Two-Step Binding of Adenosine Diphosphate to L-Glutamate Dehydrogenase. Effect on the Binding of Reduced Nicotinamide Adenine Diphosphate and on Enzymatic Catalysis†

Alan H. Colen, Dallas G. Cross, and Harvey F. Fisher*

ABSTRACT: Studies of the binding of NADPH and ADP to L-glutamate dehydrogenase by both difference spectroscopic and gel filtration methods demonstrate that a ternary complex can be formed with a negative heterotropic binding cooperativity of 6.8. The difference extinction coefficient for binding of the adenine chromophore of ADP is unaffected by the presence of bound NADPH, whereas the reduced nicotinamide difference extinction coefficient for NADPH binding is nearly doubled in the presence of bound ADP. Stopped-flow studies of ADP binding to glutamate dehydrogenase and observation of an order of addition effect of ADP on the time course of enzyme-catalyzed reactions reveal that ADP interaction with the dehydrogenase is a two-step process. A loose enzyme–ADP complex, catalytically identical with enzyme without ADP, is formed in a rapid bimolecular step. Following this step there is a relatively slow conversion to a tighter enzyme–ADP complex which shows altered catalytic efficiency. A ligand exclusion model involving competing isomerization of ADP and NADPH complexes on the enzyme surface is shown to fit the data. The demonstrated effect of ADP on NADPH binding is then related to the effect of ADP on the catalytic mechanism of glutamate dehydrogenase.

A tight complex is formed between 5′-adenosine diphosphate and L-glutamate dehydrogenase (Pantaloni and Iwatsubo, 1967; Pantaloni and Dessen, 1969; Cross and Fisher, 1970), raising the apparent dissociation constant for NADPH complexes (Pantaloni and Dessen, 1969; Cross and Fisher, 1970; Koberstein and Sund, 1973) and altering the catalytic efficiency of the enzyme (Frieden, 1959; Markau *et al.*, 1972).

At high coenzyme concentrations at any given pH, ADP activates the steady-state oxidative deamination of L-glutamate by the enzyme and $NADP^+$ (Frieden, 1959; Markau *et al.*, 1972). When concentration or pH is lowered sufficiently, ADP acts as an inhibitor (Markau *et al.*, 1972). Iwatsubo and Pantaloni (1967) have shown that ADP inhibits the rapid pre-steady-state (burst) phase of this reaction and have proposed that ADP activates the slower steady-state phase by facilitating the release of bound product NADPH. For the reverse reaction, in which α-ketoglutarate and ammonium are substrates, no measurable transient features have been reported and ADP appears simply to activate the steady-state catalysis (Frieden, 1959).

In this paper we study ADP and NADPH binding equilibria and the kinetics of ADP binding, demonstrate an order-of-addition effect of ADP on the time course of the catalytic reaction kinetics, and develop a model to explain the effects of ADP.

Experimental Procedure

Bovine liver L-glutamate dehydrogenase, NADPH, and 5′-ADP were obtained from Sigma. The ammonium sulfate suspension of the enzyme was extensively dialyzed against 0.1 M potassium phosphate buffer (pH 7.6), treated with approximately 5 mg/ml of Norit A, and filtered through a Millipore filter of 0.45-μ average pore diameter. This procedure resulted in an enzyme with a specific activity of 3.5 ± 0.5 mol of NAD^+ reduced per minute per milligram of enzyme in 0.2 M potassium phosphate buffer (pH 8.5) at 25° using 33 mM L-glutamate and 500 μM NAD^+. The ratio of 280- to 260-nm absorbance of the preparation was 1.92–1.96 indicating that 260-nm absorbing material, commonly found in glutamate dehydrogenase preparations, had been removed by the above treatment (Cross and Fisher, 1970). Enzyme concentrations were determined using 0.97 as the 280-nm extinction coefficient of a 1-mg/ml solution (Olson and Anfinsen, 1952). Coenzyme and nucleotide concentrations were determined using optical density measurements performed in a Zeiss PMQ II spectrophotometer.

Gel Filtration. The method of Hummel and Dreyer (1962) was used to determine the binding dependence of NADPH and ADP.

Three Sephadex G-25 medium columns, 0.4×110 cm, were enclosed within a single constant-temperature jacket thermostated at $20 \pm 0.2°$ with circulating water pumped from a Lauda K-2/R constant-temperature bath. The buffer for all binding studies was 0.1 M potassium phosphate (pH 7.6). Sample application, monitoring of the three effluent solutions using a Gilford Model 2000 multiple sample spectrophotometer equipped with offset control, blank compensator, and flow cells (0.2-cm path length), and the techniques associated with this equipment were similar to those described by Fairclough and Fruton (1966) with the following exception. The eluent was prepared at twice the concentration at which it was to be used and diluted 1:1 with buffer. The concentrated eluent was also diluted 1:1 with enzyme solution to prepare the sample for application. Sample sizes were from 0.15 to 0.30 ml containing 1.3–2.1 mg of enzyme. Fow rates were from 0.13 to 0.20 ml/min as measured using volumetric flasks and a stopwatch.

