Spontaneous Chemical Reversion of an Active Site Mutation: Deamidation of an Asparagine Residue Replacing the Catalytic Aspartic Acid of Glutamate Dehydrogenase†

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ABSTRACT: A mutant (D165N) of clostridial glutamate dehydrogenase (GDH) in which the catalytic Asp is replaced by Asn surprisingly showed a residual 2% of wild-type activity when purified after expression in *Escherichia coli* at 37 °C. This low-level activity also displayed Michaelis constants for substrates that were remarkably similar to those of the wild-type enzyme. Expression at 8 °C gave a mutant enzyme preparation 1000 times less active than the first preparation, but progressively, over 2 weeks' incubation at 37 °C in sealed vials, this enzyme regained 90% of the specific activity of wild type. This suggested that the mutant might undergo spontaneous deamidation. Mass spectrometric analysis of tryptic peptides derived from D165N samples treated in various ways showed (i) that the Asn is in place in D165N GDH freshly prepared at 8 °C; (ii) that there is a time-dependent reversion of this Asn to Asp over the 2-week incubation period; (iii) that detectable deamidation of other Asn residues, in Asn-Gly sequences, mainly occurred in sample workup rather than during the 2-week incubation; (iv) that there is no significant deamidation of other randomly chosen Asn residues in this mutant over the same period; and (v) that when the protein is denatured before incubation, no deamidation at Asn-165 is detectable. It appears that this deamidation depends on the residual catalytic machinery of the mutated GDH active site. A literature search indicates that this finding is not unique and that Asn may not be a suitable mutational replacement in the assessment of putative catalytic Asp residues by site-directed mutagenesis.

Elucidation of the catalytic mechanism of glutamate dehydrogenase (GDH)[1] (EC 1.4.1.2−4) was hampered for many years by the absence of a solved three-dimensional structure to provide a structural framework, despite abundant experimental evidence for the involvement of a lysine residue (e.g., refs *1*−*4*) and some indirect evidence implicating a carboxyl group in the active site (*5*). Solution of the crystal structure for the glutamate complex of *Clostridium symbiosum* GDH (*6*) made it possible to postulate a detailed mechanism and highlighted the key positions and likely roles of Lys-125 and Asp-165, putative acid−base catalysts in the mechanism. The involvement of the lysine had already been validated both by chemical modification studies on several GDHs (*1*−*4*) and by site-directed mutagenesis of the corresponding residue in *Escherichia coli* GDH (*7*). In the absence of evidence for a catalytic aspartate residue from chemical modification studies, advantage was taken of the cloning of the clostridial GDH gene (*8*) to create several mutations at the D165 position. One of these, D165S, produced the expected large decrease in catalytic activity (*9*), although, interestingly, this mutant is susceptible to catalytic rescue by fluoride (*10*). A second mutant, D165H, gave an ambiguous result, in that, though totally inactive, it was also dimeric rather than, as the wild-type enzyme, hexameric (*11*). Loss of activity therefore could not be attributed with certainty to the local requirement for an Asp residue.

A further attempt to test the requirement for Asp-165 (*12*) provides the subject of the present paper: replacement of Asp with Asn was expected to lead to a totally inactive protein, since, while retaining similar size and shape, asparagine cannot act as a donor−acceptor of protons. Puzzlingly, this mutant appeared to retain about 2% of the activity of the wild-type enzyme. Two clues suggested that this might not reflect the true activity of the D165N protein: first, K_m values for the five substrates were remarkably close to those for the unmutated enzyme (*12*), and second, a new preparation from cells grown at 27 °C instead of 37 °C produced enzyme with a specific activity about 10-fold lower (S. Coughlan and P. C. Engel, unpublished data).

In this paper we demonstrate that the D165N mutant is at least 70000 times less active than the wild-type enzyme but

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[1] Abbreviations: GDH, L-glutamate dehydrogenase; IPTG, isopropyl β-D-thiogalactoside.

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that deamidation, presumably accelerated in the environment of the GDH catalytic site, restores the natural catalytic aspartate, and with it the activity of the protein, in a chemical reversion at the protein level of a mutation at the DNA level.

MATERIALS AND METHODS

Materials. L-Glutamate (monosodium salt) and Sepharose CL-6B were purchased from Sigma. Grade II NAD^+ (free acid) was obtained from Roche. All other chemicals were of analytical grade.

Expression of Recombinant Wild-Type GDH in E. coli. E. coli TG1 cells containing ptac-wild-type *gdh* (*8*) were grown at 37 °C to an A_{600} of 0.8−0.9 in Luria−Bertani medium containing 100 μg mL^{-1} ampicillin. Expression was induced with isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM, and cultures were shaken for a further 16 h before cells were harvested by centrifugation. GDH was purified throughout according to the usual procedure (*13*).

Expression of D165N gdh in E. coli and Purification of the Mutant Protein. The plasmid ptac-D165N GDH, carrying the D165N mutation in the clostridial *gdh* gene, was produced by the Kunkel method (*14*). The mutagenic oligonucleotide 5′-TCCTGCAGGTAACCTTGGTGTAG-3′ containing the underlined Asn codon (AAC) was used to effect replacement of the GAC codon encoding Asp-165 in ptac-wild-type *gdh*.

