Mechanistic Interpretation of Tryptophan Fluorescence Quenching in the Time Courses of Glutamate Dehydrogenase Catalyzed Reactions†

Swapan K. Saha, Steven J. Maniscalco, and Harvey F. Fisher*

Laboratory of Molecular Biochemistry, Veterans Affairs Medical Center, and Department of Biochemistry, University of Kansas Medical Center, Kansas City, Missouri 64128

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ABSTRACT: We have related the ratios of the protein fluorescence quenching and nucleotide absorbance time courses for the glutamate dehydrogenase catalyzed oxidative deamination of L-glutamate to identify the occurrence and sequential location of a previously demonstrated charge-transfer intermediate. Static studies showed the major portion of the fluorescence quenching signal to be due to radiationless singlet energy transfer from tryptophan to reduced coenzyme chromophores and that conformational changes contribute little to this signal. The ratio approach applied to the transient time courses shows correspondingly that, over most of the time range, the fluorescence quenching signal provides a quantitative measure of the sum of all posthydride transfer species. However, it also indicates the very early occurrence of a species of anomalous optical properties for the reaction catalyzed by the *Clostridium symbiosum* enzyme as well as that from bovine liver. Transient-state kinetic isotope effect time courses of both the fluorescence and the absorbance signals confirm that this species must be the prehydride charge-transfer complex in both enzyme reactions. Kinetic analysis of α-deuterio- and α-protio-L-glutamate reaction time courses proves the kinetic competence of the assignments. These results also demonstrate that the intramolecular transfer of a proton from the α-amino group of the substrate to an immediately adjacent aspartate carboxylate group on the enzyme is an obligatory initial event in the reactions catalyzed by both enzyme species, even though the occurrence of protein release from a critical lysine residue to the solvent occurs at different phases in those two reactions. The abnormally low intrinsic KIE required to simulate both the α-deuterio-L-glutamate reaction and its protio counterpart implies that the transition state of the hydride transfer step must be highly asymmetric.

In a series of recent papers we have developed the use of a ratio time−course approach as a general set of tools for the dissection of specific mechanistic detail from the transient-state time courses of enzyme-catalyzed reactions. Thus, we have used the ratio of the proton release−time course to that of product formation, the ratio of two absorbance time courses measured at different wavelengths, the ratio of the bound NADPH fluorescence time course to that of its absorbance time courses (Saha et al., 1994), and the ratios of the fluorescence and absorbance time courses using protio- and deuterio-L-glutamate (transient kinetic isotope effect time courses) (Fisher et al., 1988, 1992; Fisher & Saha, 1996; Maniscalco et al., 1996). Since each specific time course ratio highlights the development of one or more reactive components and is silent on others, depending on the relative sensitivities of each signal to the properties of any given complex, it is useful to be able to employ as many different such signals as possible. The quenching of tryptophan fluorescence, presumably due to a radiationless transfer to the reduced nicotinamide chromophore in pyridine nucleotide dehydrogenase complexes, has been used both to study the nature of static complexes (Holbrook, 1972; Holbrook et al., 1972; Brochon et al., 1977; Torikata et al., 1979) and, in an *ad hoc* manner, to distinguish transient-state steps (Dunn et al., 1978; Horn & Bisswanger, 1983; Wolfe et al., 1977). Here, we explore the use of this signal in the ratio time−course mode and use it to pinpoint the location of a recently demonstrated charge-transfer complex along the reaction coordinates of the reactions catalyzed by *Clostridium symbiosum* (cs) and bovine liver (bl) glutamate dehydrogenase.