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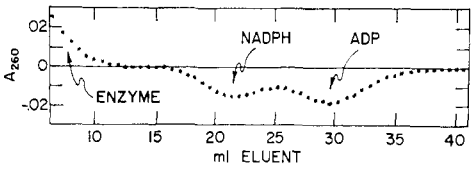


FIGURE 1: Elution profile measured at 260 nm showing troughs representing the amount of NADPH and ADP bound to glutamate dehydrogenase. Sample was 1.38 mg of glutamate dehydrogenase and eluent contained 200 μM NADPH and 100 μM ADP. The two troughs represent the removal from solution of 0.85 mol of ADP and 0.78 mol of NADPH per mol of glutamate dehydrogenase as determined by the two methods described in the text.

Only the trough portion of the elution profiles was used for determination of the amount of ligand bound to glutamate dehydrogenase. The areas of these troughs were determined by weighing tracings of the troughs on paper. The amount of each ligand bound was determined by two methods. First, the binding of NADPH in the presence of ADP was determined by monitoring the 340-nm absorbance of NADPH, where ADP has little significant absorbance. This value was used to calculate the amount of contribution of NADPH binding to the trough measured at 260 nm, where both ligands absorb. This value permitted the determination of the amount of ADP removed from solution by glutamate dehydrogenase. Second, an elution profile measured at 260 nm showed two troughs (Figure 1) which we determined spectrophotometrically to represent the amount of NADPH and ADP bound to the enzyme. The resolution of these two troughs, using a DuPont Model 310 curve resolver and an elution profile for the binding of a single ligand as a model, gave results which agreed within experimental error with the values determined using the first method. Since the second method requires fewer measurements, it was used for the data given herein.

Difference Spectra. All difference spectral experiments were performed using 0.1 M potassium phosphate buffer (pH 7.6) in 1.000-cm cells thermostated to 20°. A Cary Model 14 double-beam spectrophotometer interfaced to a Varian 620i computer was used to collect and average all spectral data. To generate difference spectra, four cells, arranged in tandem in the sample and reference compartments, were used as previously described (Cross and Fisher, 1970). The spectral data were collected at 1-nm intervals with averages over a 0.4-nm range for each datum. In addition, from three to five difference spectra were averaged for each final difference spectrum.

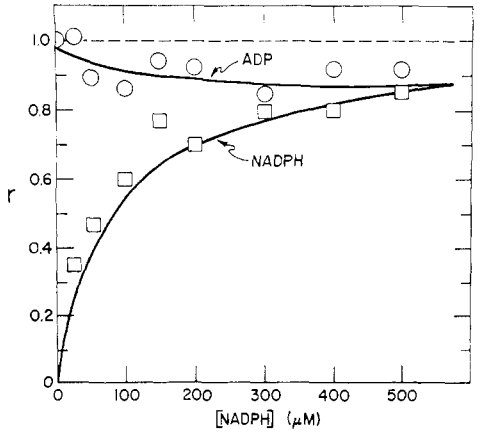


FIGURE 2: Dependence of ADP and NADPH binding on NADPH concentration determined by resolution of data such as those shown in Figure 1. The number of moles of either ligand bound per mole of glutamate dehydrogenase (mol wt 56,100) is expressed as r. The curves drawn through the data were calculated from the dissociation constants determined later in the text.

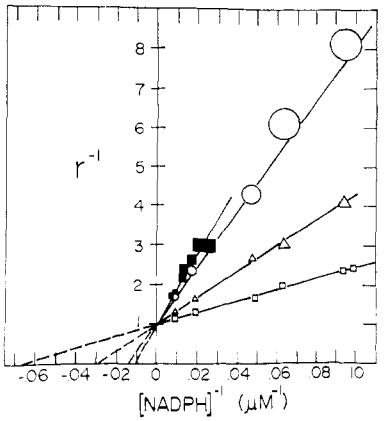


FIGURE 3: Concentration dependence of the binding of NADPH to glutamate dehydrogenase in the presence of 0 (□), 5.0 (△), 42.5 (○), and 100 (■) μM ADP. The number of moles of NADPH bound per mole of glutamate dehydrogenase (r) was measured using the gel filtration method in which the effluent was monitored at 340 nm.

Stopped Flow. Transient-state experiments were carried out at 25° on a Durrum Gibson stopped-flow spectrophotometer interfaced to a Varian 620i digital computer as described previously (Colen *et al.*, 1972). The light source was a 75-W xenon arc and the slit width was 0.3 mm. Two cuvet path lengths were used, 1.71 and 0.54 cm, in order to keep the solution absorbance in a range in which the instrument obeys Beer's law. The results obtained with the two cuvets agreed well within experimental error in overlapping concentration ranges. Both absorbance and wavelength scales were calibrated against standards. Absorbance readings agreed with those obtained on a Zeiss PMQ II spectrophotometer within 2% in the experimental absorbance range. The wavelength could be set within 1 nm for several standards in the experimental wavelength range. An error of 1 nm at 253 nm would result in at most a 4% error in the measurement of absorbance differences in our experiments. All absorbance changes we report are calculated for a 1-cm optical path length.

