Interaction of Mg$^{2+}$ with Human Liver Aldehyde Dehydrogenase

I. SPECIES DIFFERENCE IN THE MITOCHONDRIAL ISOZYME*

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The dehydrogenase activity of the mitochondrial isozyme (E2) of human liver aldehyde dehydrogenase was stimulated about 2-fold by the presence of low concentrations (about 120–140 μM) of Mg$^{2+}$ in the assay at pH 7.0 using propionaldehyde as substrate. The stimulation was totally reversible by treatment with EDTA. Maximum stimulation was dependent on the concentration of NAD$^+$ used in the assay; an increase in $K_m$ value of NAD$^+$ was observed to parallel the increase in maximal velocity with increasing Mg$^{2+}$ concentration, indicating that alterations in the catalytic properties of the E2 isozyme occur in the presence of Mg$^{2+}$. The presteady state burst of NADH product was observed to decrease in the presence of Mg$^{2+}$, suggesting that the rate-limiting step of the dehydrogenase reaction is altered by Mg$^{2+}$. No evidence for Mg$^{2+}$-induced alterations in the molecular weight properties of the E2 isozyme was observed using gel filtration column chromatography and fluorescence polarization techniques. In addition, no alterations in the inactivating properties of iodoacetamide or disulfiram were produced by Mg$^{2+}$. These results suggest that the mechanism by which human mitochondrial aldehyde dehydrogenase (E2) is stimulated by Mg$^{2+}$ is different from that of the horse enzyme, representing a significant species difference.

Recently, Takahashi and Weiner (1980) have demonstrated that the mitochondrial isozyme of horse aldehyde dehydrogenase exhibits a doubling of specific activity when assayed in the presence of low levels of MgCl$_2$ (about 500 μM) at pH 7.0, and that the increase in rate is due to dissociation of the tetrameric enzyme functioning with half of the sites reactivity to decrease in the presence of Mg$^{2+}$, the results presented demonstrate that the mechanism by which the human mitochondrial isozyme (E2) is stimulated by Mg$^{2+}$ is distinct from that reported for the horse mitochondrial isozyme. With the human enzyme, Mg$^{2+}$ alters the catalytic properties of already existing active sites and does not cause an increase in the number of functioning active sites via dissociation of the tetramer into dimers.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

It has been shown that the mitochondrial isozyme (E2) of human liver aldehyde dehydrogenase is stimulated by low levels of MgCl$_2$ (micromolar range). A 2-fold enhancement in dehydrogenase activity was observed when assayed at pH 7.0 using propionaldehyde as substrate. The stimulation was observed to occur immediately following addition of MgCl$_2$ to the assay, while complete reversal to the original reaction velocity was obtained almost instantaneously by addition of EDTA. These observations are basically consistent with data previously reported by Takahashi and Weiner (1980) from studies on the mitochondrial isozyme of horse liver aldehyde dehydrogenase. In our study, however, a buffering agent (25 mM PIPES, pH 7.0) which does not form complexes with Mg$^{2+}$ was used (Good et al., 1966), and this probably accounts for the relatively low concentration of MgCl$_2$ (about 120–140 μM) required to bring about maximum stimulation of the human enzyme when compared to the horse enzyme (about 400–500 μM; Takahashi and Weiner, 1980). Substitution of CaCl$_2$ for MgCl$_2$ caused a significantly greater fold stimulation (2.9-fold for CaCl$_2$ compared with 2-fold for MgCl$_2$) under identical assay conditions. This observation provided the first piece of evidence suggesting that the mechanisms for stimulation of the horse and human mitochondrial aldehyde dehydrogenase by divalent metal cations might be different, as both MgCl$_2$ and CaCl$_2$ produced a 2-fold enhancement in the activity of the horse enzyme (Takahashi and Weiner, 1980).

Maximum stimulation of E2-catalyzed dehydrogenase activity was dependent on the concentration of NAD$^+$ used in the assay (Figs. 1 and 2). This phenomenon was not observed

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1 Portions of this paper (including "Experimental Procedures," "Results," Table I, and Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-931, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviation used is: PIPES, 1,4-piperazinediethanesulfonic acid.

