Relationship between the Mechanisms of the Esterase and Dehydrogenase Activities of the Cytoplasmic Aldehyde Dehydrogenase from Sheep Liver. An Alternative View†

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ABSTRACT: In order to account for the low amplitude burst in 4-nitrophenoxide production (0.2–0.3 mol per dehydrogenase active site) observed during the transient phase of the esterase catalytic pathway of cytoplasmic aldehyde dehydrogenase (E) from sheep liver with 4-nitrophenyl acetate (PNPA) as substrate, an equilibrium between E·PNPA and E·acyl complexes is proposed. No release in protons was observed during the transient phase of the esterase reaction, and $MgCl_2$ which is an uncompetitive inhibitor of the dehydrogenase reaction at low concentrations (<100 μM) of propionaldehyde [Bennett, A. F., Buckley, P. D., & Blackwell, L. F. (1982) *Biochemistry 21*, 4407–4413] had virtually no effect on the hydrolysis of 4-nitrophenyl acetate in the presence of NADH. These results suggest that the esterase reaction does not involve the same kinetic mechanism as does the dehydrogenase reaction. Added NAD^+ was shown to increase the rate of the deacylation step on the esterase pathway without affecting the esterase burst rate constant or amplitude. Chloral hydrate is a competitive inhibitor of the dehydrogenase activity with respect to propionaldehyde in the presence of NAD^+ but not of the esterase activity with respect to 4-nitrophenyl acetate in the presence of NAD^+. On the basis of these results it is proposed that different binding domains (or regions) are involved for propionaldehyde (at concentrations <100 μM) and PNPA. The partially competitive inhibition pattern, obtained when the inhibition of the esterase activity by low (<100 μM) concentrations of propionaldehyde is studied in the presence of NAD^+, is evidence that propionaldehyde (at concentrations <100 μM) and PNPA can bind simultaneously to the E·NAD^+ binary complex. Pre-steady-state kinetic studies of the burst in production of NADH over a wide range of propionaldehyde concentrations above that required to saturate the high-affinity catalytic site (K_m = 1.1 μM) show no change in the burst rate constant or amplitude, despite the 3-fold enhancement of the steady-state rate at ≥20 mM propionaldehyde. A two-site model is proposed for aldehyde dehydrogenase involving in addition to the dehydrogenase active site, which contains a high-affinity propionaldehyde binding domain (P1), a modifier site which contains a low-affinity propionaldehyde binding domain (P2; K_m = 3.5 mM). In this model propionaldehyde can only bind to the P1 binding domain in the presence of NAD^+, PNPA binds only in the P2 binding domain, irrespective of the presence or absence of NAD^+, and different catalytic groups are involved in the two enzymic activities of sheep liver cytoplasmic aldehyde dehydrogenase.

It is now well established (Feldman & Weiner, 1972; Sidhu & Blair, 1975; Eckfeldt & Yonetani, 1976; Weiner et al., 1976; Hart & Dickinson, 1978; Kitson, 1978, 1982; MacGibbon et al., 1978; Duncan, 1979; Takahashi & Weiner, 1981; Vallari & Pietruszko, 1981) that the cytoplasmic and mitochondrial aldehyde dehydrogenases (EC 1.2.1.3) from a variety of mammalian sources also possess esterase activity toward 4-nitrophenyl esters. This esterase activity has been studied both because a convenient chromophore is produced during the hydrolysis which allows easy monitoring of the reaction and also because it is hoped that a study of the esterase reaction will lead to new insights into the dehydrogenase activity of ALDH.[1] The basis of this expectation is the common assumption that the dehydrogenase and esterase activities of ALDH both involve acylation of the same functional group, and hence the chemistry of the hydrolysis of the acyl-enzyme intermediates would be identical for both reaction pathways.

In order that valid mechanistic conclusions be drawn when the results obtained from a study of one type of enzymic activity are applied to the other activity, it is important to demonstrate that the kinetic and chemical mechanism of the two reactions are similar. It is also necessary to determine whether the two enzymic activities involve the same catalytic groups.

We have therefore studied the steady-state and pre-steady-state kinetics for the esterase activity of sheep liver cytoplasmic ALDH and compared them with the kinetics of the dehydrogenase reaction. We have found that the kinetic mechanism of the esterase reaction is significantly different from that reported for the dehydrogenase activity (Bennett et al., 1982) and also different from the mechanism proposed for the esterase activity of the horse liver mitochondrial enzyme (Takahashi & Weiner, 1981). We conclude for the sheep liver cytoplasmic enzyme that hydrolysis of PNPA is not taking place at the active site for the dehydrogenase reaction.

Experimental Procedures

Materials. Propionaldehyde was obtained from Koch-Light Laboratories (Colnbrook, Bucks, U.K.), 4-nitrophenyl acetate (PNPA) was from Aldrich Chemical Co. (Milwaukee, WI), NAD^+ (grade III) was obtained from Sigma Chemical Co. (St. Louis, MO), and all other chemicals were of the highest purity available. Solutions of propionaldehyde and PNPA for kinetics were prepared as previously described (Agnew et al., 1981), and cytoplasmic ALDH, which was shown by isoelectric focusing experiments to be virtually free of contamination by the mitochondrial enzyme (Agnew et al., 1981), was prepared essentially as described by MacGibbon et al. (1979).

Enzyme Assay (as a Dehydrogenase). Aldehyde dehydrogenase activity was measured by monitoring the increase in absorbance at 340 nm with a Unicam SP 1800 spectro-

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; PNPA, 4-nitrophenyl acetate; PNP, 4-nitrophenoxide ion; P1, catalytic low-K_m (1.1 μM) propionaldehyde binding domain; P2, high-K_m (3.5 mM) propionaldehyde binding domain which also binds PNPA.