The problem of protein light scatter change on dilution (Fisher and Bard, 1969; Colen *et al.*, 1972) was handled with equal success in two ways. Using ratio mixing syringes (nominally either 14:1 or 7:1) protein dilution could be minimized and no corrections were required. The syringes were calibrated with solutions of known optical density. Alternately, by using the standard 1:1 syringes, a protein dilution base line could be obtained and subtracted digitally from the data. The methods yielded identical results. Each data point reported in this paper is an average of at least five stopped-flow experiments.

Results

NADPH and ADP Binding Studies. Figure 2 shows the gel filtration results for the binding of NADPH to glutamate dehydrogenase in the presence of 100 μM ADP. These data show that at 500 μM NADPH there are 0.92 ± 0.2 mol of ADP and 0.87 ± 0.2 mol of NADPH bound to the enzyme in a ternary complex. In addition, the binding of NADPH to glutamate dehydrogenase both in the absence and in the presence of 100 μM ADP was measured using the red shift of the reduced nicotinamide chromophore characterized in difference spectra as a maximum at 383 nm and a minimum

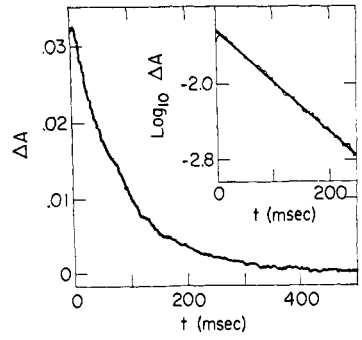


FIGURE 4: Plot of the absorbance change at 253 nm against time. In the stopped-flow experiment glutamate dehydrogenase was mixed with ADP to give final concentrations of 16.6 μM glutamate dehydrogenase and 43.1 μM ADP. The inset shows a first-order plot of the same data. In both plots, $\Delta A = A_t - A_\infty$ (defined in the text).

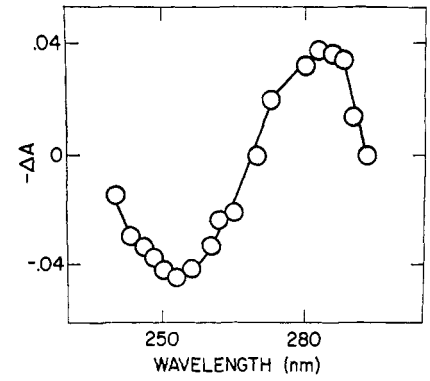


FIGURE 5: Spectrum of the signal amplitudes (see text) obtained from stopped-flow studies of ADP binding to glutamate dehydrogenase. Final concentrations after mixing were 17.8 μM glutamate dehydrogenase and 42 μM ADP.

at 333 nm (Fisher and Cross, 1966). The data were plotted in a double reciprocal plot which showed a dissociation constant increase for NADPH binding in the presence of ADP and an increase in the differential extinction coefficient of coenzyme binding from $\Delta\epsilon = 1400 \pm 100$ M^{-1} cm^{-1} in the binary complex to $\Delta\epsilon = 2300 \pm 200$ M^{-1} cm^{-1} in the ternary complex.

The binding of ADP to glutamate dehydrogenase in the absence of NADPH was also measured using the gel filtration method by monitoring the column effluent at 260 nm. These data, plotted in a double reciprocal plot, show that 1 mol of ADP binds to 1 mol of glutamate dehydrogenase (mol wt 56,100) with a binary dissociation constant of 2.67 $\pm$ 0.30 μM. Spectrophotometric titrations with ADP at saturating concentrations of NADPH were performed in the presence of 140 mM L-glutamate. The dissociation constant for NADPH from the ternary enzyme–NADPH–L-glutamate complex thus formed (Winer and Schwert, 1958; Fisher and McGregor, 1960; Cross and Fisher, 1970) is sufficiently low that the enzyme can be saturated with coenzyme at optical densities which still permit measurement of the ADP binding difference spectrum. If L-glutamate were not present, solutions with saturating coenzyme concentrations would possess prohibitively high optical densities. In the presence of L-glutamate, difference spectra resulting from the titration of the abortive enzyme–NADPH–L-glutamate complex with ADP showed little change in the 340-nm region, indicating that during the titration there was at least 95% saturation of the enzyme by NADPH. ADP binds to glutamate dehydrogenase exhibiting a difference spectrum which results from a red shift of the adenine chromophore (Cross and Fisher, 1970). The dependence of the 283-nm peak to 253-nm trough measurement of this difference spectrum on ADP concentration in the absence and presence of NADPH and L-glutamate showed that the ADP dissociation constant is increased in the presence of tightly bound NADPH and that the difference extinction coefficient remains significantly unchanged ($\Delta\epsilon = 6000 \pm 200$ M^{-1} cm^{-1}).

