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Inhibition of the Dehydrogenase Activity of Sheep Liver Cytoplasmic Aldehyde Dehydrogenase by Magnesium Ions†

Adrian F. Bennett, Paul D. Buckley, and Leonard F. Blackwell*

ABSTRACT: Magnesium chloride caused inhibition of the dehydrogenase activity of sheep liver cytoplasmic aldehyde dehydrogenase at all concentrations between pH 6 and 8 with no increase in the number of functioning subunits. There was also no decrease in the molecular weight as determined by gel filtration and laser light scattering experiments, results which are markedly different from those reported for the horse liver mitochondrial aldehyde dehydrogenase. There were changes in the spectroscopic and fluorescence properties of the enzyme, and enzyme-bound NADH, in the presence of magnesium ions. Steady-state inhibition studies revealed that magnesium ions exerted their inhibitory effect by decreasing V_{max} for the reaction by binding to a metal ion binding site which was distinct from the coenzyme and substrate binding sites. The biphasic nature of the Lineweaver-Burk plots at high (millimolar) concentrations of propionaldehyde was shown to be consistent with a steady-state model in which two binding sites (a catalytic low-K_m binding site and a high-K_m modifier binding site) for propionaldehyde exist. Pre-steady-state kinetic studies showed that $MgCl_2$ had no effect on the rates of NAD^+ or NADH binding or on the rate constants for the bursts in production of NADH or proton release. However, the dissociation constants for E·NAD^+ and E·NADH were significantly decreased in the presence of $MgCl_2$, and the rate constants for dissociation of the coenzymes were shown to be decreased. At high concentrations of propionaldehyde, the inhibitory effect of $MgCl_2$ could be almost entirely attributed to the tighter binding of NADH, but at low propionaldehyde concentrations, and for aromatic aldehydes, a more complex mechanism of inhibition must exist since the magnitude of the reduced k_{cat} values was almost an order of magnitude less than the reduced value of the decay constant for the slow step of the NADH displacement process.

Although aldehyde dehydrogenase (EC 1.2.1.3) are not metalloenzymes, a number exhibit a marked sensitivity to the presence of some metal ions. For example, Stoppani & Millstein (1959) found that yeast aldehyde dehydrogenase is inhibited by a wide range of metal ions, and Venteicher et al. (1977) showed that a variety of divalent and trivalent metal ions (such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, La^{3+}, and Gd^{3+}) affect the F_1 and F_2 isoenzymes of aldehyde dehydrogenase

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from horse liver. Most of the metal ions studied produce an initial activation of the enzyme at low metal ion concentrations (0.1–10 μM) while at higher concentrations (720 μM) the enzyme activity is inhibited.

The F_1 isoenzyme, which is believed to be of cytoplasmic origin (Eckfeldt et al., 1976), is considerably more sensitive to the presence of metal ions than is the F_2 isoenzyme, which is believed to be of mitochondrial origin. For the rat and beef liver enzymes, the effect of Mg^{2+} and Ca^{2+} ions is to activate the mitochondrial enzymes but to inhibit the cytoplasmic enzymes although the activation shows some pH dependence (Takahashi et al., 1981).

Recently (Takahashi et al., 1981), the suggestion has been made that the mitochondrial enzyme (F_2) exhibits half-of-the-sites reactivity, since in the presence of Mg^{2+} ions there is a 2-fold stimulation in the steady-state initial velocity over the range 0–800 μM. The amplitude of the pre-steady-state burst in the production of NADH is also doubled over the same concentration range. The results have been explained by proposing that, in the presence of Mg^{2+} ions, the tetrameric form of the enzyme, which functions with half-of-the-sites reactivity, is dissociated into a dimeric form, which possesses all-of-the-sites reactivity (Takahashi & Weiner, 1980; Takahashi et al., 1980).

In order to determine whether the activating effect of Mg^{2+} ions and the model proposed for the activation were common to all tissue aldehyde dehydrogenases, we have studied the effect of $MgCl_2$ on the cytoplasmic aldehyde dehydrogenase from sheep liver. We show that the cytoplasmic enzyme behaves quite differently in the presence of Mg^{2+} ions from the mitochondrial horse liver enzyme.

Experimental Procedures

Materials

Cytoplasmic aldehyde dehydrogenase (ALDH)[1] was prepared essentially as described by MacGibbon et al. (1979). Magnesium chloride was a R. P. Normapur guaranteed reagent from Prolabo (Paris, France), NAD^+ (grade III) and NADH (grade III) were Sigma (St. Louis, MO) chemicals used without further purification, propionaldehyde was obtained from Koch-Light Laboratories (Colnbrook, Bucks, U.K.), and all other chemicals were of the highest purity available.

Methods

Steady-State Kinetic Assays. These were performed either spectrophotometrically by monitoring the appearance of NADH at 340 nm or fluorometrically by monitoring the fluorescence of free NADH on excitation of the assay solution at 340 nm and measuring the emission at 455 nm. The usual conditions for V_{max} assays were 25 mM pH 7.6 NaH_2PO_4 buffer containing NAD^+ (1 mM) and propionaldehyde (20 mM), and a value of 0.25 s^{-1} (MacGibbon et al., 1977a) was used for k_{cat} to calculate the active-site concentrations. Kinetic experiments were carried out by using the same assay system, modifying the concentrations of substrates as required. Assays in the presence of Mg^{2+} ions were carried out with the appropriate additions of a stock solution of $MgCl_2$ (50 mM) in place of phosphate buffer so that the final assay volume was always 3.0 cm^3. The assay was always inititated by the addition of the aldehyde.

Stopped-Flow Measurements. Stopped-flow experiments were carried out by using a Durrum-Gibson D110 stopped-flow spectrophotometer (Durrum Instruments Corp., Palo Alto, CA) in either the absorbance or the fluorescence mode. The data were analyzed as described previously (Bennett et al., 1982).

NADH Titrations. Binding of NADH in the presence and absence of Mg^{2+} ions was carried out by using nucleotide fluorescence essentially as described previously (MacGibbon et al., 1979). The data for both NADH and NAD^+ titrations were analyzed by means of Scatchard plots and also as described by Luisi et al. (1973). Protein concentrations were determined by absorbance measurements at 280 nm by using a value of 11.3 for $E^{1\%}_{1cm}$ (Dickinson et al., 1981) and a molecular weight of 212 000 (MacGibbon et al., 1979).

NAD^+ Titrations. Equilibrium NAD^+ binding studies were carried out on an Aminco-Bowman spectrofluorometer at an excitation wavelength of 280 nm (5-nm band-pass) and an emission wavelength of 340 nm (10-nm band-pass) by monitoring the quenching of the protein fluorescence which occurs when NAD^+ binds to the enzyme (MacGibbon et al., 1979). The excitation shutter was kept closed, except when measurements were being taken, to minimize any photolytic quenching effects. NAD^+ was added in 0.01-cm^3 aliquots, by means of a micrometer-regulated Hamilton syringe, each aliquot adding about 0.5 μM NAD^+. The enzyme concentration was 1–2 μM, and usually 30 additions (ca. 15 μM) of the NAD^+ solution were added. A standard titration was always carried out first followed by titrations with added $MgCl_2$.