MATERIALS AND METHODS

Beef liver glutamate dehydrogenase was purchased from Sigma as an ammonium sulfate suspension. The enzyme was dialyzed against three changes of 0.1 M potassium phosphate at the required pH, centrifuged at 4 °C, and finally filtered with Norit A through 0.45 μm Millipore filters. The enzyme concentration was measured spectrophotometrically at 280 nm using $\epsilon = 54.4$ cm^{-1} mM^{-1}. *C. symbiosum* glutamate dehydrogenase was prepared and purified following the method of Syed (1987). The enzyme was dialyzed and filtered as was done with beef liver enzyme, and the concentration was measured using $\epsilon = 51.8$ cm^{-1} mM^{-1}. NADP and NAD were purchased from Sigma and used as obtained without any further purification. L-Glutamic acid was purchased from Calbiochem Inc. L-[^{2}H]Glutamic acid was prepared following the method of Rife and Cleland (1980). NMR analysis of the deuterated glutamic acid showed 100% conversion at the α-position.

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* Address correspondence to this author at Research Service, VA Medical Center, 4801 Linwood Blvd., Kansas City, MO 64128. Telephone, (816) 861-4700 X7156; FAX, (816) 861-1110.

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The stopped-flow experiments were carried out on a Hi-Tech SF-51 stopped-flow spectrophotometer equipped with dual photomultipliers and interfaced to an IBM compatible PC. Each fluorescence quenching or absorbance time course consists of an average of at least 20 runs. Absorbance measurements were carried out at 340 nm and converted to moles of product formed per mole of enzyme (defined as P/E in the figures) by dividing the absorbance signal by the enzyme concentration and by a value of 0.005 (under the assumption that all the enzyme-reduced nicotinamide ternary complexes have an average millimolar extiction coefficient of 5.0 (Fisher et al., 1988). Fluorescence quenching experiments were carried out by excitation at 297 nm using a combination of UG5 and WG335 filters to exclude any contribution from NADPH during fluorescence measurements. The tryptophan fluorescence quenching of free enzyme was used as the reference to calculate the relative fluorescence quenching of other enzyme complexes as well as the fluorescence time courses. Fractional fluorescence quenching was calculated as the ratio of quenching observed due to formation of enzyme complexes to the fluorescence of free enzyme and thus represents the moles of product formed per mole of enzyme. For the ratio curves, respective signals were pinned to zero at 5.5 ms. The calculation of KIE[1] time courses has been described in the text. The upper and lower limits of the error bars for the tKIE time courses were estimated by alternatively adding and subtracting the standard deviations to the fitting parameters and calculating the maximum and minimum tKIE time courses thereafter. All static and kinetic experiments were carried out at 0.1 M buffer. For the blGDH and the csGDH reactions, typical reaction conditions were pH 7.0, 10 °C, blGDH = 20 μM, NADP = 380 μM, and L-glutamate = 45 mM and pH 6.2, 5 °C, csGDH = 17.4 μM, NAD = 380 μM, and L-glutamate = 45 mM, respectively. The static fluorescence measurements were carried out at pH 7.0 and 10 °C for both of the enzymes.

RESULTS

Static Studies of Fluorescence Quenching. The fractional degree of the quenching of the fluorescence of bl and cs glutamate dehydrogenase in various binary and ternary complexes is shown in Table 1. It is clear from the results that the presence of the absorbing chromophore of the reduced nicotinamide moiety of the coenzyme (or its optical equivalent) is both necessary and sufficient to produce a major degree of quenching for both enzymes.[2] A fractional quenching of 0.91 ± 0.02 is observed in all such cases.

Table 1: Fluorescence Quenching in Stable Glutamate Dehydrogenase Complexes[a]

enzyme complex	fractional quenching	
	blGDH	csGDH
E	0.0	0.0
E−L-glutamate	0.0 ± 0.002	0.0 ± 0.002
E−D-glutamate	0.0 ± 0.002	0.0 ± 0.002
E−isophthalate	0.0 ± 0.002	0.0 ± 0.002
E−O	0.04 ± 0.005	0.04 ± 0.005
E−O−D-glutamate	0.03 ± 0.003	−0.05 ± 0.004
E−O−isophthalate	0.03 ± 0.003	0.5 ± 0.02
E−R	0.90 ± 0.03	0.90 ± 0.03
E−R−α-ketoglutarate	0.93 ± 0.01	0.93 ± 0.01
E−R−L-glutamate	0.93 ± 0.01	0.93 ± 0.01