In order to evaluate the constants involved in glutamate dehydrogenase–ADP–NADPH ternary complex formation, the gel filtration technique was used to determine the concentration dependence of NADPH binding at several ADP concentrations. The data obtained by monitoring the absorbance of the column effluent at 340 nm are shown in the double reciprocal plot in Figure 3. The negative intercept on the abscissa for the data in the absence of ADP gives a glutamate dehydrogenase–NADPH binary dissociation constant of 14.9 $\pm$ 0.4 μM.

Kinetics of ADP Binding. Figure 4 shows a stopped-flow trace obtained at 253 nm for the binding of ADP to glutamate dehydrogenase. The curve is first order and yields two measurable parameters: the signal amplitude (defined as the absorbance at time t, A_t, minus the absorbance at infinite time, A_∞, extrapolated to time zero in a plot of log $(A_t - A_\infty)$ *vs.* t) and the apparent first-order rate constant obtained from the slope of the log plot.

A spectrum of signal amplitudes is given in Figure 5. Its major component is an adenine red shift and it corresponds very closely in shape to the difference spectrum obtained in static binding experiments (Cross and Fisher, 1970).

In Figure 6 the ADP concentration dependence of the signal amplitude at 253 nm is compared with the full binding signal amplitude at 253 nm. The full amplitude curve is computed from the enzyme–ADP dissociation constant and the differential extinction coefficient at 253 nm for ADP binding from the static experiments reported above ($\Delta\epsilon_{253} = 3240 \pm 100$ M^{-1} cm^{-1}). The kinetic signal amplitudes fall significantly below the full amplitude curve (the amplitudes measured possess at most a 6% uncertainty as noted in the Experimental Procedure section). Thus, it may be concluded that we are not able to record at least one additional signal-producing process in the binding kinetics. This process must be too rapid to be observable in stopped-flow experiments since no slower signal producing processes are seen in our experiments.

The concentration dependence of the apparent first-order rate constant shown in Figure 7 indicates that we are actually observing an isomerization of an enzyme–ADP complex.

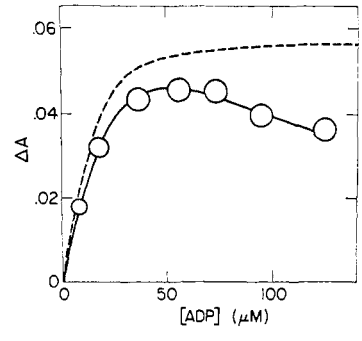


FIGURE 6: ADP concentration dependence of the stopped-flow signal amplitude at 253 nm. The binding experiments were performed with 17.8 μM glutamate dehydrogenase. The dashed line represents the signal amplitude for the 253-nm spectral feature which would be predicted on the basis of equilibrium binding experiments.

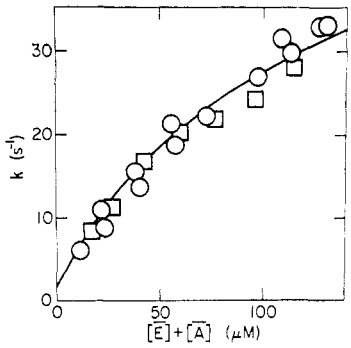


FIGURE 7: Dependence of apparent first-order rate constant for ADP binding (k) on the sum of the final equilibrium concentrations of free enzyme ([Ē]) and ADP ([Ā]). Final total enzyme concentrations after mixing: (○) 17.8 μM; (□) 36.9 μM. The solid line is a theoretical curve plotted according to eq 4.

Such a dependence is typical of an isomerization step which follows a rapid binding step which, in this case, is too fast to be observed. Isomerization of the enzyme prior to ADP binding will not of itself produce the observed concentration dependence. (The theoretical curve in Figure 7 is fitted to the data for the case in which the dissociation constant for the rapidly formed enzyme–ADP complex is 90 μM and the overall enzyme–ADP complex dissociation constant is 2.67 μM, obtained from the static binding experiments. The equation used for the fit will be presented in the Discussion.)

Raising the pH from 7.6 to 9.3 lowers the signal amplitude observed in ADP binding kinetics but has no measurable effect on the apparent first-order rate constant. Such an effect would be produced if the initial rapid binding is pH dependent in this range and the isomerization is not.

Effect of ADP on the Catalytic Reaction Kinetics. Figure 8 shows results obtained at 340 nm for the catalytic reaction kinetics. Each set of three plots follows the course of the reaction for a single set of enzyme, substrate, and coenzyme concentrations, but for a different mode of addition of ADP to the reaction mixture. The solid lines represent the time course of the reaction in the absence of ADP, the dashed lines represent experiments in which ADP was preincubated with enzyme before the start of the experiment, and the dotted lines represent cases in which ADP was added at the same time as coenzyme and substrate.