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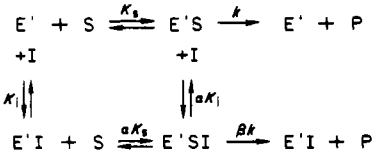
Scheme I



photometer at 25 °C. All experiments were carried out in 25 mM phosphate, pH 7.6, buffer, and the final assay volume was 3 cm^3. The concentration of NAD^+ was usually 1 mM, but the concentrations of propionaldehyde and modifiers were varied as required. When the propionaldehyde concentration was 20 mM, the enzyme active site concentration could be calculated from the initial velocity (V_{max}) by using a k_{cat} value of 0.25 s^{-1} per active site (MacGibbon et al., 1977a). Alternatively, an NADH titration (MacGibbon et al., 1979) gave the concentration of NADH binding sites directly. Thus all of our kinetic data are reported as rate constants per active site (NAD or NADH binding site) and not per enzyme tetramer as reported by Hart & Dickinson (1982).

Enzyme Assay (as an Esterase). The rate of hydrolysis of PNPA was determined spectrophotometrically in 25 mM phosphate, pH 7.6, buffer at 25 °C by following the increase in A_{400} corresponding to the production of the 4-nitrophenoxide ion as described previously (MacGibbon et al., 1978). When initial velocities were measured as a function of ester concentration, blanks in which the enzyme was omitted were always carried out in order to determine the spontaneous rate of hydrolysis which was subtracted from the enzyme-catalyzed rate. For inhibition experiments the concentration of NAD^+ in the assay was approximately 100 μM since this concentration gave the maximum stimulation of the esterase activity (MacGibbon et al., 1978) due to saturation of the coenzyme binding site.

Stopped-Flow Experiments. These were carried out on a Durrum-Gibson D110 stopped-flow spectrophotometer as described by Hardman et al. (1977) in either the fluorescence or absorbance modes. Proton release experiments were performed with enzyme which was dialyzed against 0.5 mM, pH 7.6, phosphate buffer and made up in solutions containing 0.1 M Na_2SO_4, 0.1 M $NaNO_3$, and phenol red (10–30 μM) as we have described previously (Agnew et al., 1981; Bennett et al., 1982).

Analysis of Inhibition Patterns. The inhibition patterns were analyzed as discussed by Segel (1975) based on the general modifier mechanism of Botts & Morales (1953) assuming that rapid equilibrium conditions prevail for the binding of inhibitors and substrates. Inhibition of both the esterase and dehydrogenase activity may be expressed in a general way (Scheme I).

In Scheme I K_s is the binding constant for binding of ester or aldehyde to the binary E·NAD complex (E′),[2] α is the factor by which K_s changes when the inhibitor is bound to the enzyme, K_i is the inhibitor constant for binding of I to E′, and β is the factor by which the rate constant for the breakdown of the E′S complex is altered in the presence of the inhibitor. Under rapid equilibrium conditions if K_s is altered by a factor α in the presence of the inhibitor so too will K_i become αK_i in the presence of the inhibitor. All the general cases of competitive, noncompetitive, and uncompetitive inhibition (pure, partial, or mixed) may be derived as special cases of Scheme I by assignment of appropriate values to K_s, K_i, α, and β. For example, if $\alpha = \infty$, Scheme I reduces to the usual scheme for competitive inhibition since I will not bind to E′S and S will not bind to E′I; thus the effect of the inhibitor can be overcome by sufficiently high concentrations of substrate.

Computer Simulations. These were performed on a Cromemco Z2D microcomputer as discussed previously by Bennett et al. (1983).

Results and Discussion

Mechanism of PNPA Hydrolysis at pH 7.6. A burst in the production of PNP has previously been measured by MacGibbon et al. (1978) in the absence of NAD^+, and a value of 12 ± 2 s^{-1} has been obtained for the burst rate constant, the amplitude of which corresponds to 30% of the dehydrogenase active site concentration of the enzyme. The reported burst rate constant and amplitude represent saturating values since they were unchanged over a 15-fold concentration range of PNPA. In the present work when enzyme (8 μM) premixed with NAD^+ (200 μM) was mixed with PNPA (400 μM) in the stopped-flow apparatus at pH 7.6 in 25 mM phosphate buffer, a burst in the production of PNP was again observed, with a rate constant of 11 s^{-1} and an amplitude of 0.7 μM which corresponds to 18% of the dehydrogenase active site concentration. These parameters were the same as those obtained for a control experiment in the absence of NAD^+; however, the steady-state reaction following the transient process was several times faster when NAD^+ was present (1.6 μM s^{-1} compared with 0.5 μM s^{-1}) as expected from previous work (MacGibbon et al., 1978). The steady-state rate was linear for at least 50 s following the burst, with no evidence for any slower transients such as those reported by Takahashi & Weiner (1981) for the horse liver mitochondrial enzyme. When conventional spectrophotometry was used, the steady-state assays exhibited nonlinearity after an absorbance change of 0.3, consistent with inhibition by released 4-nitrophenoxide ion as suggested by Kitson (1981).

When enzyme (27 μM) was mixed with PNPA (800 μM) in the stopped-flow apparatus in the presence of phenol red (15 μM), Na_2SO_4 (0.1 M), and $NaNO_3$ (0.1 M), no transient production of protons was observed even on instrument settings which would reveal a burst amplitude as low as 5% of the dehydrogenase active site concentration. Only a slow steady-state release of protons was detected, the rate of which was almost identical with the steady-state production of PNP as measured by assay at 400 nm. When the experiment was repeated with NAD^+ (1 mM) added, there was again no detectable burst of protons, although the steady-state rate of proton release was increased, consistent with the results obtained when the rate of PNP production was monitored.

The observation of a single transient followed by a steady state in the production of PNP during the hydrolysis of PNPA, catalyzed by sheep liver cytoplasmic ALDH, clearly indicates that at least two chemical steps are involved in the reaction and hence at least one enzyme intermediate must be present in concentrations that are significant with respect to the total enzyme concentration. Since there was no accompanying release of protons during the transient phase of the reaction, the production of acetic acid cannot be synchronous with the production of PNP but must be formed during the subsequent steady-state phase of the reaction which implies that the enzyme intermediate is an acyl-enzyme complex as has commonly been assumed. The steady-state rate-limiting step for the hydrolysis of PNPA by the sheep liver cytoplasmic enzyme must therefore be envisaged as deacylation of the enzyme rather than acylation as proposed for the horse liver mito-

[2] The esterase activity may be studied in the absence of NAD^+ (in which case E·NAD^+ or E′ in Scheme I simply becomes E), but the dehydrogenase activity of course cannot.

