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†

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Received April 24, 1995; Revised Manuscript Received June 26, 1995®

ABSTRACT: Glutamate dehydrogenases from many sources display nonclassical kinetic behavior suggestive of allosteric interaction among the six subunits of the hexamer. A three-dimensional structure now potentially offers a framework for explaining the basis of such behavior in clostridial glutamate dehydrogenase, and this paper offers evidence of extreme, all-or-none cooperativity in the binding of glutamate by this enzyme. A site-directed mutant of clostridial glutamate dehydrogenase in which Ala163 in the glutamate binding site is replaced by glycine displays a markedly sigmoid dependence of reaction rate on glutamate concentration ($S_{0.5}$ = 200 mM), with a Hill coefficient of 3.4 when assayed at pH 10.5 with 1 mM NAD^+. Under the same conditions the wild-type enzyme gave no measurable rate with glutamate concentrations in the range normally used for kinetics (0−100 mM) but gave a steep rise in reaction rate from 600 to 1200 mM glutamate. At pH 9.0, where the wild-type enzyme has previously been shown to be "inactive" in a standard assay, a study extending to much higher glutamate concentrations again revealed a sigmoid dependence, with a Hill coefficient of 5.4 and an $S_{0.5}$ at 150 mM glutamate. With the mutant A163G the apparent cooperativity was less, with a Hill coefficient of 2.3, and the affinity for glutamate was higher, with $S_{0.5}$ of 7 mM. Both proteins gave normal hyperbolic dependence on glutamate concentration at pH 7 and pH 8. At pH 9 and with saturating glutamate, both enzymes showed a hyperbolic dependence of the rate on NAD^+ concentration. The NAD^+ concentration, however, affected the observed degree of cooperativity with varied glutamate. With 40 μM NAD^+ the wild-type enzyme gave a 10-fold increase of rate for a 2-fold increase from 150 to 300 mM glutamate, and a Hill coefficient of 6.0, the theoretical maximum for absolute cooperativity in a hexamer. Succinate behaves as a classic allosteric activator, enhancing the rate at low glutamate concentrations and removing the sigmoidicity. A dicarboxylate structure, however, appears not to be essential for cooperative binding, since at pH 10.5 norvaline also gave sigmoid dependence of rate upon substrate concentration. It is concluded that the previously documented slow, pH-dependent inactivation [Syed, S. E.-H., & Engel, P. C. (1990) *Biochem J. 271,* 351−355] reflects a transition between R and T states in an allosteric system of the Monod−Wyman−Changeux type. In the presence of NAD^+ and a buffer of high ionic strength the allosteric equilibrium adjusts rapidly so that hysteretic effects are not seen on the time scale (>10 s) of the rate measurements. Instead, the equilibrium manifests itself in the form of sigmoid kinetics.

When Monod et al. (1963) enunciated the concept of allosteric control, one of their illustrative examples was that of bovine glutamate dehydrogenase. Although most glutamate dehydrogenases (GDHs)[1] are hexameric, like the bovine enzyme, a segment of about 50 amino acids in the sequence that appears to be involved in mediating heterotropic regulation by ADP and GTP in GDHs from vertebrates is missing in other GDHs that have been studied (Smith et al., 1975; Goldin & Frieden, 1971). Nevertheless, striking examples of allosteric regulation are to be found also among these smaller GDHs. The $NADP^+$ dependent GDH of *Neurospora crassa*, for instance, displays positive cooperativity in the binding of activatory dicarboxylate compounds (West et al., 1967).

Kinetic studies (Syed et al., 1991; Syed & Engel, 1990) of the NAD^+-dependent GDH of *Clostridium symbiosum*, for which a high-resolution X-ray structure has now been determined (Baker et al., 1992; Stillman et al., 1993), have revealed (a) nonclassical behavior in response to variation of NAD^+ concentration and (b) a pH -dependent activation−inactivation equilibrium which could be correlated with conformational alterations detectable by CD measurements (Syed et al., 1990). We now further report strong positive cooperativity, with Hill coefficients of up to 6 at high pH, in the dependence of the enzyme reaction on glutamate concentration. This was found initially with a mutant, A163G, constructed as part of a series of studies of the determinants of substrate specificity to be reported elsewhere [A163 is one of the residues lining the pocket that binds the side chain of the substrate glutamate (Stillman et al., 1993)]. Similar behavior, however, has now also been found with

† This research was supported by the EC through the project Biotechnology of Extremophiles and by Degussa AG.

