Chemical rescue of the catalytically disabled clostridial glutamate dehydrogenase mutant D165S by fluoride ion

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The catalytically disabled Asp¹⁶⁵ → Ser mutant of clostridial glutamate dehydrogenase shows 100 000-fold less activity than the wild-type (WT) enzyme in a standard glutamate oxidation assay and 1000-fold less activity in the reductive-amination reaction. The large reduction in the rate has been attributed to removal of the negative charge and the postulated proton-donor capacity of the aspartate carboxyl group. However, fluoride ion (1 M NaF) causes a 1000-fold activation of the mutant enzyme while simultaneously inhibiting WT activity by 20-fold in the forward reaction. For the reverse reaction, F[−] (1 M) activates the mutant 4-fold and inhibits WT activity to approx. 64%. The net result when 1 M F[−] is present is a decrease in the WT:mutant activity ratio from 100 000 to 5 for the forward reaction. None of the other halides tested, nor NO₃[−], CHCOO[−] or HCOO[−], give

comparable activation. Re-activation took 15–30 s under assay conditions, suggesting the possibility of conformational change; CD spectroscopy, however, provided no evidence of a substantial change and kinetics of modification using 5,5'-dithiobis(2-nitrobenzoic acid) suggested only subtle structural rearrangement. This phenomenon is discussed in the light of available information about the structure of the mutant enzyme. It is suggested that the F[−] ion provides a fixed negative charge at the position of the missing aspartate carboxyl group. Therefore, this appears to be an example of 'chemical rescue'.

Key words: activation, catalytic mechanism, halide, site-directed mutagenesis.

INTRODUCTION

Under certain conditions, enzymes inactivated by mutational deletion of specific amino acid side chains can be re-activated by the addition of small molecules. Examples of such 'chemical rescue' have accumulated rapidly in the literature since the introduction of the concept by Toney and Kirsch in 1989 [1], to describe the effect of exogenous amines compensating for the loss of an 'essential' lysine residue in aspartate aminotransferase [1]. Further examples include guanidinium chloride compensating for the loss of an arginine in asparagine synthetase B [2], and exogenous acetate, which can substitute for the carboxyl group of glutamate in maltodextrin phosphorylase [3]. Acetate has also been shown to be a substitute for the aspartate at position 189 in trypsin, providing an example of the addition of a ligand playing a role in substrate binding, rather than as a direct participant in the catalytic process [4]. Although the success of such rescue is variable, the phenomenon is now well established.

The widely distributed glutamate dehydrogenases (GDHs; EC numbers 1.4.1.2–4) catalyse the reversible oxidative deamination of L-glutamate to 2-oxoglutarate and ammonia using either NAD⁺ or NADP⁺ as the cofactor. The enzyme from *Clostridium symbiosum* has been purified and characterized extensively [5,6]. It is a hexameric protein [7] with six identical subunits, each with an *M_r* of 49 295 [7]. The *gdh* gene has been cloned, sequenced and overexpressed in *Escherichia coli* [8].

The X-ray crystal structure has also been solved [7,9–11]. It was proposed previously that an aspartate residue might be involved in the catalytic mechanism of GDH [12], and elucidation of the crystal structure for the clostridial enzyme highlighted Asp¹⁶⁵ as being at the heart of the active site, potentially in a