[a] E represents the native enzyme; fluorescence quenching of free E under a particular condition is considered as the baseline. Any change in fluorescence quenching due to the formation of other enzyme complexes is calculated as fractional quenching with reference to free E. O and R indicate the oxidized and the reduced coenzymes NAD(P) and NAD(P)H, respectively. Typical reaction conditions are as follows: E = 20 μM; L-glutamate = 45 mM; D-glutamate = 45 mM; isophthlate = 2 mM; NAD(P) = 380 μM; NAD(P)H = 100 μM; α-ketoglutarate = 2 mM; pH 7.0, 10 °C.

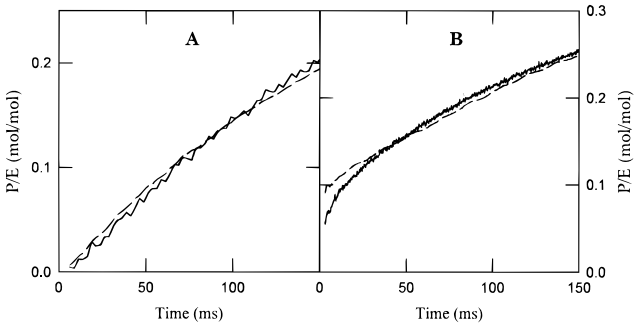


FIGURE 1: Representative transient-state time courses as observed by 340 nm absorbance and by 297 nm excited protein fluorescence quenching signals. The solid lines represent the fluorescence quenching signals, and the dashed lines represent the absorbance signals, respectively. For absorbance, the signals were converted to P/E (moles of product formed per mole of enzyme) by dividing the signal by 0.005 (average product extinction coefficient) and then by the enzyme concentration. In the case of fluorescence quenching, fractional fluorescence quenching is equivalent to the P/E signal. Panel A represents the time courses observed in the oxidative deamination of [α-$^2H_1$]-L-glutamate catalyzed by blGDH. The reaction conditions are blGDH = 20 μM, NADP = 380 μM, and [$^2H_1$]-L-glutamate = 45 mM, pH 7.0, 10 °C. Panel B represents the same reaction catalyzed by csGDH. The reaction conditions are csGDH = 17.4 μM, NAD = 380 μM, and [$^2H_1$]-L-glutamate = 45 mM.

Kinetic Studies of Fluorescence Quenching. Typical stopped-flow reaction time courses for the 297 nm excited fluorescence and for the 340 nm absorbance signals for the bl- and csGDH, respectively, are shown in Figure 1. In both cases the reactions are carried out at a relatively low pH and at a low temperature to permit facile observation of the early phase of the reaction. Under these conditions, a well-defined burst phase is observed for both signals and for both enzymes. For both enzymes, the fluorescence and absorbance signals track each other approximately but show somewhat different trends at the beginning and at the ends of the burst phases of their respective time courses. We have previously shown (Fisher et al., 1988) that the proton ratio function provides a useful approach for the resolution of transient-state time courses. We apply the same logic here, defining the function Q_{297}/A_{340} as the time-dependent scaled

[1] Abbreviations: csGDH, *Clostridium symbiosum* glutamate dehydrogenase; blGDH, bovine liver glutamate dehydrogenase; E, enzyme; O, oxidized coenzyme [NAD(P)]; R, reduced coenzyme [NAD(P)H]; K, α-ketoglutarate; KIE, kinetic isotope effect; tKIE, transient-state kinetic isotope effect; Q_{297}, fractional tryptophan fluorescence quenching (excitation 297 nm).

