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Formation of Transient Complexes in the Glutamate Dehydrogenase Catalyzed Reaction†

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ABSTRACT: The reaction of glutamate dehydrogenase and glutamate (gl) with NAD^+ and $NADP^+$ has been studied with stopped-flow techniques. The enzyme was in all experiments present in excess of the coenzyme. The results indicate that the ternary complex (E·NAD(P)H·kg) is present as an intermediate in the formation of the stable complex (E·NAD(P)H·gl). The identification of the complexes is based on their absorption spectra. The binding of the coenzyme to (E·gl) is the rate-limiting step in the formation of (E·NAD(P)H·kg) while the dissociation of α-ketoglutarate (kg) from this complex is the rate-limiting step in the formation of (E·NAD(P)H·gl). The K_m for glutamate was 20–25 m*M* in the first reaction and 3 m*M* in the formation of the stable complex. The K_m values were independent of the coenzyme. The reaction rates with NAD^+ were approximately 50% greater than those with $NADP^+$. Furthermore, high glutamate concentration inhibited the formation of (E·NADH·kg) while no substrate inhibition was found with $NADP^+$ as coenzyme. ADP enhanced while GTP reduced the rate of (E·NAD(P)H·gl) formation. The rate of formation of (E·NAD(P)H·kg) was inhibited by ADP, while it increased at high glutamate concentration when small amounts of GTP were added. The results show that the higher activity found with NAD^+ compared to $NADP^+$ under steady-state assay conditions do not necessarily involve binding of NAD^+ to the ADP activating site of the enzyme. Moreover, the substrate inhibition found at high glutamate concentration under steady-state assay condition is not due to the formation of (E·NAD(P)H·gl) as this complex is formed with K_m of 3 m*M* glutamate, and the substrate inhibition is only significant at 20–30 times this concentration.

Glutamate dehydrogenase (L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating) EC 1.4.1.3) from beef liver is one of the most extensively studied enzymes. It is an allosteric enzyme consisting of six identical protomers (Appella and Tomkins, 1966). The enzyme is activated by ADP and inhibited by GTP when glutamate is used as substrate. Since many of the complexes between glutamate dehydrogenase and its substrates and coenzymes possess characteristic spectra (Fisher, 1973), the enzyme is particularly well suited for fast kinetic studies. Iwatsubo and coworkers (Iwatsubo and Pantaloni, 1967; diFranco and Iwatsubo, 1972; diFranco, 1974) followed the spectral changes during the initial phase of the oxidative deamination of glutamate and suggested the following reaction scheme:

$$E + NAD(P)^+ + gl \rightleftharpoons (E\cdot NAD(P)\cdot gl) \rightleftharpoons (E\cdot NAD(P)H\cdot kg) \rightleftharpoons (E\cdot NAD(P)H) + kg$$

$$(E\cdot NAD(P)H) + gl \rightleftharpoons (E\cdot NAD(P)H\cdot gl) \rightleftharpoons (E\cdot gl) + NAD(P)H$$

E is enzyme and gl and kg are glutamate and α-ketoglutarate, respectively (for simplification the release of NH_3 and H^+ in the reaction sequence will not be discussed in the present paper).

The release of the reduced coenzyme from (E·NAD(P)H·gl) is assumed to be the rate-limiting step under normal assay conditions (Engel and Dalziel, 1969; D'Albis and Pantaloni, 1972; Shafer et al., 1972; diFranco 1974), while at low glutamate concentration the release of α-ketoglutarate from the ternary complex (E·NAD(P)H·kg) is the rate-limiting step (diFranco, 1974). Steady-state kinetics indicate that the enzyme binds its coenzyme and glutamate in a random manner (Engel and Dalziel, 1969, 1970; Silverstein and Sulebele, 1973). There are two binding sites for NAD^+ and $NADP^+$ per protomer, one "active site" and one "nonactive site" (Krause et al., 1974). The "nonactive site" is the same as the ADP activating site (Fisher, 1973). The "active site" binds the two coenzymes in the same manner, while the affinity of the "nonactive site" for NADPH is 10 times lower than for NADH (Krause et al., 1974). The latter finding explains why the previous authors have found only one binding site for $NADP^+$ (Frieden, 1959; Pantaloni and Dessen, 1969; Cross and Fisher, 1970). The higher activity observed with NAD^+ compared to $NADP^+$ has in part been explained by assuming that the second NAD^+ molecule activates the enzyme by binding to the ADP activating site (Pantaloni and Dessen, 1969; Cross and Fisher, 1970, Fisher, 1973; diFranco, 1974).