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® Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: GDH, L-glutamate dehydrogenase (EC 1.4.1.2−4); IPTG, isopropyl β-thiogalactopyranoside.

0006-2960/95/0434-11417$09.00/0

the wild-type enzyme, although $S_{0.5}$ values are 10−20-fold higher than for the mutant.

EXPERIMENTAL PROCEDURES

Materials. All chemicals for DNA manipulation and enzyme assay were of analytical grade and were commercially obtainable. Grade II NAD^+ (98%, free acid), grade II NADH (98%, disodium salt), and 2-oxoglutarate (disodium salt) were supplied by Boehringer Mannheim GmbH. Succinic acid and L-glutamate (monosodium salt) were from BDH, U.K.; L-norvaline was from Koch-Light Laboratories Ltd., U.K.; and other amino acids came from Sigma Chemical Co.

Escherichia coli Q100 (thr1, leuB6, gdh1, hisG1, gltB31, argH1, thi1, ara14, lacY1, gal6, malA1, xyl7, mtl2, rpsL9, tonA2, $\lambda^r\lambda^-$, supE44, hsdR2) was obtained from Dr. B. Snedecor. The expression vector $p^{tac}85$ (Marsh, 1986) and the recombinant plasmid $p^{tac}44$ (Teller et al., 1992) harboring the *gdh* gene of clostridial GDH were kindly provided by Dr. J. K. Teller.

Construction of A163G Mutant. Oligonucleotide-directed mutagenesis was performed with selection in favor of the mutated gene achieved by using template strands containing uracil (Kunkel et al., 1987). The oligonucleotide 5′-ATGTTCCT**GGA**GGTGACCTT-3′ was used as the mismatch primer hybridizing to the antisense counterpart of the clostridial *gdh* gene in the single-stranded form of M13mp19. The corresponding sequence of the *gdh* gene is 5′-ATGTTCCT**GCA**GGTGACCTT-3′. The glycine codon GGA (in bold) replaced the wild-type alanine codon (GCA). The resulting A163G mutant was directly screened and its total gene sequence was examined by using single-stranded DNA sequencing of the mutant gene inserted into M13mp19 vector according to the dideoxy chain-termination method (Sanger et al., 1977). After subcloning into the $p^{tac}85$ expression vector and transformation of *E. coli* Q100, the mutation was further confirmed by direct sequencing of the double-stranded DNA using [α-^{35}S]dATP internal labeling during the cycle sequencing reactions with the SequiTherm Kit purchased from Cambio, UK.

Enzyme Preparation. Both wild-type and A163G transformants of *E. coli* Q100 were incubated at 37 °C overnight in LB broth supplemented with 100 μg/ml ampicillin and 0.5 mM IPTG (isopropyl β-thiogalactopyranoside) and then harvested, sonicated, and centrifuged at 27000*g* for 20 min. The clarified supernatants were filtered with a 0.45-μm disposable filter for purification. The mutant enzyme, like wild-type GDH, was purified to homogeneity as described by Syed et al (Syed et al., 1991). Enzyme purity was routinely checked both by 7.5% native PAGE and 10% SDS−PAGE, and the gels were stained with Coomassie Brilliant Blue R-250. The pure enzyme, stored in 65% ammonium sulfate at 4 °C, was dialyzed before use against several changes of 0.1 M potassium phosphate buffer, pH 7.0, and clarified by centrifugation.