Cells of *E. coli* TG1 containing ptac-D165N *gdh* were grown overnight at 37 °C in 10 mL of Luria−Bertani medium containing 100 μg mL^{-1} ampicillin; 5.6 mL of that culture was used to inoculate 280 mL of Terrific Broth containing 100 μg mL^{-1} ampicillin and allowed to grow at 37 °C to an A_{600} of 0.9−1.1. In early stages of the project cells were then treated with 1 mM IPTG to induce expression of the mutant GDH. In view of the results presented in this paper, this routine was ultimately modified as follows. The rapidly growing culture at 37 °C was heat-shocked in a water bath at 42 °C for 20 min, cooled to room temperature, and left in the cold room for 1 h with vigorous stirring; contact with the magnetic stirrer maintains the temperature of the culture at about 8 °C. Expression was induced by adding IPTG to this cold suspension to a final concentration of 1 mM. Cultures were stirred in the cold for 16−18 h before cells were harvested by centrifugation. The purification of D165N GDH from the broken cells in all cases followed the same procedure as for wild-type GDH (*13*). Enzyme purity was checked by SDS−PAGE (12% gel stained with Coomassie Blue) and by LC-MS analysis of tryptic digests of the purified proteins (see below). No significant impurity was detected in wild-type GDH, but the purified preparation of D165N GDH contained trace levels of *E. coli* 3-oxoacyl-[acyl-carrier-protein] reductase. Enzyme concentrations were determined as described elsewhere (*13*).

Enzyme Assay. The activities for the forward reaction (two substrates) for the mutant were measured using a spectrophotometer (Varian Cary 50 Conc) or a fluorometer (Hitachi F-2500) by following the formation of NADH at 340 and 460 nm, respectively. Reaction mixtures (1 cm light path, 1 mL) contained 1 mM NAD^+ and 40 mM L-glutamate in 0.1 M potassium phosphate (pH 7.0) with the appropriate amount of enzyme (*13*). All values are related to a specific activity of 21.8 IU/mg for the wild-type enzyme in the standard assay with L-glutamate.

Tryptic Digestion. Each dried protein sample (see below) was redissolved in 0.025 mL of 0.4 M NH_4HCO_3, 8 M urea, and 0.0045 M dithiothreitol (reduced form) and incubated for 20 min at 50 °C. The samples were allowed to cool to 22 °C and then treated with 0.0027 mL of iodoacetamide (final concentration of iodoacetamide 0.01 M, an excess of about 1 mM over thiols in the protein and reducing agent). The samples were next incubated for 20 min at 22 °C and then diluted by addition of 0.175 mL of water. To each sample was added 0.001 mg of porcine trypsin (sequencing grade; Princeton Separations), and the samples were incubated at 37 °C for 22 h before being frozen at −20 °C until they were analyzed by LC-MS.

Liquid Chromatography−Mass Spectrometry. Peptide mapping by LC-MS was conducted by capillary HPLC using a Vydac type 218MS5.510 column (0.5 × 100 mm) running at 0.005 mL/min on an Agilent 1100 chromatograph based on a Model G1376A capillary pump and interfaced with a Thermo Finnigan LCQ ion-trap mass spectrometer. The HPLC solvents were (A) 0.02% trifluoroacetic acid and (B) 0.02% trifluoroacetic acid in acetonitrile. From initial conditions of 1.6% solvent B (0−2 min), the gradient consisted of steps from 1.6% to 35% solvent B (2−98 min) and 35% to 80% solvent B (98−108 min). The standard Finnigan electrospray interface was modified by replacing the sample needle with a coated fused silica tip (TaperTip, part number TT150-50−50-CE-5; New Objective Inc., Woburn, MA). The LCQ was controlled by Xcalibur 1.2 software (Thermo Finnigan) and set to perform data-dependent MS/MS analysis ("triple play"). Peptides were identified by searching a sequence database of bacterial proteins using the Mascot search engine (Matrix Sciences) (*15*).

RESULTS

Kinetic Study on D165N at pH 7.0. Initial results for the D165N mutant expressed at 37 °C indicated that this protein retained about 2% of the specific activity of the normal, wild-type enzyme, a surprisingly high figure in view of the postulated catalytic role for D165. To allow a direct comparison, steady-state parameters were therefore determined for both WT and D165N for all five substrates of the forward and reverse reactions (Table 1). An immediately striking feature of these results was that, while the apparent V_{max} values were decreased in each case by a factor of roughly 50, the apparent Michaelis constants were in all cases within a factor of 2 of the wild-type value. Although the agreement is not perfect, it raised the possibility that the similarity in K_m values reflected not a remarkable coincidence but rather a 2% contamination of an inactive mutant by wild-type GDH. All trivial routes to such contamination were rigorously excluded, however.