position to receive a proton from the α-amino group of the substrate [11]. This residue was replaced subsequently with serine by site-directed mutagenesis [13] to remove the negative charge and postulated proton-donor capacity while minimizing secondary structural changes. As expected, the specific activity of the mutant enzyme D165S (denoting Asp¹⁶⁵ → Ser) was decreased greatly: 10³-fold in the reductive-amination reaction and 10⁵-fold for oxidative deamination as compared with the wild-type (WT) enzyme under the same conditions. Apparent Michaelis–Menten constants for 2-oxoglutarate and NADH were reported to be unchanged [13]. The protein, like the WT GDH, is readily protected by NAD⁺ against Ellman's reagent, suggesting unimpaired binding of coenzyme. These results are consistent with the view that Asp¹⁶⁵ is required for catalysis rather than having any essential involvement in the binding of coenzyme or substrate [13]. The mutant enzyme, however, has a greatly increased *K_m* value for NH₄⁺ [13], and the attempt to distinguish between specific substrate dependence of the reaction on NH₄Cl concentration and non-specific ionic-strength effects led to a closer study of the effects of halide ions. This article documents the striking effect of the fluoride ion (F[−]) on D165S GDH and discusses the possibility that this may represent another example of chemical rescue.

EXPERIMENTAL

Materials

Sephacrose CL-6B was purchased from Pharmacia Biotech. Grade-II NAD⁺ (free acid) and grade-II NADH (disodium salt) were obtained from Boehringer-Mannheim (U.K.). L-Glutamate

Abbreviations used: GDH, glutamate dehydrogenase; WT, wild-type; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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(monosodium salt) and 2-oxoglutarate (monosodium salt) were purchased from Sigma Chemical Co. Other chemicals were supplied by BDH or Riedel-de Haen and were of analytical grade.

Expression and purification of enzyme

Crude extracts, prepared from *E. coli* TG1 cells transformed with ptac-D165S *gdh* [13], were incubated at 28 °C in Luria–Bertani medium supplemented with 100 µg/ml ampicillin and 0.5 mM isopropyl β-thiogalactopyranoside. 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 to remove cell debris. The mutant enzyme was purified to homogeneity using a dye affinity column of Remazol Brilliant Red GG-Sepharose CL-6B [5]. Column material was washed with 2 column volumes of 6 M urea/2 M NaCl, followed by 10 column volumes of distilled water, prior to equilibration with 50 mM potassium phosphate buffer at pH 7.0. The pure enzyme, stored in 65 % ammonium sulphate at 4 °C, was dialysed against several changes of 0.1 M potassium phosphate buffer (pH 7.0) and clarified by centrifugation before use. Enzyme purity was checked routinely both by native PAGE (7.5 % gel) and SDS/PAGE (10 % gel), and the gels were stained with Coomassie Brilliant Blue R-250 as described by Garfin [14]. Enzyme concentration was calculated from A_{280} measurements by using an absorption coefficient of 1.05 cm²·mg⁻¹ [6].

Enzyme assay

The activities for the forward (two-substrate) reaction for both the WT and D165S GDH were measured at 25 °C using a spectrophotometer (Kontron Uvikon 922 Plus). 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 (25 µg of D165S and 0.25 µg of WT GDH). The three-substrate reaction was also measured spectrophotometrically at 25 °C by recording a decrease in absorbance at 340 nm due to the oxidation of NADH. 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 buffer (pH 7.0). For both the forward and reverse reactions, monovalent sodium salts (0.01–1 M) were included in some reaction mixtures. In order to determine activation constants for F⁻, rates were measured with 1 mM NAD⁺ and several concentrations of glutamate (20, 40 and 60 mM) over a range of NaF concentrations (0.01–1 M). To determine kinetic parameters for both proteins, rates were measured both under standard conditions and in the presence of 1 M NaF. To determine kinetic parameters for glutamate, NAD⁺ was kept constant (1 mM) over a range of glutamate concentrations (0.02–1 M for D165S and 0.02–40 mM for WT GDH respectively). Similarly, kinetic parameters for NAD⁺ were estimated for both proteins by varying its concentration (0.025–2.5 mM) while maintaining a fixed glutamate concentration (40 mM). In the case of the three-substrate reaction, 2-oxoglutarate concentration was varied (0.025–5 mM), with standard concentrations for NADH (0.1 mM) and NH₄Cl (50 mM) maintained. In experiments where NADH concentration was varied (0.003–0.25 mM), the standard concentrations of 2-oxoglutarate and NH₄Cl were 5 and 50 mM respectively. Where NH₄Cl was varied (either 0.05–1 M with a constant ionic strength of 1 M maintained using KCl, or 0.01–0.4 M without maintenance of the ionic strength, for D165S and WT GDH, respectively), the concentrations of 2-oxoglutarate (5 mM) and

NADH (0.1 mM) were kept constant. Michaelis–Menten parameters were calculated by using the program Enzpack version 3.0 (Biosoft, Cambridge, U.K.).