Enzyme Assays. Rates were measured spectrophotometrically by recording A_{340} with 1 mM NAD^+ over a range of L-glutamate concentrations, or at fixed starting concentrations of 0.2 mM NADH and 200 mM NH_4Cl with different concentrations of 2-oxoglutarate. As buffers at pH 9.0 and 10.5, 50 mM glycine/8.8 mM NaOH and 50 mM glycine/42 mM NaOH were respectively used, since bicarbonate buffer proved insufficiently stable. Michaelis−Menten parameters

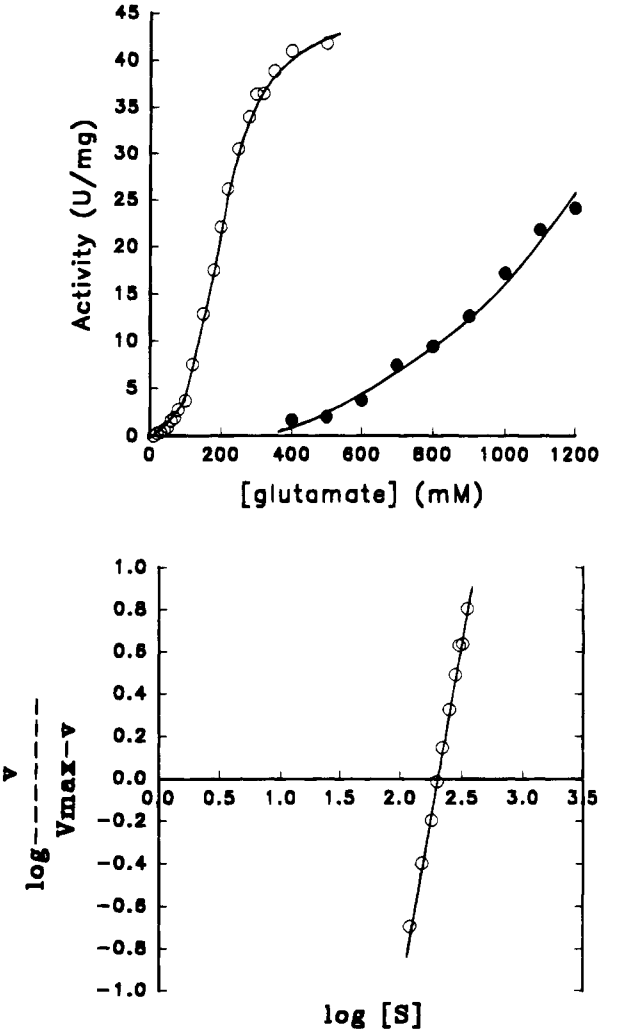


FIGURE 1: Sigmoid dependence of the rates of wild-type GDH and A163G mutant on glutamate concentration. Specific activities toward glutamate for both enzymes were measured spectrophotometrically with 1 mM NAD^+ and a range of glutamate concentrations at pH10.5. (○) A163G, (●) wild-type GDH.

were calculated where possible by using the Eadie−Hofstee plot with the program Enzpack (Biosoft Ltd., Cambridge, U.K.). The correlation coefficients were equal to or larger than 0.98. In experiments in which the NAD^+ concentration was held constant and the glutamate concentration was varied, Hill constants for both the wild-type GDH and the A163G mutant enzyme were estimated by plotting $\log[v/(V_{max} - v)]$ against log[S], on the assumption that the measured reaction rate, v, was proportional to the fractional saturation with glutamate, $\bar{Y}$, in eq 1, the Hill equation (Hill, 1910).

$$\log \frac{\bar{Y}}{1 - \bar{Y}} = h \log[S] - \log K \qquad (1)$$

Stability Test. To investigate whether the A163G mutant and the wild-type enzyme are stable at the high pH values employed in this study, the enzyme solutions (2 mg/ml) in 0.1 M potassium phosphate buffer (pH7.0) were diluted 100- or 1000-fold into glycine−NaOH buffer at pH 9.0 or 10.5, and then were kept at room temperature. Activities were measured at pH 9.0 and 10.5 by sampling 3−10 μl of diluted enzyme at intervals into 1 mL of assay solution containing 1 mM NAD^+ and glutamate at 20 or 250 mM, respectively,