When ADP is preincubated with the enzyme in the oxidative deamination of L-glutamate (the forward reaction) at pH 7.6, inhibition of the burst phase is readily discerned in addition to steady-state activation. At 50 μM ADP (Figure 8a) there is almost no inhibition of the burst when ADP, coenzyme, and substrate are mixed simultaneously with the enzyme, implying that the burst is complete before a substantial inhibitory effect is produced. The same experiment carried out with 500 μM ADP (Figure 8b) showed only a small order of addition effect, indicating that the inhibitory effect of ADP is produced rapidly enough to influence the burst kinetics, even when ADP is not preincubated with the enzyme. At pH 8.7 and 50 μM ADP (Figure 8c) the order of addition effect on the activation of the forward reaction can clearly be observed since almost no inhibition of the burst is observed even when ADP is preincubated with the enzyme. Figure 8d shows a similar effect for the reverse catalytic reaction at pH 7.6, implying that the relatively slow onset of ADP effects has introduced an observable transient feature into the progress curve for the reverse reaction.

In each case in which ADP is not preincubated with the enzyme, its effects on the catalytic reaction kinetics build up slowly in times commensurate with the relaxation times for isomerization measured in ADP binding experiments. These results suggest that isomerization of the enzyme–ADP complex is necessary for full realization of the ADP effects on the catalytic kinetics.

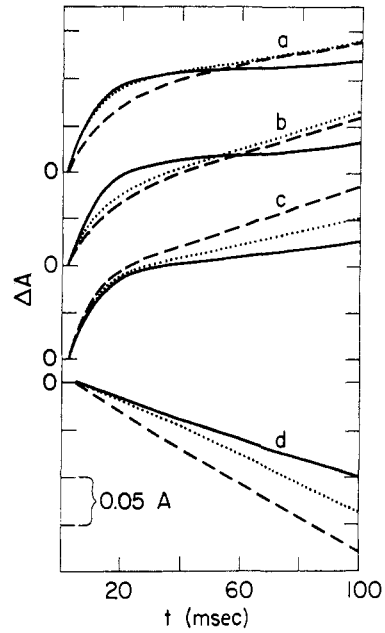


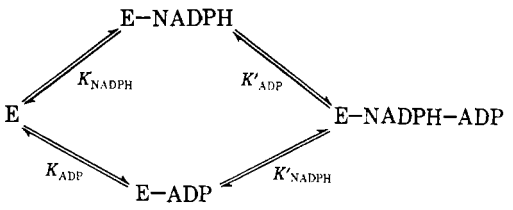
FIGURE 8: The effect of the order of addition of ADP on the catalytic reaction kinetics as observed at 340 nm. The conditions are: no ADP (—); enzyme preincubated with ADP (- - -); substrates, coenzyme, and ADP added to the enzyme simultaneously (· · ·). For the forward reaction, concentrations after mixing were: (a) pH 7.6, 50 μM ADP; (b) pH 7.6, 500 μM ADP; (c) pH 8.7, 50 μM ADP; (a,b,c) 500 μM NADP, 50 mM L-glutamate. For the reverse reaction concentrations were: (d) pH 7.6, 50 μM ADP, 37.5 μM NADPH, 1.97 mM α-ketoglutarate, 30 mM ammonium chloride. Glutamate dehydrogenase was 17.9 μM in all experiments.

Discussion

ADP and NADPH Interaction. The demonstration (Figure 2) that one molecule of ADP and one molecule of NADPH can be bound to glutamate dehydrogenase in a ternary complex eliminates a mechanism of negative interaction between these two ligands on the enzyme which is a result of the total displacement of one ligand by the other to any stoichiometric fraction of the total number of binding sites.

The dependence of the reduced nicotinamide difference spectrum on NADPH concentration in the presence of enzyme saturated with ADP showed, in addition to an increased dissociation constant for NADPH, a difference extinction coefficient for NADPH binding somewhat larger than that found for the binary complex (2300 *vs.* 1400 M^{-1} cm^{-1}). This extinction coefficient increase is consistent with a decrease in the polarizability of the environment around the reduced nicotinamide moiety of bound NADPH in the presence of bound ADP. Similar spectral phenomena have been shown to result from changes in the conformation of NADPH in solution in which the environment of the nicotinamide moiety was altered (Cross and Fisher, 1969). If ADP and NADPH were to interact as previously suggested (Cross and Fisher, 1970) through a competition of ADP for a subsite which also binds the reduced nicotinamide moiety of NADPH, a decrease rather than the observed increase in the difference extinction coefficient would result.

SCHEME I



The dependence of the ADP binding difference spectrum on ADP concentration in the presence of NADPH bound to the enzyme in an abortive complex showed that the apparent difference extinction coefficient for ADP binding remains unchanged from that found for the ADP-glutamate dehydrogenase binary complex. The implication is that the polarizability of the immediate environment and the orientation of the adenine chromophore are the same in the presence and absence of bound NADPH. This result further indicates that the environmental changes observed when NADPH binds to the enzyme–ADP complex are not a result of a direct interaction between the reduced nicotinamide moiety of the coenzyme and the adenine moiety of ADP.