Effect of the Overexpression Temperature. A clue to the possible source of the activity came from the chance observation that the D165N enzyme produced at 27 °C was about 10 times less active than the standard preparations made at 37 °C. In a more systematic investigation, overproduction of protein was induced at 37, 23, and 8 °C, and the specific activity of the mutant GDH preparation was checked after purification to homogeneity. Induction at 37 °C resulted

Table 1: Apparent Steady-State Kinetic Parameters for WT and the D165N Mutant Expressed at 37 °C[a]

	V_{app} or V_{max} (units mg^{-1})		K_m	
substrate	WT	D165N	WT	D165N
L-glutamate	22.7 ± 0.4	0.422 ± 0.025	3.97 ± 0.11	2.66 ± 0.27
NAD^+	23.7 ± 0.5	0.292 ± 0.006	140 ± 13	76.0 ± 0.7
2-OG	126 ± 2	2.54 ± 0.04	125 ± 1	86.2 ± 7.6
NADH	125 ± 2	2.82 ± 0.05	10.8 ± 0.7	5.6 ± 0.7
ammonia	92.6 ± 1.5	1.79 ± 0.02	62.8 ± 3.5	49.4 ± 2.4

[a] K_m values are in mM for L-glutamate and ammonia and μM for NAD^+, NADH, and 2-oxoglutarate. 2-OG denotes 2-oxoglutarate. Reaction rates were measured in 0.1 M phosphate buffer, pH 7.0, at 25 °C. For the two-substrate reaction, NAD^+ concentration was varied from 0.1 to 2.0 mM with L-glutamate fixed at 100 mM, and L-glutamate concentration was varied over the range 0.5−200 mM with NAD^+ fixed at 1.0 mM. For L-glutamate parameters a mutant enzyme concentration of 60 nM was used, whereas for NAD^+ 200 nM enzyme was used with 40 nM enzyme at lower substrate concentrations to maintain linearity of initial rates. In the three-substrate reaction, the concentrations of NADH, 2-OG, and ammonium ion, when fixed, were 0.1, 2, and 50 mM, respectively, and, when varied, covered the ranges 1−200 μM, 0.05−2 mM, and 10 mM−1 M, respectively. For these measurements the final enzyme concentration in general was 2 nM for the wild-type enzyme and 0.1 μM for the mutant enzyme preparation, with the exception of the experiment to determine K_m for NH_4^+, where a lower concentration of 0.25 nM was used for the wild-type enzyme. For the determination of kinetic parameters for ammonia, in view of the high K_m, a constant, high level of ionic strength was maintained by addition of KCl so that the combined contribution from NH_4Cl and KCl was 1.0 M.

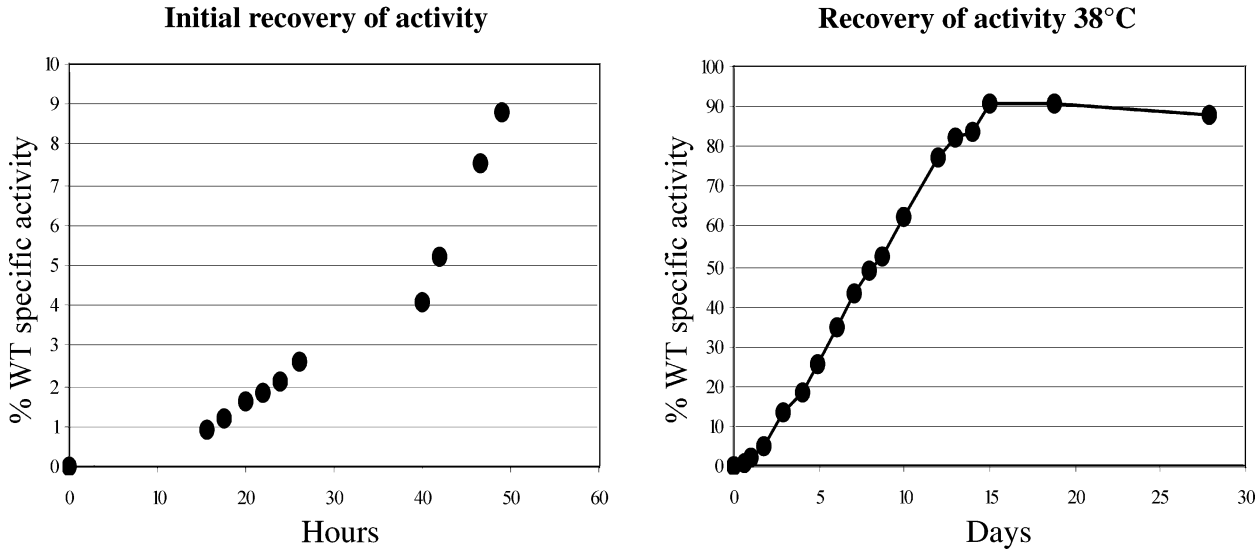

FIGURE 1: Time course of regain of GDH activity during incubation at 38 °C of a purified sample of D165N GDH initially expressed at 8 °C. Enzyme samples were prepared for incubation as described in Materials and Methods, and portions were withdrawn at intervals for assay of catalytic activity in the glutamate oxidation assay.

in a specific activity of 1.6%, similar to that originally observed (*12*). However, the experiment also confirmed that decreasing the temperature resulted in much lower activity. The cells grown and induced at 23 °C yielded enzyme about 7 times less active, but, more dramatically, protein purified from cells induced at 8 °C was 1000-fold less active than that produced at 37 °C, i.e., 70000-fold less active than the wild-type enzyme. This is very similar to the residual level of activity seen in the D165S mutant (*9*). The lower temperature also resulted in a better yield of soluble protein: at 37 °C most of the D165N GDH remained in inclusion bodies. Addition of the heat shock step (Materials and Methods) further improved the yield, and all further tests were performed on protein overexpressed at 8 °C after heat shocking the cells.