CD spectroscopy

Near- and far-UV CD spectra for both WT GDH and the D165S mutant were recorded at 20 °C in 0.1 M potassium phosphate buffer (pH 7.0), and in the same buffer with the addition of different sodium halides (1 M). The spectra were recorded with a Jasco J-600 spectropolarimeter using 1 or 10 mm fused silica cuvettes as appropriate. The GDH concentrations were 0.15 and 1.0 mg/ml for far- and near-UV spectra, respectively.

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) modification

Both WT GDH and the D165S mutant were incubated at 25 °C in 0.1 M potassium phosphate buffer (pH 7.0) with 1 mM DTNB [15] in the presence or absence of 1 M F⁻. Modification with DTNB was followed by continuous monitoring of the A_{412} of released thionitrobenzoate [15] for 40 min using a spectrophotometer (Kontron Uvikon 922 Plus). The final concentration for each enzyme was 0.1 mg/ml.

RESULTS

Effect of various monovalent salts on specific activity

The behaviour of both WT and D165S GDH enzymes was studied with a range of sodium halides. In the case of WT GDH under fixed assay conditions, 1 M NaCl, NaBr and NaI all inhibited the reverse three-substrate reaction, by 60, 70 and 97 % respectively. With these three salts the results for D165S were fairly similar to those for WT GDH: whereas 1 M NaI inhibited activity completely (no activity was detected with 40 µg of protein and when the sensitivity of the scale was increased 20-fold), 1 M NaBr and NaCl caused 90 % and 65 % inhibition respectively. Turning to fluoride, however, there was a striking contrast: whereas 1 M NaF also inhibited WT GDH (36 %) it activated D165S 4-fold.

When included in the standard forward-assay mixture, each of the four halides (1 M) inhibited the WT enzyme completely. In the case of the forward reaction for D165S, even with 25 µg of enzyme in the standard assay without halides, no detectable change was detected by a spectrophotometer over 10 min. In the presence of 1 M NaCl, NaBr and NaI, the rate remained unmeasurable. However, in the presence of NaF marked activation occurred, and with 1 M NaF activity increased to approx. 0.24 unit/mg. Although this is only 1 % of normal WT GDH activity without fluoride, it was still readily measurable. The same concentration of NaF, by contrast, caused a 20-fold decrease of the WT GDH enzyme to approx. 1.1 units/mg, so that in the presence of 1 M NaF the ratio (WT GDH activity:mutant activity) was approx. 5. This contrasts with the ratio of 100000 reported in the absence of NaF [13] and reflects an activation factor of at least 1000 for D165S in the presence of NaF. Estimation of this ratio is based on the ability to measure activity for D165S (without NaF) with a sensitive, fluorimetric method [13].

The monovalent anions NO₃⁻, formate and acetate all inhibited the activities of both WT and mutant proteins, and in both directions. For the forward reaction, 200 mM NaNO₃ decreased WT activity to approx. 4 %, while both 1 M sodium formate and sodium acetate inhibited the activity to about 6 %. In the case of the reverse reaction, whereas NaNO₃ (200 mM) caused 31 and 71 % inhibition for WT and D165S GDH respectively, both sodium formate (1 M) and sodium acetate (1 M) inhibited the

Figure 1 Initial rates for both the D165S mutant and the WT GDH as a function of NaF concentration

(A) D165S and (B) WT GDH under standard assay conditions for the forward reaction (40 mM glutamate and 1 mM NAD^+ in 0.1 M potassium phosphate, pH 7.0) with various NaF concentrations. (C) D165S and (D) WT GDH under standard assay conditions for the reverse reaction (5 mM 2-oxoglutarate, 0.1 mM NADH and 50 mM NH_4Cl in 0.1 M potassium phosphate, pH 7.0) with various NaF concentrations. U, units.

