μ l of 1 mg·ml⁻¹ wild-type (WT) or D165S protein stained with Coomassie Brilliant Blue R-250.

Figure 2 Far-u.v. c.d. spectra of wild-type and D165S mutant GDHs

Spectra were recorded at 20 °C in 0.1 M potassium phosphate, pH 7.0. The protein concentration was 1.5 mg·ml⁻¹ and the cell path length was 0.1 mm.

the mutant protein (SKYVDRVIAE) matches the wild-type sequence [8] and shows that the mutant undergoes normal removal of the N-terminal methionine.

The purified mutant and wild-type enzymes exhibit identical mobility during electrophoresis under non-denaturing conditions (Figure 1b), indicating a similar size and surface charge and thus a similar gross conformation.

In wild-type GDH, DTNB modifies only one of the two cysteine residues (Cys-320) [19,20]. Similar experiments with D165S-GDH indicate the release of 0.97 mol of thionitrobenzoate/mol of protein subunit, consistent with correct folding of the mutant protein. The modification of wild-type GDH can be prevented by the inclusion of NAD⁺ in the DTNB incubation buffer [19]. Control experiments confirmed this result (78 % protection with 5 mM, 89 % with 10 mM and 93 % with 15 mM NAD⁺). In equivalent experiments D165S was protected to a similar degree (85 % with 5 mM, 90 % with 10 mM and 93 % with 15 mM). These results suggest similar binding constants for NAD⁺ in wild-type GDH and in the mutant.

Far-u.v. c.d. spectra normalized for protein concentration (Figure 2) show similar profiles for the mutant and wild-type enzymes (approx. 10 % difference in Δε), and the spectra intersect the abscissa at an identical wavelength. The former observation indicates similar degrees of secondary structure, whilst the latter suggests that the difference in c.d. spectra may be due to an inadvertent difference in the protein concentrations. Finally, the growth of high-quality crystals under a range of conditions similar to those for the wild-type enzyme argues for overall retention of a similar tertiary structure.

D165S-GDH activities in standard two-substrate and three-substrate assays were $1.37 (\pm 0.22) \times 10^{-4}$ and 0.185 ± 0.003 μmol·min⁻¹·mg⁻¹ respectively, compared with wild-type values of 20.8 and 127 μmol·min⁻¹·mg⁻¹. This represents a dramatic reduction in the activity of D165S compared with wild-type (1.5×10^5 - and 6.9×10^2 -fold for the two- and three-substrate reactions respectively). The ratio of forward/reverse reaction rates is thus also strikingly different (220-fold) for the mutant.

Although NAD⁺ is an alternative substrate for the *E. coli* enzyme, the rate with 1 mM NADP⁺ is 1000 times higher [21]. No activity could be detected for the D165S preparation in an

Table 1 Kinetic parameters of mutant and wild-type (WT) GDH

K_m and V_{max} are the apparent Michaelis constants and maximal velocities. Units for K_m for NADH are μM; for 2-oxoglutarate and ammonium they are mM. ND, could not be determined.

	K_m		V_{max} (μmol·min ⁻¹ ·mg ⁻¹)	
	WT	D165S	WT	D165S
NADH	10.8 ± 0.7	9.7 ± 2.2	125 ± 2	0.144 ± 0.008
2-Oxoglutarate	0.31 ± 0.05	0.29 ± 0.06	191 ± 10	0.141 ± 0.010
Ammonium	61.1 ± 11.9	ND	296 ± 21	ND

assay mixture containing 1 mM NADP⁺ and 40 mM L-glutamate. This eliminates the possibility of contamination with *E. coli* GDH as an explanation of residual NAD⁺ activity. Similarly, for the reverse reaction no activity could be detected with an assay solution containing 0.1 mM NADH but lacking the other two substrates, suggesting that the observed rate with all three substrates is not due to contaminating NAD(H) oxidase.

A more detailed investigation of the diminished catalytic activity of the mutant enzyme revealed essentially unchanged apparent K_m values for 2-oxoglutarate and NADH (Table 1), despite an approximately 10^3 -fold decrease in the respective apparent V_{max} values. In the case of the third substrate, ammonium ion, it proved difficult to approach saturation, firstly because the K_m is clearly much higher than for wild-type enzyme, and secondly because of the non-specific inhibition at high ionic strengths. Likewise, it is impossible to offer a precise estimate of K_m , but this parameter for mutant enzyme is at least 300 mM. The combined parameter k_{cat}/K_m can, however, be precisely obtained for the mutant as 1.86×10^{-3} mM⁻¹·s⁻¹, decreased by a factor of 2.1×10^3 compared with 3.96 mM⁻¹·s⁻¹ for wild-type GDH.

DISCUSSION

Recent structural studies [5] have highlighted the likely central role of an aspartyl residue (Asp-165) in catalysis by clostridial GDH. Earlier mechanistic proposals [4] also invoked such a residue, but without direct evidence or specific identification. The side-chain carboxyl group of Asp-165 is thought to interact directly with the α-amino group of the substrate [5], and the choice of the optimal mutation to investigate the role of this residue purely in catalysis rather than binding was problematic. Mutation to serine may permit hydrogen bonding to the substrate through an additional internal water molecule, as in dihydrofolate reductase [22]. This provided a rationale for our first mutational replacement of Asp-165.

The large predicted decrease in catalytic activity is indeed observed in the D165S mutant. It is, however, essential to consider other possible explanations for the production of an almost inactive enzyme, e.g. gross misfolding or wild-type contamination. Data from native PAGE, protection against DTNB modification, c.d. studies and crystallization all indicate conservation of the overall native folding pattern in D165S. The detection of residual activity, albeit extremely low, also suggests essentially correct folding. The large change in the ratio of specific activities for the forward and reverse reactions implies that at least 99.5 % of the three-substrate activity is attributable to mutant rather than wild-type enzyme.