[2] Minor deviations from this generalization can be rationalized as follows: the absorbance spectrum of isophthalate does overlap the tryptophan fluorescence emission spectrum and thus permits some fluorescence quenching through a radiationless energy transfer. We cannot account for the difference in the efficiency of this process between the bl and cs enzyme forms. The slight increase in the tryptophan fluorescence of the cs E−O−D-glutamate complex over that of the free enzyme may be ascribed to the decreased exposure of the tryptophan residues to the solvent in the closed form of the enzyme in this complex. Again, we cannot explain the small but opposite behavior of the corresponding bl enzyme.

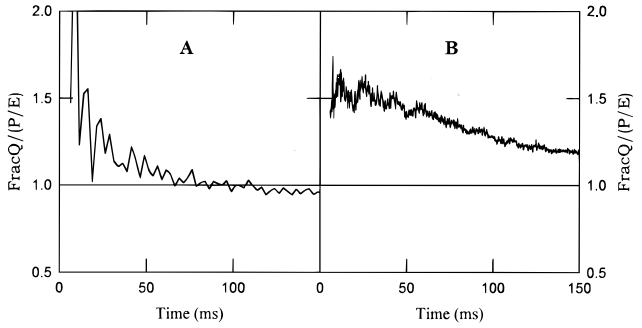


FIGURE 2: Fractional fluorescence quenching/product ratio time courses calculated from the data in Figure 1. Panel A: the blGDH reaction. Panel B: the csGDH reaction.

ratio of the fractional degree of 297 nm excited fluorescence quenching to the 340 nm absorbance. Typical Q_{297}/A_{340} time−course ratios for both enzymes are shown in Figure 2. Since the experimental Q and A values have both been scaled using the quenching and absorbance values of the enzyme−reduced coenzyme stable complexes, a horizontal straight line with a value of $Q_{297}/A_{340} = 1$ would be expected if all transient complexes had such properties. For both enzymes, however, it can be seen that $Q_{297}/A_{340} \cong 2$ at the earliest measurable point in the time course and diminishes asymptotically toward the expected value $Q_{297}/A_{340} = 1$. A high ratio could result either from an increase in the numerator, Q_{297}, or from a decrease in the denominator, A_{340}. However, since Q_{297} has been taken to have a value of 0.9 and could not exceed a value of unity by definition, this high ratio must be due to a decrease in the denominator, implying the early occurrence of a complex whose absorbance coefficient is no more than half of that of the stable model complexes but whose fluorescence quenching is nearly as complete as in those complexes. The gradual drop toward $Q_{297}/A_{340} = 1$ is presumably due to the increasing contribution to A_{340} from the subsequently formed normal E−NADPH-containing complexes.

Transient-State Kinetic Isotope Effect Studies. In a previous paper (Fisher & Saha, 1996) we have shown that observed transient-state isotope effects (tKIE's), in contrast to their steady-state counterparts, are dependent both on time and on the specific signal measured. We have defined a tKIE as

$$\text{tKIE} = (dS_H/dt)/(dS_D/dt) \qquad (1)$$

where $S_H(t)$ and $S_D(t)$ represent the ratio of any given signal obtained with α-protio-L-glutamate to that obtained in an otherwise identical experiment using α-deuterio-L-glutamate. The results of such experiments using both Q_{297} and A_{340} as simultaneously measured signals for both enzymes are shown in Figure 3. Both enzymes show very similar behavior: the tKIE_A falls from an extrapolated initial value of about 1.7 [as previously shown (Saha et al., 1994)], while tKIE_Q rises in the very early time period to a maximum of about the same value as tKIE_A and then falls at nearly the same rate. Following the rules of behavior for tKIE's which we have established (Fisher & Saha, 1996) we conclude that only posthydride transfer species make a significant contribution to the absorbance signal, while a very early prehydride transfer species must quench substantially more than it absorbs, and that its influence on the total signal decreases with time as posthydride transfer complexes accumulate.