Scheme II

$$E + PNPA \overset{K_s}{\rightleftharpoons} E\cdot PNPA \underset{}{\overset{11\ s^{-1}}{\rightleftharpoons}} E\cdot acyl + PNP \xrightarrow{0.2\ s^{-1}} E + RCOO^- + H_3O^+$$

chondrial enzyme (Weiner et al., 1976). Although the nature of the group which is acylated is currently unknown, the fact that no protons were detected during acylation of the enzyme shows that the group is already ionized at pH 7.6 or is part of a charge relay system as proposed recently by Takahashi et al. (1981) for the dehydrogenase activity of the horse liver mitochondrial ALDH.

Rather surprisingly the amplitude of the burst at saturating PNPA concentrations was only 20–30% of the dehydrogenase active site concentration, and hence the concentration of the acyl-enzyme intermediate cannot be greater than this during the transient phase of the reaction. The apparent burst magnitude (B) for a three-step Michaelis–Menten mechanism is given under saturating substrate conditions by expression 1 derived by Bender et al. (1967) where in this case E_0 is the

$$B = E_0[k_2/(k_2 + k_3)]^2 \quad (1)$$

concentration of NADH binding sites (or dehydrogenase active sites), k_2 is the rate constant for the acylation step, and k_3 is the rate constant for deacylation. If the esterase active site concentration is the same as the dehydrogenase active site concentration, then a full burst (equal to E_0) would be expected irrespective of the actual number of such sites per tetramer since k_2 (which is approximately equal to the burst rate constant of 11–12 s^{-1}) is considerably greater than k_3 (which is approximately equal to the k_{cat} value for the esterase reaction of 0.2 s^{-1}) and hence the ratio $k_2/(k_2 + k_3)$ is nearly equal to unity and $B \sim E_0$. It should be noted that if the concentration of esterase active sites was twice the number of the dehydrogenase active sites as found for the horse liver mitochondrial enzyme (Takahashi & Weiner, 1981), then the amplitude of the burst in PNP production (B) should be approximately equal to $2E_0$ in which case the observed burst amplitude would correspond to only 10–15% of the available esterase active sites.

In order to account for the observed low amplitude of the burst, significant concentrations of a nonabsorbing transient intermediate such as the binary E·PNPA complex must be in equilibrium with the acyl-enzyme intermediate throughout the transient phase of the reaction (Scheme II). In this way an equilibrium would be established during the burst phase between E·PNPA and E·acyl such that only a fraction of the available active sites are in the E·acyl form. Reversal of the acylation step logically requires that PNP can bind to E·acyl, and hence an E·acyl·PNP complex should also exist, but since this species would also absorb at 400 nm and contribute to the total burst amplitude, it obviously cannot be present in large concentrations. Computer simulations based on Scheme II confirmed that only when a reversal of the acylation step was included [which is not allowed for in the simplified scheme considered by Bender et al. (1967)] could the amplitude of the burst be reduced to the experimentally observed values without simultaneously reducing the magnitude of the burst rate constant. Any other model no matter how complex which did not include reversal of acylation always resulted either in a burst amplitude which was equal to the enzyme concentration or in a reduced burst amplitude but with a correspondingly reduced burst rate constant. The nonlinearity of the steady-state assays in the presence of NAD^+, after the increase in A_{400} is 0.3 (corresponding to 22 μM PNP), is consistent with the product inhibition expected on the basis of Scheme II due to the binding of PNP to the E·acyl species.

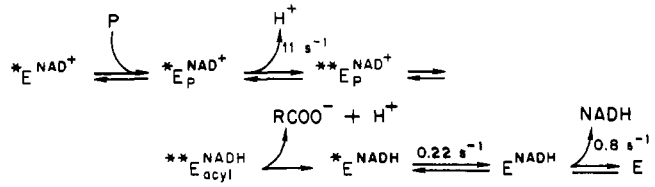
Unfortunately, since a fractional burst in the transient production of PNP was observed, it is not possible to draw any conclusions regarding either the relative number of dehydrogenase and esterase active sites or the stoichiometry of PNPA binding. There is no a priori way in which a fractional burst amplitude can be related to the actual number of PNPA molecules which are bound per enzyme tetramer (and hence presumably to the number of active sites) unless the number, kind, and concentration of all transient enzyme intermediates are known together with the rate constants for their interconversion. All that can be said in the present circumstances therefore is that the actual concentration of esterase active sites is greater than that which is observed experimentally.

The enhancement of the esterase steady-state activity which is observed for ALDH in the presence of NAD^+ has been explained in the case of the horse liver enzyme (Takahashi & Weiner, 1981) on the basis of an increased nucleophilicity of the functional group involved in the acylation step leading to a more rapid formation of the acyl-enzyme intermediate and an increase in the number of functioning subunits. That this was not the case for the sheep liver cytoplasmic enzyme was clearly shown by the fact that neither the amplitude of the esterase burst nor the burst rate constant was altered by the presence of NAD^+, but the steady-state rate immediately following the burst was stimulated 2–3-fold as previously shown by MacGibbon et al. (1978) from steady-state measurements using conventional spectroscopy. The effect of NAD^+ in our case must therefore be to increase the rate of the deacylation process, possibly by altering the environment of the PNPA binding site as a result of the conformational change which has been suggested to occur on binding of NAD^+ in its normal coenzyme binding site (MacGibbon et al., 1978; Bennett et al., 1982) in such a way that the nucleophilic attack by a water molecule is facilitated. However, it clearly does not alter the rate constant for the acylation step, nor does it increase the transient concentration of the acyl-enzyme intermediate; thus Scheme II also applies in the presence of NAD^+ (except that k_{cat} is increased to about 0.6 s^{-1}).

