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Effects of pH on Horse Liver Aldehyde Dehydrogenase: Alterations in Metal Ion Activation, Number of Functioning Active Sites, and Hydrolysis of the Acyl Intermediate†

Kojiro Takahashi, Henry Weiner,* and David L. Filmer

ABSTRACT: The reactivity of the mitochondrial (p*I* = 5) isoenzyme of horse liver aldehyde dehydrogenase was determined by studying the effects of pH on steady-state velocity, burst magnitude, and molecular weight of the enzyme in the absence and presence of Mg^{2+} ions. The Mg^{2+} ion activation of the steady-state velocity at pH 7.5 has been explained through a mechanism involving alteration of the tetrameric enzyme, functioning with half-of-the-sites reactivity, to a dimeric enzyme, functioning with all-of-the-sites reactivity [Takahashi, K., & Weiner, H. (1980) *J. Biol. Chem. 255*, 8206–8209]. With increasing pH, the tetrameric enzyme dissociated even in the absence of Mg^{2+} ions to the more active dimeric state. The pH-dependent dissociation was goverened by proton release from a group with pK = 9.5. After correcting for the increased number of functioning active sites, determined from the pre-steady-state burst, we calculated that elevated pH also caused an increase in the velocity of the rate-limiting step, hydrolysis of the acyl-enzyme intermediate. This event was governed by the ionization of two groups, with pK = 7.2 and 9.5, respectively. If these groups are directly involved in the catalytic step, a mechanism involving histidine acting as a general base can be proposed for the former group. The latter group may be involved in a charge relay activation process which only occurs at elevated, nonphysiological pH. The importance of the latter is questionable, as there is only a 3-fold increase in V_{max} when this group is involved in catalysis.

Both mitochondrial (p*I* = 5) and cytosolic (p*I* = 6) isozymes of horse liver aldehyde dehydrogenase (aldehyde:NAD oxidoreductase, EC 1.2.1.3) are isolated as tetramers (Feldman & Weiner, 1972; Eckfeldt et al., 1976). Each exhibits half-of-the-sites reactivity (Weiner et al., 1976; Eckfeldt & Yonetani, 1976) and have broad substrate specificity. The activity of the p*I* 5 isozyme is enhanced 2-fold by Mg^{2+}, Ca^{2+}, or Mn^{2+} ions at pH 7.5 (Takahashi et al., 1980a). The p*I* 6 isozyme is inhibited by these metal ions (Weiner & Takahashi, 1981) but is activated by lanthanide ions (Venteicher et al., 1977).

The molecular basis for the Mg^{2+} activation of the p*I* 5 isozyme has been explained through a mechanism relating to both a change in molecular weight and a change in the number of reacting sites. That is, in the presence of Mg^{2+} ions, the tetrameric form of the enzyme which functions with half-of-the-sites reactivity is dissociated to a pair of dimers, each possessing all-of-the-sites reactivity (Takahashi & Weiner, 1980; Takahashi et al., 1980b).

We have recently extended the study of the metal ion activation to aldehyde dehydrogenases isolated from species other than horse. With both rat and beef liver aldehyde dehydrogenases, it has been found that Mg^{2+} of Ca^{2+} ions activate the mitochondrial enzyme but inhibit the cytosol enzyme (Weiner & Takahashi, 1981). However, when assayed at pH 9.3 rather than 7.5, it has been shown that beef liver mitochondria aldehyde dehydrogenase is actually inhibited by Mg^{2+} ions but is still activated by Ca^{2+} ions (Takahashi et al., 1979). The differential effect of metal stimulation has also been observed with the sheep liver mitochondrial enzyme. Dickinson has found that this enzyme is activated by Mg^{2+} ions at pH 7.5 but not at pH 8.0.[1]

In this study, we investigated the effect of Mg^{2+} activation of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase over a wide pH range and compared it to the unactivated form. We found that the tetrameric form of the enzyme, even in the absence of Mg^{2+} ions, can dissociate to the more active dimer form at higher pHs. We also determined the effect of pH on the rate-limiting deacylation step (Weiner et al., 1976) during the enzyme-catalyzed process.

Materials and Methods

Propionaldehyde and NAD were purchased from Eastman Organic Chemicals and P-L Biochemicals, respectively. $MgCl_2$ (analytical reagent) was the product of Mallinckrodt Chemical

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[1] Dr. F. Mark Dickinson, University of Hull, United Kingdon, personal communication.