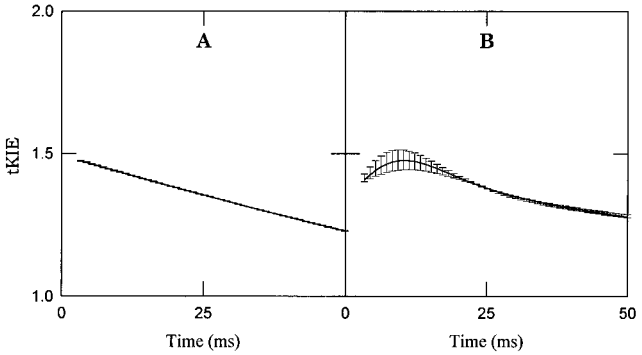


FIGURE 3: tKIE time courses for the reaction catalyzed by csGDH. Panel A represents the tKIE time courses derived from the absorbance signal whereas panel B represents the same with the fluorescence quenching signal. The estimation of error bars is described in Materials and Methods.

Thus, the isotope effect results lead independently to the same qualitative conclusions provided by the Q_{297}/A_{340} time−course ratio results described earlier.

Quantitative Relationships between the 297 nm Excited Florescence Quenching and the 340 nm Absorbance Signals. To derive a formal relationship between the absorbance and the fluorescence quenching signals, we assume the linear model shown in Scheme 1 for the interconversions between

Scheme 1

$$\text{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{B} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{C} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{D} \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{E}$$

the known and suspected central complex intermediates. Here A represents the first ternary complex formed, and k_3 and k_4 are the rate constants for the isotopically sensitive steps. k_1 and k_2 are therefore assigned to the prehydride steps, whereas k_5, k_6, k_7, and k_8 correspond to the posthydride transfer steps shown in Scheme 1. We express the time courses of the reaction shown in Scheme 1 as measured by absorbance or by fluorescence quenching as

$$P(t) = b[\text{B}] + c[\text{C}] + d[\text{D}] + e[\text{E}] \qquad (2)$$

where b, c, d, and e are the signal coefficients for absorbance and

$$P(t) = b_1[\text{B}] + c_1[\text{C}] + d_1[\text{D}] + e_1[\text{E}] \qquad (3)$$

where b_1, c_1, d_1, and e_1 are the signal coefficients for fluorescence quenching. At pH 7.0, for beef liver enzyme, the absorbance signal can be fitted by using the equation

$$P = 0.035[\text{B}] + 1.0[\text{C}] + 1.0[\text{D}] + 1.1[\text{E}] \qquad (4)$$

using a given set of rate constants. For the fluorescence quenching signal, using identical rate constants, the data can be fitted by the equation

$$P = 0.1[\text{B}] + 0.9[\text{C}] + 0.9[\text{D}] + 0.9[\text{E}] \qquad (5)$$

using the identical set of rate constants. The agreement between the solutions for the two equations establishes the kinetic competence of the reaction scheme itself and of the properties assigned to the various species.

We now impose an additional set of constraints on the solution to eqs 2 and 3 by requiring that an intrinsic primary KIE be confined solely to two rate constants, k_3 and k_4, which