Whether stimulation of the steady-state esterase activity is actually observed depends on the ratio of the NAD^+ and PNPA concentrations. Stimulation will only be observed when the enzyme is predominantly in the E·NAD^+·PNPA form, but since NAD^+ can also bind to a second site so that binding of PNPA is prevented (MacGibbon et al., 1978), stimulation is not observed at high NAD^+ concentrations. Since Takahashi & Weiner (1981) reported that for horse liver mitochondrial ALDH a greater stimulation by NAD^+ is found at lower concentrations of PNPA than at saturating levels, the stimulatory effect of NAD^+ (57 μM) on the sheep liver esterase activity was also measured at two low concentrations of PNPA (7.8 and 23 μM). However, for the sheep liver cytoplasmic enzyme there was no activation at all at 7.8 μM PNPA and only a slight (9%) activatory effect of NAD^+ at 23 μM PNPA; hence, at low ester concentrations E and E·NAD^+ hydrolyze PNPA at virtually the same rate. This result is to be expected for sheep liver cytoplasmic ALDH since the apparent K_m value for PNPA is increased in the presence of NAD^+, and hence the greater intrinsic reactivity of the E·NAD^+·PNPA complex is counterbalanced by a lower degree of saturation of the PNPA binding site.

It is clear therefore that the esterase activity of sheep liver cytoplasmic ALDH is quite different in many respects from the behavior reported for horse liver mitochondrial ALDH by Takahashi & Weiner (1981). In addition to the differences

Scheme III



already mentioned for the sheep liver enzyme, the steady state immediately following the burst was linear both in the presence and in the absence of NAD^+. Also, for the sheep liver enzyme the Lineweaver–Burk plots were linear even at 800 μM PNPA and provided no evidence for two states of the enzyme with respect to the hydrolysis of PNPA or for two nonequivalent sets of PNPA binding sites. These differences in behavior between the two enzymes may reflect a species difference since the esterase activity of sheep liver mitochondrial ALDH (Agnew et al., 1981) appears to be generally similar to that observed for the sheep liver cytoplasmic enzyme.

Relationship between the Esterase and Dehydrogenase Kinetic Pathways at pH 7.6. We have recently proposed (Bennett et al., 1982) a mechanism for the dehydrogenase activity at low concentrations of propionaldehyde (<100 μM) which is shown in Scheme III. In Scheme III P represents propionaldehyde, and the reaction sequence is shown for convenience as commencing with the conformationally rearranged binary *E·NAD^+ complex. The essential features of the mechanism involve an isomerization of the $*E_P^{NAD^+}$ ternary complex (accompanied by a proton loss) which controls the rate of both the proton and NADH bursts (Bennett et al., 1982), the formation of an $**E_{acyl}^{NADH}$ ternary complex combined with a conformational rearrangement to form the *E·NADH binary complex which, together with an isomerization of the binary *E·NADH complex, controls the steady-state rate. Since NADH dissociation is controlled by this isomerization step with a rate constant of 0.22 s^{-1} (MacGibbon et al., 1977b) and the hydride transfer step is fast, the rate constant for the hydrolysis of the $**E_{acyl}^{NADH}$ species must be about 0.13 s^{-1} to account for the observed k_{cat} value (0.082 s^{-1} per active site) as discussed by Bennett et al. (1983). Thus at low concentrations of propionaldehyde the predominant enzyme form will be $**E_{acyl}^{NADH}$ in the steady-state phase of the reaction. The direct spectrophotometric observation of the buildup of such acyl-enzyme intermediates on the reaction pathway with chromophoric aldehydes has recently been reported (Buckley & Dunn, 1982).

Scheme II and Scheme III are therefore similar in that both involve the slow hydrolysis of an acyl-enzyme intermediate, but this is really the only step which could possibly be considered common to both pathways. For example, the conformational change and rapid release of a proton (Bennett et al., 1982) which follow propionaldehyde binding (Scheme III) were not observed in the present work when PNPA bound to the enzyme, and the rates of formation of the acyl-enzyme intermediates at pH 7.6 are clearly different. The rate constant for the burst in PNP production (11 s^{-1}) is significantly less than the rate constant (23 s^{-1}) for the burst in production of NADH during the oxidation of acetaldehyde (MacGibbon et al., 1977c) although in both cases an acetyl-enzyme intermediate is presumably formed. The first steps in the oxidation of propionaldehyde would yield the $**E_{XCOCH_2CH_3}^{NADH}$ intermediate rather than the $E_{XCOCH_3}^{NADH}$ intermediate (as would be formed by initial reaction by E^{NADH} and PNPA). Since the burst amplitudes for the two acetylation processes are also different, it is apparent that the esterase reaction does not (nor should it be expected to) involve the same transient enzyme species as does the dehydrogenase reaction, and different conformational states of the enzyme are probably involved.

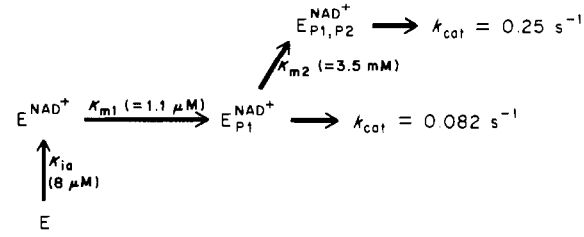
Although it is a common assumption for aldehyde dehydrogenases that from the point of acylation onward the kinetics of the two enzymic activities are identical, results obtained with magnesium ions showed that this was not the case for cytoplasmic sheep liver ALDH irrespective of whether the two activities involve the same catalytic groups. The addition of $MgCl_2$ (1.6 mM) had no effect on the rate of hydrolysis of PNPA at saturating ester concentrations, and increasing the concentration of $MgCl_2$ to 15 mM was equally ineffective. However, when $MgCl_2$ (1.6 mM) was added to an assay containing enzyme (2 μM), PNPA (300 μM), and NAD^+ (100 μM), there was an immediate inhibition of the esterase activity. When the same concentration of $MgCl_2$ was added to an assay containing NADH (100 μM), which like NAD^+ has been reported to give stimulation of the esterase activity (MacGibbon et al., 1978), only a small (4.9%) inhibitory effect was observed.[3] Addition of higher concentrations of $MgCl_2$ did not result in any further inhibition. It is, however, clear that magnesium ions bind in the same site in both reactions since the K_i values for magnesium as determined from inhibition of both the esterase and dehydrogenase activities are 320 μM and 240 μM, respectively.

