Location of Deuterium Oxide Solvent Isotope Effects in the Glutamate Dehydrogenase Reaction*

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Stopped flow studies of D₂O kinetic solvent isotope effects on the reaction catalyzed by L-glutamate dehydrogenase reveal, in addition to several effects apparently attributable simply to pKₐ shifts, a 2-fold pH-independent effect on the velocity of the steady state oxidative deamination of L-glutamate by enzyme and NADP. Comparable pH-independent D₂O kinetic solvent isotope effects are seen both in a transient phase of the reaction in which α-ketoglutarate is displaced by L-glutamate from an enzyme-NADPH-α-ketoglutarate (product) complex and in an analogous model reaction in which α-ketoglutarate is displaced by D-glutamate. These results suggest that α-ketoglutarate dissociation from an enzyme-NADPH-α-ketoglutarate complex is rate-limiting in the steady state.

Transient state kinetic studies of the reaction catalyzed by bovine liver L-glutamate dehydrogenase have produced a detailed picture of the enzyme mechanism in terms of both catalytically productive and dead-end complexes of enzyme, coenzyme, and substrate (1–6). The three kinetic phases (7) of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP (hereafter called the forward reaction) are shown schematically in Fig. 1, along with a summary of the mechanism as it is currently understood. The complexes associated with each phase are located below that phase in the figure. The point of entry of ammonium ion into the catalytic scheme has not yet been elucidated. No significant transient features have been detected for the reverse catalytic reaction (8).

It has commonly been assumed that the rate-limiting step in Phase 3 (Fig. 1) of the forward reaction is the release of bound NADPH from the dead-end enzyme-NADPH-L-glutamate complex (3, 4, 6). There is evidence from isotope exchange experiments, however, that α-ketoglutarate dissociation may be rate-limiting under similar conditions (8).

In the present work, we determine the phenomenological location and pH dependence of deuterium oxide (D₂O) kinetic solvent isotope effects in the glutamate dehydrogenase-catalyzed reaction. As was true of the original study of this type by Kosicki and Srere on the steady state kinetics of citrate-condensing enzyme (9), we find that some of the D₂O kinetic solvent isotope effects observed here are attributable simply to pKₐ shifts; but in addition, we find a pH-independent effect which provides evidence that α-ketoglutarate dissociation is rate-limiting in Phase 3 of the forward reaction.

MATERIALS AND METHODS

NADP, NADPH, D₂O, and L-glutamate dehydrogenase (EC 1.4.1.3) were obtained from Sigma Chemical Co. L-Glutamic acid and α-ketoglutaric acid were obtained from Calbiochem, and D-glutamic acid from Aldrich Chemical Co. All solutions were prepared in 0.1 M potassium phosphate buffer as described previously (5, 10). pD values were computed using a linear interpolation (11) according to the equation:

\[
pD = \text{pH meter reading} + 0.004 \times \% \text{D}_2\text{O}
\]

Kinetic studies were performed at 20° on a Durrum-Gibson stopped-flow spectrophotometer interfaced either to a Varian 620i or to a Varian 620L digital computer (5, 12). All concentrations reported are final concentrations after mixing in the stopped flow apparatus. Additional experimental details are given in the figure legends.

In the discussion which follows, the term “isotope effect” refers to the ratio of reaction velocity or rate constant in H₂O to that measured in the H₂O-D₂O mixture. The effects are extrapolated to 100% D₂O only when noted in the text.

RESULTS

\(D_2O\) Solvent Isotope Effects on Steady State Velocities—Stopped flow studies of the steady state phase (Phase 3 in Fig. 1) of the forward reaction show a 2-fold pH-independent D₂O kinetic solvent isotope effect (Fig. 2a). In contrast, the initial

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NADPH than does L-glutamate (14, 15), yet is not a substrate which forms a more stable ternary complex with enzyme and 4b). The displacement was carried out using n-glutamate shows an approximately 2-fold deceleration in 83% D,O (Fig. 4a).

The principal effect appears to arise from a pK, solvent isotope effects (Fig. 2b). The pH dependence of the pseudo-first order rate constants for all three phenomena and which could be responsible for the mechanical location of the pH-independent D,O effects on the reverse reaction originate.