Recently, Shafer et al. (1972) studied the oxidative deamination of glutamate with enzyme present in excess of the coenzyme. Since the affinity of the "active site" for the

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two coenzymes is several times greater than the affinity of the "nonactive site" for the coenzymes (Krause et al., 1974), the coenzymes will under such conditions only interact with the "active site". Moreover, only a fraction of the enzyme molecules will be involved in the enzymatic reaction and the enzyme molecules participating will only catalyze one cycle of the reaction. Upon mixing of the reactants the absorption at 310 nm showed a rapid increase followed by a more slow decrease. These results were interpreted in accordance with the above reaction sequence and it was assumed that the rapid increase in absorption was caused by formation of the ternary complex (E·NADH·kg), while the decrease in absorption was due to formation of (E·NADH·gl).

In the present paper we have studied in further detail the oxidative deamination of glutamate with the enzyme in excess of the coenzyme. Furthermore, the difference between the two coenzymes in the reaction as well as the effect of the allosteric effector molecules GTP and ADP on the reaction rates have been investigated.

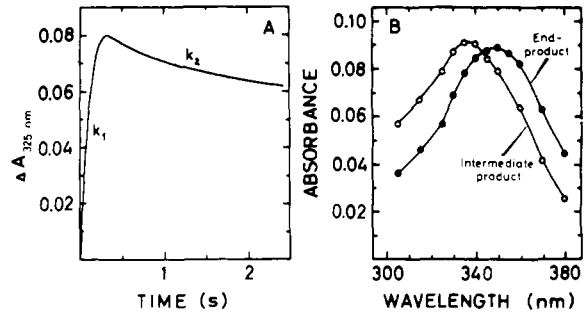

FIGURE 1: Change in absorption after mixing of glutamate dehydrogenase with glutamate and NAD^+. (A) Effect of time on the absorption at 325 nm. (B) Absorption spectra of the rapidly formed intermediate and the final product. The time curves obtained were decomposed as described in the text. The experiments were carried out with stopped-flow spectroscopy. The final concentrations were: 50 μM enzyme, 100 mM glutamate, and 8.5 μM NAD^+ in 0.05 M phosphate buffer (pH 7.6) containing 0.1 mM EDTA. Glutamate was preincubated with the enzyme.

Experimental Procedures

Enzyme and Coenzymes. Glutamate dehydrogenase was dialyzed overnight at 4°C against 0.05 M phosphate buffer (pH 7.6) containing 0.1 mM EDTA. The concentration of the enzyme solution was determined from its absorbance at 279 nm using a value of 0.97 $cm^2\ mg^{-1}$ for the absorption coefficient (Olson and Anfinsen, 1952) and a molecular weight of 56000 per protomer (Smith et al., 1970). The NAD^+ and $NADP^+$ concentrations were determined from the absorbance at 260 nm using 18.0 $cm^2\ \mu mol^{-1}$ as the molar absorption coefficient. The NADH concentration was determined from the absorption at 340 nm using 6.2 $cm^2\ \mu mol^{-1}$.