Effect of Enzyme Storage Conditions. Comparison between a sample of D165N GDH immediately after purification (in 0.05 M phosphate buffer, pH 7.0, 1 M KCl) and the same sample dialyzed for 24 h against 0.05 M phosphate buffer, pH 7.0, in the cold room, after precipitation in 60% ammonium sulfate, showed a 10-fold increase in specific activity. This was the first clear indication that the activity of the mutant protein was not constant and led to the hypothesis of deamidation, possibly induced by local heating during the precipitation process. On the other hand, enzyme maintained in the cold without precipitation and dialysis remained at the same low activity level for many days.

Monitoring Recovery of Activity. A sample of purified D165N GDH (0.86 mg/mL), preserved in 0.1 M phosphate buffer, pH 7.0, containing 1 M NaCl, was filtered through a sterile 0.45 μm Acrodisc filter. The solution was then divided into 50 μL aliquots which were sealed and incubated at 38 °C (a higher temperature caused denaturation).

The activity was monitored (*13*) initially with a fluorometer and later on spectrophotometrically. Before each reading, samples were centrifuged and the protein concentrations (A_{280} and $\epsilon = 1.05$ cm^2 mg^{-1}) redetermined. Each time point was determined from the average activity for two different samples. At first the activity increased quite slowly, so that after 15 h the mutant protein had recovered about 1% of the activity of wild-type GDH. However, instead of following the normal pattern of a first-order reaction, this recovery process accelerated, resulting in the sigmoid time course seen in Figure 1. Ultimately, after 2 weeks, the activity far exceeded anything seen previously with the mutant protein, approaching that of the normal, unmutated enzyme.

Table 2: Specific Activities Measured Spectrophotometrically for the Mutant Overexpressed at 37 and 23 °C[a]

growth temp (°C)	specific activity (unit/mg)	relative activity (%)
37	3.4×10^{-1}	1.6
23	4.7×10^{-2}	0.22
8	3×10^{-4}	0.0014

[a] The mutant overexpressed at 8 °C showed extremely low activity (measured fluorometrically), and it was not possible to estimate this to better than single-significant-figure accuracy. Relative activity refers to the percentage of the normal wild-type specific activity.

Table 3: Sample Preparation and Concentration

enzyme	concn (mg/mL)	additive	aliquots (μL)
D165N (a)	0.70		7×200
D165N (b)	0.42	Gdn-HCl (6M)	3×300
D165N (c)	0.70	L-glutamate	1×500
D165N (d)	0.70	L-aspartate	1×500
WT	1.33		7×150

Mass Spectrometry. Mass spectrometry was used in order to test the deamidation hypothesis and also to explore the effects of destroying the local catalytic environment of the active site by denaturation. Wild-type GDH was also included in order to follow the deamidation of other asparagines present in the enzyme. In the same experiment the effects on the reactivation process of active site ligands were tested by including either L-glutamate at a concentration (500 mM) at which it should at least partially occupy the active site (*6*) or L-aspartate (500 mM), an inactive substrate analogue. All samples were prepared as for the reactivation experiment above, appropriately treated (Table 3), and then sealed and incubated at 38 °C. The activity of D165N (a) was monitored as described above on matching samples, and at specific times samples of (a), the denatured sample (b), and wild-type GDH were desalted on Macro spin columns (Harvard Bioscience) eluting with 0.5 mL of 0.1% TFA in H_2O:CH_3-CN (1:2). Samples were then dried under vacuum. When the activity shown by D165N was 47% of the reference value, samples (c) and (d) were also checked and found to be 36% and 20%, respectively. Both glutamate and aspartate thus moderated the rate of reactivation but by no means prevented it, and these samples were therefore not included in the mass spectrometry study.

The predicted products of tryptic digestion of clostridial GDH are listed in Table 4. Residue 165, the site of mutation in the D165N protein, is located in an octadecapeptide designated T15 in the wild-type version or D165N-T15 in the mutant case. When LC-MS analysis of digests (Figure 2) with data-dependent MS/MS was followed by computer-based peptide identification using Mascot, sequence coverage typically was 78% of the GDH polypeptide and included T15 and its mutant form. Additional products could be identified by studying the data directly.

As indicated in Figure 2A and shown in detail in Figure 3, digests of the D165N protein at successive time points during 37 °C incubation showed progressive loss of D165N-T15 and a progressive compensating gain of T15. The two peptides could be distinguished by a 1 Da difference in their