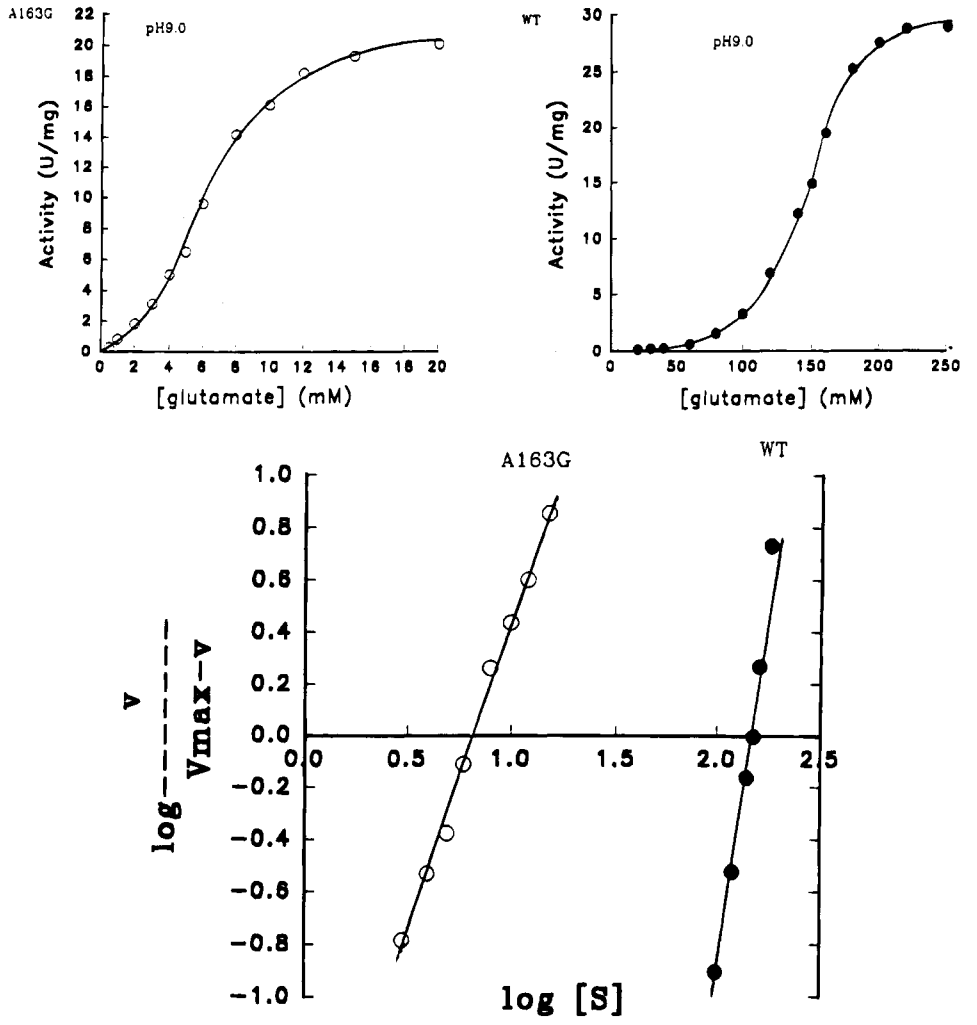


FIGURE 2: Initial rates plotted against glutamate concentration at pH 9.0 and the Hill plots. Activities for the wild-type and A163G mutant were monitored at fixed concentration of 1 mM NAD^+ over the range of glutamate concentrations at this pH. (○) A163G; (●) wild-type GDH.

for A163G at pH 9.0 and 10.5 or 200 mM for wild-type enzyme at pH 9.0. The final concentration of each enzyme was 0.01−0.02 μg/ml. As a control, measurements were performed under the same conditions for enzyme diluted 100- or 1000-fold in 0.1 M potassium phosphate at pH 7.0. These showed no significant decline in activity over 4 h at pH 7.0. Likewise the samples diluted at pH 9.0 (both wild type and mutant) retained their activity over this period. Only the mutant sample diluted at pH 10.5 (the wild-type enzyme was not checked in view of its very low activity with glutamate at this pH) showed a clear loss of activity, decreasing to 84% of the initial activity after 2 h. These levels of stability are entirely satisfactory in view of the short time course (a few minutes) of individual rate measurements.