Ultraviolet Absorption Difference Spectrum. The ultraviolet difference spectrum between enzyme-bound and free NADH with and without $MgCl_2$ present was determined by using a Shimadzu MPS-5000 spectrophotometer. Two quartz UV cells were mounted in tandem in both the sample and reference compartments. One cell in the reference compartment contained enzyme (40 μM) and the other NADH (26 μM), while in the sample compartment one cell contained enzyme and NADH and the other buffer only. After the difference spectrum had been determined with these solutions, $MgCl_2$ was added to all the cells, and the difference spectrum was redetermined.

The enzyme, NADH, and $MgCl_2$ were added to the cells by using a glass syringe attached to a micrometer which delivered 0.01-cm^3 aliquots. A blank was also run with all four cells containing buffer only, to determine the difference between the two sets of cells. This difference was subsequently subtracted from the difference spectrum obtained with the various samples present.

Gel Filtration Chromatography. Gel filtration chromatography was carried out by using a 70 cm × 2.5 cm Sephacryl S300 column. The column was equilibrated with 0.022 M pH 7.3 phosphate buffer containing 0.1% v/v β-mercaptoethanol for 24 h prior to use. Samples (7 cm^3) were loaded onto the column which was eluted at a rate of 9.0 cm^3/h with the equilibration buffer. Samples were collected by using an LKB 7000 Ultrorac fraction collector with a drop counter attachment set at 70 drops per fraction.

An initial run was carried out with ALDH only, then the column was washed with elution buffer (2 L) containing 5 mM $MgCl_2$, and an enzyme sample containing 5 mM $MgCl_2$ was run through the column. A standard, yeast alcohol dehydrogenase, with a molecular weight of 150 000 was also run. The presence of ALDH was detected by assay, and the yeast alcohol dehydrogenase was detected by absorbance measure-

[1] Abbreviations: ALDH, aldehyde dehydrogenase; P1, catalytic low-K_m propionaldehyde binding site; P2, high-K_m propionaldehyde binding site; λ, observed first-order decay constant; EDTA, ethylenediaminetetraacetic acid.

ments at 280 nm. Where $MgCl_2$ was present, the ALDH assays were carried out in 33 mM pH 5.6 citrate buffer, which removes the inhibitory effect of $MgCl_2$. The elution volume for the gel filtration experiments was taken as the point at which the concentration of the enzyme had reached 50% of its maximum value.

Laser Light Scattering Experiments. The laser used in the light scattering experiments was a Spectra Physics Model 125A helium–neon laser with a power output of 55 mW at 623 nm which was coupled to a Precision Devices 4300 spectrometer and an ITT FW 130 photomultiplier tube. Signals from the photomultiplier tube were stored in a correlator which was interfaced to a pdp 11/03 computer (Digital Equipment Corp.) as described by O'Driscoll & Pinder (1980).

Cells polished on four sides were sonicated in hot radioactive decontamination detergent (Decon 75) for 90 s with an MSE 100-W ultrasonicator and then washed 10 times with hot water, twice with acetone and ethanol, and then with filtered distilled deionized water. Analar cyclohexane (16 cm^3) was centrifuged at 11000*g* for 2 h in stoppered glass tubes and then used to rinse both the laser cells and a glass syringe. The rinsing was carried out in an atmosphere of air which was filtered through a 0.22-μm Millipore filter. The enzyme and enzyme–$MgCl_2$ samples were centrifuged for 2 h at 11000*g* in stoppered plastic centrifuge tubes and then transferred to the glass cells in the filtered atmosphere, using the cyclohexane-rinsed syringe. The cells were immediately stoppered and then kept under refrigeration until needed.

Intensity fluctuations in the laser light, caused by scattering from macromolecules in solution, with time are characterized by the formation of a normalized intensity autocorrelation function, or NIAF (Jakeman & Pike, 1969), which can be formed by photocount autocorrelation. A plot of ln (NIAF − 1) against $^1/_2[4\pi n_0 2 \sin(\theta/2)/\lambda_0]^2$, were θ is the scattering angle, λ_0 is the wavelength of the incident radiation, and n_0 is the refractive index of the sample solution, yields a straight line of slope D_0 (the macromolecular diffusion coefficient at infinite dilution) which is proportional to the inverse of the molecular weight to the power of one third ($M^{-1/3}$).

Computer Simulations. Computer simulations of the steady-state behavior of ALDH in the presence of $MgCl_2$ were carried out by using a Cromenco Z2D minicomputer. Equations were derived for a number of possible mechanistic models by assuming that rapid equilibrium conditions prevailed (Segel, 1975). The computer was programmed in BASIC to calculate the reaction velocity at varying substrate concentrations by using the derived equations and various steady-state parameters which were entered into the program.

Results

Effect of $MgCl_2$ on V_{max}. When $MgCl_2$ was added as a solid or in solution to an ALDH assay with propionaldehyde (20 mM) as the substrate, a substantial decrease in the steady-state activity was observed in pH 7.6 25 mM phosphate buffer. The inactivation occurred within the time taken to add the $MgCl_2$ to the assay, and the recorder traces obtained after the $MgCl_2$ addition were linear for at least 10 min. The order of addition of $MgCl_2$ and substrates had no effect on the degree of inhibition produced, and plots of the percentage of initial velocity remaining vs. $MgCl_2$ concentration at both high (>1 mM) and low (<100 μM) propionaldehyde concentrations resulted in hyperbolic inhibition curves, with greater than 90% inhibition being obtained at $MgCl_2$ concentrations greater than 2 mM (Figure 1). The steady-state activity could not be reduced to zero even at much higher concentrations of $MgCl_2$. Even at very low concentrations of $MgCl_2$ (1–100 μM), there was

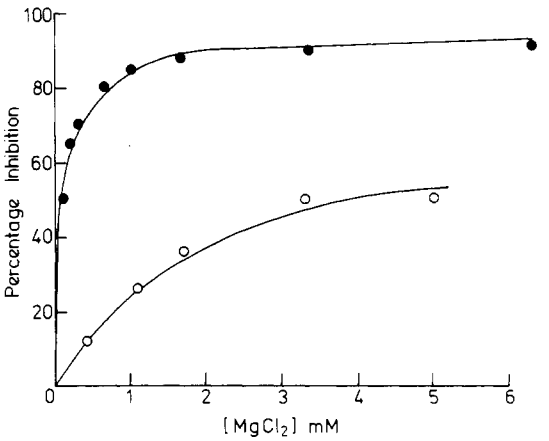


FIGURE 1: Inhibitory effect of $MgCl_2$ on the steady-state initial velocity for ALDH at pH 7.6. The assay mixture contained ALDH (2 μM), NAD^+ (2 mM), and propionaldehyde (20 mM). The open circles are replots of the data at low $MgCl_2$ concentrations (one-fiftieth of the values shown in the figure).

no evidence for any activation of the enzymic activity (see Figure 1), and over the pH range 6.0–8.0 in 35 mM phosphate buffers, the percentage inhibition obtained when $MgCl_2$ (1.6 mM) was added to enzyme assays remained constant at 88–90%.