Stopped-Flow Experiments. The presteady state kinetic experiments were carried out with a Durrum stopped-flow spectrophotometer, Model 110, fitted with a 20-mm cuvet. All experiments were performed at 20°C in 0.05 M phosphate buffer (pH 7.6) containing 0.1 mM EDTA. The change in transmission was displayed on a Tektronix storage oscilloscope. Photographs of the curves were taken with a Polaroid camera. The changes in transmission were measured and converted to absorption by the use of a Hewlett Packard calculator combined with an XY plotter and an external memory as previously described (Sanner, 1971). Unless otherwise indicated the change in absorption was measured at 325 nm. The curves were fitted to first-order reaction kinetics by maximizing the correlation coefficient. The total change in absorption as well as the reaction rate were obtained from the calculations. In the experiments with enzyme in excess of the oxidized coenzyme, the absorption curve could be decomposed into two first-order reactions. In most cases, the fast reaction rate was calculated from experiments with a time scale of 20 msec/cm on the oscilloscope, while the rate of the slow reaction was determined from experiments with a time scale of 0.5 sec/cm on the oscilloscope disregarding the first 0.5 sec of the curve.

Materials. Glutamate dehydrogenase from beef liver, NAD^+, $NADP^+$, and NADH were obtained from Boehringer, GmbH, Mannheim, Germany. Glutamate, ADP, and GTP were purchased from Sigma Chemical Company, St. Louis, Mo.

Results

The oxidative deamination of glutamate by glutamate dehydrogenase is studied with the enzyme present in excess of the coenzyme. Figure 1A shows the change in absorption as a function of time after mixing of the enzyme with NAD^+ and glutamate. The initial rapid increase in absorption is followed by a more slow decrease. The curve can be decomposed into two first-order reactions. The first-order rate constant of the rapid reaction is denoted k_1. The increase in absorption associated with the rapid reaction is denoted ΔA_1. The corresponding constants for the slow reaction resulting in a decrease in absorption are denoted k_2 and ΔA_2, respectively. The rate constants and the changes in absorption involved in these two reactions were determined as described in Experimental Procedures. In the results below, the reaction rates as well as the changes in absorption involved in the two reactions have been determined under different experimental conditions.

In order to characterize the processes responsible for the two reactions, the spectra of the enzyme complexes have been determined (Figure 1B). The spectrum of the rapidly formed intermediate has a maximum absorbance at about 335 nm with E_{335} 5.4 $cm^2\ \mu mol^{-1}$. This spectrum agrees closely with that of (E·NADPH·kg) (Cross, 1972; diFranco and Iwatsubo, 1972). As the spectra for the intermediates with NADH and NADPH are very similar in the region studied (Cross and Fisher, 1970), the present data support the assumption (Shafer et al., 1972) that the rapid increase in absorption is due to the formation of the ternary complex (E·NADH·kg).

The spectrum of the final product has an absorption maximum at 350 nm with E_{350} 5.3 $cm^2\ \mu mol^{-1}$. Cross (1972) has previously found that (E·NADPH·gl) has a maximum at 348 nm with E_{348} 4.8 $cm^2\ \mu mol^{-1}$, while diFranco and Iwatsubo (1972) found a maximum at approximately 345 nm with E_{340} 5.5 $cm^2\ \mu mol^{-1}$. It is assumed that the final absorption is due to the ternary complex (E·NADH·gl).

From the results it follows that k_1 represents the apparent first-order rate constant for the reaction:

$$(E\cdot gl) + NAD(P)^+ \rightleftharpoons (E\cdot NAD(P)^+\cdot gl) \rightleftharpoons (E\cdot NAD(P)H\cdot kg)$$

while k_2 represents the apparent rate constant for the reaction:

$$(E\cdot NAD(P)H\cdot kg) \underset{kg}{\overset{kg}{\rightleftharpoons}} (E\cdot NAD(P)H) \underset{gl}{\overset{gl}{\rightleftharpoons}} (E\cdot NAD(P)H\cdot gl)$$