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by Takahashi and Weiner (1980) with the mitochondrial isozyme of horse aldehyde dehydrogenase. In addition, stimulation curves obtained with E2 isozyme at both concentrations of NAD+ (570 μM and 3 mM; Fig. 1) were different from the curve obtained for the horse enzyme. With E2, a biphasic ascending pattern was observed before maximum stimulation was reached at about 120-140 μM Mg2+, which was then followed by a gradual, steady decrease in specific activity as the concentration of Mg2+ was further increased. In contrast, the horse enzyme showed a hyperbolic stimulation curve with a leveling off pattern after maximal stimulation was reached (Takahashi and Weiner, 1980).

An increase in the Km value of NAD+ with E2 isozyme was observed to parallel the increase in maximal velocity with increasing levels of Mg2+ (Fig. 2; Table I), indicating that alterations in the catalytic properties of E2 isozyme occur in the presence of Mg2+. That the positions of the stimulation peaks were the same at both levels of NAD+ (Fig. 1), suggests that Mg2+ probably does not combine with NAD+ to form a "true substrate" as a means for stimulation. This observation is consistent with the results of Burkhard (1981), who has calculated the dissociation constants for divalent metal cations with NAD+ to be in the millimolar range. Thus, the requirement for increased concentrations of NAD+ to achieve maximum fold stimulation appears not to be related to formation of an NAD-·Mg2+ substrate complex, but it is instead related to an increased value for the Km of free NAD+ in the presence of Mg2+.

The parallel series of lines (Fig. 2) generated in the double reciprocal plots of data obtained with varied NAD+ at changing levels of MgCl2 and saturating propionaldehyde suggest that Mg2+ does not combine with free enzyme. It is possible, however, that Mg2+ may combine with the enzyme-NAD binary complex, as suggested by the pattern of intersecting lines (Fig. 3) generated in the double reciprocal plots of data obtained for varied glycolaldehyde, constant NAD+, and changing levels of Mg2+.

The presence of a presteady state burst of NADH product indicates that the rate-limiting step of the E2-catalyzed dehydrogenase reaction occurs after ternary complex interconversion. In the case of horse mitochondrial aldehyde dehydrogenase, the amplitude of this burst was observed to increase in the presence of Mg2+ (Takahashi and Weiner, 1980), and has been used as evidence to support an increase in number of functioning active sites as the mechanism of Mg2+ stimulation for that enzyme. The amplitude of the presteady state burst of NADH product formed with the E2 isozyme was, however, observed to decrease, while the steady state reaction velocity increased in response to increasing concentrations of Mg2+ (Fig. 4). In addition, the stimulation constants obtained for Mg2+ from the steady state (about 15 μM) and presteady state (about 16 μM) are in good agreement, as are the coefficients for maximum change in maximal velocity (2.25) and burst amplitude (2.30) obtained by extrapolation of the data plotted in Figs. 2 and 4, respectively. Since Mg2+ showed no effect on the unstimulated esterase burst amplitude, and no burst was detected for the coenzyme-stimulated reactions (with or without Mg2+), information regarding esterase active site number, in the presence and absence of Mg2+, using presteady state techniques could not be obtained. Since the decrease in dehydrogenase burst amplitude virtually parallels the increase in specific activity (Fig. 4), it appears unlikely that Mg2+ stimulates the E2 isozyme by increasing the number of functioning active sites; these data are more consistent with a mechanism in which Mg2+ alters the properties of already existing sites.

No evidence for Mg2+-induced alterations in the molecular weight properties of the E2 isozymes was observed. Gel filtration chromatography experiments indicated a molecular weight of about 260,000 in the presence and absence of Mg2+, a value which is in fairly good agreement with that determined previously for the tetrameric form of the enzyme (Pietruszko et al., 1977), and which is precisely the same value reported by Takahashi and Weiner (1980) for the tetrameric form of the horse mitochondrial enzyme. Presence of dehydrogenase substrates (and products) together with Mg2+ also appeared to have no effect on the molecular weight of the E2 isozyme, as determined by gel filtration chromatography. In the study by Takahashi et al. (1981), a decrease in the polarization of fluorescence (P = 0.34 to P = 0.26) determined for enzyme-bound NADH was observed to occur in response to Mg2+ and correlated to a decrease in enzyme molecular weight (from tetramer to dimer). In fluorescence polarization experiments on the E2 isozyme (carried out under conditions identical with those of Takahashi et al., 1981), however, no decrease in P value was observed. A value of 0.32 was determined in both the absence and presence of Mg2+, a number closely resembling the value of 0.34 determined for the tetrameric form of the horse enzyme (Takahashi et al., 1981). Since the active site number of the E2 isozyme cannot be determined by conventional titration methods, it is impossible to duplicate experiments of Takahashi et al. (1980) which clearly demonstrate an increase in the number of NADH binding sites. Finally, no alterations in the inactivating properties of either disulfiram or iodoacetamide were observed with E2 isozyme when incubations were carried out in the presence of Mg2+.