Table 4: Predicted Products >500 Da of Tryptic Digestion of *C. symbiosum* GDH (Wild Type and D165N)

peptide	$[M + H]^+$[a]	residues	sequence
T1	552.3	3−6	YVDR
T2	787.5	7−13	VIAEVEK
T3	4059.0	15−50	YADEPEFVQTVEEVLSSLGPVVDAHPEYEEVALLER
T4	744.4	51−56	MVIPER
T5	663.4	57−61	VIEFR
T6	1059.5	62−70	VPWEDDNGK
T7	945.5	71−78	VHVNTGYR
T8	1193.6	79−89	VQFNGAIGPYK
T9	1206.7	94−104	FAPSVNLSIMK
T10	1086.6	105−113	FLGFEQAFK
T11	1190.6	114−125	DSLTTLPMGGAK
T12	993.4	126−135	GGSDFDPNGK
T13	534.3	139−142	EVMR
T14	1465.7	143−153	FCQAFMTELYR
T15	1758.9	154−171	HIGPDIDVPAGDLGVGAR (wild type only)
D165N-T15	1757.9	154−171	HIGPDIDVPAGNLGVGAR (D165N mutant only)
T16	1279.6	172−181	EIGYMYGQYR
T17	1324.7	183−195	IVGGFYNGVLTGK
T18	2667.3	198−222	SFGGSLVRPEATGYGSVYYVEAVMK
T19	1012.5	223−231	HENDTLVGK
T20	1532.8	232−247	TVALAGFGNVAWGAAK
T21	701.4	249−255	LAELGAK
T22	2355.1	256−277	AVTLSGPDGYIYDPEGITTEEK
T23	1069.5	278−285	INYMLEMR
T24	838.4	292−298	VQDYADK
T25	1751.9	299−313	FGVQFFPGEKPWGQK
T26	2160.0	314−332	VDIIMPCATQNDVDLEQAK
T27	757.5	334−340	IVANNVK
T28	1884.9	341−356	YYIEVANMPTTNEALR
T29	1589.8	357−370	FLMQQPNMVVAPSK
T30	2052.0	371−390	AVNAGGVLVSGFEMSQNSER
T31	1264.6	391−401	LSWTAEEVDSK
T32	1921.9	402−419	LHQVMTDIHDGSAAAAER
T33	1884.0	420−437	YGLGYNLVAGANIVGFQK
T34	1277.6	438−449	IADAMMAQGIAW

[a] $[M + H]^+$ theoretical values are monoisotopic. Masses for Cys-containing peptides are calculated on the basis of *S*-carbamoylmethylcysteine, as the protein was reduced and S-alkylated with iodoacetamide before digestion.

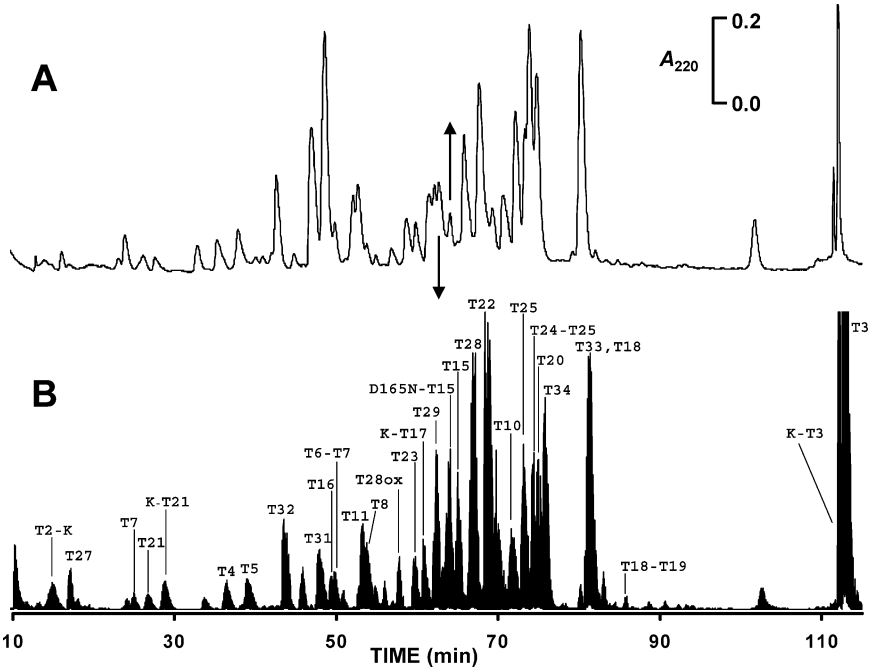

FIGURE 2: LC-MS analysis of a representative tryptic digest of D165N GDH partly reactivated by incubation at 37 °C. (A) HPLC fractionation of the digest with the scale of absorbance at 220 nm as indicated. (B) Base peak chromatogram from the ion trap spectrometer. Arrows show the peaks that declined (downward arrow) and increased (upward arrow), respectively, with incubation at 37 °C. Peak labels refer to tryptic peptides (see Table 4). Terminology: T6-T7 is the product of incomplete cleavage composed of the sequences making up T6 and T7; K-T21 indicates the T21 peptide with an additional N-terminal Lys; T28ox denotes the T28 peptide containing Met as the *S*-oxide.