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Works. Ampholines were from LKB Produkter AB. Buffer solutions, prepared with deionized, distilled water, were 10 mM sodium acetate (pH 2.9–6.8), 10 mM sodium phosphate (pH 5.1–8.1), and 10 mM sodium pyrophosphate (pH 8.7–10.3); each buffer contained 100 mM NaCl.

Isolation of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase and determination of specific activity and concentration were performed as previously reported (Feldman & Weiner, 1972). The purified enzyme obtained after isoelectric focusing was used without dialysis in kinetic studies since the specific activity did not change in the presence of ampholine and sucrose. The preparation was dialyzed at 4 °C for 12 h against 0.1 M sodium phosphate, pH 6.0, prior to molecular weight determinations.

Kinetic studies were performed at 25 °C with a Gilford Model 240 spectrophotometer connected to a Sargent recorder. The reaction was initiated by addition of 50 μL of enzyme solution to 1 mL of assay mixture (the final concentrations of enzyme, propionaldehyde, and NAD were 0.39, 128, and 105 μM, respectively), and Mg^{2+} ions were added to the assay mixture when required. The steady-state velocity was obtained from the slope of the linear absorbance change at 340 nm due to formation of NADH which occurred between 1 and 2 min after the reaction was initiated (Takahashi & Weiner, 1980). The pre-steady-state burst magnitude, an indicator of the number of functioning active sites, was estimated by extrapolating from the linear portion of the absorbancy change obtained from the steady-state velocity assays to time zero.[2] NAD and NADH concentrations were determined spectroscopically by using extinction coefficients of 18×10^3 (260 nm) and 6.22×10^3 M^{-1} cm^{-1} (340 nm), respectively. Propionaldehyde concentration was determined enzymatically by measuring the complete oxidation of the substrate with liver aldehyde dehydrogenase.

The molecular weight of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase was determined by sedimentation equilibrium with a Beckman Model E ultracentrifuge equipped with an ultraviolet scanner at 6000 rpm, 8 °C, for 24 h as described previously (Feldman & Weiner, 1972; Takahashi & Weiner, 1980). The enzyme solution, dialyzed at pH 6.0, was added to the buffer solution at selected pHs (the final enzyme concentration was 0.8 μM). The pH of the enzyme solution was measured prior to the ultracentrifugation, and the enzyme activity was measured prior to and after the run.

Fluorescence polarization measurements were performed with an Aminco-Bowman spectrofluorometer at 25 °C by exciting the aldehyde dehydrogenase bound NADH at 340 nm and measuring the emisson at 450 nm (Takahashi et al., 1980b). All solutions were filtered through a Millipore filter before use. Fluorescence polarization determinations at desired pHs were performed with the emission and excitation polarizers in both the perpendicular and parallel positions. It is possible to calculate the percentage polarization as

$$\% P = \frac{F_{BB} - F_{EB}(F_{BE}/F_{BB})}{F_{EE} + F_{EB}(F_{BE}/F_{BB})} \times 100 \qquad (1)$$

where F is the fluorescence value and E and B refer respectively to the vertical and horizontal positions of the polarizers (Azumi & McGlynn, 1962).

[2] In previous studies, the magnitude of the pre-steady-state burst of NADH formation has been determined from stopped-flow tracings (Takahashi & Weiner, 1980). In this study, however, a stopped-flow apparatus was not used. It was found that a simple extrapolation to time = 0 could be made from spectrophotometric tracings in order to determine the magnitude of the pre-steady-state burst. This technique has been previously used to assess the magnitude of the burst when the rate constants were not investigated (Weiner et al., 1976).

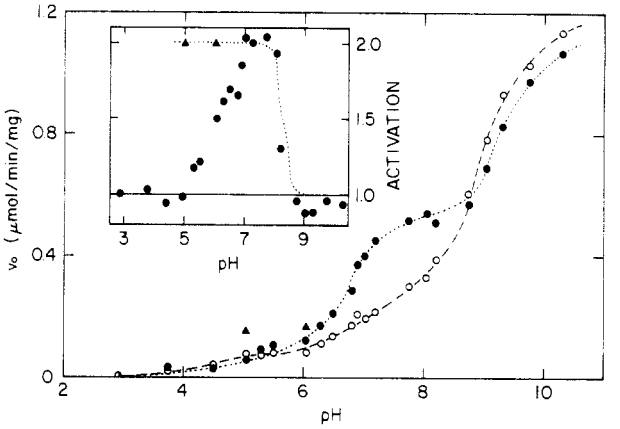


FIGURE 1: Steady-state velocity of aldehyde dehydrogenase as a function of pH in the absence and presence of Mg^{2+} ions. Mg^{2+} ion concentration was 0 (○), 0.7 mM (●), 1.2 mM at pH 6.0 (▲), and 2 mM at pH 5.1 (△). In the insert, the activation values were calculated by dividing the velocities in the presence of Mg^{2+} ions by those in its absence.