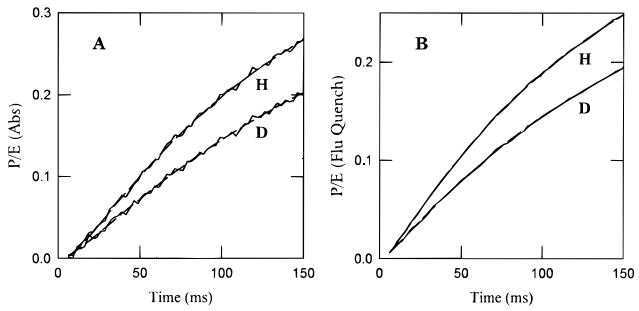


FIGURE 4: (A) Reaction time courses of reduced coenzyme product formation for protio-L-glutamate (H) and deuterio-L-glutamate (D) obtained from the absorbance signal in the blGDH catalyzed reaction. The bold lines represent the product time courses whereas the dashed line represents the simulated curves. The simulation curves were generated using the constants $k_1 = 10.2$, $k_2 = 20$, $k_3 = 13$, $k_4 = 30$, $k_5 = 22$, $k_6 = 10$, $k_7 = 30$, and $k_8 = 25$. (B) The same reaction time courses for protio-L-glutamate (H) and deuterio-L-glutamate (D) obtained from the tryptophan fluorescence quenching signal shown in panel A. The fluorescence time courses are represented by bold lines. The simulated curves represented by the dashed line were generated by using the same rate constants as in panel A.

alone are assumed to represent the hydride transfer step. In panels A and B of Figure 4 we show the reaction time courses of reduced coenzyme formation as measured by absorbance and protein quenching, respectively. Each of the dashed lines in the figures represents the time courses of the corresponding reactions as calculated by eqs 2 and 3 assuming an intrinsic KIE of 2.85 ± 0.1 for k_3 and k_4 alone and using the values for k_3, k_4, k_5, k_6, k_7, and k_8 as reported in an earlier section. The extremely close agreement of each of the four calculated curves with their experimental conditions under this extensive range of numerical constraints satisfies a severe test of the assignments of species and of the various signal coefficients we have determined.[3]

Utility of the Protein Fluorescence Quenching Signal in the Mechanistic Resolution of Enzymatic Transient-State Time Courses. The results shown here appear to provide an answer to the question of whether tryptophan fluorescence quenching observed in catalytic reaction time courses is due to increased exposure of the residues to solvent or to radiationless singlet energy transfer. It is clear from the results shown in Table 1 that the opening and closing of the active site cleft produce changes in fluorescence of only 10%, while the presence of the reduced nicotinamide chromophore in any enzyme complex produces a 90% quenching. The Q_{297} time course, therefore, provides some quantitative constraints which can be useful in interpreting transient-state time courses obtained from A_{340} absorbance or A_{340} excited fluorescence signals. In the postburst phases of such pyridine nucleotide dehydrogenase reactions, eventually some amount of free reduced coenzyme is released from the enzyme. As such, it continues to contribute to both the 340 nm absorption and fluorescence excitation spectral signals but makes no contribution to the tryptophan fluorescence quenching signal. This latter signal, therefore, sets an upper limit to the molar concentration of enzyme−reduced coenzyme complexes present at any given point in time. The lower limit of this value is somewhat imprecise because of possible minor contributions to quenching from the presence of charge-transfer complexes as we have shown here. On the other hand, the quenching contribution of such a prehydride charge-transfer complex, occurring as it must before significant amounts of reduced coenzyme complexes have been formed, provides a sensitive method for the detection of such entities. At this time, charge-transfer complexes have been demonstrated in pyridine nucleotide dehydrogenase reactions only in the cases of glyceraldehyde phosphate and glutamate dehydrogenase. It will be of some interest to determine whether they occur generally in this class of enzyme reactions and may play some as yet undefined mechanistic role or whether they merely reflect the chance location of an unshared electron pair constrained by the particular geometry of a given substrate.