In order to characterize fully the negative heterotropic cooperativity of NADPH and ADP binding to glutamate dehydrogenase, the constants describing the dissociation of the two ligands from the ternary complex were evaluated using the minimum assumption of four demonstrable enzyme species in solution at equilibrium as shown in Scheme I. The K's in Scheme I are apparent dissociation constants from binary and ternary complexes. Since the gel filtration method indicates the total number of ligand molecules bound, the following equation can be derived from the general binding expression

$$\frac{E_t}{N} = 1 + \frac{K_{NADPH}}{[NADPH]} \frac{(1 + [ADP]/K_{ADP})}{(1 + [ADP]/K'_{ADP})} \quad (1)$$

where N = total moles of bound NADPH and E_t = moles of glutamate dehydrogenase (mol wt 56,100). The data in Figure 3, plotted as E_t/N *vs.* 1/[NADPH], yield linear plots with an intercept of one at the ordinate. According to eq 1 the slopes of these plots are given by

$$m = K_{NADPH} \frac{(1 + [ADP]/K_{ADP})}{(1 + [ADP]/K'_{ADP})} \quad (2)$$

Using the values of the slopes in Figure 3 plus the values of K_{NADPH} and K_{ADP} obtained independently, K'_{ADP} was evaluated from the negative intercept on the abscissa in Figure 9 and found to be 18.3 μM. Knowing K_{NADPH} = 14.9 μM and K_{ADP} = 2.67 μM and the identity, $K_{NADPH}\, K'_{ADP} = K_{ADP} \cdot K'_{NADPH}$, K'_{NADPH} was determined to be 102 μM. The ratio of ternary to binary dissociation constants is 6.8 and is a measure of the negative heterotropic cooperativity of binding between ADP and NADPH on glutamate dehydrogenase.

ADP Binding Mechanism. Our data support a simple phenomenological mechanism for ADP binding in which a rapid binding step to form a loose complex, EA, is followed by a slower isomerization to form the tight complex, EA′

$$\begin{aligned} &E + A \underset{K_A}{\rightleftharpoons} EA \qquad \text{fast step} \\ &EA \underset{k'_{-A}}{\overset{k'_A}{\rightleftharpoons}} EA' \qquad \text{slow step} \end{aligned} \quad (3)$$

The slow isomerization step is the one which we observe in stopped-flow experiments. The concentration dependence of the apparent first-order rate constant for the isomerization step in this mechanism (eq 3) is given by

$$k_{app} = \frac{1}{\tau} = k'_{-A} + k'_A \frac{[\bar{E}] + [\bar{A}]}{K_A + [\bar{E}] + [\bar{A}]} \quad (4)$$

where τ is the observed relaxation time, K_A is the dissociation constant for the rapidly formed loose complex, k'_A and k'_{-A} are the forward and reverse rate constants for the isomerization step, and $[\bar{E}]$ and $[\bar{A}]$ are the final equilibrium values of the enzyme and ADP concentrations. The constants in this equation are related to the apparent dissociation constant measured in static binding studies as shown in eq 5, where

$$K_{ADP} = K_A/(1 + K'_A) \quad (5)$$

$K'_A = k'_A/k'_{-A} = [\overline{EA'}]/[\overline{EA}]$, the ratio of the isomerized enzyme–ADP complex to the loose complex in the final equilibrium mixture.

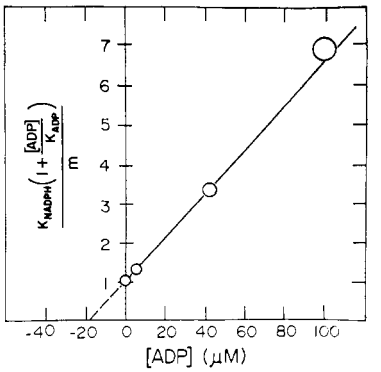


FIGURE 9: Graphical determination of K'_{ADP}. See eq 2 in the Discussion.

In order to obtain the theoretical curve in Figure 7, values of K_A, k'_A, and k'_{-A} were chosen for eq 4 which were consistent both with the constraint imposed by eq 5 and the experimental data. These values are: K_A = 90 ± 20 μM, k'_A = 50 ± 10 sec^{-1}, and k'_{-A} = 1.5 ± 0.2 sec^{-1}. Thus, the isomerization constant for the enzyme–ADP complex, K'_A, is 33 ± 7, indicating that the isomerized complex, EA′, is considerably more stable than the looser complex, EA.

This mechanism provides not only a fit for the stopped-flow ADP binding data, but also a simple interpretation in terms of ADP binding for the glutamate dehydrogenase–NADPH–ADP binding experiments and for the observed slow onset of ADP effects on the enzyme-catalyzed reactions. The shape and amplitude of the spectrum obtained from ADP binding kinetics show that both EA and EA′ possess red-shifted adenine spectra. If the effect of NADPH binding is to reduce the fraction of enzyme–ADP complex in the tighter, isomerized form, we will observe a netative heterotropic binding cooperativity for the two ligands, as reported above. The fact that the difference extinction coefficient for ADP binding remains unchanged in the presence of NADPH implies, for this mechanism, that EA and EA′ have identical difference extinction coefficients for ADP binding.