WT enzyme 50% while decreasing D165S activity 60 and 33% respectively.

The striking result with NaF prompted a closer study of the effect on both the WT and D165S GDH enzymes.

Dependence of specific activity on NaF concentration

In the standard forward reaction for D165S, hyperbolic activation occurred with increasing fluoride concentration (Figure 1A), giving a half-maximal activation with 0.2 M NaF. However, in the forward assay for the WT GDH, NaF produced a steady decline to a mere 5% of the original activity (Figure 1B). In assay (forward) mixtures containing relatively low concentrations of NaF (< 80 mM), there was a slight lag phase for D165S, i.e. the observed rate of reaction increased during the period of observation, only reaching a steady maximum value after 15–30 s. With higher NaF concentrations (> 200 mM) no lag was observed, i.e. any transient increase in rate must have been complete within the 5–10 s between mixing and first observation. Even with low fluoride concentrations, preincubating the mutant with NaF for 10 min prior to initiating the reaction with coenzyme (NAD^+) eliminated the lag.

When NaF was included over the same range of concentrations (0.01–1 M) in the reverse reaction for D165S, hyperbolic activation

occurred again, with a steep rise in the rate over the range 0.03–0.1 M, and with half-maximal activation at 0.04 M NaF (Figure 1C). The WT enzyme was again inhibited by NaF (Figure 1D) and, with 1 M NaF, the activity for the reverse reaction was reduced to 64% of the original value. In the case of the reverse reaction for both the enzymes, the extent of the changes (activation of the mutant and inhibition of WT) in the presence of 1 M NaF was 1–2 orders of magnitude lower than those in the forward reaction, possibly implying that some process that is rate limiting in the two-substrate reaction is much less important in determining the three-substrate rate.

Measurement of activation constants of D165S GDH for the F^- ion

The dependence of activation on fluoride concentration was investigated by measuring the forward reaction with 1 mM NAD^+ and several fixed concentrations of glutamate (20, 40 or 60 mM) over a range of NaF concentrations (0.01–1 M). Although at $[F^-] = 0$ the rate is not exactly zero, the present experiments were carried out on a spectrophotometer that gave no measurable rate under these conditions. The activation factor was so great that the Enzpack program could be used with negligible error to determine the kinetic parameters. As the

Table 1 Comparison of kinetic parameters between WT and D165S mutant GDH in the presence or absence of 1 M F⁻

To determine kinetic parameters for glutamate, NAD⁺ was kept constant (1 mM) over a range of glutamate concentrations (0.02–40 mM for WT GDH and 0.02–1 M for D165S). Similarly, a fixed glutamate concentration (40 mM) was maintained while determining kinetic parameters for NAD⁺ (0.025–2.5 mM for both proteins). In the case of the three-substrate reaction, the 2-oxoglutarate concentration was varied (0.025–5 mM), with standard concentrations for NADH (0.1 mM) and NH₄Cl (50 mM). In experiments where both NADH (0.003–0.25 mM) and NH₄Cl (either 0.05–1 M for D165S or 0.01–0.4 M for WT GDH) were varied, the standard concentrations for the remaining substrates were used. When determining the kinetic parameters of NH₄Cl for D165S, a constant ionic strength of 1 M was maintained using KCl. All experiments were repeated in triplicate and the kinetic parameters and their standard errors were calculated by a non-linear regression method [22] with the Enzpack version 3.0.