These results, coupled with the fluorescence polarization, gel filtration, steady state, and presteady state data, indicate that the stimulation of the E2 isozyme by Mg2+ is probably not related to enzyme dissociation or an increase in the number of functioning active sites.

As discussed previously, the presence of a presteady state burst of NADH in the dehydrogenase reaction indicates that the rate-limiting step is after interconversion of the ternary complex (e.g. deacylation or NADH product release). A Mg2+-induced acceleration in the rate of NADH release or deacylation could shift the rate-limiting step to a step which precedes ternary complex interconversion, thereby causing a decrease in the magnitude of the presteady state burst. The 4-fold higher maximal velocity obtained with glycolaldehyde (0.8 μmol/min/mg) than with propionaldehyde (0.2 μmol/min/mg) as substrate suggests further that deacylation rather than NADH dissociation may be rate-limiting in the case of the E2 isozyme, since the greater electron withdrawing capacity of the R group of glycolaldehyde (when compared to propionaldehyde) facilitates this nucleophilic step in the reaction (Weiner, 1979). Decacylation has previously been determined to be the rate-limiting step for horse liver mitochondrial aldehyde dehydrogenase (Weiner et al., 1976). As discussed above, Mg2+ appears to effect the amplitude of the presteady state burst (Fig. 4), with propionaldehyde as substrate, by shifting the rate-limiting step to a step preceding ternary complex interconversion. The relatively small degree of stimulation of the E2 isozyme by Mg2+ with glycolaldehyde as substrate (1.6-fold), coupled with an already accelerated rate of dehydrogenation for the same substrate in the absence of added Mg2+, suggests that the rate of decacylation for glycolaldehyde is already quite fast and another step in the reaction sequence may already be rate-limiting. Preliminary studies revealed a presteady state burst of NADH product with glycolaldehyde that appears to be even smaller in magnitude than the one obtained with propionaldehyde and Mg2+.
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(80 μM), indicating that this is indeed a good possibility.

In summary, these results suggest that Mg²⁺ causes E₂-catalyzed dehydrogenase activity to increase by altering the catalytic properties of already existing active sites rather than by causing an increase in their functional number. Mg²⁺ changes the kinetic properties of the enzyme relative to $K_m$ values of NAD⁺, but does not appear to cause alterations in the molecular weight properties of the enzyme. This finding represents a significant species difference between mitochondrial aldehyde dehydrogenases from horse and man.

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Human Liver Aldehyde Dehydrogenase I

1. SPECIES DIFFERENCES IN THE MITOCHONDRIAL ISOZyme
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EXPERIMENTAL PROCEDURES

Material: Micromoles of adenosine diphosphate (ADP) was obtained from Sigma Chemical. Enzyme from the human liver was obtained from Worthington (free 11-100 from Sigma Chemical, Co., while NAD+; adenosine diphosphate, and 1,2-propionic acid were obtained from Sigma Chemical Co. All other reagents were of the highest grade available. The mitochondrial (E2) isozyme of aldehyde dehydrogenase was purified from bovine liver by the procedure of Tabor and Tabor (1968). The mitochondrial isozyme of liver aldehyde dehydrogenase from the horse (E1) was also obtained from the mitochondria of liver, as described in the previous paper (Wolf et al., 1968). The enzyme was isolated from horse liver microsomes by a modification of the method of Fildes and Weiler (1971), while protein concentrations were determined by the method of Lowry et al. (1951) using BSA as a standard, and by measuring the absorbance at 280 nm using extinction coefficients of 1.038 mg/ml (Bradford, 1976).