We have previously shown (Bennett et al., 1983) that magnesium ions do not affect either the rate constants or burst amplitudes of the bursts in PNP production (during the hydrolysis of PNPA) and NADH production (during the oxidation of propionaldehyde) and hence that only the postacylation steps are being affected. Therefore, if the same enzyme intermediates were involved in the two enzymic reactions from the point of acylation onward, then on the basis of our previous work (Bennett et al., 1983) on the dehydrogenase reaction at low concentrations of propionaldehyde (<100 μM) in the presence of $MgCl_2$ a decrease in k_{cat} for the esterase reaction would be predicted (especially in the presence of NADH with presumably the same E–NADH–acyl intermediate being formed as in the dehydrogenase reaction). At these low concentrations of propionaldehyde, inhibition of the acyl-enzyme hydrolysis step (Scheme III) by magnesium ions is required to account for the observed inhibition of the dehydrogenase activity (Bennett et al., 1983), and thus the esterase reaction would be similarly inhibited. As there is virtually no inhibition of the esterase reaction by magnesium ions in the presence of NADH, we must conclude that for cytoplasmic sheep liver ALDH the postacylation reaction also does not proceed by a common kinetic pathway for the esterase and dehydrogenase activities. Thus, for the cytoplasmic ALDH from sheep liver a study of the kinetics and mechanism of the enzyme-catalyzed hydrolysis of PNPA does not lead to any new information regarding the kinetics and mechanism of propionaldehyde oxidation.

Evidence for Separate PNPA and Propionaldehyde Binding Domains. The fact that the kinetic pathways for the esterase and dehydrogenase activities of cytoplasmic sheep liver ALDH are not the same does not of course exclude the possibility that both reactions involve acylation of the same nucleophilic center. For example, the obligatory conformational change which we have reported (Bennett et al., 1982) for the dehydrogenase pathway (Scheme III) might indicate that the acylation step requires a physical movement of the nonchemically bound

[3] The stimulation of the esterase activity caused by the presence of NADH is only a factor of 1.2 whereas it was incorrectly reported previously (MacGibbon et al., 1978) as 2–3.

Scheme IV



propionaldehyde molecule to bring it into proximity with the catalytic group. Since the same conformational change does not occur along the esterase pathway (no proton being released), it is conceivable that PNPA binds in a different region, or domain, of the dehydrogenase active site but ultimately acylates the same catalytic group. This picture necessitates a conceptual distinction between an active site, which can be a relatively large and structurally nonhomogeneous region of a protein, and a binding domain which is a smaller area within the active site with a special affinity for the substrate(s). The active site will of course contain a catalytic group which performs the chemistry of the enzyme-catalyzed reaction. When the differences between sites, domains, and catalytic groups are taken into account, there appear to be three possible ways in which the dehydrogenase and esterase activities of ALDH can be related. Case 1: PNPA and propionaldehyde bind in the same region (or domain) of the dehydrogenase active site and utilize the same catalytic groups. Case 2: PNPA and propionaldehyde bind in different domains within the dehydrogenase active site but utilize the same set of catalytic groups. Case 3: PNPA and propionaldehyde do not bind in the same active site on the protein and (necessarily) utilize different sets of catalytic groups. It is our aim in subsequent sections of this paper to distinguish between these three possible cases for ALDH. However, before the evidence concerning the three cases listed is discussed, it is helpful to consider the accepted steady-state mechanism for the oxidation of propionaldehyde at low (<100 μM) and high (ca. 20 mM) concentrations.

MacGibbon et al. (1977a) have shown that the substrate activation which is observed when the steady-state initial velocity is measured over a wide range of propionaldehyde concentrations can be explained if ALDH possesses two types of binding domain for propionaldehyde. At low concentrations (<100 μM) the steady-state kinetics can be described in terms of a binding domain (P1) with a high affinity for propionaldehyde which has a low (1.1 μM) K_m value and a k_{cat} value of 0.082 s^{-1}, whereas at high concentrations (>20 mM) the data can be fitted if a second low-affinity propionaldehyde binding domain (P2) is assumed which has a high K_m value (3.5 mM). When both the P1 and P2 binding domains are occupied, the k_{cat} value is increased 3-fold to 0.25 s^{-1}. A high-affinity propionaldehyde binding domain and a low-affinity propionaldehyde binding domain are also required to fit the magnesium inhibition data which we recently reported (Bennett et al., 1983) for the dehydrogenase activity of cytoplasmic sheep liver ALDH at both low (<100 μM) and high (1–20 mM) propionaldehyde concentrations. The general mechanism proposed on the basis of this work together with the appropriate kinetic constants is shown in Scheme IV.

In Scheme IV the coenzyme binding domain is shown as a superscript, the high-affinity propionaldehyde binding domain is shown in the first subscript position, and the low-affinity propionaldehyde binding domain is shown in the second subscript position; however, no specific spatial relationships between the domains were implied by this scheme. At low concentrations of propionaldehyde (<100 μM) the normal pathway involves the $E^{NAD^+}_{P1}$ complex whereas at high concentrations (20 mM) the active form is mainly $E^{NAD^+}_{P1,P2}$.