Effect of Chelating Agents on $MgCl_2$ Inhibition. When trisodium citrate (6.6 mM) and tetrasodium pyrophosphate (6.6 mM) were added to assays, a 30% increase in the initial velocity was observed, but EDTA (6.6 mM) had no effect. All three substances, when added to assays which were inhibited by the addition of $MgCl_2$, caused an immediate increase in the steady-state activity provided that the concentration of the chelator was in excess of the $MgCl_2$ concentration. Continued addition of those substances eventually resulted in the complete removal of all inhibitory effects due to $MgCl_2$, showing that the effect was reversible.

The competitive inhibition pattern obtained when varying citrate ion concentrations were added to steady-state assays containing different levels of $MgCl_2$ and fixed levels of NAD^+ (2 mM) and propionaldehyde (20 mM) was consistent with the view that the chelators are competing with the enzyme for the Mg^{2+} ions. The slope replot was linear with a K_i value for magnesium ions of 330 μM.

NADH Titrations. The stepwise addition of aliquots (0.01 cm^3) of NADH (130–150 μM) to an ALDH solution (typically 1–2 μM) in a fluorometer cuvette resulted in a smooth titration curve (Figure 2), the replots of which resulted in a K_D value of 1.0 μM and a fluorescence enhancement factor of 5.8. These values are in good agreement with those previously reported from this laboratory (MacGibbon et al., 1979) of 1.2 and 5.6 μM, respectively. When $MgCl_2$ (1.6 mM) was added to the titration mixture, a significant increase in the fluorescence of the enzyme-bound NADH occurred, whereas the control titration showed that the fluorescence of free NADH was unaffected (Figure 2). Despite the increase in fluorescence of the E·NADH species in the presence of Mg^{2+} ions, Scatchard plots of the data showed that the concentration of NADH binding sites was unchanged (Figure 3). The dissociation constant (K_D) had, however, decreased to 0.2 μM as shown by the increase in the slope (Figure 3), and the average fluorescence enhancement factor in the presence of $MgCl_2$ showed a more than 3-fold increase (to a value of 18).

Trisodium citrate (6.6 mM) caused the fluorescence enhancement which is produced on binding of NADH to ALDH to decrease from 5.6 to 2.8 although the fluorescence of free

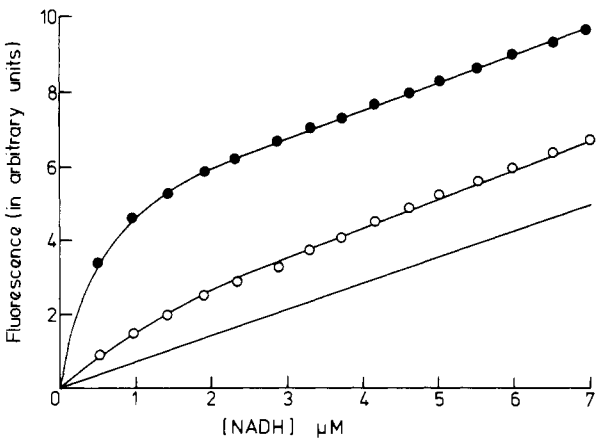


FIGURE 2: Effect of $MgCl_2$ on the number of NADH binding sites. The bottom line shows the fluorescence of NADH only in the presence and absence of $MgCl_2$, the open circles show the control titration in which ALDH was titrated with NADH, and the solid circles show the same titration in the presence of magnesium chloride (1.6 mM).

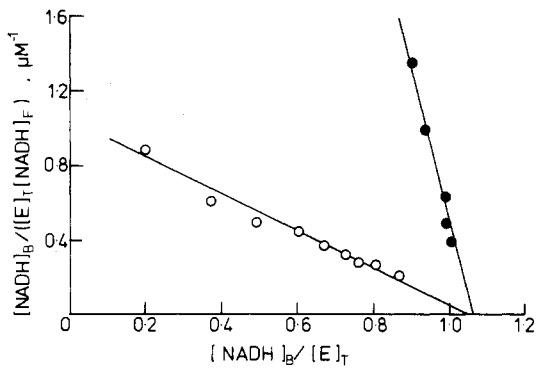


FIGURE 3: Scatchard plot of NADH titration data in the presence and absence of $MgCl_2$. The open circles show the replot for the control titration from which K_D was determined as 1.1 μM. The closed circles show the replot in the presence of $MgCl_2$ (1.6 mM) for which K_D was determined as 0.20 μM. Both lines extrapolate to give the same number of binding sites. The effect of dilution on the fluorescence intensity was corrected.

NADH was unaffected in the presence of citrate ions. While the NADH binding site concentration was unchanged from the value determined from the control titration, the dissociation constant increased to 1.8 μM. However, EDTA (6.6 mM) did not affect either the dissociation constant or the NADH binding site concentration.

NAD^+ Titrations. When NAD^+ (0.01-cm^3 aliquots of 300 μM NAD^+) was added to a cuvette containing ALDH (1–2 μM) in a fluorometer, the protein fluorescence was quenched, and a titration curve was obtained. After all the NAD^+ binding sites were occupied, there was a more or less linear quenching of the fluorescence upon further additions of NAD^+, partly as a result of inner filter effects from the absorption of the excitation energy by NAD^+ and partly as a result of a photolytic quenching effect caused by prolonged exposure of the enzyme to the exciting radiation. If ALDH was continually exposed to the exciting radiation over a 2–3-h period, considerable quenching of the protein fluorescence occurred (80%) with concomitant loss in ALDH activity. This effect could be minimized by leaving the excitation shutter closed except when measurements were being taken.

When the data were replotted as a Scatchard plot (Figure 4), a straight line was obtained, whose slope gave a dissociation constant of 3.2 μM and an NAD^+ binding site concentration equal to the NADH (or active site) binding site concentration. This K_D value was in reasonable agreement with the value of

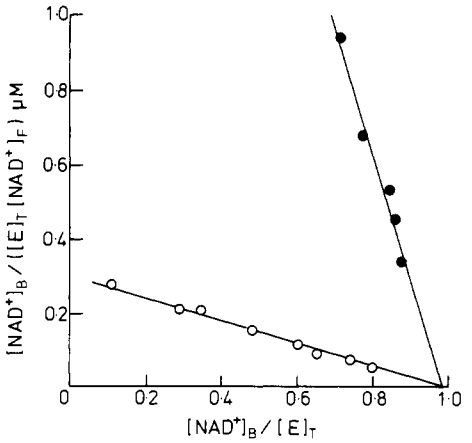


FIGURE 4: Effect of $MgCl_2$ on the number of NAD^+ binding sites. The data were obtained from the quenching of protein fluorescence which results from the binding of NAD^+. The open circles show the data for the control titration for which K_D was determined as 3.2 μM and ΔF_{max} was 12.6 fluorescence units. In the presence of 3.3 mM $MgCl_2$ (●), the dissociation constant was decreased to 0.31 μM, and ΔF_{max} increased to 27.4 fluorescence units. Both lines extrapolated to give the same number of NAD^+ binding sites.