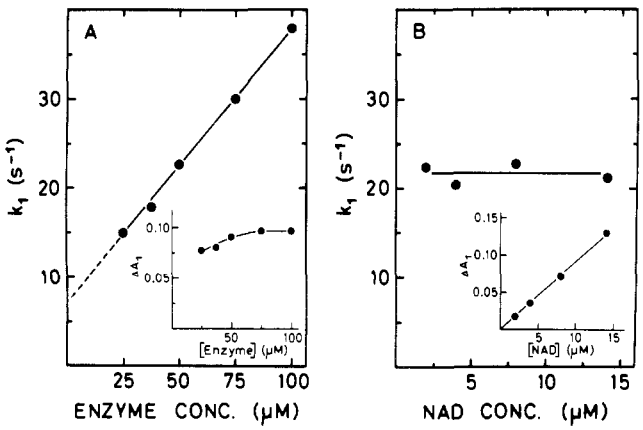

FIGURE 2: Effect of enzyme (A) and NAD^+ (B) concentration on the formation of (E·NADH·kg). The first-order rate constant was determined from the increase in absorption at 325 nm. The inserted figures show the concomitant change in absorption. The final concentrations were: 50 μM enzyme (in panel B), 100 mM glutamate, and 8.5 μM NAD^+ (in panel A). The enzyme was premixed with glutamate.

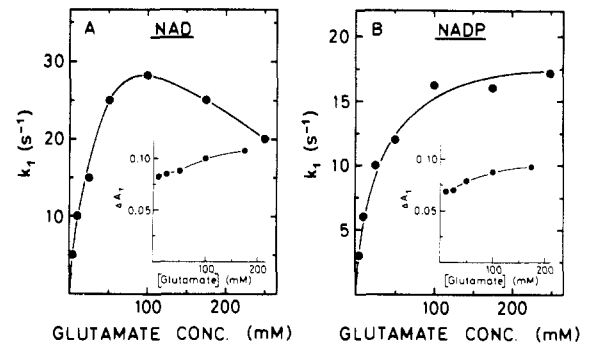

FIGURE 3: Effect of glutamate on the formation of (E·NAD(P)H·kg). The first-order rate constant was determined from the increase in absorption at 325 nm measured with NAD^+ (A) and $NADP^+$ (B) as a function of the glutamate concentration. The inserted figures show the concomitant change in absorption. The final concentrations were: 50 μM enzyme and 8.5 μM NAD^+ or $NADP^+$. Glutamate was present in the concentrations indicated.

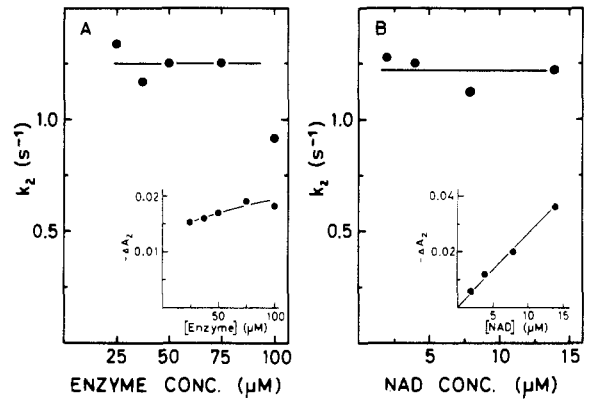

FIGURE 4: Effect of enzyme (A) and NAD^+ (B) concentration on the formation of (E·NADH·gl). The first-order rate constant was determined from the decrease in absorption at 325 nm. The inserted figures show the concomitant change in absorption. The final concentrations were: 50 μM enzyme (in panel B), 100 mM glutamate, and 8.5 μM NAD^+ (in panel A). The enzyme was premixed with glutamate.

