Nicotinamide Adenine Dinucleotide Activation of the Esterase Reaction of Horse Liver Aldehyde Dehydrogenase†

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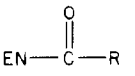
ABSTRACT: The esterase reaction catalyzed by horse liver aldehyde dehydrogenase is activated with NAD(H) by factors of 2 under a V_{max} assay and of 6.8 at low ester concentrations (Feldman, R. I., & Weiner, H. (1972) *J. Biol. Chem. 247*, 267–272). Stopped-flow experiments suggested that an initial burst of 0.4 mol followed by a second burst of 1 mol of nitrophenol per mol of tetrameric enzyme occurred in the absence of NAD, while the magnitudes increased to 2 and 4 mol/mol of enzyme in its presence. If the enzyme was incubated for 1 min with NAD, the burst phase was 4 mol/mol of enzyme. Nonlinear Lineweaver–Burk plots were found in the absence and presence of NAD, but incubation with NAD for 1 min abolished the biphasic response. Mg^{2+} ions activate the dehydrogenase reaction of horse liver aldehyde dehydrogenase (Takahashi, K., & Weiner, H. (1980) *J. Biol. Chem. 255*, 8206–8209). The metal neither increased the esterase reaction nor affected the NAD activation. The rate-limiting step for the esterase reaction was thought to be the formation of an acyl intermediate, while that for the dehydrogenase reaction was deacylation (Weiner, H., Hu, J. H. J., & Sanny, C. G. (1976) *J. Biol. Chem. 251*, 3853–3855). Finding that a full burst exists for the esterase reaction in the presence of NAD shows that the deacylation step or product dissociation can become rate limiting. The major kinetic alteration produced by NAD is to increase the rate of acylation while not affecting deacylation. The presence of NAD appears to activate the attack of the active-site nucleophile on the carbonyl group of the substrate.

Horse liver ALDH[1] (aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3) is composed of four subunits (Feldman & Weiner, 1972a; Eckfeldt et al., 1976), as are the enzymes isolated from other eucaryotes (Weiner, 1979). Recent investigations have shown that the enzyme functions with half-of-the-sites reactivity (Weiner et al., 1976; Eckfeldt & Yonetani, 1976; Takahashi & Weiner, 1980a) which can be changed to all-of-the-sites reactivity in the presence of Mg^{2+} ions when the enzyme is catalyzing the dehydrogenase reaction (Takahashi & Weiner, 1980a). In addition to the dehydrogenase reaction, ALDH also hydrolyzes esters such as *p*-nitrophenyl acetate (Feldman & Weiner, 1972b; Sidhu & Blair, 1975a; Eckfeldt & Yonetani, 1976; Duncan, 1977; MacGibbon et al., 1978). Although it has not been proven unequivocally that the dehydrogenase and esterase reactions occur at the identical catalytic site, in all probability the same active site catalyzes both reactions (Feldman & Weiner, 1972b; Sidhu & Blair, 1975a).

The esterase reaction catalyzed by ALDH is not of physiological significance. Understanding the mechanism of the reaction, however, can lead to a better understanding of the dehydrogenase reaction. Both reactions proceed through a common acyl intermediate (Feldman & Weiner, 1972b; Weiner et al., 1976)



where N is the nucleophilic amino acid at the active site. Even though both reactions proceed through the same intermediate and the hydrolysis of the acyl intermediate is presumably by an identical mechanism, the overall kinetics of the two reactions differ. Where for the dehydrogenase reaction hydrolysis of the intermediate is rate limiting, for the esterase reaction formation of the intermediate is rate limiting (Weiner et al., 1976).

NAD and NADH enhance the rate of the esterase reaction catalyzed by horse liver ALDH (Feldman & Weiner, 1972b). Though this observation has been extended to the enzymes isolated from livers of other mammalian sources (Sidhu & Blair, 1975a; Eckfeldt & Yonetani, 1976; MacGibbon et al., 1978), the mechanism of the activation has not been studied yet in depth.

Mg^{2+} ions activate the dehydrogenase reaction of horse liver by ALDH by increasing the number of functioning catalytic sites from 2 to 4 per molecule of tetrameric enzyme (Takahashi & Weiner, 1980a; Takahashi et al., 1980). The effect of a change in the number of functioning dehydrogenase catalytic sites produced by Mg^{2+} ions was now investigated for the esterase reaction. Furthermore, an investigation into the mechanism of the NAD activation of the esterase reaction catalyzed by horse liver ALDH was undertaken. The results of these findings are reported in this paper.

Experimental Procedures

Materials. NPA was obtained from Aldrich Chemical Co. NAD and ADP-ribose were from P-L Biochemical and Sigma Chemical Co., respectively. $MgCl_2$ (analytical reagent) was from Mallinckrodt Chemical Works. Deionized, distilled water was used to prepare solutions.

Preparation of ALDH. Isolation of the p*I* 5 isozyme of horse liver ALDH as well as the determinations of specific activity and concentration was performed as previously reported (Feldman & Weiner, 1972a). A second isoelectric focusing step was employed to produce a preparation with a specific activity of 900 nmol min^{-1} mg^{-1} [the highest value previously reported was 720 nmol min^{-1} mg^{-1} (Feldman & Weiner, 1972)]. The purified enzyme was dialyzed at 4 °C

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[1] Abbreviations used: ALDH, aldehyde dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NPA, *p*-nitrophenyl acetate; E_I and E_{II}, the conformational states of ALDH having low and high K_m values, respectively.