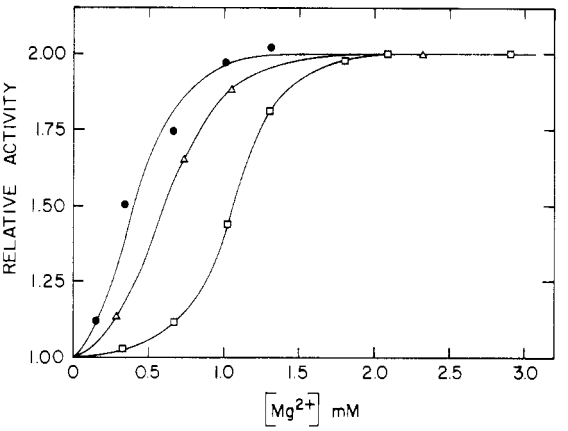


FIGURE 2: Mg^{2+} ion stimulation of aldehyde dehydrogenase at acid pHs. The steady-state velocity was obtained at pH 5.1 (□), pH 5.6 (△), and pH 6.0 (●). In all cases. a 2-fold activation was obtained, but a different Mg^{2+} in concentration was required. The data was used to complete the experiments described in Figure 1.

Results

pH–Velocity Profiles in the Absence and Presence of Mg^{2+} Ions. Our initial study with the p*I* 5 isozyme of horse liver aldehyde dehydrogenase has shown that compared to the activity at pH 9, the relative activity is 26% at pH 7, 51% at pH 8, and 165% at pH 10 (Feldman & Weiner, 1972). These overall results were verified and expanded by repeating the pH–velocity profile in the absence and presence of Mg^{2+} ions (Figure 1). The pH–velocity profile in the absence of added Mg^{2+} ions cannot be fitted to a simple sigmoidal curve. This indicates that the events are not governed by just one proton dissociation. This type of pH–velocity profile has been observed also for the p*I* 6 isozyme of horse liver aldehyde dehydrogenase (Eckfeldt & Yonetani, 1976).

Assays performed in the presence of a fixed concentration of Mg^{2+} ions as a function of pH revealed a more complex pattern (Figure 1). Below pH 7.5, less than the 2-fold activation was observed with 0.7 mM Mg^{2+} ions. However, when the concentration of Mg^{2+} ions was increased, a 2-fold activation could be found below pH 7.5; the required concentration of Mg^{2+} ions was approximately 1.2 and 2.0 mM at pH 6.0 and 5.1, respectively, as shown in Figure 2. It was possible

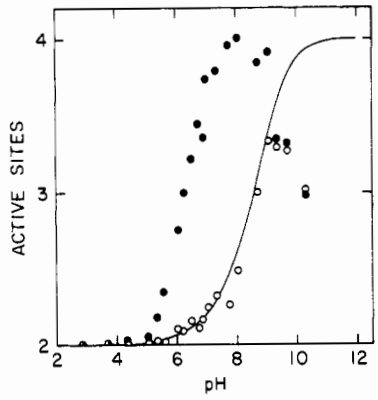


FIGURE 3: Number of functioning active sites of aldehyde dehydrogenase found from the pre-steady-state burst magnitude as a function of pH in the absence and presence of Mg^{2+} ions. The symbols ○ and ● represent the experimental data in the absence and presence (0.7 mM) of Mg^{2+} ions, respectively. The theoretical curve in the absence of Mg^{2+} ions was obtained with $n = 1$ and $pK_H = 9.5$ by using eq 4.

to construct a metal activation curve as a function of pH by dividing the velocity of the metal-activated enzyme by the nonactivated velocity. This is shown in the insert to Figure 1. Above pH 7.5, the stimulation decreased such that above pH 8.7 no stimulation was observed.

pH Dependencies of the Burst Magnitude of NADH Production in the Absence and Presence of Mg^{2+} Ions. Analysis of the pre-steady-state burst kinetics performed with a stopped-flow apparatus has shown that the magnitudes of the NADH burst was 2 mol/mol of enzyme in the absence of Mg^{2+} ions and 4 mol/mol of enzymne in the presence of 0.7 mM Mg^{2+} when assayed at pH 7.5 (Takahashi et al., 1980a; Takahashi & Weiner, 1980). The magnitude of the burst was redetermined in the absence and presence of Mg^{2+} ions over the wide pH range, and the data are presented in Figure 3.