Rate of Formation of (E·NAD(P)H·kg). Figure 2 shows the effect of the enzyme and NAD^+ concentration on k_1 and ΔA_1. The enzyme concentrations were in all cases much higher than the NAD^+ concentrations. The rate constant increases linearly with the enzyme concentration, while ΔA_1 is nearly independent of the enzyme concentration (Figure 2A). As expected, k_1 is independent of the NAD^+ concentration, while ΔA_1 increases linearly with the coenzyme concentration (Figure 2B). The results suggest that the rate-limiting step in the reaction is the bimolecular interaction between the enzyme–glutamate complex and NAD^+. If the rate-limiting step were some isomerization occurring after the binding of NAD^+, k_1 would be expected to be independent of the enzyme concentration.

The effect of glutamate concentration on k_1 and ΔA_1 is shown in Figure 3. With NAD^+ (Figure 3A), k_1 increased with the glutamate concentration until a maximum was reached at about 100 mM. The reaction rate decreased again at higher glutamate concentrations. With $NADP^+$ (Figure 3B), a normal saturation curve was obtained with increasing glutamate concentration. The increase in reaction rate with the glutamate concentration is probably due to the fact that at low glutamate concentration only part of the enzyme will be complexed with glutamate and thus the concentration of the binary complex (E·gl) reacting with the coenzyme increases with increasing glutamate concentration. This is in agreement with the finding that the change in absorption is nearly independent of the glutamate concentration. If the results are plotted in a double reciprocal plot, a K_m for glutamate of 20–25 mM is obtained both with NAD^+ and $NADP^+$ as coenzyme. The rate constants with $NADP^+$ are significantly smaller than those obtained with NAD^+.

diFranco (1974) has recently observed in other types of experiments a much higher rate constant for the formation of (E·NADPH·kg) than observed here. The discrepancy is probably due to the fact that she used much higher concentration of the coenzyme. Conceivably, under such conditions some isomerization steps after binding of the coenzyme may be the rate-limiting step.

Rate of Formation of (E·NAD(P)H·gl). Figure 4 shows that k_2 is independent of both the enzyme and the NAD^+ concentration. The change in absorption (ΔA_2) is nearly independent of the enzyme concentration (Figure 4A), while it increases linearly the NAD^+ concentration (Figure 4B).

The data in Figure 5 show the effect of glutamate concentration on k_2 with NAD^+ (Figure 5A) and $NADP^+$ (Figure 5B) as coenzyme. The rate constant increases with the glutamate concentrations and K_m for glutamate in the reaction was 3 mM for both coenzymes. The rate constant obtained with NAD^+ was considerably greater than the one obtained with $NADP^+$. In contrast to the result for the first reaction, it was found that the increase in k_2 with the glutamate concentration was accompanied by an increase in ΔA. The glutamate concentration dependence of this reaction probably reflects the trapping of glutamate with the formation of (E·NAD(P)H·gl).

In order to obtain further information on the last steps of the reaction, experiments were carried out in which (E·NADH) was allowed to interact with glutamate. The results are shown in Figure 6. Only one reaction rate was apparent, and the change in absorption corresponded closely to that expected for the convertion (E·NADH) to (E·NADH·gl) (Figure 6A). Thus, the difference between the two spectra is very similar to that previously found with NADPH as coenzyme (diFranco and Iwatsubo, 1972). The rate constant was independent of the NADH concentration, while the change in absorption increased proportionally

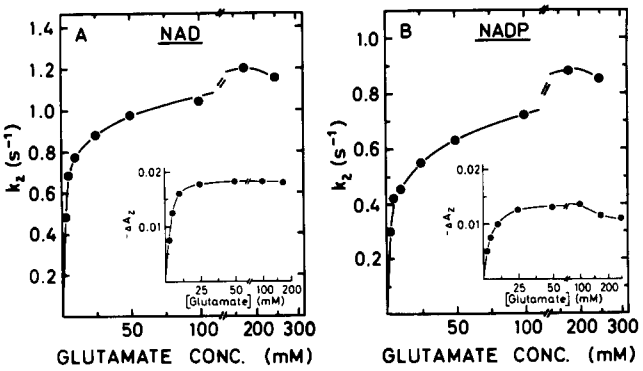

FIGURE 5: Effect of glutamate on the formation of (E·NAD(P)H·gl). The first-order constant was determined from the decrease in absorption at 325 nm measured with NAD^+ (A) and $NADP^+$ (B) as a function of the glutamate concentration. The inserted figures show the concomitant change absorption. The final concentrations were: 50 μM enzyme and 8.5 μM NAD^+ or $NADP^+$. Glutamate was present in the concentration indicated.