We have previously shown (MacGibbon et al., 1978) that high concentrations of propionaldehyde (ca. 20 mM) competitively inhibit PNPA hydrolysis in the absence of coenzymes which is consistent with case 1, but inhibition experiments were not previously carried out in the presence of NAD^+ since it was believed that the NADH produced during the concurrent aldehyde oxidation would interfere with the measurement of the rate of PNP production at 400 nm. However, when low concentrations of propionaldehyde (90 μM), which are sufficient to saturate the high-affinity (K_m = 1.1 μM) propionaldehyde binding domain (P1) but too low to bind in the low-affinity (K_m = 3.5 mM) propionaldehyde binding domain (P2), were added to an assay containing enzyme (1.5 μM), NAD^+ (257 μM), and PNPA (185 μM) at pH 7.6 in 25 mM phosphate buffer at 25 °C while the ester hydrolysis was monitored at 400 nm, the initial velocity (0.5 μM s^{-1}) was reduced by 64.3%. The kinetic traces were linear, and a control assay in which the ester was omitted showed no absorbance change at 400 nm due to the concurrent production of NADH. At this level of propionaldehyde no inhibition of the esterase activity was observed if NAD^+ was not present. On the other hand, when the oxidation of the propionaldehyde was monitored at 340 nm under the same conditions (and order of mixing as above), the apparent rate of reaction was greater than for the control in which the ester was omitted. However, when the contribution due to the absorbance of 4-nitrophenoxide ion at 340 nm (40% of the absorbance at 400 nm) was substracted, it was apparent that the enzyme-catalyzed oxidation of propionaldehyde was inhibited by about 30% in the presence of PNPA under the conditions specified above.

When a concentration of propionaldehyde (27.4 mM) which was sufficient to saturate both the high- and low-affinity aldehyde binding domains was added to an assay containing enzyme (1.5 μM), NAD^+ (257 μM), and PNPA (37 μM), the ester hydrolysis was inhibited by 94.3%. There was no significant inhibition of the aldehyde oxidation when the same assay mixture was monitored at 340 nm.

The results of a systematic study of the inhibition of the esterase activity in the presence of NAD^+ at concentrations of propionaldehyde less than 100 μM are shown in Figure 1. An apparently competitive inhibition pattern is observed, with K_m(app) for PNPA being increased in the presence of propionaldehyde but V_{max} being unaffected. However, K_m(app) did not increase to infinity as expected for true competitive inhibition but approached a limit at a propionaldehyde concentration of approximately 100 μM (see inset to Figure 1) when the high-affinity binding domain should be saturated. As expected for partially competitive inhibition, a replot of $1/\Delta$(slope) vs. 1/[propionaldehyde] was linear (see inset to Figure 1), from which a K_i value of 1.44 μM was derived for the binding of propionaldehyde to the E·NAD^+ binary complex. The apparent ester binding constant (K_s) was 25 μM in the absence of propionaldehyde but was increased (α = 5.3) in its presence whereas the maximum velocity was unaltered (β = 1).

The inhibition constant (1.44 μM) for propionaldehyde confirms that propionaldehyde is causing inhibition by binding in the usual P1 binding domain. However, even at levels of propionaldehyde which were sufficient to saturate the P1 binding domain, in the presence of PNPA the slope of the Lineweaver–Burk plot did not approach infinity as must be

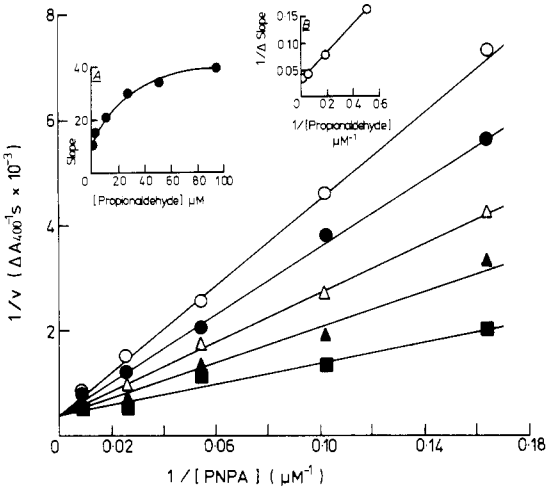


FIGURE 1: Inhibition of esterase activity at low concentrations of propionaldehyde. ALDH (0.19 μM), NAD^+ (37 μM), and varying concentrations of propionaldehyde [(■) 0, (▲) 5.1, (△) 24.1, (●) 48.2, and (○) 96.4 μM] were premixed in a spectrophotometer cuvette at 25 °C in 25 mM, pH 7.6, phosphate buffer. The reaction was initiated by adding PNPA to give the final assay concentrations shown in the figure. (Inset A) Slope (ΔA_{400}^{-1} μM s × 10^{-3}) vs. propionaldehyde concentration. (Inset B) Δslope^{-1} (s^{-1} ΔA_{400} μM^{-1} × 10^3) vs. [propionaldehyde]$^{-1}$ replot of the data shown in inset A.

the case if the P1 and PNPA binding domains were the same or overlapped (case 1). Analysis of the inhibition pattern in terms of Scheme I showed that increasing levels of saturation of the P1 binding domain with propionaldehyde resulted in an increase in the apparent K_m value for PNPA, but once the P1 binding domain is saturated with propionaldehyde, there can be no further effect on the esterase activity, thus accounting for the partial nature of the inhibition. Since V_{max} for the esterase reaction was unaltered by the presence of propionaldehyde in the P1 binding domain, high concentrations of PNPA overcome the inhibitory effect even though propionaldehyde and PNPA can simultaneously bind to the enzyme.

There is now available for the cytoplasmic sheep liver aldehyde dehydrogenase a considerable body of other evidence which also requires that separate nonoverlapping ester and aldehyde binding domains exist (Table I).