In the absence of Mg^{2+} ions, the burst magnitude was essentially 2 mol/mol of enzyme in the acidic range. The magnitude, though, above neutral pH increased to a value of 3.3 at pH 9. Above pH 9, the burst magnitude decreased, presumably due to alkaline denaturation of the enzyme. In the presence of a fixed concentration of Mg^{2+} ions, the burst magnitude increased from 2 to 4 mol/mol of enzyme above pH 5. Above pH 8.5, the magnitude started to decrease such that by pH 9 it was nearly identical with that found in the absence of Mg^{2+} ions.

It has been previously shown that the increase in burst magnitude correlates with a decrease in molecular weight of the enzyme (Takahashi & Weiner, 1980). The increase in burst magnitude found in the absence of Mg^{2+} ions may also be related to a pH-dependent dissociation of the enzyme from a tetramer to a pair of dimers, each exhibiting all-of-the-sites reactivity.

Molecular Weight of Aldehyde Dehydrogenase as a Function of pH. The molecular weight of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase, as determined by sedimentation equilibrium, decreases from 260 000 in the absence of Mg^{2+} ions to 130 000 in the presence of 0.4 mM Mg^{2+} ions (Takahashi & Weiner, 1980). If the change in burst magnitude with increasing pH in the absence of Mg^{2+} ions (Figure 3) correlates with a change in the state of aggregation of the enzyme, the molecular weight should decrease with increasing pH. Between pH 5.5 and 8, there was only a slight decrease in the average molecular weight, as shown in Figure 4. Unfortunately, it was not possible to determine the molecular weight by sedimentation equilibrium in the

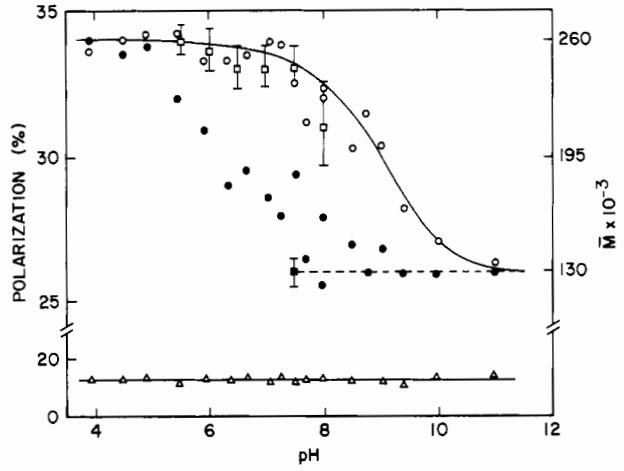


FIGURE 4: Apparent molecular weight of aldehyde dehydrogenase estimated from fluorescence polarization and sedimentation equilibrium studies as a function of pH in the absence and presence of Mg^{2+} ions. Fluorescence polarizations of NADH (2.3 μM) (△), NADH (2.3 μM) + enzyme (0.8 μM) (○), and NADH (2.3 μM) + enzyme (0.8 μM) + Mg^{2+} (0.7 mM) (●). Molecular weight from two or three equilibrium sedimentation runs with 0.8 μM enzyme in the absence of Mg^{2+} ions (□) and with 1.1 μM enzyme and 0.7 mM Mg^{2+} ions (■) [latter from Takahashi and Weiner (1980)]. The theoretical curve (solid line) was obtained by using eq 8, in which the values of Y from Figure 3 were used. The dashed line indicates the apparent end point for the polarization values. It also is to show that this corresponds to the molecular weight of the dimer.

alkaline pH range due to denaturation of the enzyme. We found that at pH 8 there was a 5% loss in catalytic activity after 24 h at 8 °C, but this increased to approximately 30% at pH 8.5 and above 50% inactivation at pH 9. Sedimentation equilibrium data obtained above pH 8.5 gave random values presumably due to denaturation of the enzyme and possible aggregation of denatured enzyme forms.