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for 12 h against 0.1 M sodium phosphate buffer, pH 7.5, before being used.

Kinetic Measurements. All assays were performed in 0.1 M sodium phosphate, pH 7.5, at 25 ± 0.5 °C. Esterase activity was followed by monitoring the absorbance or transmittance at 400 nm due to *p*-nitrophenol production with a Gilford Model 240 spectrophotometer or a Durrum–Gibson stopped-flow spectrophotometer, respectively (Weiner et al., 1976). A molar extinction coefficient of 16 mM^{-1} cm^{-1} (Kezdy & Bender, 1962) for the product at pH 7.5 was used. The exact concentration of substrate employed was determined by performing a total hydrolysis of the substrate in 2 N NaOH by using a molar extinction coefficient of 18.3 mM^{-1} cm^{-1} at 400 nm (Kezdy & Bender, 1962). The concentration of NAD was determined spectroscopically by using a molar extinction coefficient of 18 mM^{-1} cm^{-1} at 260 nm. Steady-state assays were performed by adding enzyme solution (10–50 μL) to 1 mL of substrate or substrate + effector solution. In runs which required a preincubation of the enzyme with NAD, the enzyme + NAD solution was incubated for 1 min prior to the addition of 5–100 μL of substrate solution (the final reaction volumes were 1.05 mL). The stopped-flow experiments were performed with three sets of mixing combinations: syringe A enzyme, syringe B NPA; syringe A enzyme, syringe B NPA + NAD; and syringe A enzyme + NAD, syringe B NPA. The change in transmittance as a function of time was presented on a teletype after being stored on an Aminco Dasar-TM digital computer. For monitoring of the wide time range for any one reaction, two time-range switches on the digital computer were used: one point at intervals of 50 ms for the fast initial 100 points and one at intervals of 1 s for the remaining 100 points. Control reactions were performed in the absence of enzyme, and the slow rate of spontaneous hydrolysis of substrate was subtracted from that observed in the assays performed in the presence of enzyme.

Results

Estimation of Steady-State Velocity. When the esterase activity of ALDH was assayed in the absence of added NAD, the initial measured velocity was faster than the ultimate steady-state velocity. Though this pattern was observed also in the presence of NAD when ALDH was not preincubated with the coenzyme, a preincubation of the enzyme with NAD for 1 min abolished the phenomenon. Hence, the linear portion, which was obtained after the initial 10 or 20 s of the reaction, was assumed to be the steady-state velocity of the esterase reaction. This phenomenon has been observed previously in the dehydrogenase reaction catalyzed by horse liver ALDH (Takahashi & Weiner, 1980b) as well as a reaction catalyzed by honey bee GAPDH (Gelb et al., 1970).

Extrapolation from the steady-state phase to time zero for the assays performed in the absence and presence of NAD yielded different results. In the absence of NAD, the line extrapolated to nearly zero absorbance. In the presence of NAD, though, the value was extrapolated to an absorbance of 0.019 which corresponds to a pre-steady-state burst magnitude equivalent to 4 mol of product formed per mol of tetrameric enzyme. Analysis of the initial time course, as measured by the stopped-flow experiment, revealed that in the absence of NAD a very small burst of about 10% of theoretical magnitude[2] was observed (Figure 1, line a). The trace showed an additional transient phase occurring after the first set of

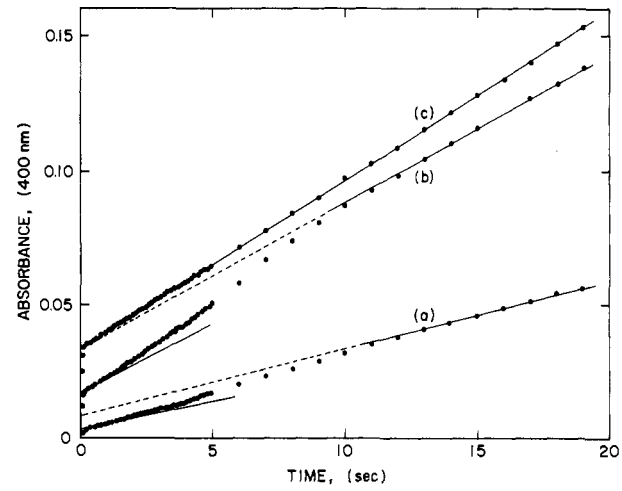

FIGURE 1: Product formation of the esterase reaction of horse liver ALDH as measured with the stopped-flow apparatus. (ALDH) = 0.52 μM and (NPA) = 160 μM for three curves. (NAD) = 0 μM for (a) and 140 μM for (b) and (c). Preincubation of ALDH with NAD was performed for (c) only. The points show the measured absorbance (see text). The dashed lines are the extrapolated line to time zero. The short straight lines on (a) and (b) were extrapolated from the linearr portions during the initial 1 or 2 s.

burst and steady-state phases. By the extrapolation to time zero from the ultimate steady-state phase, the total burst magnitude was now determined to be ~25% of total concentration of subunit[2] in the absence of NAD. In the presence of added NAD, a biphasic burst pattern occurred (Figure 1, line b). Only one set of burst and steady-state phases was obtained if the enzyme was preincubated with NAD (Figure 1, line c). The extrapolated burst magnitude in the presence of NAD was 2 and 4 mol per mol of tetrameric enzyme in the absence of preincubation, but 4 if preincubated. The hydrolysis of NPA by α-chymotrypsin was performed to check the spectrophotometric stability of the stopped-flow apparatus over the relatively long time period used. For 3 min after the burst occurred, the absorbance change was still linear (data not shown).

