Magnesium Stimulation of Catalytic Activity of Horse Liver Aldehyde Dehydrogenase

CHANGES IN MOLECULAR WEIGHT AND CATALYTIC SITES

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The addition of ~0.5 mM Mg$^{2+}$ ions enhances the specific activity of the pl 5 isozyme of horse liver aldehyde dehydrogenase by a factor of 2. The mechanism of the Mg enhancement of the activity was investigated by means of presteady state and steady state kinetic assays and molecular weight determination. The magnitude of the presteady state burst formation of NADH increased from 2 mol/mol of tetrameric enzyme to 4 mol/mol of tetrameric enzyme in the presence of 0.7 mM Mg$^{2+}$. Both the steady state velocity and the burst magnitude increased to the same degree if less than 0.7 mM Mg$^{2+}$ was employed. The curves of the burst magnitude and steady state velocity, as determined by the linear portion of the stopped flow run, were sigmoidal with respect to increasing Mg$^{2+}$ ion concentrations. In contrast, the steady state velocity as determined by nonstopped flow (static) spectrophotometric assays was hyperbolic.

The molecular weight of aldehyde dehydrogenase, as determined by sedimentation equilibrium, decreased from 280,000 in the absence of Mg$^{2+}$ to 130,000 in the presence of 0.4 mM Mg$^{2+}$. The dependency of apparent molecular weight on Mg$^{2+}$ ion concentrations was hyperbolic and essentially parallel to that found for the steady state assay.

The fact that the presteady state burst doubled in the presence of Mg$^{2+}$ suggests that the enzyme exhibits half-of-sites reactivity in the absence of metal and all-of-sites reactivity in its presence. The metal also caused a dissociation of tetrameric enzyme into dimers. Assuming that the tetrameric and dimeric forms of the enzyme can function with half-of-sites and all-of-sites reactivities, respectively, we can show that an excellent correlation exists between the apparent molecular weight and the specific activity of the enzyme at any Mg$^{2+}$ concentration.

Horse liver aldehyde dehydrogenase (1, 2), like the enzymes purified from other mammalian sources (3–8), is isolated as a tetrameric enzyme. In the presteady state, only 2 mol of NADH are produced per mol of enzyme (9), suggesting that the enzyme functions with half-of-sites reactivity, and thus can be viewed as an example of extreme negative cooperativity (10). Further evidence for half-of-sites reactivity comes from equilibrium-binding studies which revealed that only 2 mol of product, NADH, bind to the tetrameric enzyme while 4 mol of NAD were found to bind (11). Two moles of NAD were found to bind tightly ($K_d = 7 \mu M$) while the second two bind more weakly ($K_d = 45 \mu M$).

Disulfiram is an irreversible inhibitor of the enzyme (12). Recently, it was shown that when 2 mol of the inhibitor bind per mol of tetrameric enzyme, 50 to 75% of the catalytic activity of the enzyme is lost. The observation has been made with the enzyme isolated from either sheep (13) or horse liver (14). The human enzyme, which is also a tetramer, is inactivated when just 2 mol of iodoacetate are attached per mol of the enzyme.1 Thus, it appears that mammalian aldehyde dehydrogenase, though composed of four subunits, may function by a half-of-sites mechanism.

No modulators of enzyme activity have been reported other than some steroids which inhibit the rabbit liver enzyme (5). Recently, though, it has been shown that various cations stimulate the enzyme activity. We showed (15, 16) that the pl 5 form of the horse liver enzyme was activated in the presence of millimolar concentrations of Mg$^{2+}$, Mn$^{2+}$, and Ca$^{2+}$. The pl 6 form of the horse liver enzyme was found to be activated by micromolar concentrations of rare earth metals and millimolar concentrations of Mg$^{2+}$ and Ca$^{2+}$ (17). The mechanism of the divalent ion activation is not known. Since the rate-limiting step for the pl 5 form of horse liver aldehyde dehydrogenase is deacylation of the covalent intermediate (acyl enzyme) formed during the dehydrogenase reaction (9), it is possible that the acceleration in velocity observed in the presence of metal is related to an increase in this slow step. Alternatively, the metal could cause an alteration in structure such that the enzyme exhibits all-of-sites rather than half-of-sites reactivity. In this communication, we will show that alterations of molecular weight of the pl 5 horse liver enzyme occur in the presence of Mg$^{2+}$ and will discuss how this may be related to the stimulation of catalytic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Propionaldehyde was obtained from Eastman Organic Chemicals. NAD was purchased from P-L Biochemicals. Deionized, distilled water was used in the buffer made from sodium phosphate salts obtained from Mallinckrodt Chemical Works. MgCl$_2$ (analytical reagent) was from Mallinckrodt. EDTA was the product of Matheson Coleman and Bell.

**Preparation of Aldehyde Dehydrogenase**—Isolation of the pl 5

1 R. Pietruszko, Rutgers University, personal communication.
isozyme of horse liver aldehyde dehydrogenase as well as the determination of specific activity and concentration were performed as previously reported (1). The purified enzyme was dialyzed at 4°C for 12 h against 0.1 M phosphate buffer, pH 7.5, before use.