It also was not possible to use gel permeation chromatography to determine the molecular weight of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase, because the molecular weight obtained at pH 7.5 with Sephadex G-150 or G-200 corresponded to that of the dimer (data not shown). Conceivably, on the column, the dimers and tetramers separate, so that the species run not as the weight average form but as the lower molecular weight component due to its interactions with the gel.

Polarization of fluorescence can be used to estimate the molecular weight of a protein (Weber, 1953). The advantage of this technique is that measurements can be performed relatively quickly, such that the enzyme will not be denatured during the time course of the measurement. For these determinations, the fluorescence of NADH bound to the enzyme were measured. In the absence of added Mg^{2+} ions where the molecular weight is 260 000 (Takahashi & Weiner, 1980), the polarization of fluorescence at pH 7.5 was 34%,[3] while in the presence of Mg^{2+} ions where the molecular weight is 130 000, the polarization of fluorescence was 26%. A complete pH titration was performed, and the data are presented also in Figure 4. The polarization of fluorescence for free NADH is independent of pH, but for the NADH bound to the enzyme, it decreased from 34% at low pH to 26% at high pH. This lower value corresponds to the molecular weight of the dimer, while the higher value corresponds to that for the tetramer.

[3] The value of 34% is due to the fact that some free NADH is present during the titration. It can be calculated by using $K_d = 2$ μM and polarization of bound NADH = 38% (Takahashi et al., 1980b) that the polarization would equal 34% under the condition of the experiment.

Thus, in a qualitative manner, it can be observed that the tetrameric enzyme, even in the absence of Mg^{2+} ion, dissociates with increasing pH. In both the presence and absence of Mg^{2+} ions, there appeared to be a comparable change in burst magnitude (as shown in Figure 3) with the change in polarization of fluorescence (Figure 4). A linear correlation exists among the increase in pH, increase in burst magnitude, and decrease in polarization of fluorescence, the latter indicating a molecular weight change.

Discussion

pH Dependency of the Number of Functioning Active Sites. The oxidation of aldehydes by the p*I* 5 isozyme of horse liver aldehyde dehydrogenase proceeds through an acyl intermediate (Weiner et al., 1976). When the formation of acyl intermediate is rapid compared to its decomposition and $[S]_0 \gg K_m$, the observed magnitude of the initial burst phase corresponds to the number of functioning active sites (Bender et al., 1967). For the p*I* 5 isozyme of horse liver aldehyde dehydrogenase, the magnitude of the initial burst is 2 and 4 mol of NADH per mol of tetrameric enzyme in the absence and presence of Mg^{2+} ions, respectively, at pH 7.5 (Takahashi et al., 1980a; Takahashi & Weiner, 1980). This increase in burst magnitude produced by Mg^{2+} ions means that an enzyme functioning with half-of-the-sites reactivity in the absence of the metal ions has changed to a form functioning with all-of-the-sites reactivity in its presence. We have found an excellent correlation between the apparent molecular weight and the number of functioning active sites of the enzyme (Takahashi & Weiner, 1980). This correlation may be valid for the pH-dependent change of burst magnitude presented in Figure 3 since the molecular weight of the enzyme appears to decrease from that of the tetrameric to that of the dimeric with increasing pH (Figure 4).

The most simple equilibrium for the pH-dependent dissociation–association process of the enzyme is

$$E_T \overset{K_H}{\rightleftharpoons} 2E_D + nH^+ \qquad (2)$$

where E_T and E_D are the tetrameric and dimeric forms, respectively, of the enzyme, n is the number of the protons, and K_H is the equilibrium constant. This leads to

$$K_H = ([E_D]^2[H^+]^n)/[E_T] \qquad (3)$$

$$\log ([E_D]^2/[E_T]) = n\text{pH} - \text{p}K_H \qquad (4)$$

The total number of active sites, Y, per unit of enzyme concentration has been expressed by (Takahashi & Weiner, 1980)

$$Y = 2X + 2[2(1 - X)] \qquad (5)$$

where X is the fraction of enzyme in the tetrameric form, functioning with half-of-the-sites reactivity, and $1 - X$ is the fraction in the dimeric state, functioning with all-of-the-sites reactivity. Combining eq 4 and 5 to estimate n and pK_H, we can obtain