8.0 μM determined from steady-state kinetic studies by MacGibbon et al. (1977a).

When $MgCl_2$ (3.3 mM) was added to the titration mixture, the maximum quenching of the protein fluorescence, obtained on saturation of the enzyme binding sites by NAD^+, was twice that obtained for the control titration. However, as was the case for the NADH titrations, although the dissociation constant determined from the Scatchard plot of the data (Figure 4) was decreased (by a factor of 10), the concentration of NAD^+ binding sites was unchanged by the presence of $MgCl_2$, being still equal to the NADH binding site concentration as determined for the original enzyme sample.

Effect of $MgCl_2$ on ALDH Subunit Composition. Since it has been demonstrated that Mg^{2+} ions cause the horse liver mitochondrial ALDH to dissociate into dimers (Takahashi & Weiner, 1980), attempts were made to determine whether $MgCl_2$ affected the degree of aggregation of cytoplasmic sheep liver ALDH. ALDH was eluted from a Sephacryl S300 column in pH 7.6 25 mM phosphate buffer in 58 h with an elution volume of 454 ± 2 cm^3 while under the same conditions yeast alcohol dehydrogenase was eluted in 59 h with an elution volume of 463 ± 2 cm^3. If it is assumed that there is a linear relationship between the elution volume and the logarithm of the molecular weight, a protein with a molecular weight of 106 000 (half the tetramer molecular weight) would have an elution volume of 471 cm^3. However, when the Sephacryl column was preequilibrated with $MgCl_2$ (5 mM) in the pH 7.6 25 mM phosphate buffer, the ALDH sample, also in the presence of 5 mM $MgCl_2$, again resulted in an elapsed time of 58 h and a similar elution volume of 456 ± 2 cm^3.

Laser light scattering experiments were also carried out to confirm that no dissociation of the ALDH tetramer was occurring in the presence of $MgCl_2$. A plot of ln (NIAF − 1) against $[\sin(\theta/2)]^2$ for ALDH (70 μM) in 35 mM pH 7.6 phosphate buffer was linear with a slope [equal to $^1/_2 D_0 \cdot (4\pi n_0/\lambda_0)^2$] of 28 000. When the experiment was repeated with 10 mM $MgCl_2$ added, there was a small decrease in the slope of the line to 24 500. Since λ_0 was constant and n_0 was unchanged in the presence of $MgCl_2$, the decrease in the slope corresponds to a decrease in D_0 and hence an increase in the molecular weight of the scattering particles. If the tetramer had been split in two by the presence of $MgCl_2$, the slope would be expected to increase to a value of 35 280 which is clearly

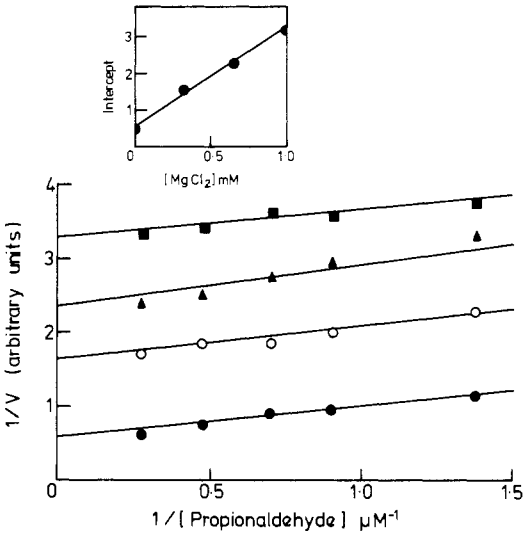


FIGURE 5: Lineweaver–Burk plot for inhibition by $MgCl_2$ at low concentrations of propionaldehyde (<60 μM). The effect of various concentrations of $MgCl_2$ [(●) 0 mM; (○) 0.33 mM; (▲) 0.66 mM; (■) 1.0 mM] was studied in pH 7.6 25 mM phosphate buffer at 25 °C. [NAD^+] was 2 mM, and K_i as determined from the intercept replot (inset) was 240 μM. The initial velocity was monitored by the nucleotide fluorescence as described under Methods.

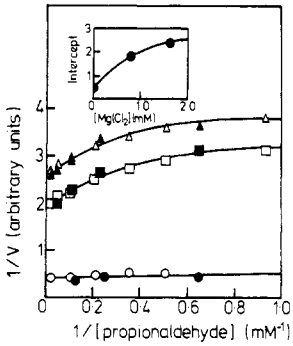


FIGURE 6: Inhibition of the steady-state initial velocity by $MgCl_2$ at high propionaldehyde concentrations at pH 7.6. Various concentrations of $MgCl_2$ [(●) no $MgCl_2$; (■) 0.83 mM; (▲) 1.6 mM] were added to steady-state assays which contained NAD^+ (2 mM) and a range of propionaldehyde concentrations in the millimolar range. The same initial velocity in the presence of 1.6 mM $MgCl_2$ was obtained even at 100 mM propionaldehyde. The initial velocity is expressed in arbitrary nucleotide fluorescence units. Also shown for comparison are the data points simulated on the basis of Scheme II [(○) no $MgCl_2$; (□) 0.83 mM $MgCl_2$; (△) 1.6 mM $MgCl_2$].

contrary to experiment.

Initial Velocity Inhibition Patterns with $MgCl_2$. The inhibition by $MgCl_2$ at low propionaldehyde concentrations (<100 μM) appeared to be uncompetitive (Figure 5), and the replot of the ordinate intercepts of the double-reciprocal plot vs. the $MgCl_2$ concentration was linear (Figure 5, inset) with an intercept inhibition constant (K_i) of 240 μM.[2] V_{max} was lowered at all concentrations of $MgCl_2$, and the apparent K_m for propionaldehyde was decreased.

At higher concentrations of propionaldehyde (2–20 mM), the inverse initial velocity plots were nonlinear (Figure 6), curving downward toward faster rates at very high propionaldehyde concentrations. The inhibition pattern appeared to be either mixed or noncompetitive but was clearly not competitive, since the initial velocity with 100 mM propionaldehyde and 1.6 mM $MgCl_2$ was almost identical with that obtained at 20 mM propionaldehyde and 1.6 mM $MgCl_2$. The replot of the intercepts was nonlinear (Figure 6, inset), curving downward, characteristic of hyperbolic mixed-type inhibition (Segel, 1975) which makes the estimation of inhibition constants difficult.