The principal finding of the present study is the probable mechanistic location of the pH-independent D,O solvent isotope effects on the steady state velocity (Phase 3) as the source of the pH-independent effects observed for the steady state velocity (Phase 3). The pH-independent effects of Phase 3, then, must involve steps which follow the hydride transfer step for the forward reaction.

The mechanical event which immediately follows Phase 1 is the conversion of the blue-shifted enzyme-NADPH-a-ketoglutarate complex to the red-shifted enzyme-NADP-l-glutamate dead-end complex (Phase 2). Indeed, this phenomenon shows a 2-fold pH-independent D,O kinetic solvent isotope effect. The model reaction in which p-glutamate is used to displace a-ketoglutarate from its ternary complex with enzyme and NADPH also shows a 2-fold pH-independent D,O kinetic solvent isotope effect. Thus, the D,O kinetic solvent isotope effects for Phase 3, Phase 2, and a-ketoglutarate displacement are identical, suggesting that these processes share a common mechanistic feature. In addition, the pH dependences of the rates for these three processes are quite similar, considering the mechanistic complexity of the observed phenomena, and the isotope effects for all three show a linear dependence on the percentage of D,O.

One step in the enzymatic mechanism which is common to all three phenomena and which could be responsible for the

**DISCUSSION**

The observed D,O solvent isotope effects on the initial velocity of the reverse reaction appear to arise principally from pK, shifts with change of solvent composition. Since pK, increases in D,O of 0.3 to 0.6 unit are quite common for weak acids which ionize in the experimental pH (pD) range (16), it is not yet possible to say where specifically the D,O effects on the reverse reaction originate.
FIG. 2. pH (pD) dependence of velocities measured at 340 nm in the stopped-flow experiments in the presence and in the absence of D₂O. a, steady state velocity of Phase 3 of the forward reaction; [L-glutamate] = 50 mM; [NADP] = 500 μM; O, H₂O; □, 90% D₂O. b, initial velocity of the reverse reaction; [α-ketoglutarate] = 2 mM; [NADPH] = 30 μM; [NH₄Cl] = 50 mM; O, H₂O; □, 88.5% D₂O. c, initial velocity of Phase 1 of the forward reaction; conditions are the same as in a. In all experiments, each syringe contained 1 mg/ml of L-glutamate dehydrogenase and the substrates were in one syringe and coenzyme in the other, thus avoiding the problem of protein light scatter changes on dilution during Phase 1 of the reaction (5). The enzyme concentration, [E], is calculated using a molecular weight of 56,100 per polypeptide chain. The velocities are computed using the millimolar extinction coefficient for free coenzyme (6.2) in a and b and the millimolar extinction coefficient for the enzyme-NADPH-α-ketoglutarate complex (5.0) in c (see Ref. 5).

observed behavior is the release of α-ketoglutarate from its ternary complex with enzyme and reduced coenzyme. This step is thought to be rate-limiting in Phase 2 and in the α-ketoglutarate displacement by α-glutamate and is certainly involved in the mechanism of the steady state production of free NADPH (Phase 3). Thus, the observations above provide direct evidence that α-ketoglutarate dissociation is a rate-limiting factor in the steady state phase (Phase 3) of the forward reaction. This conclusion gains additional support from the isotope exchange data (8) and is consistent with transient state data obtained in Tris buffer which have led to the suggestion that coenzyme release from the enzyme-NADPH-L-glutamate dead-end complex is actually rate-limiting in the steady state under conditions similar to the ones employed in the present work (6).²

Unfortunately, the rate of NADPH dissociation from the dead-end ternary complex with enzyme and α-glutamate cannot be obtained either directly or indirectly with sufficient accuracy to determine whether or not that process alone can account for the D₂O solvent isotope effect and pH dependence of the velocity of the steady state phase of the catalytic reaction.

FIG. 3. Time dependence of the absorbance for the forward reaction at three wavelengths and low NADP concentration. [L-GLUTAMATE DEHYDROGENASE] = 1 mg/ml; [NADP] = 10 μM; [L-glutamate] = 50 mM; pH = 8.23. The optical path length of the cuvette is 2 cm.