RESULTS

Cooperative Response to Glutamate at High pH. Detailed kinetic studies designed to obtained apparent K_m values for various amino acids with a fixed concentration of 1 mM NAD^+ and at different pH values produced a striking result. The rate of reaction observed with A163G mutant enzyme at pH 10.5 showed a markedly sigmoid dependence on glutamate concentration (Figure 1 upper panel, open circles). A Hill plot of these data (Figure 1, lower panel, open circles) gave a good straight line for the central portion, with a Hill coefficient, h, of 3.4. In similar measurements at pH 10.5 for the wild-type GDH (Figure 1, upper panel, solid circles) the saturation curve was shifted to a 10-fold higher range of substrate concentration, and saturation was not attainable. Nevertheless, a steep rise in the rate was observed over the range 600−1200 mM, whereas no activity was detectable over the normal range (0−40 mM). This remarkable result prompted a reinvestigation of the enzyme's behavior at lower pH values. It has been reported (Syed & Engel, 1990) that the wild-type enzyme undergoes a conformational change from an "active" to an "inactive" form between pH 7.0 and 8.8. The standard "high" glutamate concentration used for assay purposes in those studies was 40 mM. Accordingly the rate behavior as a function of glutamate concentration has now been examined in detail at pH 9.0. There is indeed very low activity (<1 unit/mg) below 80 mM glutamate (Figure 2, upper panel, solid circles), but between 100 and 200 mM there is a steep rise, and the enzyme is effectively saturated at about 300 mM glutamate. The Hill coefficient is equal to 5.4 (Figure 2, lower panel, solid circles).

The corresponding results with the A163G mutant (Figure 2, upper panel, open circles) show that, as at pH 10.5, the affinity for glutamate is higher than with the wild-type enzyme; again the saturation curve is sigmoid, but $S_{0.5}$ is 7 mM as compared with 150 mM for wild-type GDH. The

Hill coefficient (Figure 2, lower panel, open circles) of 2.3 indicates lower cooperativity in the mutant.

In all these experiments good linearity was observed in individual reaction time courses; i.e., the level of activity did not obviously change during the assay procedure.

In view of the very high concentrations of glutamate required for the measurements with the wild-type enzyme, it appeared possible that the results were artifactual. An experiment was therefore performed (data not shown) in which the combined molarity of monosodium glutamate and sodium chloride was maintained constant (400 mM) as the glutamate concentration was varied between 0 and 400 mM. Although the results differed in detail from those without addition of compensating salt, qualitatively they were similar.

In contrast to the results at higher pH, both wild-type GDH and A163G mutant showed typically hyperbolic dependence of reaction rate on the glutamate concentration at pH 7.0 (0.1 M potassium phosphate) and at pH 8.0 (0.1 M Tris-HCl). Apparent K_m values for the wild-type GDH with glutamate are 3.04 mM at pH 7.0 and 4.59 mM at pH 8.0 with 1 mM NAD^+, and those for the mutant are 18.4 mM at pH 7.0 and 2.0 mM at pH 8.0.

Effect of Coenzyme Concentration. The rates for both wild-type GDH and the A163G mutant were measured at pH 9.0, with effectively saturating concentrations of glutamate (20 mM for A163G, and 250 mM for wild-type enzyme) over a range of NAD^+ concentrations (50−1000 μM). The rates of reactions observed with both enzymes gave clearly hyperbolic curves rather than sigmoid dependence. The apparent K_m value (95 μM) of the mutant enzyme for NAD^+ at pH 9.0 is slightly higher than that (61 μM) at pH 7.0. The wild-type GDH shows similar K_m values at both pH values (K_m = 86 μM at pH 7.0, and K_m = 89 μM at pH 9.0).