The inhibition pattern for chloral hydrate inhibition of the esterase activity in the presence of NAD^+ was also inconsistent with the idea that the PNPA and P1 binding domains are the same or overlap (case 1). Chloral hydrate competes with low concentrations of propionaldehyde for the E·NAD^+ binary complex with a K_i value of 19 μM (MacGibbon et al., 1977a) and also competes with PNPA for E (in the absence of NAD^+) with a K_i value of 287 μM (MacGibbon et al., 1978). However, in the presence of NAD^+, chloral hydrate did not inhibit the esterase activity in a competitive fashion, and even at very high concentrations of PNPA the inhibitory effect could not be completely removed (Figure 2), 59% inhibition remaining at a level of 165 μM chloral hydrate. The inhibition pattern appeared to be mixed, with a linear slope replot and a hyperbolic intercept replot (see inset to Figure 2). If the PNPA and P1 binding domains were identical or overlapping, then a competitive inhibition pattern would have been expected since chloral hydrate would be binding in the same domain both with and without NAD^+. Since this is not in fact observed, it must again be concluded that PNPA is not binding in the P1 propionaldehyde binding domain.

The competitive inhibition of the esterase activity which is observed when the coenzyme is not present can be understood

Table I: Summary of Inhibition Patterns for the Esterase Activity of ALDH[a]

modifiers (and concn)	K_i (μM)	propionaldehyde binding domain involved	inhibition type
chloral hydrate (1.14, 2.28 mM)	287	P2	competitive[b]
chloral hydrate (0–165 μM) + NAD^+		P1	mixed or partial[c]
propionaldehyde (9.4, 18.8 mM)	4200	P2	competitive[b]
propionaldehyde (0–100 μM) + NAD^+	1.44	P1	partial competitive[c]
NAD^+ (478, 956 μM)	107		competitive[b,d]

[a] Data are for sheep liver cytoplasmic enzyme at pH 7.6. [b] Data from MacGibbon et al. (1978). [c] Present work. [d] The dissociation constant (K_D) for binding of NAD^+ in the normal coenzyme binding site is 8 μM.

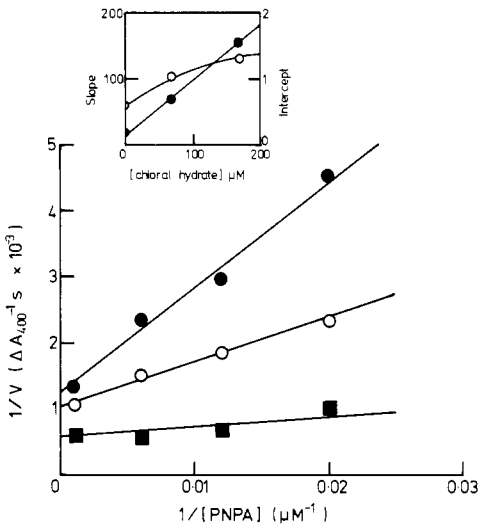


FIGURE 2: Inhibition of the esterase activity by chloral hydrate in the presence of NAD^+. ALDH (0.20 μM), NAD^+ (101 μM), and varying concentrations of chloral hydrate [(■) 0, (○) 66, and (●) 165 μM] were preincubated in phosphate buffer (25 mM), pH 7.6, at 25 °C before the reaction was initiated by addition of PNPA. (Inset) Slope (●) (ΔA_{400}^{-1} μM s × 10^{-3}) and intercept (○) (ΔA_{400}^{-1} s × 10^{-3}) replots.

if under these conditions the P1 binding domain either does not exist or cannot bind chloral hydrate, and hence chloral hydrate now binds only in the PNPA binding domain for which it has a lower affinity. The suggestion that choral hydrate can bind to both the PNPA and P1 binding domains (depending on whether NAD^+ is present or not) is not unreasonable since both the esterase and dehydrogenase activities probably involve a transition state which structurally resembles chloral hydrate.

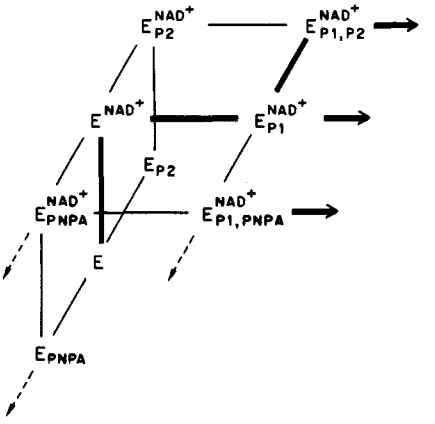
Relationship between the PNPA Binding Domain and the Low-Affinity (P2) Propionaldehyde Binding Domain. Although it is clear from the results presented that the PNPA and P1 propionaldehyde binding domains are distinct, we have not so far addressed ourselves to the question of the relationship between the PNPA and P2 propionaldehyde binding domains. We have previously shown (MacGibbon et al., 1977c) that a concentration of propionaldehyde of 500 μM is sufficient to saturate the high-affinity propionaldehyde (K_m = 1.1 μM) binding domain and the maximum burst rate constant is obtained (11 s^{-1}) at this concentration. The amplitude of the

burst process also reaches a value equal to the dehydrogenase active site concentration at this level of propionaldehyde.

Although high levels of propionaldehyde (20 mM) stimulated the steady-state initial velocity by a factor of approximately 3, there was no significant change in the burst rate constant or amplitude when the propionaldehyde concentration was changed from 100 μM to 50 mM, at pH 7.6, and no new transient was observed. Nor was there any change in the burst rate constant or amplitude if the coenzyme was not premixed with ALDH as reported by Hart & Dickinson (1982). There was no evidence for any premixing effects either at high or at low propionaldehyde concentrations at pH 7.6 unless the NAD^+ concentration was less than about 30–40 μM, in which case the only observable difference was a lag phase at the beginning of the transient. However, the same rate constant was always obtained for the transient within experimental error irrespective of whether it was monitored in the absorbance, nucleotide fluorescence, or protein fluorescence modes. If the P2 binding domain was just part of a low-affinity catalytic binding site, it would be expected that catalytic activity would be observed when the concentration of propionaldehyde was raised high enought to saturate the binding domain and hence either a second transient would be observed (contrary to experiment) or the original burst would show an increase in amplitude (again contrary to experiment). Therefore, we suggest that the P2 binding domain acts as a modifier site, occupation of which increases the steady-state velocity by increasing the rate of acyl-enzyme hydrolysis so that the isomerization of the binary E·NADH complexes (Scheme III) becomes largely rate determining. It is therefore interesting to note that similar high concentrations of propionaldehyde also inhibit the esterase activity (in a competitive manner) (see Table I) in the absence of NAD^+ with an inhibition constant of 4.2 mM (MacGibbon et al., 1978) which is similar to the K_m value for binding of propionaldehyde in the P2 binding domain.[4] This suggests that the P2 and PNPA binding domains are in fact the same (or overlap) and indicates that the presence of NAD^+ may not be necessary for optimal binding of propionaldehyde in this region of the protein.[5]