Although these two ADP complexes, which are formed at different rates, are spectrally indistinguishable, they differ significantly in their effects on the catalytic reaction. The complex, EA, forms during the apparatus dead time (the first 3 msec) in stopped-flow experiments, yet its formation has no observable effect on the catalytic reaction kinetics. It is only when significant quantities of the tighter EA′ complex are formed that inhibition of the burst phase of the forward reaction and activation of the steady-state phases of the for-

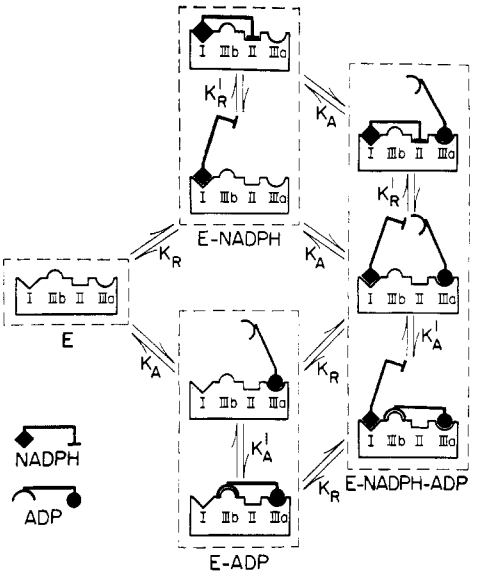


FIGURE 10: Ligand exclusion model of NADPH and ADP binding to glutamate dehydrogenase. The diamond represents the reduced nicotinamide moiety of NADPH and the circle represents the adenine moiety of ADP. K_R and K_A are the microscopic dissociation constants for binding in the monodentate mode signaled by chromophore perturbation. K'_R and K'_A are microscopic equilibrium constants for the mutually exclusive isomerization steps for each ligand, going from monodentate to tighter bidentate interaction. The dashed boxes enclose species of the same binding stoichiometry, corresponding to the species shown in Scheme I used to analyze the gel filtration binding data.

ward and reverse enzyme-catalyzed reactions are observed. It may be concluded, therefore, that only EA′ possesses altered catalytic efficiency, whereas EA is catalytically equivalent to enzyme in the absence of ADP.

We conclude that an interaction of the adenine moiety of ADP with enzyme, producing a red shift of adenine absorbance, is simply an index of ADP binding but bears no direct relationship to ADP's action as an allosteric effector. It is the additional interaction achieved through isomerization of the enzyme–ADP complex which is of catalytic importance.

Ligand Exclusion Model. The simplest model of NADPH and ADP interactions which will account quantitatively for the results presented here requires not only the isomerization of the enzyme–ADP complex discussed above, but an isomerization of an enzyme–NADPH complex as well.[1] Figure 10 shows a two-dimensional schematic ligand exclusion model (Fisher *et al.*, 1970) of ADP and NADPH binding to glutamate dehydrogenase. Each ligand can interact in either a monodentate or a bidentate manner with two subsites on the enzyme surface, but the geometrical and chemical connectivity of these subsites is such that both ligands cannot bind in a bidentate manner simultaneously.

Binding of NADPH at subsite I is signaled by a red shift of the reduced nicotinamide spectrum and binding of ADP at subsite IIIa is accompanied by a red shift of the adenine spectrum. Binding of NADPH at subsite II has no effect on the spectrum of adenine bound at subsite IIIa, but binding at subsite II excludes ADP binding at subsite IIIb, presumably by blocking interaction of the enzyme with some portion of the ribose phosphate moiety of ADP. Conversely, when ADP is bound to subsite IIIb, it prevents NADPH interaction with subsite II without any interference with subsite I, but the reduced nicotinamide binding signal is somehow enhanced, either through a medium effect in the presence of the ribose phosphate of ADP or simply because NADPH binding in the monodentate mode possesses a larger difference extinction coefficient (possibly stronger chromophore perturbation). Of course, there is always the possibility that these effects are mediated through an enzyme conformation change.

The microscopic constants noted in Figure 10 are unaffected by binding of the second ligand, with one exception. Bidentate binding of one ligand renders isomerization to a tighter bidentate complex impossible for the second ligand, essentially reducing the second ligand's isomerization constant to zero. The negative heterotropic cooperativity computed earlier is given in terms of the microscopic isomerization constants by eq 6. We have already obtained K_A and K'_A

$$C = \frac{K'_{NADPH}}{K_{NADPH}} = \frac{K'_{ADP}}{K_{ADP}} = \frac{(1 + K'_A)(1 + K'_R)}{1 + K'_A + K'_R} \quad (6)$$

from our fit of the ADP binding rate constants. Employing the relationship

$$K_{NADPH} = \frac{K_R}{1 + K'_R} \quad (7)$$

plus eq 6 above, we obtain $K_R = 108\ \mu M$ and $K'_R = 7.2$. Nearly identical constants have been obtained elsewhere for a postulated two-step binding of NADPH in the presence of GTP (Huang and Frieden, 1972). It is interesting to note that, in this model, since the isomerization constant for ADP ($K'_A = 33$) is much larger than that for NADPH, the observed cooperativity is essentially determined by the NADPH isomerization.