Dissohion States Enzymes. Aldehyde dehydrogenase activity was evaluated spectrophotometrically by monitoring NAD+ production at 340 nm using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Enzyme Assay Kinetics. The apparent K\text{m} for ethanol of the mitochondrial isozyme was determined by following the rate of NAD+ production at 340 nm using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Effect of NAD+ Concentration on the Kinetics of the Human Liver Aldehyde Dehydrogenase Activity. The effect of NAD+ concentration on the kinetics of the human liver aldehyde dehydrogenase activity was examined using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Results

Effect of NAD+ on the Dehydrogenase Activity of the E2 Isozyme. The effect of increasing concentrations of NAD+ on the rate of NAD+ production was determined spectrophotometrically at 340 nm using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Discussion

The effect of increasing concentrations of NAD+ on the rate of NAD+ production was determined spectrophotometrically at 340 nm using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Figure 1. Effect of NAD+ concentration on the characteristics of \text{NAD}+ induced stimulation of the mitochondrial aldehyde dehydrogenase activity. The data were obtained using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

Comparison of the Kinetics of the Human Liver Aldehyde Dehydrogenase Activity with that of the Mitochondrial Isozyme of Liver Aldehyde Dehydrogenase. The effect of NAD+ concentration on the kinetics of the human liver aldehyde dehydrogenase activity was examined using a Varian Instruments Model 110B spectrophotometer. An extinction coefficient of 6.34 mmol/mg/cm was used for NAD+. All assays were carried out at 25°C in 10 mM phosphate, pH 7.0, and were incubated at room temperature for 10 min.

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Figure 2. Stimulation of human mitochondrial aldehyde dehydrogenase (E2) activity at several constant levels of NAD+, and varied concentrations of 2-propanol. The reaction was initiated by the addition of NAD+ to a final concentration of 0.14 mM, while the concentration of 2-propanol was varied from 0.05 to 0.5 mM. The reaction was at room temperature and the enzyme was incubated for 10 min.

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**Table 1**

| MgCl₂ (μM) | NaN₃ (μM) | Maximal Velocity (μmol/min/mg) |
|------------|-----------|-------------------------------|
| 0          | 98        | 0.20                          |
| 15         | 146       | 0.28                          |
| 40         | 171       | 0.20                          |
| 90         | 188       | 0.38                          |
| "infinite" | 220       | 0.45                          |

**Figure 4.** Relation between MgCl₂ concentration, steady state burst amplitude, and maximal steady state velocity of the E2-catalyzed dehydrogenase reaction. Burst amplitudes (Δ) and steady state velocities (●) were determined using a stopped flow spectrophotometer as described in Materials and Methods. The MgCl₂ and NaN₃ concentrations were determined separately from the stopped flow data by linear regression (r = 0.993).

**Figure 3.** Stimulation of human mitochondrial aldehyde dehydrogenase (E2) activity at several constant levels of NADH and variable concentrations of glyceraldehyde. Concentrations of MgCl₂ were 0, 5.32, 11.7, or 23.3 μM E2, 5.52, 11.0, or 22.0 μM at 200 μM NaN₃ and the desired amount of MgCl₂. This method was used to select the optimal condition for E2-catalyzed reaction. The steady state procedure was the same as that described in the legend for Figure 4.

**Figure 2.** Effect of MgCl₂ on Presteady State Burst Amplitude of NADH in the E2 Isomeric-Catalyzed Dehydrogenase Reaction. Using a stopped flow spectrophotometer, the presteady state formation of NADH in the E2-catalyzed dehydrogenase reaction was investigated in the absence of presence (50 μM) of added MgCl₂. Although we were unable to record the entire presteady state phase of the reaction, we were able to obtain a recording of the steady state phase reaction immediately following the burst phase. This method has been used successfully with other dehydrogenase isoenzymes to examine the effect of metal ions on the reaction. This technique was used to examine the effect of MgCl₂ on the presteady state event in the E2-catalyzed reaction.

**Figure 1.** Effect of MgCl₂ on the Presteady State Burst of p-Nitrophenol Produced in the Esterase Reaction. Evidently, for a steady state burst of p-nitrophenol produced at ca. 0.16 to 0.20 active sites per tetramer, no evidence could be found; however, for E2-catalyzed reactions, the rate of decrease in p-nitrophenol concentration was not observed. These data are consistent with those of Takahashi and Neiner (1981), who observed a burst amplitude corresponding to ca. 0.3 active sites per tetramer of horse mitochondrial aldehyde dehydrogenase, and also with those of Moccia et al. (1978), who calculated a burst amplitude corresponding to ca. 0.3 active sites per tetramer of horse liver aldehyde dehydrogenase. There is no evidence for an intact burst. In the presence of NaN₃, the steady state amplitudes were determined.
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