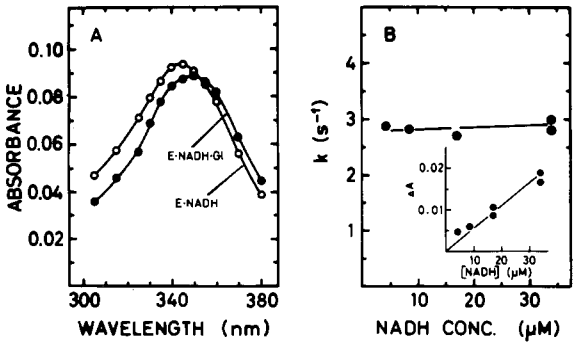

FIGURE 6: Formation of (E·NADH·gl) from (E·NADH). (A) Absorption spectra of (E·NADH) and (E·NADH·gl). The spectra were determined from the change in absorption upon mixing with glutamate. (B) Effect of NADH concentration on the rate and change in absorption at 310 nm (inserted figure). The initial concentrations were: 50 μM enzyme, 100 mM glutamate, and 34 μM NADH (in panel A). The enzyme was preincubated with NADH.

with the coenzyme concentration (Figure 6B). Interestingly, the rate constant for binding of glutamate to (E·NADH) with the formation of (E·NADH·gl) was several times higher than k_2. These results suggest that the release of α-ketoglutarate from (E·NAD(P)H·kg) is the rate-determining step in the formation of (E·NAD(P)H·gl).

Effects of ADP and GTP on k_1 and k_2. Figure 7 shows the effect of the allosteric effector molecules GTP and ATP on the rate of formation of (E·NADH·kg) (Figure 7A) and (E·NADH·gl) (Figure 7B). Low concentrations of GTP enhanced k_1. This enhancement was most pronounced at high glutamate concentrations. When higher concentrations of GTP were added, k_1 decreased again. k_1 decreased strongly with increasing concentration of ADP up to a concentration of about 0.2 mM. At higher ADP concentration no further inhibition was observed. Interestingly, it was not possible to inhibit the reaction rate completely with ADP. The percentage reduction in k_1 by ADP was independent of the glutamate concentration (data not presented).

k_2 was strongly reduced in the presence of GTP (Figure 7B); 75 μM GTP reduced k_2 by approximately 50%. The effect of GTP was independent of the glutamate concentration. ADP increased k_2. The increase was most pronounced at high glutamate concentration. With 250 mM glutamate the reaction rate increased by a factor of nearly 3 in the presence of 0.06 mM ADP. Both with ADP and GTP the results obtained with $NADP^+$ as coenzyme were very similar to those shown with NAD^+ (data not presented).

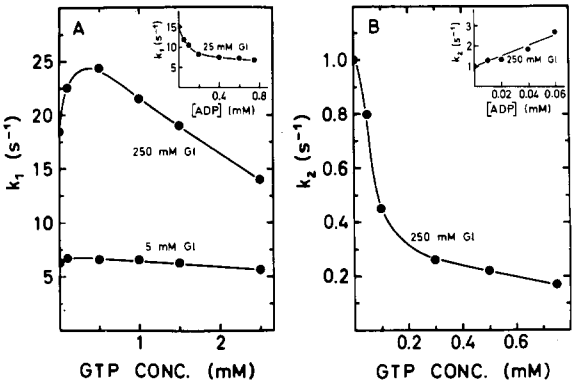

FIGURE 7: Effect of GTP and ADP on the formation of (E·NADH·kg) (A) and (E·NADH·gl) (B). The rate constants were determined from the change in absorption at 325 nm with time for different concentrations of glutamate with the effector concentrations indicated. The final concentrations were: 50 μM enzyme, 8.5 μM NAD^+, and other concentrations as indicated.