[2] The K_i values quoted in this work are expressed in terms of the total concentration of magnesium. The concentration of free magnesium is about 8% of the total under our experimental conditions, and hence all of the K_i values should be multiplied by 0.08 to give an estimate of the true K_i. The levels of both total magnesium and free magnesium used in this work are similar to those experienced physiologically.

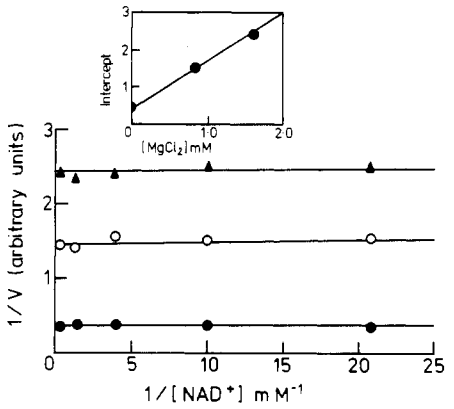


FIGURE 7: Effect of $MgCl_2$ on the $1/V$ vs. $1/[NAD^+]$ plot. The concentration of propionaldehyde was kept constant at 60 μM while the concentration of $MgCl_2$ was varied as follows: (●) no $MgCl_2$; (○) 0.83 mM; (▲) 1.67 mM. The K_i value determined from the intercept replot (inset) was 250 μM. The reactions were monitored by the nucleotide fluorescence as described under Methods.

The inhibition pattern when NAD^+ was the varied substrate at a fixed low concentration of propionaldehyde (60 μM) and with various levels of $MgCl_2$ also appeared to be simple uncompetitive (Figure 7) with a decreasing V_{max}, and an apparent K_m value for NAD^+, as the concentration of $MgCl_2$ was increased. The intercept replot was linear and gave an intercept inhibition constant of 250 μM (Figure 7, inset).

Ultraviolet Difference Spectrum. When NADH binds to ALDH, there is a shift of the absorption maximum to longer wavelengths which results in a decrease in absorbance of the enzyme-bound NADH in the region 300–350 nm. When $MgCl_2$ (3.3 mM) was added to each of the cuvettes, the difference spectrum was significantly altered with the absorbance difference between enzyme-bound and free NADH being approximately twice the values obtained in the absence of $MgCl_2$ at each wavelength from 300 to 350 nm.

Effect of $MgCl_2$ on NADH Displacement from the E·NADH Binary Complex. When NAD^+ (3 mM) was mixed with ALDH (3 μM) and NADH (18 μM) in the stopped-flow apparatus, a biphasic displacement curve was obtained when the fluorescence change was monitored at 435 nm. The decay constants, λ_f and λ_s, for the two exponentials (0.8 and 0.22 s^{-1}) were in good agreement with those previously obtained from this laboratory (MacGibbon et al., 1977b). The amplitude of the displacement was equal to 85–100% of the enzyme active-site concentration as determined by an NADH titration, also in agreement with MacGibbon et al. (1977b). The addition of $MgCl_2$ (3.5 mM) to the enzyme syringe (3 μM) containing NADH (12.8 μM) resulted in marked changes in the displacement curve when this solution was rapidly mixed with NAD^+ (3 mM). The decay constants for the fast process (λ_f) was reduced to 0.3 s^{-1} while the decay constant for the slow process (λ_s) was reduced to 0.025 s^{-1}. Due to the very slow nature of the second process, it was necessary to follow the reaction for more than 250 s to obtain the fluorescence value at infinite time, and at sweep times of this magnitude, it was difficult to distinguish the displacement signal from machine drift. The fluorescence change on displacement of NADH in the presence of $MgCl_2$ was greater than in the

absence of $MgCl_2$, being on average 1.5 times larger. Identical results were obtained when $MgCl_2$ was added to the syringe which contained NAD^+.

The displacement experiment was also performed by utilizing the difference in absorption between free and enzyme-bound NADH at 328 nm as described by MacGibbon et al. (1979). In the absence of $MgCl_2$, a biphasic trace was obtained as expected with rate constants of 0.65 and 0.2 s^{-1} for λ_f and λ_s, respectively. When $MgCl_2$ (3.3 mM) was added to either the ALDH syringe or the NAD^+ syringe, the displacement rate constants (λ_f and λ_s) were again decreased (to 0.2 and 0.02 s^{-1}, respectively). The amplitude of the displacement process was larger in the presence of $MgCl_2$, due to the change in the extinction coefficient at 328 nm (being 813 L mol^{-1} cm^{-1} as compared with a value of 481 L mol^{-1} cm^{-1} in the absence of $MgCl_2$).

When trisodium citrate (60 mM) was added to the NAD^+ syringe in NADH displacement experiments as described above, an increase in the displacement rates was observed with λ_f increasing to 2.9 s^{-1} and λ_s increasing to 0.5 s^{-1}.

Effect of $MgCl_2$ on NADH Association. When ALDH (3 μM) was mixed with NADH (18 μM) in the stopped-flow spectrophotometer, a biphasic binding curve was observed when the fluorescence emission at 435 nm was monitored. The rate constants (5 ± 2 s^{-1} for λ_f and 1 ± 0.2 s^{-1} for λ_s) were similar to those reported previously (MacGibbon et al., 1977b), and the amplitude of the association process was greater than 90% of the NADH binding site concentration. The addition of $MgCl_2$ (3.5 mM) to either the NADH or the ALDH solution resulted in a slight increase in the rate constants for both λ_f (8 ± 2 s^{-1}) and λ_s (1.3 ± 0.25 s^{-1}), but the amplitude was 2–3 times greater than in the absence of $MgCl_2$.

The rate of binding of NADH to ALDH was also monitored by the quenching of protein fluorescence which occurs on coenzyme binding (MacGibbon et al., 1977c). When ALDH (3 μM) was mixed with NADH (18 μM), a biphasic quenching curve was again observed (λ_f = 4 ± 1 s^{-1} and λ_s = 0.8 ± 0.2 s^{-1}), and in the presence of $MgCl_2$ (3.3 μM), there was a slight increase in the magnitude for both of these rate constants (to 8 ± 2 and 1.15 ± 0.2 s^{-1}, respectively), but the amplitude of the overall quenching process was twice that obtained in the absence of $MgCl_2$.

The rate of NAD^+ binding was also monitored by protein fluorescence quenching techniques (MacGibbon et al., 1977c), but the addition of $MgCl_2$ (3.3 mM) to the enzyme solution had no apparent affect on the rate constant for the process.

Effect of $MgCl_2$ on the NADH Burst. When ALDH (3 μM) was mixed with saturating concentrations of NAD^+ (3 mM) and propionaldehyde (40 mM), a burst in the production of NADH was observed when the reaction was monitored in fluorescence at 435 nm as we have previously reported (MacGibbon et al., 1977c). The burst rate constant was 9.3 s^{-1}, and the amplitude of the process was equal to the enzyme active-site concentration (1.48 μM or 804 mV). When the experiment was repeated with $MgCl_2$ (3.3 mM) added to the ALDH solution, the burst rate constant was 12.3 s^{-1}, and the amplitude of the process was virtually unchanged (720 mV).