The observation that apparent Michaelis constants for NADH and 2-oxoglutarate are virtually unchanged in the mutant enzyme suggests that the replacement of Asp-165 by serine has little effect

on the binding of either of these substrates. In the case of coenzyme this is also borne out by the results of protection studies with DTNB. Since Asp-165 is believed to contact the ammonium ion in the catalytic complex, its replacement might be expected to affect binding of this substrate. The apparent K_m for ammonium ion is certainly much increased, but the precise extent of the effect remains uncertain.

Ideally, to establish the involvement of Asp-165, not merely in substrate binding, but more specifically in rate enhancement within the fully formed central complex, a comparison is needed of extrapolated maximum rates for wild-type and mutant enzymes under conditions of saturation with all substrates. This has proved experimentally inaccessible to date, and it remains possible in theory that the very large decreases in rate are entirely attributable to impaired binding. However, overall the striking decrease in catalytic activity of D165S-GDH is fully consistent with the view that Asp-165 is a catalytic residue.

Asp-165 is fully conserved in all GDH sequences obtained so far [8], except for early protein sequences for chicken and bovine liver enzymes [23,24], where the equivalent residue was reported as asparagine. More recently, the determination of the amino acid sequences from the DNA for the highly similar enzymes from mouse, rat and human have shown that the corresponding residue is aspartate ([8], and references cited therein). Finally, in the hyperthermophilic archaeon, ES4, the equivalent residue in GDH was originally designated as a histidine [25]; however, this has now been confirmed as aspartate (Dr. F. Robb, personal communication).

It seems likely that the significance of this finding extends beyond the family of GDHs, since a structure-based alignment of the sequences for the wider superfamily of amino acid dehydrogenases [26], including for example leucine and phenylalanine dehydrogenases, reveals an equivalent aspartic acid residue throughout. However, in a recent study of the pH-dependence of catalysis in leucine dehydrogenase [27], no group with a pK ascribable to aspartate was found, but we note that the lower limit of pH in this investigation was 7.5 and therefore such a group would not be detected. Our work therefore suggests a universal role for aspartate in catalysis of the oxidative deamination/reductive amination of amino acids/oxo acids. The present study offers the first direct evidence for such a role. It will be interesting to study the kinetics of Asp-165 mutants in more detail, in particular by rapid-reaction kinetics, in order to elucidate the role of this residue more fully.

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REFERENCES

- 1 Von Euler, H., Adler, E., Gunter, G. and Das, N. B. (1938) Hoppe-Seyler's Z. Physiol. Chem. **254**, 61–103
- 2 Strecker, H. J. (1953) Arch. Biochem. Biophys. **46**, 128–140
- 3 Hochreiter, M. C., Patek, D. R. and Schellenberg, K. A. (1972) J. Biol. Chem. **247**, 6271–6276
- 4 Rife, J. E. and Cleland, W. W. (1980) Biochemistry **19**, 2328–2333
- 5 Stillman, T. J., Baker, P. J., Britton, K. L. and Rice, D. W. (1993) J. Mol. Biol. **234**, 1131–1139
- 6 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 7 Perbal, B. (1988) A Practical Guide to Molecular Cloning, John Wiley and Sons, New York
- 8 Teller, J. K., Smith, R. J., McPherson, M. J., Engel, P. C. and Guest, J. R. (1992) Eur. J. Biochem. **206**, 151–159
- 9 Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 488–492
- 10 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Methods Enzymol. **154**, 367–382
- 11 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
- 12 Marsh, P. (1986) Nucleic Acids Res. **14**, 3603
- 13 Syed, S. E.-H., Engel, P. C. and Parker, D. M. (1991) Biochim. Biophys. Acta **1115**, 123–130
- 14 Garfin, D. E. (1990) Methods Enzymol. **182**, 425–441
- 15 Wilkinson, G. N. (1961) Biochem. J. **80**, 324–332
- 16 Ellman, G. L. (1959) Arch. Biochem. Biophys. **82**, 70–74
- 17 Matsudaira, P. T. (1989) A Practical Guide to Protein and Peptide Purification for Microsequencing, Academic Press, New York
- 18 Stillman, T. J., Baker, P. J., Britton, K. L., Rice, D. W. and Rodgers, H. F. (1992) J. Mol. Biol. **224**, 1181–1184
- 19 Syed, S. E.-H., Hornby, D. P., Brown, P. E., Fitton, J. E. and Engel, P. C. (1994) Biochem. J. **298**, 107–113
- 20 Wang, X.-G. and Engel, P. C. (1994) Protein Eng. **7**, in the press
- 21 Dean, J. L. E. (1992) M.Phil. Thesis, University of Sheffield
- 22 Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J. and Kraut, J. (1986) Science **231**, 1123–1128
- 23 Moon, K., Piszkiwicz, D. and Smith, E. L. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 1380–1383
- 24 Landon, M., Piszkiwicz, D. and Smith, E. L. (1971) J. Biol. Chem., **246**, 2374–2399
- 25 DiRuggiero, J., Robb, F. T., Jagus, R., Klump, H. H., Borges, K. M., Kessel, M., Mai, X. and Adams, M. W. W. (1993) J. Biol. Chem. **268**, 17767–17774
- 26 Britton, K. L., Baker, P. J., Engel, P. C., Rice, D. W. and Stillman, T. J. (1993) J. Mol. Biol. **234**, 938–945
- 27 Sekimoto, T., Matsuyama, T., Fukui, T. and Tanizawa, K. (1993) J. Biol. Chem. **268**, 27039–27045