**Steady-State Assays**—Steady state assays were performed in 0.1 M phosphate buffer, pH 7.5, at 25°C, with a Gilford model 240 spectrophotometer connected to a Sargent recorder. Assays contained 0.24 mM NAD, 129 μM propionaldehyde and 1.12 μM enzyme with variable amounts of Mg²⁺ as indicated in Figs. 1 to 5.

**Stopped Flow Studies**—Stopped flow experiments were performed in 0.1 M phosphate buffer, pH 7.5, at 25°C. A Durrum-Gibson stopped flow spectrophotometer, with a 2-cm light path and equipped with a pneumatic drive attachment was used. One of the drive syringes contained NAD and propionaldehyde at concentrations of 724 and 280 μM, respectively, and Mg²⁺ when required. The other drive syringe contained 2.66 μM enzyme in the same buffer. The rate of formation of NADH was followed at 340 nm. The transmittance as a function of time was presented on a teletype after being stored on a Dasar-TM (Amino) digital computer.

The concentrations of NAD and NADH were determined spectrophotometrically using extinction coefficients of 18 x 10⁴ M⁻¹ cm⁻¹ (260 nm) and 6.22 x 10⁴ M⁻¹ cm⁻¹ (340 nm), respectively.

**Molecular Weight Determination**—Sedimentation equilibrium studies were performed as described (1) in a Beckman model E ultracentrifuge equipped with an ultraviolet scanner, at 6000 rpm, 8°C, for 24 h in 0.1 M phosphate buffer, pH 7.5. Enzyme concentration was 1.1 μM and Mg²⁺ was varied as indicated in Fig. 1.

**RESULTS**

The Effect of Mg²⁺ on the Steady State Velocity—As the concentration of Mg²⁺ was increased, the steady state velocity also increased. The maximum degree of stimulation was a factor of 2. The concentration of Mg²⁺ producing half the maximal activation (Kₐc) is 0.05 mM (Fig. 1). In assays performed with the stopped flow spectrophotometer (to be discussed below), a 2-fold stimulation of Vₜₕₜₑₚₑₚₑ in the presence of Mg²⁺ is also obtained.

The reversibility of the effect of Mg²⁺ was examined by complexing the metal (0.7 mM) with 2 mM EDTA. Adding EDTA to the Mg²⁺-activated enzyme caused the velocity to return to essentially the Mg²⁺-free value as shown in Fig. 2.

Effects of Mg²⁺ on the Presteady State Burst Magnitude and the Steady State Velocity—It was previously shown that the magnitude of the presteady state burst was 2 mol of NADH formed per mol of tetrameric enzyme (9); this result was verified by repeating the stopped flow experiment. In the presence of Mg²⁺, the magnitude of the burst increased from essentially 2 to 4. These results are illustrated in Fig. 3. In the same figure is included the value for the steady state portion of the reaction. The increase in steady state velocity essentially parallels the magnitude of the burst. This implies that the increase in specific activity of the enzyme is related to an increase in the number of functioning active sites on the enzyme.

Even though there is an increase in the magnitude of the steady state velocity, the actual rate constants, based on the number of active subunits determined by the magnitude of the presteady state burst, are the same in the absence and presence of Mg²⁺. Furthermore, the rate constants for the presteady state portion, when corrected for the increased number of functioning active sites, also are the same. Thus, there is no alteration in individual kinetic parameters of the enzyme in the presence of Mg²⁺. In the steady state assays

**Fig. 1.** Dependencies of relative specific activity and apparent molecular weight of aldehyde dehydrogenase on Mg²⁺ concentration. ○ and ■, relative specific activity and apparent molecular weight, respectively. Data of sedimentation equilibrium studies are the average values from two or three runs at each concentration of Mg²⁺.

**Fig. 2.** Reversibility of catalytic activation of aldehyde dehydrogenase by Mg²⁺. The dehydrogenase reaction of horse liver aldehyde dehydrogenase was initiated by adding 25 μl of 23 μM enzyme solution to 1 ml of assay mixture containing 120 μM NAD and 90 μM propionaldehyde at arrows I. Ten microliters of 72 mM MgCl₂ solution and then 100 μl of 22.7 mM EDTA solution were added to the reacting solution at arrows II and III, respectively. The numbers on the solid line are the relative slopes (velocity) and show that the Mg²⁺ effect is reversible. The dashed line indicates a time course (control) without Mg²⁺ and EDTA. The final velocity is not exactly equal to the initial one due to the dilution effect and possible product inhibition.

**Fig. 3.** Effects of Mg²⁺ on burst magnitude and steady state velocity of aldehyde dehydrogenase obtained by stopped flow experiments. ○, burst magnitude; ■, steady state velocity. Inset, time courses at 0 (lower) and 0.7 (upper) mM of Mg²⁺. The steady state phase was assumed to be the linear portions that occurred within approximately 2 s after the presteady state phase. The burst magnitudes were obtained from the intercepts of the linear portions extrapolated to time zero. The dead time of stopped flow apparatus was about 5 ms under the experimental conditions.