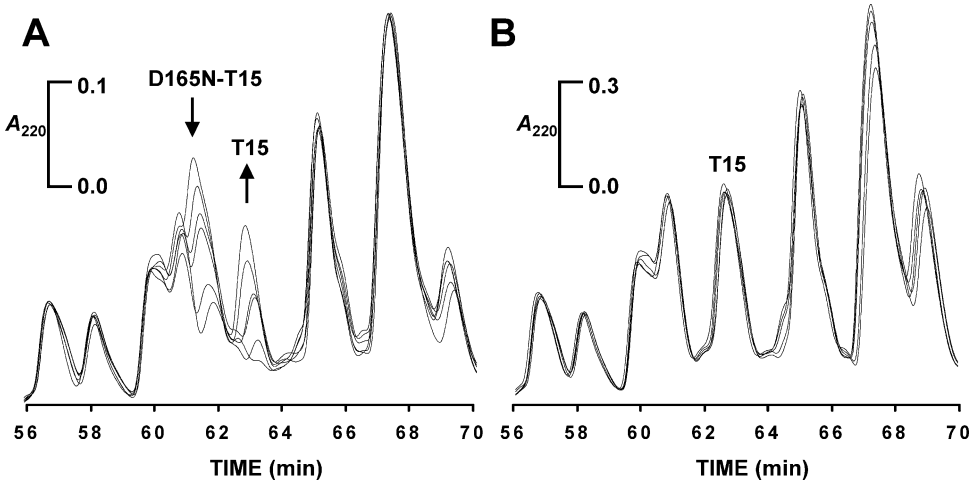

FIGURE 3: Overlaid HPLC chromatograms of tryptic digests of (A) D165N GDH and (B) wild-type GDH sampled at time intervals during incubation at 37 °C. In D165N GDH, there is a progressive decline in the peak at 61.5 min (D165N-−T15) and a corresponding increase in the peak at 63 min (T15). In the wild-type protein, the D165N-T15 peptide is absent and T15 remains at a constant level. Each set of chromatograms was normalized with respect to time and to the absorbance of an invariant peak to correct for minor variations due to experimental factors.

mass (data not shown) and with additional specificity by their respective MS/MS spectra. Theoretical b and y sequence ions for the wild-type and mutant forms are shown in Figure 4, and the experimental data for the declining D165N-T15 peak (Figure 5A) and increasing T15 peak (Figure 5B) allowed the structural basis of the HPLC changes to be attributed to deamidation of the mutant N165 residue.

Deamidation at Asn frequently gives a mixture of aspartyl and β-aspartyl residues in a ratio of about 1:3. These residues are isobaric, but peptides differing by the presence of an Asp or a β-Asp are usually resolved by reversed-phase HPLC. In the present case, only a single product was obtained (Figure 3). When a tryptic digest of maximally reactivated D165N GDH was further treated with AspN, all of the newly generated T15 peptide was cleaved, with HIGPDIDVPAG as a major product (not shown). This was consistent with the single deamidation product of D165N-T15 having Asp (and not β-Asp) at position 165 of the GDH sequence.

DISCUSSION

The results obtained here clearly show, first, that the D165N mutant of GDH is effectively inactive, with specific activity at least 5 orders of magnitude lower than that of wild-type GDH, if not even less. This further validates the view (*6*, *9*) that D165 is an essential catalytic residue. Second, the level of apparent residual activity previously seen when the mutant is expressed at 37 °C is a function of the conditions of growth, purification, and storage. The relatively

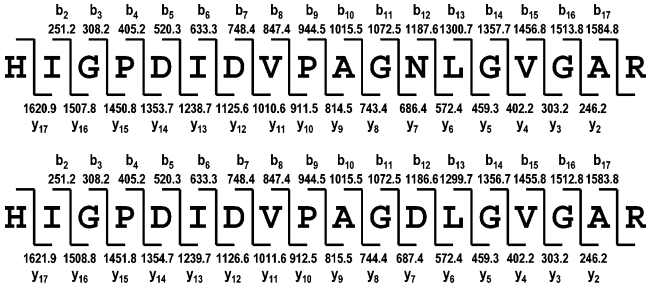

FIGURE 4: Theoretical b and y sequence ions derived from the tryptic peptide of GDH that includes residue 165 of the protein. Upper panel: mutant form of the peptide containing Asn-165 (D165N-T15). Lower panel: native or deamidated form of the peptide containing Asp-165 (T15). The two versions of the peptide are distinguished by differences in the $b_{12}-b_{17}$ and y_7-y_{17} ions, as well as by the 1 Da difference in their masses.

reproducible figure of about 2% of the wild-type activity previously obtained (*9*) merely reflects fairly constant procedures and time scales. Likewise, the fact that this activity remained unchanged over many months of storage at 4 °C reflects the temperature sensitivity of further changes in the protein. Equally, however, the potential for progressive reactivation suggests that even the very low level of activity (0.0014%) seen in the protein expressed at 8 °C may merely reflect early stages of reactivation of a totally inactive protein.

The kinetic parameters determined for the mutant overexpressed at 37 °C are strikingly similar to those of the wild-type enzyme and suggest that such activity as is seen may be due to the presence of subunits containing the catalytic Asp in place at position 165. The slight differences in some K_m values may reflect the difficulty of determining them accurately with a protein of such low activity. Also, though, it is likely that any normal subunits would be isolated active units embedded among five inactive D165N subunits in the hexameric structure. It is not necessarily obvious that such subunits would show precisely the same behavior as when they are one among six identical active subunits.