Effects of NAD, ADP-ribose, and Mg^{2+} Ions of the Steady-State Velocity. It has been previously shown that NAD enhances the steady-state velocity of the esterase reaction of horse liver ALDH by a factor of ~2 in a V_{max} assay (Feldman & Weiner, 1972b). Inasmuch as we (Takahashi & Weiner, 1980a; Takahashi et al., 1980) have recently demonstrated that Mg^{2+} ions enhance the rate of dehydrogenase reaction, but not the esterase reaction (C. S. Brown, and H. Weiner, unpublished results), the NAD activation for the esterase reaction was reinvestigated in the absence and presence of added Mg^{2+} ions. The presence of Mg^{2+} ions did not in any way affect the esterase reaction. It neither caused an increase in the velocity nor altered the NAD stimulation of the reaction (Figure 2). In the absence or presence of the metal, a 2.8-fold enhancement of activity was obtained when the concentration of NAD was between 120 and 200 μM.

ADP-ribose is a competitive inhibitor of the dehydrogenase reaction of horse liver ALDH (K_i = 280 μM, data not shown). Even at a concentration of 400 μM, it did not inhibit the esterase reaction (also in Figure 2). It did, though, reduce the NAD stimulation of activation of the hydrolysis, suggesting that weak competition exists between ADP-ribose and NAD in the esterase reaction.

Effect of Substrate Concentrations on the Steady-State Velocity. In the initial study on the esterase reaction catalyzed by horse liver ALDH, the concentration of NPA was varied

[2] The theoretical burst magnitude was calculated by assuming that four active sites per tetrameric enzyme are involved.

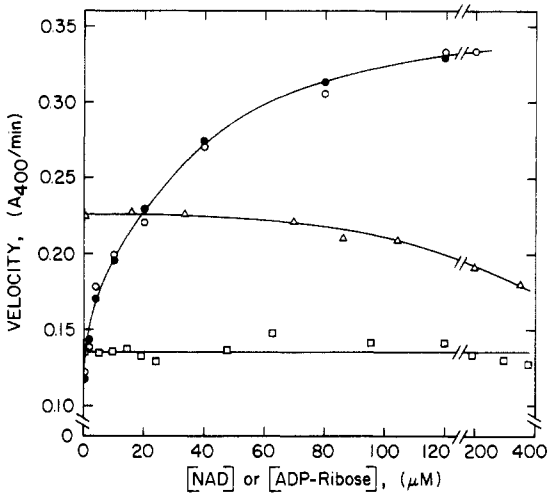

FIGURE 2: Effects of NAD, ADP-ribose, and Mg^{2+} ions on steady-state velocity of esterase reaction of horse liver ALDH. At the NAD concentration shown, assays were performed in the absence (○) and in the presence (●) of 0.69 mM Mg^{2+} ions at (ALDH) = 3.7 μM and (NPA) = 125 μM. Assays performed with ADP-ribose were at (ALDH) = 0.43 μM and (NPA) = 183 μM (□) and at (ALDH) = 0.30 μM, (NPA) = 128 μM, and (NAD) = 29.3 μM (△).

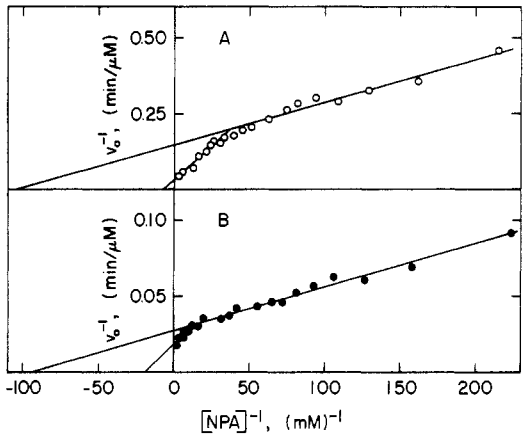

FIGURE 3: Double-reciprocal plots for velocity as a function of NPA conentration in esterase reactio of horse liver ALDH. (○) (ALDH) = 0.43 μM in the absence of NAD. (●) (ALDH) = 0.43 μM in the presence of 120 μM NAD.

between 1 and 25 μM (Feldman & Weiner, 1972b). We extended the range of substrate concentration to 300 μM. Assays were also performed in the presence of NAD, and the double-reciprocal plots are presented in Figure 3.

The data presented in Figure 3 lead one to conclude that ALDH has two sets of active sites, each possessing separate values of K_M and V_{max}. One can write eq 1 by expressing the

$$V_0 = \frac{k_{cat\,I}(E_I)(S)_0}{(S)_0 + K_{MI}} + \frac{k_{cat\,II}(E_{II})(S)_0}{(S)_0 + K_{MII}} \quad (1)$$

steady-state velocity, V_0, as a function of substrate concentration. In eq 1, I and II refer to the individual class of sites and (E_I) and (E_{II}) are the concentrations of the high- and low-affinity forms of the enzyme, respectively. Since (E_0), the total concentration of ALDH, equals the sum of (E_I) and (E_{II}), eq 1 can be rewritten to give

$$V_0 = (E)_0(S)_0\left[\frac{\alpha k_{cat\,I}}{(S)_0 + K_{MI}} + \frac{(1-\alpha)k_{cat\,II}}{(S)_0 + K_{MII}}\right] \quad (2)$$

where $\alpha = (E_I)/(E)_0$ and $(1 - \alpha) = (E_{II})/(E)_0$.