In order to test whether the extent of cooperativity in the binding of glutamate depends on NAD^+ concentration, activities of both enzymes were also monitored at a relatively low fixed concentration of NAD^+ (40 μM) with different concentrations of glutamate at pH 9.0. The wild-type GDH gave very low activity (<0.5 unit/mg) below 150 mM glutamate, but there was a steep rise in the rate over the range 150−300 mM, giving a Hill coefficient of 6 (Figure 3). To our knowledge, this is the highest such coefficient yet demonstrated and corresponds to the theoretical maximum value for a six-site enzyme showing the absolute cooperativity (eq 2) implied by the Hill equation.

$$E + 6(\text{glutamate}) \leftrightarrow E(\text{glutamate})_6 \qquad (2)$$

Clostridial GDH is hexameric (Rice et al., 1985), and this observation thus suggests that at high pH the binding of glutamate to a single subunit transforms all six subunits to the active (i.e., presumably glutamate-binding) conformation.

A decrease of the coenzyme concentration (about $K_m/2$) thus enhanced the extent of cooperativity in the wild-type GDH. A similar phenomenon was observed with A163G mutant under the same conditions, with a steep rise in the rate over the range 15−30 mM glutamate. The Hill coefficient of 3.6 is again higher than that (2.3) obtained with a saturating concentration of coenzyme.

Effect of Succinate. One of the characteristics of allosteric enzymes is that heterotropic effects may be observed if substances other than the varied substrate can perturb the allosteric equilibrium (Monod et al., 1965). Syed and Engel reported that the "reactivation" of clostridial GDH upon

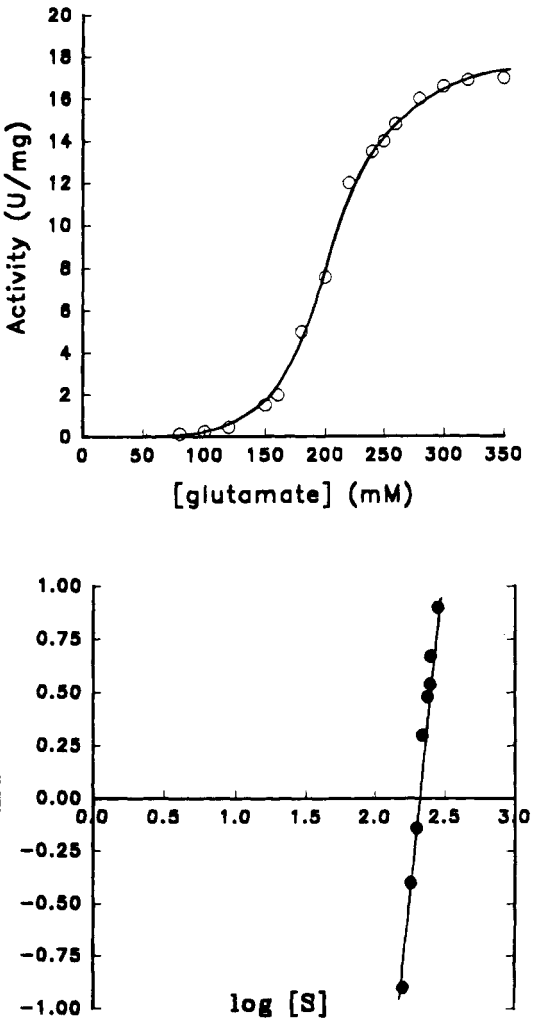


FIGURE 3: Plot of initial rates for wild-type GDH against glutamate concentration and its Hill plot. Activities toward glutamate were measured at a fixed concentration of 40 μM NAD^+ over a range of glutamate concentrations at pH 9.0.

transfer from pH 8.8 to 7.0 was potentiated by succinate, and accordingly this seemed an appropriate candidate as a possible allosteric activator. Figure 4 shows that with the wild-type GDH the sigmoidicity was gradually removed by succinate. The Hill coefficients decrease from 5.4 to 2.9, 1.2, and finally 1.0 with an increase of succinate concentration from 0 to 10, 50, and 100 mM. The sigmoid dependence of the rate upon glutamate concentration is thus replaced with a normal hyperbolic curve in the presence of 100 mM succinate. The Eadie−Hofstee plot gave an apparent K_m value of 38.8 mM for glutamate under these conditions.