When a proton burst experiment was carried out as described by Bennett et al. (1982) in the presence of $MgCl_2$ (1.6 mM), the burst rate constant was 11 s^{-1}, and the observed burst amplitude remained equal to 90% of the enzyme active-site concentration.

Discussion

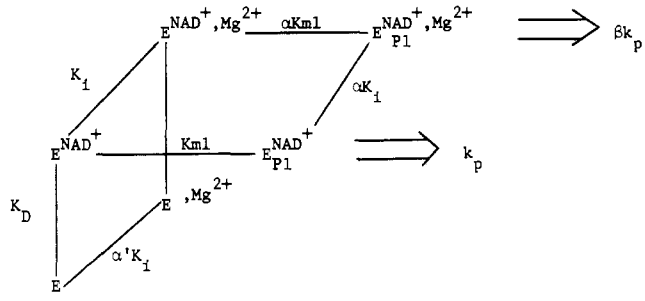
Effect of $MgCl_2$ on Physical Properties of ALDH. The effect of Mg^{2+} ions on the cytoplasmic ALDH from sheep liver was markedly different from that reported by Takahashi & Weiner (1980) and Venteicher et al. (1977) for the mitochondrial ALDH from horse liver. There was no evidence for activation of the steady-state activity at any concentration of $MgCl_2$ at pH 7.6, and the inhibitory effect of a fixed concentration of $MgCl_2$ was unchanged between pH 6 and pH 8. The number of NADH binding sites per tetramer for sheep liver ALDH ranged from 1 to 3 when determined for a large number of enzyme preparations; hence, it is not certain whether there are two or four functional subunits per tetramer, and therefore, it is not clear whether ALDH functions with half-of-the-sites reactivity. However, it was quite clear from the Scatchard analysis of both the NADH and NAD^+ titration data that the number of NADH and NAD^+ binding sites were identical and that neither value was changed by the presence of Mg^{2+} ions, as would have been expected if tetramer dissociation was occurring with an increase in the number of functioning subunits. Thus, even if the enzyme is functioning with half-of-the-sites reactivity, there is certainly no change to all-of-the sites reactivity in the presence of $MgCl_2$ as found for the horse liver enzyme.

In agreement with these conclusions, neither the gel filtration nor the laser light scattering experiments showed any evidence for a change in the molecular weight of ALDH, although both of these techniques would be expected to detect a halving of the molecular weight if dissociation of the ALDH tetramer into a pair of dimers was in fact occurring. Thus, it must be concluded that Mg^{2+} ions had no effect on the subunit composition of sheep liver cytoplasmic ALDH, in marked contrast with the reports (Takahashi & Weiner, 1980; Takahashi et al., 1981) for the horse liver enzyme. This fundamental difference in behavior between the two enzymes is probably related to their different intracellular localizations since Takahashi et al. (1981) have reported that for both rat and beef liver ALDH, Mg^{2+} ions activate the mitochondrial enzyme but inhibit the cytoplasmic enzyme. The physiological significance of this difference is, however, not clear.

Important changes in the physical properties of sheep liver cytoplasmic ALDH were, however, produced by the presence of $MgCl_2$ which, in the absence of contradictory evidence from coenzyme titrations or from molecular weight studies, could be incorrectly interpreted in terms of an increase in the number of functioning subunits. For example, there were significant increases in the amplitudes of both the NAD^+ and NADH titrations, which appeared to correlate with the change in magnitude of the calculated dissociation constants, and also for the NADH displacement experiments in either absorbance or nucleotide fluorescence. The amplitude of the burst in NADH (measured by the nucleotide fluorescence) was virtually unchanged in the presence of Mg^{2+} ions, and the amplitude of the proton burst (measured by the absorbance at 540 nm) was also unchanged, being equal to the active-site concentration both with and without $MgCl_2$, thus confirming that there was no change in the concentration of significant transient species. Therefore, it is obvious that the observed amplitude increases in the other experiments are due to changes in the intrinsic fluorescence and spectroscopic properties of ALDH in the presence of $MgCl_2$ probably as a result of changes in the tertiary structure.

Effect of $MgCl_2$ on the Steady-State Mechanism at Low Propionaldehyde Concentrations. When the propionaldehyde concentration was kept sufficiently low (60 μM) so that only the high-affinity (P1) propionaldehyde binding site (MacGibbon et al., 1977a; Bennett, 1982) was saturated, the inhibition pattern with respect to NAD^+ was apparently linear

Scheme I



uncompetitive. Since the inhibitory effect of $MgCl_2$ could not be overcome even at infinitely high NAD^+ concentrations, the observed inhibition pattern was not consistent with the formation of an $NAD^+ \cdot Mg^{2+}$ inhibitor complex in some preequilibration step. If this were so, as the concentration of NAD^+ was increased, an increasing concentration of free NAD^+ would be available which could compete with the inhibitor complex for the coenzyme binding site until the inhibitory effect was removed. Thus, a competitive inhibition pattern would be expected, contrary to experiment. Also, while NAD^+ can complex Mg^{2+} ions, the presence of 25 mM phosphate buffer in all of the assays would result in the majority of the Mg^{2+} ions being present as phosphate complexes. Using a dissociation constant of 20 mM for the $NAD^+ \cdot Mg^{2+}$ complex (Apps, 1973) and a dissociation constant of 3.16 mM for the magnesium phosphate complex (Dawson et al., 1969), it can be calculated that less than 1% of the NAD^+ will be present as an $NAD^+ \cdot Mg^{2+}$ complex at the concentrations of $MgCl_2$ used here. However, using the K_i value determined from the inhibition data, and assuming rapid equilibrium conditions, it can be shown that at 2 mM $MgCl_2$ most of the enzyme will be present as an $E \cdot NAD^+ \cdot Mg^{2+}$ complex. That rapid equilibrium conditions do prevail was confirmed by the ready reversibility of the inhibition by citrate ions (and also by pyrophosphate ions and EDTA) which reactivated the enzyme in a competitive fashion. The inhibition constant of 330 μM obtained for the reactivation process suggests that the reactivation is due to the removal of the Mg^{2+} ion from its binding pocket on the enzyme by chelation with the citrate ions.