Extrapolations from the straight lines of Figure 3 allow for the calculation of the kinetic parameters presented in Table I. In the absence of NAD, K_M is 9.4 μM for the high-affinity site and 125 μM for the low-affinity site. This value for the high-affinity site is in good agreement with that (6 μM) previously reported (Feldman & Weiner, 1972b). In the presence of NAD, K_M for the low-affinity site is reduced by about 50% while the high-affinity K_M remained constant. The ratio of k_{cat} in the presence and absence of NAD is approximately 6 for the high-affinity site and about 2 for the low-affinity site.

Table I: Apparent Kinetic Constants for the Esterase Reaction of Horse Liver ALDH in the Absence and Presence of NAD

type[a]	kinetic constants[b]	−NAD	+NAD
I	K_{MI} (μM)	9.4	10.6
	V_I (μM/min)	6.8	37.0
	$k_{cat\,I}$ (s^{-1})	0.26	1.44
II	K_{MII} (μM)	125.0	52.6
	V_{II} (μM/min)	31.3	57.0
	$k_{cat\,II}$ (s^{-1})	1.21	2.21

[a] Types I and II refer to the low and high K_M forms, respectively (see eq 1). [b] All data were obtained from Figure 3.

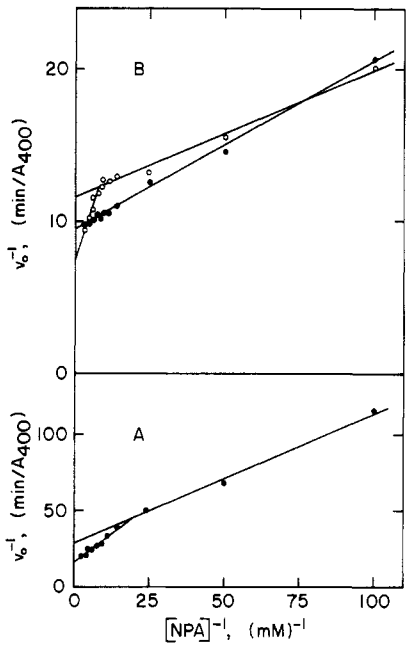

FIGURE 4: Effect of NAD preincubation with horse liver ALDH on the esterae reaction. (A) (●) (ALDH) = 0.32 μM in the absence of NAD (as a control for calculating the degree of activation by NAD, which will be shown in Figure 5). (B) (○) (ALDH) = 0.32 μM and (NAD) = 160 μM. The reaction was initiated by addition of enzyme to the substrate + NAD solution. (●) The same condition as (○), but initiation of the reaction was performed by addition of substrate to the enzyme + NAD solution after 1-min preincubation.

Effect of Preincubation of ALDH with NAD on the Steady-State Velocity. When the nonlinear double-reciprocal plots shown in Figure 3 were obtained, the velocity profile as the function of substrate concentration revealed that an intermediary plateau region existed. These types of curves have been previously reported for such diverse enzymes as CTP synthetase (Levitzki & Koshland, 1969), phosphoenolpyruvate carboxylase (Corwin & Fanning, 1968), glutamic dehydrogenase (LeJohn & Jackson, 1968), honey bee GAPDH (Gelb et al., 1970), and the dehydrogenase reaction catalyzed by horse liver ALDH (Takahashi & Weiner, 1980b). As has been demonstrated with GAPDH (Gelb et al., 1970) and the dehydrogenase reaction of horse liver ALDH (Takahashi & Weiner, 1980b), this intermediary plateau region disappears if the enzymes are preincubated with NAD for 1 min. As

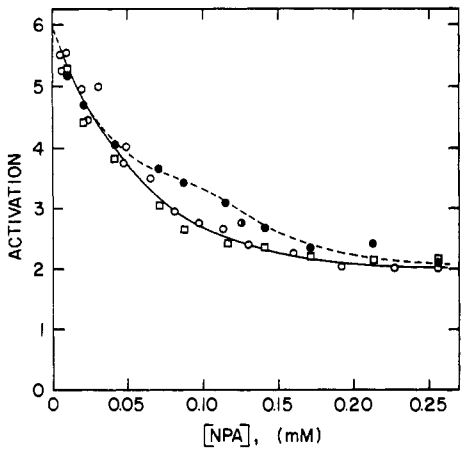

FIGURE 5: Degree of activation of NAD on steady-state velocity of esterase reaction of horse liver ALDH. Data O from Figure 3, □ and ● from Figure 4, and ◑ from Figure 2.

revealed in Figure 4, a linear double-reciprocal plot was found over the entire concentration range of substrates if 1 min of preincubation occurred.

Degree of NAD Stimulation of the Esterase Reaction. The conditions of the assay will depict the actual degree of NAD stimulation of the esterase reaction. The degree of activation of the enzyme as a function of substrate concentration is illustrated in Figure 5. When the substrate concentration was lower than 200 μM, the degree of activation produced by NAD increased in a hyperbolic manner with a decrease in substrate concentration. In essentially a V_{max} assay using greater than 200 μM substrate, the degree of activation was just 2-fold.