$$\log [(Y - 2)^2/2(4 - Y)] = n\text{pH} - \text{p}K_H \qquad (6)$$

Figure 5 illustrates a plot of eq 6, where the value of Y at each pH was obtained in the absence of Mg^{2+} ions from Figure 3. The data fits well to the straight line with the values of $n = 1$ and p$K_H = 9.5$ ($r = 0.95$). Alternatively, when the values of n and pK_H are substituted into eq 4, it is possible to calculate the value of $[E_D]^2/[E_T]$ as a function of pH. From the value of $[E_D]^2/[E_T]$ and the relation of $[E_O] = [E_T] + 2[E_D]$, Y at different pHs can be determined by using eq 5. The calculated curve is superimposed on the experimental data

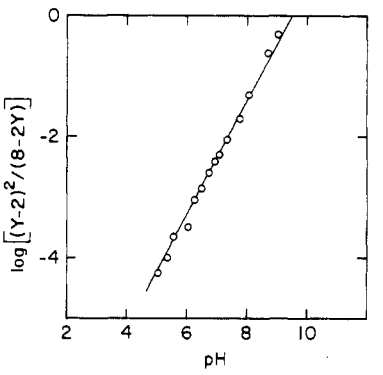


FIGURE 5: Determination of n and pK_H in pH-dependent association-dissociation equilibrium of aldehyde dehydrogenase in the absence of Mg^{2+} ions. The data were calculated with the experimental number of active sites, Y, in Figure 3 by using eq 6.

presented in Figure 3. In the absence of Mg^{2+} ions, the experimental data fit well with a theoretical value of p$K_H = 9.5$, if the portion in the alkaline denaturation region is not considered. The physical significance of the pK_H value obtained in the absence of Mg^{2+} ions may be related to the pK_a of a group involved in the tetramer–dimer dissociation process. On the basis of a pK_H of 9.5, the amino acid residues involved in this transition could possibly be a tyrosine or lysine. Alternatively, it may be the α-amino group of the N terminus.

Correlation between the Apparent Molecular Weight and the Number of Active Sites. An equation relating the total number of active sites per unit concentration and the apparent molecular weight ($\bar{M}$) of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase has been presented (Takahashi & Weiner, 1980):

$$Y = 4 - \frac{2(\bar{M} - 130\,000)}{(390\,000 - \bar{M})} \qquad (7)$$

which rearranges to

$$\bar{M} = \frac{1\,820\,000 - 390\,000Y}{6 - Y} \qquad (8)$$

Substituting the values of Y, used in obtaining the theoretical curves in Figure 3, into eq 8 allowed for the calculation of $\bar{M}$ as a function of pH. This curve is superimposed on the polarization of fluorescence data in Figure 4. The polarization of 34% corresponds to the tetramer region while the value of 26% found at high alkaline pH corresponds to the dimeric region. The polarization data fits well with the curve of pK_H = 9.5 in the absence of Mg^{2+} ions. Thus, an excellent correlation exists between the number of functioning active sites and the molecular weight when it is assumed that all events are regulated by a group with pK_H of $\simeq$ 9.5. That is, the enzyme appears to dissociate from the less active tetramer to the more active dimers at alkaline pHs, and these dimers function with all-of-the-sites reactivity in the presence of absence of Mg^{2+} ions.

Other researchers have found that Mg^{2+} ions do not activate a beef liver mitochondrial aldehyde dehydrogenase at high pH (Takahashi et al., 1979). The reason is related to the fact that the enzyme already exists in the more active dimeric form. The possible physiological significance of the metal ion activation of liver aldehyde dehydrogenases has been discussed (Weiner & Takahashi, 1981).