Mechanism of ADP Effects on the Catalytic Reaction. The action of ADP in the glutamate dehydrogenase system can now be explained in terms of its effect on coenzyme binding. In the steady-state phase of the oxidative deamination of L-glutamate, where formation of tight enzyme–NADPH complexes appears to slow the course of the catalytic reaction, ADP speeds up the reaction by loosening these tight complexes through an isomerization step. The same mechanism may operate in the reverse catalytic reaction if the tight complexes differ significantly from the enzymatic transition state.

At chosen conditions of pH and coenzyme concentration, ADP can act either as an activator or as an inhibitor of the steady-state kinetics for the forward reaction. The recent findings by Markau *et al.* (1972) of steady-state inhibition of the forward reaction by ADP can readily be explained by our results. At lower pH inhibition of the burst by ADP is pronounced. When coenzyme concentration is sufficiently low to lower the rate of the burst substantially and render the steps associated with the burst rate limiting, then ADP will inhibit the glutamate dehydrogenase reaction even in steady-state experiments. At higher coenzyme concentrations, however, release of product NADPH from tight enzyme–NADPH complexes becomes the rate-limiting factor and ADP acts as an activator. The inhibitory effect of ADP on the burst kinetics is diminished at higher pH. Thus, at higher pH, inhibition of the steady-state rate by ADP is achieved by lowering the concentration of coenzyme such that inhibition of the burst over-

[1] As noted earlier in the Results section, isomerization of the enzyme alone would not produce the observed concentration dependence for the apparent first-order rate constant for ADP binding. In addition, if the isomerization of the E–ADP complex were solely responsible for the negative heterotropic binding cooperativity of ADP and NADPH, we would observe a value of 33, not 6.8 for that cooperativity. We conclude, therefore, that there must be two forms of NADPH–enzyme complex which differ in stability by a factor of approximately seven.

rides the activation of the later phase of the catalytic reaction. It will then appear that progressively lower concentration is required to produce inhibition as the pH is raised.

Thus, the mechanism we propose for the effects of ADP on the rapid and steady-state kinetics of glutamate dehydrogenase is sufficient to explain the variety of effects reported here and in the literature using only direct interactions between ADP and other ligands on the enzyme surface.

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The Role of the Internal Cross-Link in Oxytocin. Preparation of Mercury Mercaptide Oxytocin Derivatives†

Ruth Sperling* and Marian Gorecki

ABSTRACT: The disulfide bond of oxytocin was reduced using an insoluble reducing reagent, dihydrolipoyl attached to polyacrylamide P-6. The sulfhydryl groups thus formed were bridged by bivalent mercury ion with the formation of an S–Hg–S bridge. The monomeric product obtained was shown to be homogeneous and contained one mercury atom per peptide molecule. Further addition of mercury ions led to the formation of a dimercurated open-chain derivative. The mercuration had a marked effect on the ultraviolet and circular dichroic spectral features of these derivatives in comparison with oxytocin. The tryosine's fluorescence intensity of mono- and dimercurated oxytocin derivatives was quenched in comparison with that of oxytocin and oxytoceine, respectively. The mercury–oxytocin derivatives were found to have negligible biological activity as muscular-contracting agents of isolated rat uteri. Elongation of the internal cross-link of oxytocin by 3 Å, thus dramatically affects the biological activity of the hormone, which is intimately connected with the native conformation of the peptide.

Internal disulfide cross-links play a decisive role in maintaining the three-dimensional conformation of proteins which possess such linkages (Anfinsen, 1965–1966). Yet not all the disulfide bonds are crucial for the stabilization of the biologically active conformation (Azari, 1966; Kress and Laskowski, 1967; Neumann *et al.*, 1967; Sperling *et al.*, 1969; Arnon and Shapira, 1969). It would be interesting, therefore, to know what demands there are on the length and the geometry of the internal cross-links which are essential for maintaining the biological active conformation of proteins.

Introduction of a mercury atom between the sulfur atoms of a disulfide cross-link results in the formation of an S–Hg–S bridge known to be linear (Grdenic, 1965). This insertion of mercury lengthened the disulfide bridge by about 3 Å (Yakel and Hughes, 1954; Pauling, 1960; Bradley and Kunchur, 1965). This approach was successfully applied to various proteins where a single disulfide bond was modified. Thus, insertion of mercury into the IV–V bond of RNase (Sperling *et al.*, 1969), 43–152 bond of papain (Arnon and Shapira, 1969), and A6–A11 bond of insulin (Sperling and Steinberg, 1974) did not affect the biological properties of these proteins. Elongation of all the disulfide bridges of RNase resulted in a marked decrease in the biological activity (Sperling and Steinberg, 1971).

In the small hormonal cyclic peptides, oxytocin and vaso-

† From the Department of Chemical Physics (R. S.) and the Department of Chemistry (M. G.), The Weizmann Institute of Science, Rehovot, Israel. *Received October 17, 1973.* This research was supported in part by Grant AM05098 from the National Institutes of Health, U. S. Public Health Services.