With the A163G mutant at pH 9.0, the sigmoidicity is entirely removed by addition of 10 mM succinate (h = 1.01).

To further investigate how the concentration of succinate affects the rate of reaction, the activity of wild-type GDH was measured at a fixed concentration of 20 mM glutamate and 1 mM NAD^+ with a range of succinate concentrations at pH 9.0. A sigmoid dependence of the rate of reaction upon the concentration of succinate was observed (Figure 5).

Concentration Dependence for Other Substrates. It was of interest to discover whether cooperativity depends upon the intact glutamate structure. The wider substrate specificity of the A163G mutant (X.-G. Wang, unpublished results) allows this to be examined. Figure 6 shows results

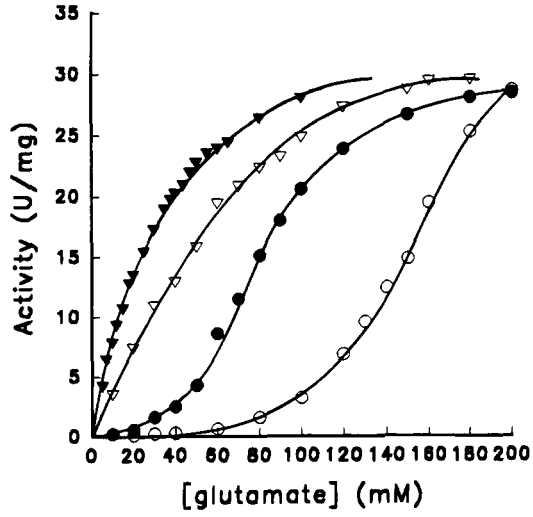


FIGURE 4: Effect of succinate on the sigmoid dependence of the rates of the wild-type GDH upon glutamate concentration at pH 9.0. Initial rates for the wild-type enzyme were measured at a fixed concentration of 1 mM NAD^+ over a range of glutamate concentrations with or without addition of succinate. (○) 0 mM succinate; (●) 10 mM succinate; (△) 50 mM succinate; (▲) 100 mM succinate.

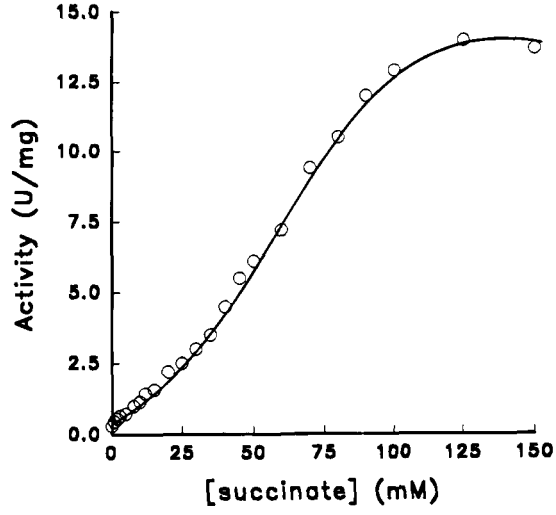
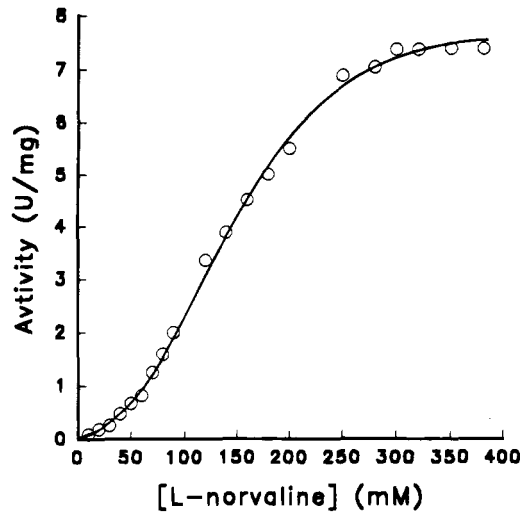