When propionaldehyde was the varied substrate (at concentrations from 0.8 to 5 μM) at saturating levels of NAD^+, the inhibition pattern again appeared to be uncompetitive and gave an apparently linear replot for the intercepts against the $MgCl_2$ concentration. The fact that virtually the same inhibition constant was obtained from both the NAD^+ and low propionaldehyde inhibition data is consistent with the view that ALDH possesses a unique binding site for magnesium ions which is separate from both the coenzyme and low propionaldehyde (P1) binding sites. Binding of Mg^{2+} ions to this site decreased the V_{max} for the reaction and increased the apparent affinity of the $E \cdot NAD^+ \cdot Mg^{2+}$ species for propionaldehyde. The simplest model which can account for both the NAD^+ and low propionaldehyde inhibition data is shown in Scheme I. In Scheme I, ALDH is envisaged as possessing three binding sites: a coenzyme binding site shown as a superscript, a magnesium ion binding site also shown as a superscript separated from the NAD^+ binding site by a comma, and a high-affinity binding site for propionaldehyde (P1) shown as a subscript. So that the validity of Scheme I could be confirmed, calculations were made by assuming that rapid equilibrium conditions applied to all steps except the catalytic steps. The value of 8.0 μM obtained from steady-state kinetics (MacGibbon et al., 1977a) was assigned to K_D, and in the absence of a value for the dissociation constant of propionaldehyde, the low K_m1 value of 1.2 μM reported by MacGibbon et al. (1977a) was used. The K_i was 320 μM, the k_p was set equal to 1.0, β was 0.075 since the steady-state rate was inhibited to the extent of 93% at saturating $MgCl_2$ concentrations, and α and α' were treated as adjustable parameters. An uncompetitive pattern was only obtained from Scheme I if it was assumed (as indicated by the experimental data) that Mg^{2+} bound more tightly to $E_{P1}^{NAD^+}$ than to E^{NAD^+} and thus (as required by the principle of microscopic reversibility) propionaldehyde bound more tightly to $E^{NAD^+,Mg^{2+}}$ (i.e., $\alpha < 1$). A good fit to the data was obtained when α was 0.25 for both NAD^+ and propionaldehyde as the variable substrates, but the slope and intercept replots were nonlinear. However, the extent of the predicted nonlinearity would have been hard to detect experimentally over the concentration ranges of $MgCl_2$ used in this study, and hence, this prediction does not represent a significant difference between the calculations based on Scheme I and the experimental data. The calculations were not very sensitive to the value of α', and hence, it was unclear whether $MgCl_2$ binds to the free enzyme with the same K_i value as required for binding to E^{NAD^+}. What is clear, however, is that the inhibitory effect of Mg^{2+} ions is due to their effect on the catalytic steps of the reaction and not to a decrease in the affinity of ALDH for its substrates.

Evidence for Distinct Magnesium Ion and Modifier Binding Sites. At levels of propionaldehyde greater than 1 mM [the concentration region where there is substrate activation in the absence of Mg^{2+} (MacGibbon et al., 1977a)], the inhibition patterns in the presence of $MgCl_2$ also showed substrate activation and were significantly different from the inhibition patterns observed at low (<100 μM) propionaldehyde concentrations. We have previously suggested (MacGibbon et al., 1977a) that this nonlinearity can be explained if it is assumed that a propionaldehyde binding site exists for which propionaldehyde has a low affinity, although it is not clear whether such sites are on different isoenzymes, the same enzyme, or the same location on the enzyme as the site with a high affinity for aldehydes. Bennett (1982) has recently provided evidence that this second propionaldehyde binding site (designated P2) is in fact a modifier site, occupation of which increases the steady-state initial velocity 3-fold, which indicates (although it does not demand) that the two sites may be on the same subunit. Since the inhibitory effect of $MgCl_2$ could not be removed even at very high (40–100 mM) propionaldehyde concentrations, it is obvious that the Mg^{2+} ion binding site cannot be identical with the modifier site, and hence, the metal ion binding site (shown in Scheme I) must be regarded as separate from the other binding sites which have been proposed for ALDH (MacGibbon et al., 1977a; Bennett, 1982).

If Scheme I is extended by the addition of the modifier (P2) site (Scheme II) which is shown for convenience as a second subscript (no spatial connotations are intended by the scheme), the experimental data can be satisfactorily fitted by the model, with the same parameters as required to fit the low propionaldehyde concentration data together with the parameters appropriate for the P2 binding site. For calculations based on Scheme II, rapid equilibrium conditions were again assumed except for those steps which lead directly (and irreversibly) to products. K_m2 was assigned a value of 3.5 mM (MacGibbon et al., 1977a), and the rate of formation of products from $E_{P1,P2}^{NAD^+}$ was set equal to $3k_p$ since the k_{cat} value for the steady-state reaction increases by a factor of 3 at high propionaldehyde concentrations (MacGibbon et al., 1977a). The

Scheme II

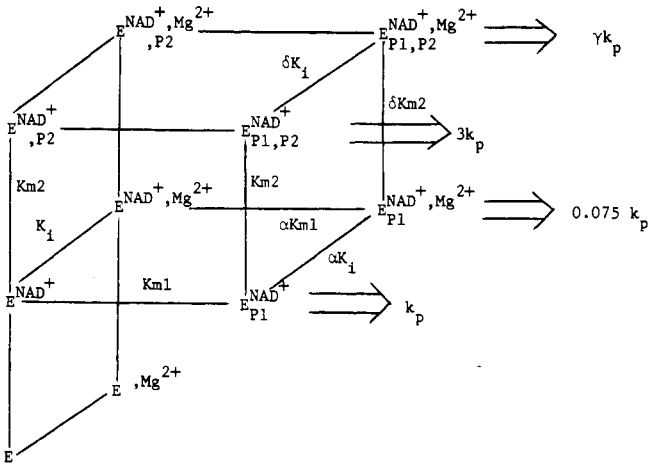


Table I: Kinetic Constants and Parameters Used in the Simulations of Schemes I and II

kinetic constants (pH 7.6)	parameters
$K_{m1} = 1.1\ \mu M$[a]	$\alpha = 0.25$
$K_{m2} = 3.5$ mM[a]	$\beta = 0.075$
$K_i = 320\ \mu M$[b]	$\gamma = 0.2$
$K_D = 8\ \mu M$[a]	$k_p = 0.082\ s^{-1}$[a]

[a] MacGibbon et al. (1977a). [b] This constant was determined as described in the text and is defined with respect to the total concentration of magnesium ions, although the concentration of free magnesium ions is very much less in phosphate buffers.

rate of formation of products from $E_{P1,P2}^{NAD^+,Mg^{2+}}$ was allowed to vary by allocating different values to γ on the basis of the known percentage inhibition at high propionaldehyde concentrations, and all other parameters were kept the same as for Scheme I. Although Scheme II looks complex, the rapid equilibrium assumption allows the required equations to be easily derived without knowledge of all of the equilibrium constants, and using these equations, it was possible to reproduce all of the experimental data to a good degree of approximation (see Figure 6). To achieve a good fit to the data, it was necessary to increase K_m2 for the binding of propionaldehyde to ALDH forms which contain magnesium (in contrast to the behavior at low propionaldehyde concentrations) and to assign a value of 0.2 to the parameter γ. (A summary of the constants which were used to fit Scheme II to the experimental data is given in Table I). The model correctly predicts a nonlinear hyperbolic relationship between the intercepts of the Lineweaver–Burk plot and the $MgCl_2$ concentration, since at sufficiently high concentrations of $MgCl_2$ it is possible to convert all of the enzyme into the $E_{P1,P2}^{NAD^+,Mg^{2+}}$ form and thereafter $MgCl_2$ can have no further effect. Thus, Scheme II is a good approximation of the actual mechanism which is operating at both high and low propionaldehyde concentrations and confirms that the inhibitory effect of magnesium is caused by changes in the catalytic steps.