	K_m (mM)		V_{max} (units/mg)	
	WT	D165S	WT	D165S
Glutamate	3.22 ± 0.15		31.9 ± 0.5	
Glutamate + 1 M F ⁻	6.06 ± 0.32	110 ± 6	3.15 ± 0.04	0.966 ± 0.013
NAD ⁺	0.141 ± 0.012		33.3 ± 0.6	
NAD ⁺ + 1 M F ⁻	0.144 ± 0.013	0.186 ± 0.016	2.22 ± 0.04	0.259 ± 0.005
2-Oxoglutarate	0.053 ± 0.002	0.316 ± 0.026	160 ± 2	0.194 ± 0.005
2-Oxoglutarate + 1 M F ⁻	0.274 ± 0.012	0.075 ± 0.004	104 ± 1	0.659 ± 0.006
Ammonium	70 ± 5	550 ± 4	315 ± 8	1.80 ± 0.06
Ammonium + 1 M F ⁻	71 ± 6	198 ± 11	267 ± 7	4.15 ± 0.07
NADH	0.011 ± 0.001	0.0031 ± 0.0003	163 ± 3	0.190 ± 0.003
NADH + 1 M F ⁻	0.0060 ± 0.0004	0.0010 ± 0.0001	101 ± 2	0.649 ± 0.012

glutamate concentration increased the concentration of fluoride required for 50% activation also increased somewhat, from 0.33 mM with 20 mM glutamate to 0.49 mM with 60 mM glutamate.

Measurement of kinetic constants

Kinetic parameters were estimated under standard conditions and in the presence of 1 M NaF as described above. The results are summarized in Table 1. Without NaF, it was impossible to determine kinetic constants of D165S for either glutamate or NAD⁺. However, kinetic parameters were determined for glutamate in the presence of 1 M NaF for the D165S mutant enzyme (Table 1). Although the high apparent K_m value (110 mM) may suggest weak binding of the substrate to the mutant enzyme, an inhibition experiment (results not shown) in the reverse reaction for D165S, using several concentrations of glutamate as the inhibitor and 2-oxoglutarate as the varied substrate, showed that glutamate inhibited D165S competitively with a K_i value of 24 mM, almost identical with the K_i value for WT GDH (23.5 mM) reported previously [16]. The apparent K_m values of WT GDH for glutamate were also determined by varying the glutamate concentration (0.02–40 mM) and maintaining a fixed NAD⁺ concentration (1 mM). In the presence of 1 M NaF the apparent K_m for glutamate increased 2-fold for the WT protein (6.06 mM).

The K_m values of WT GDH for NAD⁺ were practically identical both with (0.144 mM) and without (0.141 mM) 1 M NaF. It was impossible to determine the K_m of D165S for NAD⁺ in the absence of NaF, but when 1 M NaF was present, the mutant enzyme had a similar K_m to WT GDH (0.186 mM), suggesting that D165S binds NAD⁺ in much the same way as WT GDH. The modification of WT GDH by DTNB can be blocked by the inclusion of NAD⁺ [17,18], and it has been reported previously in equivalent experiments that D165S was protected to a similar degree, also suggesting similar binding constants for NAD⁺, and thus unimpaired binding of the coenzyme [13]. In the forward reaction, therefore, the inhibitory effect of fluoride on WT GDH is attributable mainly to a large decrease in the apparent V_{max} values.