DISCUSSION

Physical Basis of Protein Fluorescence Quenching in Pyridine Nucleotide Dehydrogenase Reaction Time Courses. It is clear from the results presented here that the tryptophan fluorescence quenching observed throughout the reaction time course of both enzyme-catalyzed reactions is largely accounted for by radiationless transfer to the reduced nicotinamide moiety present in all posthydride transfer complexes. This conclusion disagrees with that reached by Basso and Engel. However, the Q_{297}/A_{340} ratio time−course studies we have described do indicate the presence of a rapidly formed species which absorbs only very weakly at 340 nm but which quenches tryptophan fluorescence quite effectively. The tKIE studies confirm this finding and show that this species must be a prehydride transfer complex. Allowing for the overall greater velocity of the cs enzyme, both the cs and the bl enzymes show qualitatively similar behavior in this regard. In a previous paper (Saha et al., 1994) we demonstrated the existence of an enzyme−oxidized coenzyme−L-glutamate charge-transfer complex in the early phase of the bl enzyme catalyzed reaction time course. This complex is characterized by a very small absorbance coefficient but yet exhibits a strong 355 nm excited fluorescence. These properties are precisely those required to explain the anomalous Q_{297}/A_{340} values of the prehydride transfer complexes detected in the work reported here. On the basis of the structural coordinates of the cs enzyme (Stillman et al., 1993) we concluded that the location of the unprotonated α-amino nitrogen atom of the bound substrate molecule above the face of the oxidized nicotinamide moiety of the bound coenzyme must provide the unbonded electron pair required for the charge-transfer phenomenon. This argument was strengthened by the strict requirement of a substrate α-amino group−D-glutamate and isophthalate, both of which bind cooperatively with NADP and produce a kinetically competent proton release but do not produce a charge-transfer phenomenon with bl glutamate dehydrogenase. It can be seen from Table 1 of this paper that these same two compounds fail to produce the tryptophan fluorescence quenching phenomenon with *either* the bl *or* the cs enzyme. These observations provide the necessary logical connectivity required to show that the formation of a kinetically competent prehydride charge-transfer complex contributes to tryptophan

[3] We neither make nor require for our purpose here any claim that these particular sets of rate constants are unique solutions to eqs 2−5. Therefore, no physical significance should be attached to any specific rate constant. The fact that there exists at least one set of such values in each case whose use permits a suitably close fit to the experimental data does constitute a proof of the kinetic competence of the scheme itself, and this fact alone is required by our subsequent argument.

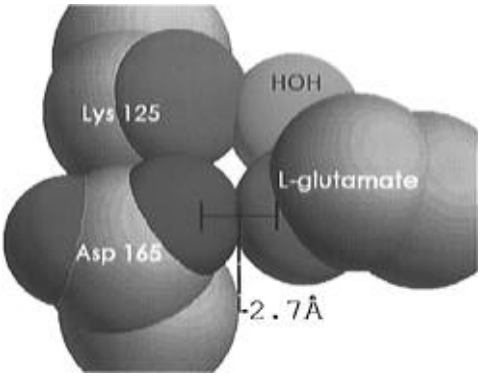


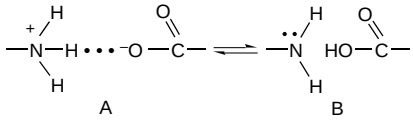
FIGURE 5: Spatial relationships between the enzyme-bound substrate (L-glutamate) and two nearby enzyme functional groups in csGDH. For clarity, we show here only the α- and β-carbon atoms and the α-nitrogen atom of the substrate molecule, the conserved aspartyl carboxylate moiety, and the conserved Lys 125 ϵ-amino group holding a single isolated water molecule. This water molecule is seen to be poised for a nucleophilic attack on the substrate α-carbon atom required later in the reaction sequence (Singh et al., 1993). All atoms are portrayed in terms of their van der Waals radii. The interatomic distance shown has been calculated by the RASMOL program using atomic coordinates provided by David Rice (personal communication).

fluorescence quenching in both bl and cs glutamate dehydrogenase complexes.

Basso et al. (1993) recently reported the results of a study of stopped-flow kinetic measurement of Q_{297} of the csGDH catalyzed reaction. They concluded that there is no burst phase in the fluorescence quenching signal and that the fluorescence quenching is due entirely to the formation of a prehydride enzyme−NAD complex. As a result of the work reported here, we interpret their results quite differently. Their failure to observe the burst reported here in Figure 1 was because at the higher pH and temperature used by Basso et al. (1993) the burst phase is nearly complete in the instrumental dead time. tKIE studies (not shown here) demonstrate that the signal observed by Basso and Engel was entirely due to the accumulation of posthydride transfer (reduced coenzyme) complexes. Basso et al.'s deduction of the existence of a prehydride complex, on the other hand, is quite correct even though they did not observe it directly, because they based their conclusion on the *concentration dependence* of the fluorescence quenching signal.