Mechanism of Inhibition by $MgCl_2$. Stopped-flow experiments showed that $MgCl_2$ had no effect on the transient kinetics of propionaldehyde oxidation and therefore that the inhibitory effect of Mg^{2+} ions cannot be accounted for in terms of a decrease in any of the rate constants for substrate binding steps, or for the aldehyde-induced conformational change which controls the rate of both the proton and NADH bursts (Bennett et al., 1982). The fast (λ_f) and slow (λ_s) decay constants for the biphasic binding of NADH to the enzyme were also both unaltered, within experimental error, in the presence of $MgCl_2$, but the corresponding values for the biphasic displacement of NADH from the binary E·NADH complexes were both significantly reduced. The reduction in λ_s (from 0.22 s^{-1} in the absence of $MgCl_2$ to 0.02 s^{-1} in its presence) was approximately the same as the reduction in k_{cat} (0.25–0.03 s^{-1}) at high propionaldehyde concentrations, which suggests that when both the P1 and P2 propionaldehyde binding sites are occupied the inhibition may be entirely due to a decrease in the rate constants for the biphasic displacement process (Scheme III). (In Scheme III, *E·NADH·Mg^{2+} represents a conformationally rearranged form of the E·NADH·Mg^{2+} complex which is first formed when NADH binds to ALDH.)

Scheme III

$$*E\cdot NADH\cdot Mg^{2+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot NADH\cdot Mg^{2+} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + NADH + Mg^{2+}$$

Since λ_f and λ_s are quite complex functions of the rate constants for Scheme III,[3] it is not a simple matter to assign absolute values to the individual rate constants when Mg^{2+} ions are present. However, for satisfaction of the experimentally obtained values of λ_f and λ_s for both the NADH displacement and association experiments (both of which are still biphasic), the relative amplitudes of the fast and slow processes of both of these experiments, and the mathematical relationships between λ_f, λ_s, and the individual rate constants,[3] good estimates may in fact be made. The set of values k_1 = 0.075 s^{-1}, k_{-1} = 0.15 s^{-1}, k_2 = 0.1 s^{-1}, and $k_{-2} = 5 \times 10^5$ L mol^{-1} s^{-1} gave a satisfactory agreement with the experimental data and when substituted into the expression for K_D[3] yielded a value of 0.13 μM, which is similar to the experimentally determined value (0.2 μM). Thus, when both propionaldehyde binding sites are occupied, Mg^{2+} ions caused inhibition of the steady-state rate, largely by reducing the magnitude of k_1 (from 0.22 to 0.075 s^{-1}) and k_2 (from 0.8 to 0.1 s^{-1}) and by increasing the magnitude of k_{-1} (from 0.05 to 0.15 s^{-1}). However, since the NADH association rate constant (k_{-2}) was virtually unaltered in the presence of $MgCl_2$, it seems likely that Mg^{2+} ions are not involved in this step. Since the binding of Mg^{2+} ions is apparently fast, these results may be understood if Mg^{2+} ions can only bind, to any significant extent, to the E·NADH species (as would be the case if K_i were much greater than 350 μM for the E·Mg^{2+} species). In this case, NADH would bind to ALDH with its usual rate constant following which Mg^{2+} ions would rapidly bind to form the E·NADH·Mg^{2+} species and all further changes (including the dissociation step, k_2) would involve magnesium.[4]

Like other dehydrogenases which have been studied, ALDH appears to undergo conformational changes (Bennett et al., 1982) during its catalytic cycle. One occurs when NAD^+ binds to the enzyme, and a second one is caused by the binding of propionaldehyde in the P1 binding site, and the data presented

[3] The decay constants for Scheme III are given by the expressions $\lambda_f = (P + Q)/2$ and $\lambda_s = (P - Q)/2$ where $P = k_1 + k_{-1} + k_2 + k_{-2}$ and $Q = [P^2 - 4(k_1k_2 + k_{-1}k_{-2} + k_1k_{-2})]^{1/2}$. For the displacement experiments, $k_{-2} = 0$ since the presence of high concentrations of NAD^+ prevents the recombination of enzyme and NADH. For Scheme III, the dissociation constant (K_D) is given by the expression $k_2k_1/(k_{-2}k_{-1} + k_{-2}k_1)$, and hence a further check on the validity of the assigned values for the individual rate constants is possible.

[4] It is interesting to note that since $MgCl_2$ decreases the K_D value for dissociation of NAD^+ from the binary E·NAD^+ complex without increasing the rate constant for NAD^+ binding, the rate constant for the NAD^+ dissociation must also be decreased.

in this paper show that Mg^{2+} ions do not interfere with this aldehyde-induced conformational change. However, binding of Mg^{2+} ions to ALDH clearly does cause changes in the tertiary structure as evidenced by the changes in the spectroscopic and fluorescence properties associated with the enzyme. It seems evident that these changes are associated with the reduced rates of coenzyme dissociation (and hence tighter binding) and consequent decrease in the steady-state rate. A possible mechanism for these effects can be envisaged on the basis of the crystal structure which has been reported for lactate dehydrogenase (Holbrook et al., 1975). For lactate dehydrogenase, there is a cavity near the NAD^+ binding site, which is lined with carboxylate groups and a single tyrosine residue, which is ideally suited as a binding site for Mg^{2+} ions. If for ALDH a similar cavity were to be formed as a result of the NADH binding step (k_{-2} in Scheme III), then binding of Mg^{2+} ions in this cavity would lead to a decrease in the dissociation step (k_2) as is experimentally observed. If the subsequent isomerization of the E·NADH complex (steps k_1 and k_{-1} of Scheme III) involved the movement of a further carboxylate group into the region of the cavity (as is the case for lactate dehydrogenase), then the tighter binding of NADH and the associated decrease in the steady-state rate would be explained.

At low concentrations of propionaldehyde (when only the P1 binding site is occupied), and for aromatic aldehydes, the high degree of inhibition of the steady-state rate cannot be entirely accounted for on the basis of the effect of $MgCl_2$ on λ_s for displacement of NADH for the binary E·NADH complex, since the inhibited k_{cat} values were significantly less than the reduced value for λ_s. Magnesium chloride must therefore be affecting other steps after hydride transfer, and although several possibilities may be envisaged, in the absence of crystallographic data for ALDH, the precise manner by which Mg^{2+} ions exert their inhibitory effect must remain a matter for speculation.

Acknowledgments

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Registry No. Aldehyde dehydrogenase, 9028-86-8; Mg, 7439-95-4; NAD, 53-84-9; NADH, 58-68-4; propionaldehyde, 123-38-6.

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