Turning to the kinetic analysis of the three-substrate reaction; in the absence of 1 M F⁻, there was an approx. 6-fold difference between the K_m values of WT GDH and D165S for 2-oxoglutarate. However, when 1 M F⁻ was present, the K_m for D165S decreased from 0.316 to 0.075 mM (approx. 4-fold), whereas the K_m for WT GDH increased from 0.053 to 0.274 mM (approx. 5-fold). A greatly increased K_m value for NH₄⁺ for the mutant has been reported previously, and for this substrate the approach to saturation inevitably entailed a large increase in the ionic strength contribution of NH₄Cl. This problem was overcome by fixing ionic strength at a constant value by addition of KCl, so that the combined contribution of both salts to the ionic strength was 1 M [13]. The K_m of D165S for NH₄⁺ was estimated to be 550 mM compared with the WT GDH value of 70 mM, representing an approx. 8-fold increase. However, in the presence of 1 M NaF, the K_m for WT GDH was essentially unchanged (71 mM), whereas the value for D165S decreased 2.5-fold to 198 mM. In the case of NADH, the K_m values shown by both proteins decreased when 1 M F⁻ was present. For D165S, the K_m value decreased approx. 3-fold (0.001 mM), whereas the K_m value for the WT protein decreased approx. 2-fold (0.006 mM).

In summary, in contrast with the situation for the forward reaction, where the major effect of fluoride on WT GDH was on the maximum rate, in the reverse reaction the inhibition mainly manifested itself in an increased apparent K_m for 2-oxoglutarate. For D165S the activation was attributable to several-fold favourable changes in both apparent K_m and apparent V_{max} values.

CD spectroscopy

The time dependence of the response to fluoride by the D165S mutant in the forward reaction suggested that a conformational change might be occurring. Therefore, CD spectroscopy was employed to investigate this more closely. Both far- and near-UV CD spectra were recorded for WT GDH and the D165S mutant protein (results not shown). The far-UV CD spectra of the two proteins were nearly identical, and showed that there was no obvious change in the presence of 1 M F⁻. There was, therefore, no evidence for a fluoride-induced, time-dependent, change in the secondary structure of D165S GDH. The near-UV CD

spectra of WT GDH and the D165S mutant were also closely similar, and were unchanged in the presence of 1 M F⁻. Both spectra showed the feature at 275 nm described previously [19]. The latter observation suggests that there is also no fluoride-induced change in protein tertiary structure, at least as assessed by near-UV CD.

DTNB modification

Since the CD spectra showed no obvious conformational change, chemical modification was used as an alternative tool to explore local changes in the active-site cleft. Cys³²⁰, located close to the coenzyme-binding site of clostridial GDH [9,10], is the sole residue that reacts with DTNB [17,20], resulting in complete loss of activity. In the present experiments, the WT enzyme was inactivated over 40 min by 1 mM DTNB, in agreement with earlier results [17,18,20] and gave a rate constant of 0.094 s⁻¹. When 1 M F⁻ was present it gave superimposable results (with an identical rate constant). In the same experiment, D165S GDH, in the absence of 1 M F⁻, gave a somewhat smaller rate constant of 0.079 s⁻¹. In the presence of 1 M F⁻ the rate constant was measured as 0.076 s⁻¹. This value was very close to that obtained with fluoride, but the difference appeared to be reproducible (each experiment was repeated five times). Clearly, however, even at the local level any structural change was subtle, since the change in rate of chemical modification was so slight.

DISCUSSION

Our results clearly show that F⁻ is capable of activating an otherwise almost inactive protein. In the presence of 1 M NaF the activity is increased approx. 1000-fold for the forward reaction and 4-fold in the case of the reverse reaction. Whereas F⁻ activates the mutant, it simultaneously has an inhibitory effect on the WT GDH, decreasing the activity to a mere 5% in the case of the forward reaction, and to approx. 64% in the three-substrate reverse reaction. The net result is that the WT:mutant activity ratio, which is 100000 for the forward reaction under standard conditions, decreases dramatically to only 5 when 1 M NaF is present. Since NaF is the only sodium halide to cause this striking activation of D165S GDH, it is reasonable to assume that it is the anion that is responsible. It seems likely that because of their larger ionic radii, chloride (1.81 Å), bromide (1.95 Å) and iodide (2.16 Å) ions are unable to produce the same effect as the smaller F⁻ ion (1.36 Å). Similarly, NO₃⁻, formate and acetate, all larger anions, fail to activate the mutant. In view of this specificity for the anion, activation probably results from binding at one particular site on the enzyme rather than a general ionic-strength effect.