Discussion

Feldman & Weiner (1972b) have originally reported that a p*I* 5 isozyme of horse liver ALDH possesses esterase activity. Sidhu & Blair (1975a) have proved unequivocally that human liver ALDH catalyzes both the esterase and the dehydrogenase reactions. Though it has not been proven if both reactions occur at the same active site, circumstantial evidence points strongly to the fact that the enzyme contains an active site which will hydrolyze the acyl intermediate produced from either the dehydrogenase or esterase reaction.

It has been recently demonstrated that Mg^{2+} ions activate by 2-fold the dehydrogenase reaction catalyzed by the p*I* 5 isozyme of horse liver ALDH (Takahashi & Weiner, 1980a; Takahashi et al., 1980). It has been further shown that in the presence of Mg^{2+} ions, the tetrameric enzyme that exhibits half-of-the-sites reactivity dissociates into the dimers exhibiting all-of-the-sites reactivity (Takahashi & Weiner, 1980a,b). Our finding, as reported in this paper, that Mg^{2+} ions do not alter the esterase activity of ALDH was truly unexpected. It leads us to suggest that the esterase reaction is independent of the degree of aggregation of the enzyme.

For the esterase reaction, a pre-steady-state burst of 4 mol of nitrophenol per mol of tetrameric enzyme exists when NAD was present. Thus it appears that all four active sites are simultaneously functioning in the esterase reaction while only two are functioning in the dehydrogenase reaction. We have no explanation at this time for the observations of the apparent all-of-the-sites reactivity of the enzyme in the esterase reaction while half-of-the-sites reactivity occurs in the dehydrogenase reaction.

ADP-ribose was ineffective in activating the esterase reaction, though it was a very weak inhibitor against the NAD activation. The fact that the esterase reaction is activated by either NAD or NADH but not by ADP-ribose suggests that

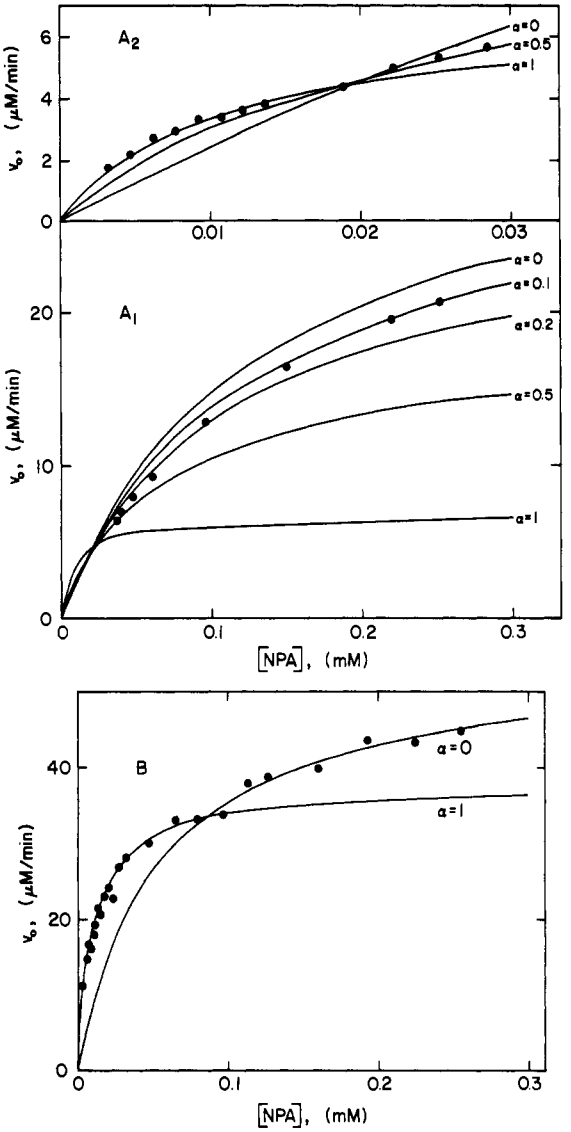

FIGURE 6: Theoretical substrate saturation curves of esterase reaction of horse liver ALDH calculated by eq 2. The calculations were performed by using the kinetic constants in Table I and eq 2. α is the fraction of low k_m form of the enzyme. The superimposed data are from Figure 3. (A) In the absence of NAD. (A_1) is for data at high substrate concentration, and (A_2) is for data at low substrate concentration. (B) In the presence of NAD.

the nicotinamide ring of the coenzyme must play an important role in the activation process. If the nicotinamide ring was interacting with the SH group of the active site in ALDH as was shown in the crystallographic studies of GAPDH (Buehner et al., 1974; Harris & Waters, 1976), one would expect that the presence of NAD would cause tighter binding of both the aldehydes in the dehydrogenase reaction and the ester in the esterase reaction. This is indeed what has been found: the value of K_i for propionaldehyde as an inhibitor in the esterase reaction was 490 μM to free ALDH (Feldman & Weiner, 1972b), while the K_m of the aldehyde was 0.2 μM to an ezyme–NAD complex as measured by the dehydrogenase reaction (Feldman & Weiner, 1972a).

The effect of substrate on the initial velocity, as presented in Figure 3, was biphasic. The enzyme seems to exist in two states, and the ratio, α, as defined in eq 2 appears to be a function of substrate concentration. By incorporation of the kinetic constants from Table I into eq 2, theoretical curves can be calculated for the velocity as a function of α.