If the activity indeed reflects the presence of wild-type subunits, their presence has to be both proven and explained. The results in Figure 1 clearly show that mutant enzyme, expressed and purified at low temperatures and virtually inactive, is capable at higher temperatures of progressively acquiring not only 2% of wild-type activity but ultimately almost full activity. (The maximum 91% recovery is not corrected for the possibility of a small amount of denaturation over 2 weeks at 37 °C.) The activity is thus not a result of contamination at either the DNA level or the protein level but rather the result of a progressive chemical change in the mutant protein.

Specifically what is required to explain the results is a spontaneous reversion of the mutation at the protein level. The deamidation reaction of glutaminyl and asparaginyl residues occurs naturally in biological molecules and has been studied in synthetic peptides and some proteins such as cytochrome *c* (*16*). pH, temperature, and ionic strength are factors that contribute to setting the rate of deamidation. Under acidic conditions, for instance, there is a quantitative conversion of amides to carboxylic acids. However, under the mild conditions of both pH and temperature in the present study the rate of conversion postulated here is unexpected for Asn followed by any residue other than Gly; the mutant Asn-165 of GDH is followed by Leu-166. The evidence of mass spectrometry (Figures 2−5) is thus crucial in providing a direct demonstration that in this mutant protein the Asn residue introduced at position 165 reverts to Asp and that this roughly parallels the observed recovery of activity.

Other asparagine residues in the protein may be divided into those followed by a residue other than Gly and the four Asn that exist in Asn-Gly dipeptide sequences, predicted to show enhanced lability to deamidation (*16*). Mass-specific plots for three randomly picked peptides (T9, T29, and T33) containing four Asn not followed by Gly showed no variations through the course of incubation of either wild-type or D165N GDH that would indicate the occurrence of significant deamidation (data not shown).

The four peptides predicted to contain Asn-Gly sequences are T6, T8, T12, and T17. Extracted-ion plots of the respective mass ranges for the incomplete digestion product T6-T7, T8, and the incomplete digestion product K-T17, and their respective deamidated forms, showed appreciable deamidation in each case, but its extent was approximately constant throughout the set of digests spanning the time course of the incubation (data not shown). This suggested that deamidation at these Asn-Gly sequences had occurred mainly during tryptic digestion in ammonium bicarbonate solution at 37 °C, although a lower level of progressive deamidation during the experiment could have been masked by the extensive deamidation resulting from the digestion step. T12 was not accounted for in the peptide map.

The exceptionally rapid deamidation of Asn-165 raises the likelihood that the vestigial catalytic machinery, tailored to promote, in normal circumstances, the cleavage of a substrate imine intermediate to release ammonia, is able to promote a similar cleavage of the asparagine amide. The results show that the rapid deamidation is indeed specific to Asn-165, but it is at least theoretically possible that the effects of neighboring residues in the sequence are responsible. To exclude this possibility beyond doubt, the D165N mutant was also incubated in the denatured state and under these conditions did not deamidate. This establishes that the reaction depends on the three-dimensional geometry of the intact active site. Perhaps the most obvious candidate residue to account for the catalysis is Lys-125, which is also a postulated catalytic base in the enzymatic oxidative deamination reaction (*6*). However, there are also two other essential lysine residues in the close vicinity, K113 and K89, responsible respectively for anchoring the α- and γ-carboxylate groups of the substrate molecule. Either of these could also be seen as potentially available to assist the deamidation reaction. This is under current investigation by further site-directed mutagenesis.

Another aspect of this phenomenon is the mechanism of the chemical deamidation. The deamidation of asparaginyl peptides in free solution usually proceeds via cyclization to form a succinimide intermediate (*16*). This is semisymmetrical and can ring-open on either side of the ring oxygen. One cleavage yields an L-aspartyl residue; the other produces the β-aminoacyl residue of isoaspartate, and in general the latter cleavage is favored over the former by a factor of about 3 to 1 (*17*). On this basis one might predict that deamidation of N165 could lead to a maximal reactivation of 20−30%. The reactivation was almost complete, however, and furthermore the mass spectrometry gave no sign of the predicted