Effect of pH on the Rate-Limiting Step. For the dehydrogenase reaction of the p*I* 5 isozyme of horse liver aldehyde dehydrogenase, deacylation of the acyl intermediate is rate limiting, while for the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate, formation of the acyl intermediate is

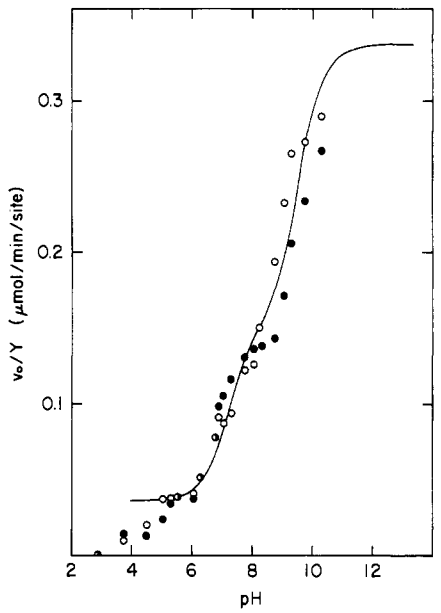
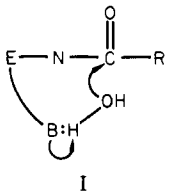


FIGURE 6: Effect of pH on deacylation of the acyl-enzyme intermediate formed per active site of aldehyde dehydrogenase. All experimental data were obtained by dividing the steady-state velocity in Figure 1 by the number of active sites found from Figure 3 at each pH. The symbols ○ and ● corresponded to the absence and presence of Mg^{2+} ions, respectively, as described in Figure 1 or 3. The theoretical curve was obtained by assuming pK_as of 7.2 and 9.5 for the first and second proton dissociation steps, respectively, in Scheme I, and a V_{max} of $k_H[E_o]$ = 0.1 and $k[E_o]$ = 0.3 μmol min^{-1} per site. (For the calculation, the data below pH 5 were not considered. pK_a or V_{max} was obtained by trial and error.)

rate limiting (Weiner et al., 1976). In the presence of NAD, the deacylation step for the esterase reaction also becomes rate limiting (Takahashi & Weiner, 1981). It is possible to study the effect of pH on the deacylation step by correcting the increased reaction rates found as a function of pH for the change in the number of functioning active sites. The calculated velocity per subunit as a function of pH is presented in Figure 6. Finding here that the data obtained for the effect of pH on the velocity in the absence and presence of Mg^{2+} ions fit well to the same titration curve provides further evidence that the Mg^{2+} activation is not due to alterations of the individual rate constants involved in the overall process. It is due to an increase in the number of functioning active sites, as has been suggested previoulsy (Takahashi & Weiner, 1980).

The hydrolysis of acyl intermediate is a well-characterized process. For all enzymes, the reaction appears to proceed by a general base catalyzed reaction (Jencks, 1969):

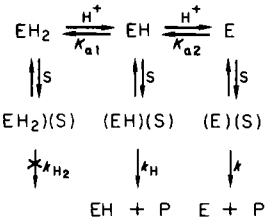


I

where B: is the general base and N is a nucleophilic side chain of an amino acid such as the oxygen of serine for chymotrypsin (Segal et al., 1971; Henderson et al., 1971) and the sulfur of cysteine for papain (Drenth et al., 1976) or glyceraldehyde phosphate dehydrogenase (Moras et al., 1975).

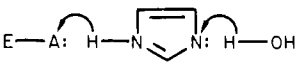
A comparison of the geometries of the active site of the three diverse enzymes, chymotrypsin, papain, and glyceraldehyde phosphate dehydrogenase, has been made (Garavito et al., 1977). For all three, the base (B) as shown in structure I is a histidine. Furthermore, with all three, the histidine is in close

Scheme I[a]



[a] EH_2 and EH are the di- and monoprotonated forms, respectively, while E is the highly active unprotonated form, and S and P are substrates and products, respectively.

proximity to an electron sink such that a "charge relay" system can function:



II

where A: is the electron sink (aspartate–carboxylate with chymotrypsin, asparagine–carboxyl oxygen with papain, and tyrosine–phenol with glyceraldehyde phosphate dehydrogenase).