Discussion

Stopped-flow experiments have been carried out in which NAD^+ and $NADP^+$ are mixed with an excess of glutamate dehydrogenase and glutamate. Under these conditions the stable product formed is (E·NAD(P)H·gl). The ternary enzyme complex (E·NAD(P)H·kg) has been identified as an intermediate in the reaction. The identification of the enzyme complexes is based on their absorption spectra. The results indicate that the binding of $NAD(P)^+$ to (E·gl) is the rate-limiting step in the formation of the intermediate, while the liberation of α-ketoglutarate is the rate-limiting step in the formation of (E·NAD(P)H·gl). Since the enzyme complex (E·NAD(P)H) has not been identified as an intermediate in the reaction, it is not known whether glutamate interacts directly with the ternary complex (E·NAD(P)H·kg) or whether it interacts with (E·NAD(P)H). ADP enhanced while GTP reduced the rate of (E·NAD(P)H·gl) formation. The rate of formation of (E·NAD(P)H·kg) was inhibited by ADP, while it increased at high glutamate concentration when small amounts of GTP were added.

K_m for glutamate measured from the formation of (E·NAD(P)H·kg) was approximately 25 mM. This value is assumed to represent K_d for (E·gl) and is similar to that obtained by Prough et al. (1972) from observation of the spectral changes occurring upon mixing of the enzyme with glutamate. The apparent K_m for glutamate in the formation of the final product (E·NAD(P)H·gl) was 3 mM. This value is assumed to represent K_d for (E·NAD(P)H·gl) and is only slightly greater than that previously obtained in other types of experiments by diFranco and Iwatsubo (1972).

In steady-state experiments it is found that the kinetics are more complexed with NAD^+ than with $NADP^+$ as coenzyme. Moreover, higher activity is found with NAD^+ than with $NADP^+$. It has been suggested that the higher activity is caused by binding of NAD^+ to the ADP activating site (Pantaloni and Dessen, 1969; Cross and Fisher, 1973; diFranco, 1974). Krause et al. (1974) found that both NAD^+ and $NADP^+$ can be bound to the ADP activating site, but as the affinity of the ADP binding site is rather weak for binding of $NADP^+$ interaction of this coenzyme with the activating site can probably be neglected under

most steady-state conditions. The present results show that also under conditions where NAD^+ does not bind to the ADP activating site, the rate constants obtained with NAD^+ as coenzyme were approximately 50% greater than those obtained when $NADP^+$ was present. The finding that high glutamate concentrations reduce the rate of (E·NADH·kg) formation while it has no inhibitory effect on the (E·NADPH·kg) formation shows another difference between the two coenzymes in the enzyme reaction.

Under steady-state conditions it is found that high concentrations of glutamate inhibit the reaction (Engel and Dalziel, 1969; Sanner and Pihl, 1972). The possibility that this substrate inhibition can be accounted for by formation of (E·NAD(P)H·gl) appears unlikely. Thus, this complex is formed with an apparent K_m for glutamate of 3 m*M* while the substrate inhibition is only significant at 20–30 times this concentration. The possibility that the substrate inhibition is associated with the inhibitory effect of high glutamate concentration on the formation of (E·NADH·kg) mentioned above is unlikely. Since this inhibition occurs only with NAD^+ while the substrate inhibition under steady-state conditions occurs with both coenzymes. Previous results have shown that the substrate inhibition observed under steady-state conditions is extremely sensitive to radiation damage, and most probably caused by some allosteric interaction (Sanner and Pihl, 1972).

Acknowledgment

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