The kinetic analysis presented in the Results section shows not only the contrasts just mentioned between the effect of fluoride on WT and D165S GDH, and between the effects on forward and reverse reactions, but also striking differences in the effects on various kinetic parameters. At this stage, however, the current kinetic analysis of the mechanism of the reaction catalysed by clostridial GDH does not allow us to identify clearly particular kinetic parameters with individual rate constants, dissociation constants or ratios thereof. In the long run, the observed differences should be informative, but to attempt to interpret them in detail at this stage would be premature, especially since the $V_{\max}$ and K_m values determined here are 'apparent' values rather than extrapolated constants for saturating concentrations of fixed substrates.

In an attempt to explain at a chemical level the activation of the D165S mutant enzyme by the specific binding of F⁻, there are

perhaps two possible mechanisms to envisage. Asp at position 165 was replaced with Ser to remove the negative charge and the postulated proton-donor capacity but at the same time to minimize secondary structural changes [13]. The mutant enzyme has a greatly reduced activity under our normal assay conditions, and this has been attributed to removal of the proton-donor capacity [13]. However, the data presented here suggest that the F⁻ ion is capable of restoring the protein to a state in which it can offer as much as 20% of the catalytic ability of the WT enzyme under the same conditions. If indeed this is due to the specific binding of a F⁻ ion at the active site, this calls into question the view that Asp¹⁶⁵ (D165) normally serves as a proton donor/acceptor. A halide ion seems unlikely to be able to operate efficiently as a proton acceptor at pH 7.0, and it therefore seems more probable that the F⁻, and by implication the side chain of Asp¹⁶⁵, offers a fixed negative charge in the active site. It remains to be established what the function of a fixed charge might be, but the indications, both from the three-dimensional structure and from the kinetic data presented here, are that the charge is not primarily involved in binding the coenzyme or the dicarboxylate substrate. It might, however, have a role in enforcing the specific requirement for NH₄⁺ as a substrate.

One puzzling feature of the results is the time dependence of recovery of activity. Although the relative slowness of the reactivation suggested a protein conformational change, possibly implying that the D165S mutation causes some disordering of the structure, the CD experiments provide no evidence for any substantial change in the secondary structure. Also, several earlier experiments [13] indicated that the mutant protein was properly folded. Likewise, the kinetic data of DTNB modification suggest no major structural change. This raises an obvious question as to why the binding of a halide ion should be such a slow process.

The pH-dependent inactivation described by Syed et al. [21] was reversed by different anions and cations, presumably owing to a general ionic-strength effect. This was also a relatively slow process but was associated with a substantial conformational change affecting the CD signature, which distinguishes it from the fluoride effect described here.

In addition to the remarkable effect of F⁻ ion on the D165S mutant, there is also the inhibitory effect of all sodium halides tested on the WT enzyme and of all halides other than NaF on D165S GDH. It seems reasonable to assume that these inhibitory effects are unconnected with the specific-site binding of fluoride. In particular, it seems unlikely that the WT enzyme, which already has the negative charge of Asp¹⁶⁵ in place, would accept a second negative charge in close proximity. It is apparent that the inhibition is most potent with the larger halide ions and the possibility that the inhibition is due to a bulk solvent effect certainly cannot be excluded. This implies that the net activity achieved by D165S GDH in 1 M NaF may be the result of two countervailing effects, so that without the superimposed inhibition the activity might be still higher than that observed.

In conclusion, it appears that the D165S mutation in GDH may be amenable to chemical rescue with fluoride at high concentrations, restoring the missing negative charge and, with it, the activity. We are not aware of a previous example of a halide ion substituting for a carboxylate side chain and believe that this pattern suggests strongly that the role of Asp¹⁶⁵ is as a source of fixed charge rather than an acceptor/donor of protons.

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