An estimation of the pK_a of the group(s) involved in the hydrolysis of the acyl intermediate can be made by analyzing the pH profile curve presented in Figure 6. It was not possible to fit the data to one simple dissociation. The data could fit to a two-functional groups model (Scheme I). The data for the region of pH 5–8 appear to be governed by an event associated with a group possessing pK_{a1} = 7.2. The data above pH 8 is governed by this event plus that from a group with pK_{a2} = 9.5. The values for $k_H[E_o]$ and $k[E_o]$ where ($[E_o]$ = total enzyme concentration), are ca. 0.1 and 0.3 μmol min^{-1} per site, respectively. The groups with these pK_as could be those of general base (B) shown in structure I and the electron rich component (A:) shown in structure II. If the pK_as identified from the data in Figure 6 represent the groups involved in the rate-determining step, it is possible to suggest that a general base catalyzed reaction for the hydrolysis of the acyl intermediate exists. At physiological pHs, only the group with pK_{a1} $\simeq$ 7.2, presumably a histidine, analogous to histidine-176 in glyceraldehyde phosphate dehydrogenase, would be involved (structure I). In the nonphysiological alkaline region, the charge relay system may function (structure II). The group involved in this possible relay system could be a tyrosine, analogous to tyrosine-311 in glyceraldehyde phosphate dehydrogenase, or any other group with pK_a = 9.5. If this mechanism is correct, the hydrolysis of the acyl intermediate at physiological pHs does not require the action of the charge relay system. There would be just a 3-fold increase in the turnover number per active site with the second ionization which would permit the charge relay system to function. Hence, the overall role of a charge relay mechanism in the hydrolysis of acyl intermediates is of dubious importance. This challenge has been also made for the serine proteases as reviewed by James (1980).

This study revealed that two different events in the action of aldehyde dehydrogenase are governed by groups possessing approximately the same pK_a. One event influenced the increase in magnitude of the pre-steady-state burst, i.e., the number of functioning active sites. This event appears to be related to a dissociation from the tetrameric state to the dimeric state, analogous to the mode that Mg^{2+} ions stimulate (Takahashi & Weiner, 1980). The other event is involved in the hydrolysis of the acyl intermediate in the alkaline region. Though the same residue could be responsible for both processes, it is more likely that two separate amino acids are

actually involved in the two very different processes. Detailed structural work will have to be performed with aldehyde dehydrogenase to determine the groups whose p*Ks* were identified.

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1α,25,26-Trihydroxyvitamin D_3: An in Vivo and in Vitro Metabolite of Vitamin D_3†

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ABSTRACT: A new metabolite of vitamin D_3 has been isolated from the plasma of vitamin D_3 treated cows and has been generated from 25(*S*),26-dihydroxyvitamin D_3 with homogenates of vitamin D deficient chick kidney. This metabolite has been identified as 1,25,26-trihydroxyvitamin D_3 by comigration with synthetic 1,25(*S*),26-trihydroxyvitamin D_3 in four chromatographic systems, ultraviolet spectroscopy, mass spectrometry, and high-pressure liquid chromatography and mass spectrometry of derivatives. 1,25(*S*),26-Trihydroxyvitamin D_3 is one-tenth as effective as 1,25-dihydroxyvitamin D_3 in binding to the chick intestinal cytosol 1,25-dihydroxyvitamin D receptor. Either 25(*S*),26-dihydroxyvitamin D_3 or 1,25-dihydroxyvitamin D_3 can serve as precursor for in vitro production of 1,25,26-trihydroxyvitamin D_3 by chick kidney tissue.

Vitamin D undergoes extensive metabolism. One of its metabolites, 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$],[1] is a hormone that stimulates intestinal absorption of calcium and phosphate (Omdahl & DeLuca, 1973; Napoli & DeLuca, 1979; Stern, 1980). Possible functions of the other characterized metabolites are the subject of controversy. Moreover, the existence of many additional metabolites has been reported, but they remain to be isolated, identified, and studied (Norman, 1979). The extensive modification of this secosterol, which occurs to a large extent in the kidney, is reminiscent of cholesterol metabolism in the adrenal. Therefore, continued investigation of vitamin D metabolism promises to be interesting.

During the development of a protein-binding assay for 1,24,25-$(OH)_3D_3$ (Reinhardt et al., 1981), a previously unidentified vitamin D_3 metabolite, more polar than 1,24(*R*),25-$(OH)_3D_3$, was detected in the plasma of vitamin D_3

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[1] Abbreviations used: 25-OHD_3, 25-hydroxyvitamin D_3; 24(*R*),25-$(OH)_2D_3$, 24(*R*),25-dihydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 1,24(*R*),25-$(OH)_3D_3$, 1,24(*R*),25-trihydroxyvitamin D_3; 1,25(*S*),26-$(OH)_3D_3$, 1,25(*S*),26-trihydroxyvitamin D_3; 25(*S*),26-$(OH)_2D_3$, 25(*S*),26-dihydroxyvitamin D_3; HPLC, high-pressure liquid chromatography; Me_3Si, trimethylsilyl; Tris, tris(hydroxymethyl)-aminomethane.

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