Structure−Function Relationships. The formation of the L-glutamate−oxidized coenzyme−enzyme complex requires the prior deprotonization of the substrate α-amino nitrogen. The crystallographic structure data for the csGDH−L-glutamate complex provided by the Sheffield group provide a physical basis for just such a chemical event in the cs enzyme catalyzed reaction. The structure shown in Figure 5 indicates that the internuclear distance between the α-amino nitrogen atom of the substrate and one of the γ-carboxyl oxygen atoms of the highly conserved Asp 165 residue is 2.7 Å. This distance and geometry should force the formation of an N···H···O−C hydrogen bond linkage between the substrate amino group and the enzyme's aspartyl carboxyl group. Since the p*K* of the α-amino group of glutamic acid in aqueous solution at 25 °C is 9.5 and that of the carboxyl group in free aspartic acid is less than 4.0 (Christensen et al., 1976), the zwitterionic form portrayed as structure A in Scheme 2 should predominate and the concentration of the deprotonated amino form required for

Scheme 2



charge transfer to the reduced nicotinamide ring would be negligible. However, it has been shown theoretically and experimentally that as the dielectric constant of the solvent decreases, amino group p*K* values decrease and carboxylate p*K* values increase. The work of Stillman et al. (1993) shows that in the forced structure of the cs glutamate dehydrogenase crystal the enzyme active site cleft is closed and that the substrate is in a nearly anhydrous medium. The authors also present convincing evidence that in the enzyme−substrate−NAD ternary complex this closure must also occur without any substantial difference in protein conformation from that of the corresponding enzyme−substrate binary complex. Under these conditions and given the known N−O interatomic distance, it is obvious that the simple transfer of charge required to effect the reaction will be driven far to the right (if not completely so), producing the electrically neutral form B, and will proceed at a rate limited by that of the cleft closing itself. Since the nuclear movement of the proton in this reaction should be no more than 0.7 Å, it is quite possible that the proton transfer occurs directly and concomitantly with the binding of the substrate and the closure of the cleft and that species A of Scheme 2 never forms at all. Since D-glutamate, which cannot form an Asp 165 L-glutamate hydrogen bond, is a very effective competitive inhibitor for the reaction and actually binds more tightly both to free enzyme and to the bl-E−R complex (Subramanian et al., 1978), this interaction appears to provide very little to the overall $\Delta G°$ of substrate binding (Rife & Cleland, 1980). These facts and the findings reported here support the conclusions reached by Basso et al. (1994), using a csGDH mutant lacking Asp 165, that the role of this residue is largely catalytic rather than substrate binding. Since the same phenomena are observed in both the bl and cs enzymes and all of the required functional groups are conserved, we conclude that this same process occurs in the reactions catalyzed by both enzymes.

While this deamination process is necessary to account for the phenomena reported here, chemical reasoning requires that it must precede the hydride transfer step in either enzyme catalyzed reaction. The tKIE results reported here confirm such a location in both reaction time courses. While chemical intuition suggested that removal of the amino proton must precede hydride transfer, the prehydride location of the formation of the unprotonated substrate amine demonstrated here for both enzymes now provides direct evidence to that point.

Intrinsic KIE of the bl Glutamate Dehydrogenase Catalyzed Reaction. The requirement of the assumption of an intrinsic primary KIE of less than 3 for even a reasonable fit to the four curves is of particular significance in itself. While this value is much higher than any steady-state KIE reported for the oxidative deamination of L-glutamate and is even somewhat higher that the extrapolated tKIE's reported here, it is nearly 2-fold smaller than that anticipated by the classical zero-point energy KIE for the breaking of a covalent carbon−hydrogen bond. The results described here, there

fore, imply that the transition state for this step may be highly asymmetric.

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