²The abbreviations used are: Kₐc, the concentration of Mg²⁺ necessary to cause a 50% change. M, apparent molecular weight.
The fraction of enzyme molecules existing as tetramers can be calculated from the molecular weight data presented in Fig. 1 using Equation 1, which has been used by Levitzki and Koshland (20).

\[
\frac{M}{M_0} = \frac{(260,000)^2 X + (130,000)^2(1 - X)}{260,000 X + 130,000(1 - X)}
\]  

(1)

where \( M \) is the apparent molecular weight and \( X \) is the fraction of enzyme in the tetrameric form. This plot is presented in Fig. 4. In the presence of 0.04 mM \( \text{Mg}^{2+} \), half of the tetrameric enzyme molecules have dissociated into dimers. This is about the same concentration of metal necessary to cause a 50% increase in specific activity of the enzyme.

If it is assumed that the tetrameric and dimeric forms can function with the half-of-sites and all-of-sites reactivities, respectively, the total number of active sites (\( Y \)) per unit of enzyme concentration can be expressed by the following equation:

\[
Y = 2(1 - X) + 2(1 - X)
\]

\[= 4 - 2X
\]  

(2)

where \((1 - X)\) is the fraction of enzyme in the dimeric form. Furthermore, we can obtain Equation 3 by substituting Equation 1 into Equation 2:

\[
Y = 4 - \frac{2(M - 130,000)}{(260,000 - M)}
\]  

(3)

As shown in Fig. 5, the theoretical curve based on Equation 3 fits well with the experimental data of relative activity taken from Fig. 1. This means that an excellent correlation exists between the apparent molecular weight and the specific activity of the enzyme.

**DISCUSSION**

The physiological concentration of \( \text{Mg}^{2+} \) is \(~0.5\ \text{mM}\) in mitochondria of various mammalian tissues (21–23). This concentration of \( \text{Mg}^{2+} \), even in 100 mM phosphate buffer, caused a 2-fold stimulation in the catalytic activity of the \( \text{pI} \) 5 form of horse liver aldehyde dehydrogenase. The apparent \( K_{\text{act}} \) for metal was \(~0.05\ \text{mM}\). If only free \( \text{Mg}^{2+} \) binds to enzyme, the \( K_{\text{act}} \) would be \(~1\ \mu M\) since at \( \text{pH} \) 7.5 only 2% of the \( \text{Mg}^{2+} \) is in the uncomplexed state.\(^3\)

Analysis of the magnitude of the burst formed in the pre-steady state revealed that the number of functioning catalytic sites increased from two per tetramer in the absence of \( \text{Mg}^{2+} \) to four per tetramer in its presence. Furthermore, the rate constant for the pre-steady state portion of the reaction and the turnover number per active subunit were the same in the presence and absence of the metal. Thus, it appears that the mechanism for activation of the enzyme by metal must be associated with a change in the number of functioning subunits and not with an alteration in the catalytic property of any existing active site.

The apparent molecular weight of aldehyde dehydrogenase is dependent upon the \( \text{Mg}^{2+} \) concentration. The proportion of enzyme existing in the tetrameric state (i.e. \( M = 260,000 \)) decreases from 100% in the absence of \( \text{Mg}^{2+} \) to essentially zero in the presence of 0.4 mM \( \text{Mg}^{2+} \). As the enzyme is transformed from the tetrameric to the dimeric state, there is an excellent correlation between the increase in specific activity and the apparent molecular weight. In the dimeric state, where the enzyme is more active, both subunits must be catalytically functioning, while in the tetrameric state only two subunits per tetramer are active at any time. Thus, in the presence of

\(^3\) The concentration of free \( \text{Mg}^{2+} \) was approximated from the dissociation constant for \( \text{MgHPO}_{4} \), of \( 1.2 \times 10^{-7} \) M (24).
Mg$^{2+}$, the enzyme changes from having half-of-sites reactivity to having all-of-sites reactivity.

Though it has not been proven that the four subunits of aldehyde dehydrogenase are identical, it appears that when the enzyme exists as a dimer, all subunits are capable of being active. When the enzyme is in the tetrameric state, the subunits apparently are arranged such that only two sites can function. The fact that 2 mol of inhibitor inactivate the bulk of the enzymatic activity (13-14) is suggestive, as is the binding of only 2 mol of product (NADH) (11), that the tetrameric enzyme has only two functioning sites, as do other enzymes exhibiting half-of-sites reactivity (10). If this model of Mg$^{2+}$ activation is correct, then the stoichiometry of coenzyme binding must increase in the presence of the metal. Binding studies reveal that this occurs (15).

It is not known how metal causes the tetrameric enzyme to dissociate. It is possible that the enzyme, even in the absence of metal ions, exists in a dynamic equilibrium between tetramer and dimer. Metal ions, then, could bind to the dimeric form, disrupting the equilibrium. Alternatively, metal could bind to the tetrameric enzyme, causing it to dissociate into dimers. These possibilities are currently being investigated.

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REFERENCES