FIGURE 5: Sigmoid dependence of the rate upon succinate concentration. Activities for the wild-type GDH were measured at fixed concentrations of 1 mM NAD^+ and 20 mM glutamate over the range of succinate concentrations at pH 9.0.

for L-norvaline as substrate at pH 10.5. The Hill coefficient of 2.5 is smaller than that (h = 3.4)with glutamate under the same conditions, but nevertheless the binding of this substrate is also clearly cooperative, and the $\gamma-COO^-$ of the glutamate dicarboxylate structure is thus not a crucial determinant of the allosteric interaction.

DISCUSSION

The results in this paper clearly demonstrate that, although clostridial GDH lacks the extra 50 amino acids found toward the C-terminus in GDH of vertebrates and likewise lacks the associated heterotropic regulation by purine mononucleotides, this enzyme nevertheless has the capacity for cooperative interaction between its subunits. This manifests itself at high pH in an all-or-none interconversion between a catalytically inert and a catalytically competent state governed by the apparent inability of the first state to bind the substrate glutamate. This fits perfectly with the requirements



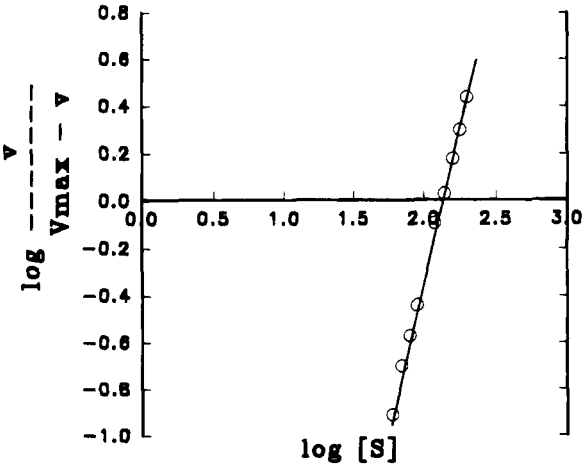


FIGURE 6: Plot of initial rates of A163G mutant against L-norvaline concentration. Activities toward norvaline were monitored spectrophotometrically at a fixed concentration of 1 mM NAD^+ over a range of L-norvaline concentrations at pH 10.5.

of the allosteric equilibrium between R and T states postulated by Monod et al. (Monod et al., 1965). The Hill coefficient of 6 seen in Figure 4 is consistent with a "symmetry model" in which one conformational state of the subunit is unable to bind glutamate.

These findings place the earlier study of time-dependent inactivation at high pH (Syed & Engel, 1990) in a new light. This conversion, associated with a directly observable conformational change (Syed et al., 1990), evidently represents the pH dependence of the allosteric equilibrium. Syed et al. noted that the speed of reactivation was strongly dependent on ionic strength and in particular on the effect of substrates and their analogues (Syed & Engel, 1990). In the present study no upward curvature was seen in reaction time courses, and clearly under the conditions of assay readjustment of the allosteric equilibrium was complete within the period (30 s) between mixing and commencement of recording of A_{340}.

It is noteworthy that at a high concentration of NAD^+ (1 mM) cooperativity in response to glutamate was less pronounced than at 40 μM NAD^+. This is consistent with the observation (Syed & Engel, 1990) that NAD^+ promotes reactivation to some extent. Thus high concentrations of NAD^+ presumably swing the allosteric equilibrium back toward the active state even in the absence of glutamate.

Until now, it has not been possible to offer a structural basis (beyond the obvious fact of oligomeric structure) for the documented allosteric properties of numerous GDHs. In the case of the clostridial enzyme, however, a structural framework exists, and the clear demonstration of strong binding cooperativity in this enzyme presents a challenge for X-ray crystallography. If clearly differentiated structures can be obtained for both R and T states, it may be possible to infer the basis of allosteric interaction both in this GDH and possibly in other, homologous members of the family.

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BI950904A