In Figure 6 are presented the calculated curves superimposed on actual data. In the absence of NAD, at low substrate

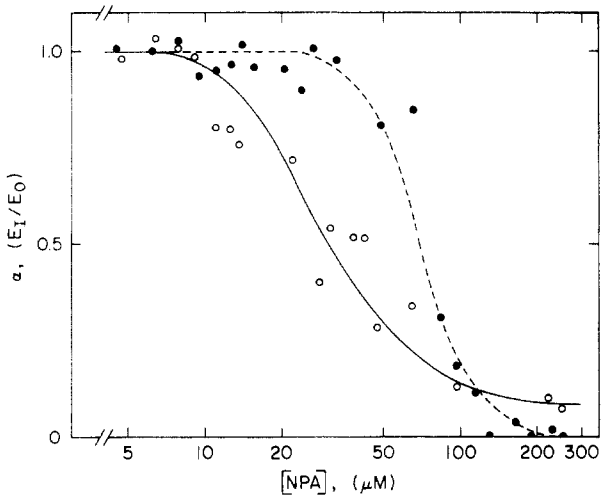

FIGURE 7: Effect of NAD on substrate-induced transition of horse liver ALDH. The fraction α (defined in eq 2) of type I form in ALDH was calculated from data in Figure 3 by using the kinetic constants in Table I and eq 3. (ALDH) = 0.43 μM. Assays in the absence (○) and in the presence of 120 μM NAD (●).

concentrations, the experimental data fit well on the theoretical curve of $\alpha = 1$ but approach the curve of $\alpha = 0.1$ at high substrate concentrations (Figure 6A). In the presence of NAD, the experimental data fall on the curve of $\alpha = 1$ and $\alpha = 0$ at low and high substrate concentrations, respectively (Figure 6B).

Equation 2 can be rearranged to solve for α, as presented in eq 3. By substitution of the kinetic constants in Table I

$$\alpha = \frac{[(S)_0 + K_{MI}]V_0[(S)_0 + K_{MII}] - (E)_0(S)_0k_{cat\,II}}{(E)_0(S)_0k_{cat\,I}[(S)_0 + K_{MII}] - k_{cat\,II}[(S)_0 + K_{MI}]} \quad (3)$$

and by use of the value of V_0 against each $(S)_0$ obtained from Figure 3, the dependency of α on the substrate concentration can be obtained (Figure 7). In the absence of NAD, there appears to be a sigmoidal dependence of α on the substrate concentration. In the presence of NAD, it appears that as the concentration of substrate is raised to above 30 μM the dependency becomes more sharp.

From the calculation presented in Figure 7, it appears that as the substrate concentration is increased the enzyme may undergo a transition from one type of reactive species to another. This transition is a substrate-induced cooperative phenomenon. Furthermore, after ~10 s of the steady-state phase of the reaction proceeded with a different k_{cat} as shown in Figure 1. This can be explained by invoking a coenzyme-induced hysteretic effect. This type of hysteretic effect may be similar to what has been described by Frieden (1970, 1979) and by Neet & Ainslie (1980). Preincubation of ALDH with NAD causes both the substrate-induced kineic cooperativity (Figure 4) and hysteresis (Figure 1) to change; the non-Michaelis–Menten saturation curve becomes a normal hyperbolic curve, and the two-step burst changes to a simple one-step burst. It is not known if these alterations in kinetic cooperativity and potential hysteresis can be correlated with each other. If such a correlation exists, then the kinetic data obtained for the enzyme would not fit the normal models for cooperativity (Monod et al., 1965; Koshland et al., 1966) but would have to be analyzed by modified hysteresis models. These problems are now being investigated.

The coenzyme activation on the esterase reaction has been first reported for the horse liver enzyme (Feldman & Weiner, 1972b), and the phenomenon has been studied subsequently with other mammalian ALDH; the degree of activation is species dependent. These data are summarized in Table II.

Table II: Degrees of NAD and NADH Activation of the Steady-State Velocity of Esterase Hydrolysis for Mammalian ALDHs

source	activation (x-fold) +NAD	+NADH	reference
horse liver			
p*I*5 (mitochondria)	2[a]	2	Feldman & Weiner (1972b)
p*I*6 (cytoplasma)	8	11	Eckfeldt & Yonetani (1976)
human liver	5.2	2.1	Sidhu & Blair (1975a)
sheep liver	1.7[b] 3[c]	1.8[b] 7[c]	MacGibbon et al. (1978)
rabbit liver	inhibition	inhibition	Duncan (1977)

[a] The degree of activation was 6.8-fold when the assay was not a V_{max} assay (Feldman & Weiner, 1972b). [b] These values were calculated from k_{cat} in Table I of the paper by MacGibbon et al. (1978). [c] These values were from Figures 1 and 3 of the paper by MacGibbon et al. (1978).

It is not possible at this time to state whether the differences are characteristic of each enzyme or are simply due to different experimental conditions being employed. We show here that for the p*I* 5 isozyme of horse liver ALDH the degre of activation could be as high as 6 at low substrate concentrations but is only a factor of 2 under a V_{max} assay. The rabbit liver enzyme, for which the esterase reaction is inhibited by coenzyme, also differs in other ways from enzymes isolated from the liver of other mammalian species. That enzyme is sensitive to the presence of steroidal hormones (Maxwell & Topper, 1961; Koivula & Koivusalo, 1975), a property not observed with other ALDHs (Takio et al., 1974; Sidhu & Blair, 1975a,b).