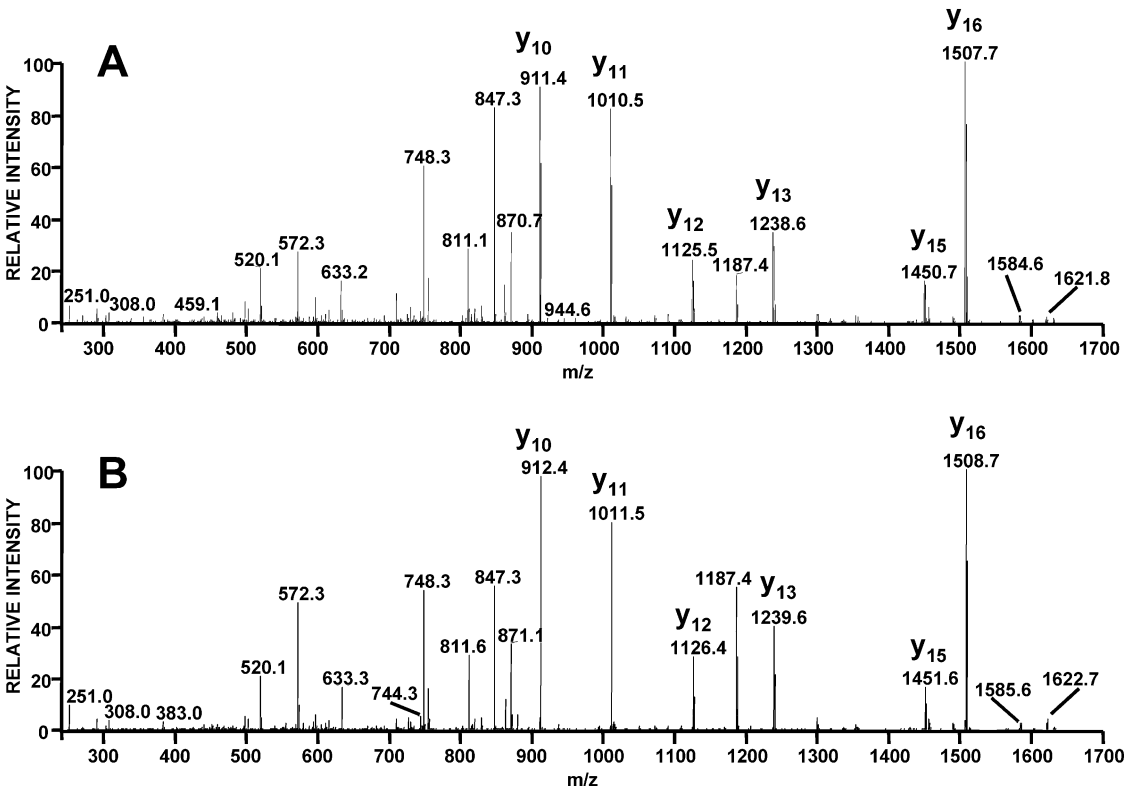

FIGURE 5: MS/MS spectra of the peptide containing the mutant residue Asn-165 in two forms: (A) D165N-T15, the mutant form HIGPDIDVPAGNLGVGAR, which progressively disappears as D165N GDH is incubated at 37 °C, and (B) T15, which progressively appears as D165N-T15 disappears. The y ions labeled in each panel are those which distinguish the two forms of the peptide from each other (see Figure 4).

majority isoaspartyl product. Moreover, the susceptibility of the deamidated peptide to cleavage by AspN is consistent with the restoration of T15 and not with a variant containing isoaspartate. This leads to the conclusion either that the succinimide intermediate is so sterically constrained in the active site cleft that only one cleavage route is feasible or else that in this case the deamidation proceeds by a different route, e.g., direct hydrolysis.

The observed time course for the recovery of activity also requires comment as it is strikingly different from what might be expected for a simple first-order process, showing a strong sigmoidicity. As subunits start to deamidate, it appears that the remaining ones are able to deamidate more rapidly.[2] This implies a cooperative process. Clostridial GDH is indeed a cooperative, allosteric enzyme (*18*, *19*), and it has previously been observed in the case of another D165 mutant, D165H, that a change in the charge state at this position can be sensed at the subunit interfaces and therefore presumably also relayed across them (*11*). This clearly merits closer examination in the future.

In site-directed mutagenesis experiments, Asn is frequently chosen as an ideal replacement for Asp since it retains the same size and shape and some degree of polarity though no charge. However, one of the commonest reasons for replacing an Asp residue in this way is the suspicion of a catalytic role. In the present case it is precisely the catalytic environment of the GDH active site that has led to the facile deamidation of the introduced Asn residue, and this raises an obvious question as to whether in a wider sense Asn may be an unsuitable replacement for putative catalytic Asp residues in other enzymes. A search of the literature reveals that the present example is not unique. Thus Pries et al. (*20*) report an even more rapid deamidation of Asn replacing an active site Asp in haloalkane dehalogenase, and similarly Xiang et al. (*21*) observed a deamidation of Asn in the active site of 4-chlorobenzoyl-CoA dehalogenase which was accelerated 36000-fold by the presence of the ionic product of the normal catalytic reaction. A further interesting case is that of the chemotaxis response regulator CheY, which is thought to undergo an internal dephosphorylation involving a succinimide intermediate (*22*). As noted earlier, such an intermediate can also readily lead to deamidation of an amide side chain, and again in this case when the active site Asp was replaced by Asn, a rapid deamidation was seen. Very recently, another example has been reported for a dehalogenase, in this case fluoroacetate dehalogenase, and good evidence is provided for recovery of Asp without any formation of isoaspartate (*23*).

In all of the examples just cited, the deamidation has been both noticed and clearly documented. We have, however, also found several other recent examples of enzymes in which a putative essential Asp has been replaced by Asn and where a low but nonzero activity has resulted (*24*, *25*). In some cases the residual activity is associated with remarkably unchanged K_m values. It appears possible,

[2] In view of the possibility, suggested by a referee, that the very low concentrations of released ammonia accelerated further deamidation, an experiment was carried out in which two parallel samples were incubated, differing only in the addition to one of a molar concentration of ammonium chloride equal to that of the enzyme subunits (12.4 μM). The time courses of recovery of activity were both sigmoid and were indistinguishable.

therefore, that postmutational deamidation of amides may be widespread, that the results of Asp → Asn or Glu → Gln mutations in active sites need to be interpreted with caution, and that, contrary to general belief and practice, the acid to amide mutation may not be the advisable choice for testing possible catalytic carboxylate groups.

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