FIG. 4. pH (pD) dependence of the apparent first order rate constants measured at 320 nm for the conversion from a blue-shifted to a red-shifted reduced nicotinamide spectrum. a, in Phase 2 of the forward reaction. [L-glutamate dehydrogenase] = 9 mg/ml; [L-glutamate] = 100 mM; [NADP] = 10 μM; O, H₂O; □, 82% D₂O. b, in the displacement of α-ketoglutarate from the enzyme-NADPH-α-ketoglutarate complex by α-glutamate; [L-glutamate dehydrogenase] = 2 mg/ml; [NADPH] = 20 μM; [α-ketoglutarate] = 200 μM; [β-glutamate] = 100 mM; O, H₂O; □, 83% D₂O.

In conclusion, the following simple mechanistic picture for Phase 3 of the forward reaction is consistent with all the experimental evidence presently available. Under the experimental conditions reported here, the presence of the dead-end enzyme-NADPH-L-glutamate complex renders a fraction of the enzyme active sites catalytically inaccessible. The remainder of the sites catalyze the oxidative deamination of L-glutamate at a velocity limited by the rate of dissociation of α-ketoglutarate from a tight enzyme-NADPH-α-ketoglutarate complex (17). The linear dependence of the rate of α-ketoglutarate dissociation on D₂O concentration suggests the possibility that only a single proton is transferred during α-ketoglutarate dissociation (18). It has been pointed out, however, that in a complex system such as this, with a multiplicity of exchangeable hydrogens, an apparently linear dependence can be generated by a combination of secondary isotope effects (19). If we assume that only a single proton is involved, the isotope effect for α-ketoglutarate dissociation corresponds either to a primary deuterium kinetic isotope effect with a minimum value of 2.7 ± 0.3 or to a pKₐ shift of at least 0.4 for a group with a pKₐ above 10.
REFERENCES

1. Iwatsubo, M., and Pantaloni, D. (1967) Bull. Soc. Chim. Biol. 49, 1569-1572
2. Fisher, H. F., Bard, J. R., and Prough, R. A. (1970) Biochem. Biophys. Res. Commun. 41, 601-607
3. di Franco, A., and Iwatsubo, M. (1971) Biochimie 53, 153-159
4. di Franco, A., and Iwatsubo, M. (1972) Eur. J. Biochem. 30, 517-532
5. Colen, A. H., Prough, R. A., and Fisher, H. F. (1972) J. Biol. Chem. 247, 7905-7909
6. di Franco, A. (1974) Eur. J. Biochem. 45, 407-424
7. Fisher, H. F. (1973) Adv. Enzymol. 39, 369-417
8. Silverstein, E., and Sulebele, G. (1973) Biochemistry 12, 2164-2172
9. Kosicki, G. W., and Srere, P. A. (1961) J. Biol. Chem. 236, 2566-2570
10. Cross, D. G., and Fisher, H. F. (1970) J. Biol. Chem. 245, 2612-2621
11. Glasoe, P. K., and Long, F. A. (1960) J. Phys. Chem. 64, 188-190
12. Colen, A. H., Cross, D. G., and Fisher, H. F. (1974) Biochemistry 13, 2341-2347
13. Henderson, R. F., and Henderson, T. R. (1969) Arch. Biochem. Biophys., 129, 86-93
14. Iwatsubo, M., Lécuyer, B., Di Franco, A., and Pantaloni, D. (1966) C. R. Acad. Sci. (Paris) 263, 558-561
15. Pantaloni, D., and Lécuyer, B. (1973) Eur. J. Biochem. 40, 381-401
16. Laughton, P. M., and Robertson, R. E. (1969) in Solute-Solvent Interactions (Coetzee, J. F., and Ritchie, C. D., eds) pp. 399-538, Marcel Dekker, New York
17. Cross, D. G. (1972) J. Biol. Chem. 247, 784-789
18. Pollock, E., Hogg, J. L., and Schowen, R. L. (1973) J. Am. Chem. Soc. 95, 968-969
19. Kresge, A. J. (1973) J. Am. Chem. Soc. 95, 3065-3067
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J. Biol. Chem. 1975, 250:5243-5246.

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