An explanation for the "2-fold" and "6-fold" activations by NAD can be made. Under the conditions where $(S)_0 \gg K_{MII}$, eq 2 reduces to eq 4 because α now equals 0. This equation

$$V_0 = k_{cat\,II}(E_{II}) \quad (4)$$

shows that the steady-state velocity correlates linearly with enzyme concentration. Since the value of $k_{cat\,II}$ in the presence of NAD is about twice that in its absence (Table I), the degree of activation due to NAD should be 2-fold at constant enzyme concentration. Under the condition of $\alpha = 1$ where only the E_I form of ALDH exists, the ratio of the steady-state velocities in the presence and absence of NAD should be about 6 at constant enzyme and low substrate concentrations since the value of K_{MI} is the same in the absence and presence of NAD (Table I).

The mechanism of the hydrolysis of NPA by ALDH can be depicted by the three-step mechanism proposed originally for chymotrypsin (Bender et al., 1967):

$$E + NPA \rightleftharpoons E{:}NPA \xrightarrow{k_a} E{-}\overset{O}{\overset{\|}{C}}CH_3 + NP \xrightarrow{k_d} E + CH_3CO_2^-$$

where NP is *p*-nitrophenol. In this scheme, E-$COCH_3$ is the acyl intermediate, and k_a and k_d are the rate constants for the formation (acylation) and decomposition (deacylation) of the intermediate, respectively. Previously, we have reported that no burst occurred in the esterase reaction catalyzed by the horse liver enzyme when an extrapolation from the steady-state phase to time zero was made (Weiner et al., 1976). We then concluded that the rate-limiting step for the reaction must have been the acylation step, k_a. Subsequently, MacGibbon et al. (1978) have observed a very small burst when the esterase

Table III: Rate Constants for Acylation and Deacylation Steps of Esterase Reaction of Horse Liver ALDH in the Absence of NAD

	10% burst[a]			25% burst[b]		
reactivity model	k_d/k_a[c]	k_a (s^{-1})[d]	k_d (s^{-1})[d]	k_d/k_a[c]	k_a (s^{-1})[d]	k_d (s^{-1})[d]
half-of-the-sites	1.2	2.2	2.7	0.41	4.1	1.7
all-of-the-sites	2.2	1.8	3.8	1.0	2.4	2.4

[a] Based on the initial small burst shown in Figure 1. [b] Based on the second burst shown in Figure 1. [c] These ratios were calculated from the observed burst magnitude and the assumed active-sites (half-of-the-sites or all-of-the-sites) concentration per tetrameric enzyme by using eq 6. [d] These values were calculated from the equation of $k_{cat} = k_a k_d/(k_a + k_d)$ by using the ratio of k_d/k_a and $k_{cat} = 1.2\ s^{-1}$ (at high substrate concentration in Table I).

reaction catalyzed by a sheep liver ALDH was measured with a stopped-flow apparatus. As shown in Figure 1, a very small initial burst plus an additional small burst were observed also in the esterase reaction of p*I* 5 isozyme of horse liver ALDH when the reaction was measured in the absence of NAD with the stopped-flow apparatus. This finding leads us to suggest tha the extrapolation to time zero from the initial portion of the time course performed in the steady-state assay may be unsuitable for measuring the presence or absence of a burst, as was pointed out by MacGibbon et al. (1978). We then must reconsider our previous conclusion that for the esterase reaction the acylation step is the rate-limiting step.

It is possible to determine the rate-limiting step for the enzyme from the stopped-flow experiments if one knows the theoretical number of active sites. The apparent burst magnitude, B, for the three-step mechanism is expressed by (Bender et al., 1967)

$$B = (E)_t[k_a/(k_a + k_d)]^2/[1 + (k_M/(S)_0)]^2 \qquad (5)$$

where $(E)_t$ is the total concentration of active sites. When $(S)_0 \gg K_M$ as was employed in these experiments, eq 5 can be simplified to yield eq 6. Only in the case where $k_a \gg k_d$ will

$$B = (E)_t/[1 + (k_d/k_a)]^2 \qquad (6)$$

the burst be equivalent to the total concentration of active sites. Depending upon the ratio of k_d/k_a, a burst magnitude less than the number of active sites of enzyme can be found. From the data presented in Figure 1, it can be estimated that the magnitude of the bursts observed in the absence of NAD is about 10% and 25% of the total subunit concentration for the first and second bursts, respectively. The ratio of k_d/k_a can be estimated from the observed burst magnitude for the enzyme functioning with half-of-the-sites or with all-of-the-sites reactivity in the esterase reaction (Table III). Finding a ratio near unity implies that the value of the rate constant of the acylation step is nearly equal to or the same as that of the deacylation step. This is in contrast to the dehydrogenase reaction where the deacylation step is very much slower than the acylation step (Weiner et al., 1976). The values of the two rate constants, k_a and k_d, can be estimated from the ratio of k_d/k_a and the k_{cat} presented in Table I since $k_{cat} = [k_a k_d/(k_a + k_d)]$ for the three-step model of ester hydrolysis (Bender et al., 1967). The individual rate constants calculated are presented also in Table III.

In the presence of NAD, the burst magnitude was 4 mol of product per mol of tetrameric enzyme (Figure 1). Thus, under these assay conditions, all four subunits are catalytically functioning. The fact that the burst magnitude is 100% of the total subunit concentration means $k_a \gg k_d$. With the assumption then that k_{cat} in the presence of NAD is equal to k_d, a value for k_d is 2.2 s^{-1} at high substrate concentrations. This value for k_d in the presence of NAD is essentially identical with the value found in the absence of NAD for the model of all-of-the-sites reactivity at high substrate concentration. It appears then that NAD is increasing the rate of total esterase reaction by increasing the magnitude of the rate constant of the acylation step, not the deacylation step. Thus, we have a mechanistic explanation as to the activation of the esterase reaction by coenzyme, though we cannot yet explain the detailed chemistry. The presence of coenzyme appears to increase the nucleophilicity of the active site amino acid and hence increase the velocity of the attack of the nucleophile on the ester carbonyl group to form a covalent intermediate. A logical extension, then, is to explain why in the dehydrogenase reaction the coenzyme binds prior to the substrate. Aldehyde also forms a covalent adduct, and apparently in the presence of NAD, the active-site nucleophile is chemically more reactive. Thus, tetrahedral formation with the carbonyl carbon is favored in the presence of NAD.

It has not yet been determined what amino acid is acting as a nucleophile at the active site of ALDH. In the case of GAPDH, which also catalyzes the hydrolysis of NPA (Park et al., 1961; Bond et al., 1970), the amino acid is a cysteine residue (Harris & Waters, 1976). However, it has been also reported that the esterase reaction catalyzed by GAPDH is inhibited rather than activated by NAD (Mathew et al., 1967). We are investigating the active-site amino acid of p*I* 5 isozyme of horse liver ALDH and the reason for the difference of the coenzyme activation of the esterase reaction catalyzed by the two enzymes.

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Inactivation of the RTEM β-Lactamase from *Escherichia coli*. Interaction of Penam Sulfones with Enzyme†

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ABSTRACT: The characteristics of the reaction of a number of mechanism-based inactivators of the RTEM β-lactamase have suggested that a common mechanistic pathway may be followed by many of these compounds. These ideas have been tested by the synthesis and evaluation of some penam sulfones as β-lactamase inactivators. The sulfones of poor β-lactamase substrates are, as predicted, potent inactivators of the enzyme. A unique serine residue (Ser-70) is labeled by quinacillin sulfone, and it is likely that this serine acts nucleophilically in the normal hydrolytic reaction of the β-lactamase to form an acyl-enzyme intermediate.

β-Lactam antibiotics exert their lethal effect on growing cells by interfering with the enzymes responsible for the synthesis and integrity of the bacterial cell wall. Resistance to the bacteriocidal action of β-lactams may develop, however, and this resistance is most frequently due to the production by the organism of a β-lactamase which catalyzes the hydrolytic cleavage of the β-lactam ring in penicillins and cephalosporins. The gene that encodes the β-lactamase is often on a plasmid, and the promiscuous transfer of plasmids among bacterial populations has sharply increased the incidence of β-lactam-resistant infections. This problem is of obvious clinical importance and was first attacked by developing β-lactam antibiotics [for example, semisynthetic penams (e.g., Doyle & Nayler, 1964), the cephamycins (Nagarajan et al., 1971), thienamycin (Kahan et al., 1979), and moxalactam (Komatsu & Nishikawa, 1980)] that were less sensitive to the hydrolytic action of the β-lactamases. More recently, however, a second approach to the β-lactamase problem has been taken; it utilizes the synergistic action of reagents that inhibit the β-lactamase when applied simultaneously with a good (though β-lactamase-sensitive) antibiotic. The first report of a β-lactamase inactivator, clavulanic acid (Brown et al., 1976), has been followed by the isolation and synthesis of a number of natural and semisynthetic β-lactam derivatives, including carbapenems (Brown et al., 1977; Maeda et al., 1977; Okamura et al., 1980), penicillanic acid sulfone (English et al., 1978; Labia et al., 1980), 6β-bromopenicillanic acid (Pratt & Loosemore, 1978; Knott-Hunziker et al., 1979a,b), 6-chloropenicillanic acid sulfone (Cartwright & Coulson, 1979), and the sulfenimines of various cephems and penams (Gordon et al., 1980). The mechanism of action of many of these materials has been subjected to some scrutiny (Fisher et al., 1980a), and it seems likely that, in common with at least one substrate (Fisher et al., 1980b), the interaction with the enzyme involves the formation of an acyl-enzyme. The acyl-enzyme may then collapse to a more stable species, with concurrent inactivation of the enzyme. We suggested, therefore, that most, if not all, of the above β-lactam inactivators are "suicide" or "mechanism-based" inactivators of the β-lactamase (Fisher et al., 1980a).

The simplest mechanistic postulate that accommodated the kinetic and chemical behavior of clavulanic acid and the penicillanic acid derivatives [carbapenems such as the olivanates are considered elsewhere (Charnas & Knowles, 1981)] was that the first-formed acyl-enzyme suffered a β elimination across C-5 and C-6 (Scheme I). Abstraction of the 6α proton and cleavage of the bond between C-5 and the heteroatom would lead to a relatively stable β-aminoacrylate chromophore and concurrent inactivation of the enzyme. It seemed that the acyl-enzyme could partition between this β elimination and the normal hydrolysis of the ester linkage (Scheme I), and we suggested (Fisher et al., 1980a) that this class of inactivators

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