Evidence That PNPA Is Not Hydrolyzed at the Dehydrogenase Active Site. Although it is clear that the P1 and P2/PNPA binding domains do not overlap (excluding case 1), both the second and third cases still remain. There are, however, difficulties with case 2 (for which the P1 and P2/PNPA binding domains are both situated in different regions of the dehydrogenase active site, but the same catalytic groups are involved in both enzymic activities). There may well be structural features of the second propionaldehyde binding domain (such as a very hydrophobic pocket) which allow PNPA to bind tightly [K_S = 5 μM in the absence of NAD^+ (MacGibbon et al., 1978)]; however, if PNPA can then be physically transported from this domain to the catalytic group and hydrolyzed, it is difficult to understand why the second molecule of propionaldehyde could not be oxidized by the catalytic group (in the presence of NAD^+) exactly as is the first (tightly bound) molecule of propionaldehyde. In this case a second transient would be observed (as discussed above) which is contrary to experiment.

Scheme V



The partially competitive inhibition of PNPA hydrolysis which was obtained at low levels of propionaldehyde (<100 μM) when only the P1 binding domain is occupied is also inconsistent with case 2. Inspection of this inhibition data (shown in Figure 1) reveals that V_{max} for PNPA hydrolysis was unaffected by the binding of propionaldehyde in the P1 binding domain even at saturating levels of PNPA. If both propionaldehyde and PNPA were acylating the same nucleophilic group on the protein, as must be the case if both the propionaldehyde P1 and P2/PNPA binding domains were in the dehydrogenase active site, there would be an internal competition with respect to acylation of this group. Since as the burst rate constants for propionaldehyde oxidation (12 s^{-1}) and for PNPA hydrolysis (11–12 s^{-1}) are similar at saturating levels of both substrates, there must be an almost equal chance of forming $^{**}E^{NADH}_{acyl,PNPA}$ along the dehydrogenase pathway or $^{*}E^{NAD^+}_{P1,acyl}$ along the esterase pathway. If the two acyl groups (propionyl and acetyl, respectively) were attached to the same functional group, then obviously $^{**}E^{NADH}_{acyl,PNPA}$ could not hydrolyze PNPA, and therefore a significant fraction of the enzyme (at least 50%) would always be unavailable for ester hydrolysis in the presence of propionaldehyde. Thus the apparent V_{max} for PNPA hydrolysis would always be reduced when propionaldehyde was bound in the P1 binding domain, but this is clearly contrary to experiment. Thus, we are led to the conclusion that case 3 is the most likely for the cytoplasmic ALDH from sheep liver, which therefore probably possesses a catalytic or active site which binds aldehydes in the presence of NAD^+ and converts them to carboxylic acids, and a separate modifier site which also binds propionaldehyde and hydrolyzes PNPA via a catalytic group which is different from the one involved in the dehydrogenase reaction.[6] However, the relationship between the active site(s), the modifier site(s), and the subunits of the enzyme is completely unknown.

Two-Site Model for the Esterase and Dehydrogenase Activities of Sheep Liver Cytoplasmic ALDH at pH 7.6. The kinetic consequences of the two-site model for the esterase and dehydrogenase activities are summarized by the model shown in Scheme V. In this scheme, as in Scheme IV, the coenzyme

[4] The presence of NAD^+ is, however, required for the binding of propionaldehyde in the P1 domain since there was no effect of 100 μM propionaldehyde on the esterase activity if NAD^+ was not added to the assay mixture. This suggests that the P1 propionaldehyde binding domain may not exist until NAD^+ is bound to the enzyme.

[5] The presence of a reactive thiolate anion in the PNPA (or P2) binding domain could explain the fact that high concentrations of propionaldehyde can bind in a competitive fashion with PNPA in the absence of NAD^+. Recent studies of hemithioacetal formation with a series of thiol anions (Gilbert & Jencks, 1977) show that a propionaldehyde concentration of 20 mM would probably be sufficient to convert an esterase active site thiol anion almost completely into the hemithioacetal derivative.

[6] The observation that a small amount (about 1%) of NAD^+ and acetaldehyde is produced when rabbit liver ALDH, NADH, and PNPA are mixed together has been interpreted in terms of a common acyl-enzyme intermediate (Duncan, 1979). However, similar experiments with sheep liver ALDH (R. L. Motion, unpublished results) did not produce a detectable (≥1%) amount of NAD^+ or acetaldehyde.

Table II: Summary of Kinetic Constants for Scheme V[a]

enzyme form involved	ligand	inhibition constant (μM)	K_m (μM)
E	PNPA		5[b]
E	NAD^+		8[c]
E	propionaldehyde	4200[b]	
E^{NAD^+}	PNPA		5 (1 + [NAD^+]/107)[b]
E^{NAD^+}	propionaldehyde	1.44	1.1[c]
$E^{NAD^+}_{P1}$	propionaldehyde		3500[c]
$E^{NAD^+}_{P1}$	PNPA		26.5 (αK_m)[d]
$E^{NAD^+}_{PNPA}$	propionaldehyde		5.83 (αK_m)[d]

[a] These constants were used in all calculations based on Scheme V by using rapid equilibrium random calculations as described by Segel (1975). Either the inhibition constant or K_m value was used (as appropriate) as the dissociation constant for the equilibrium EL $\rightleftarrows$ E + L where E is one of the enzyme forms listed in the table. [b] MacGibbon et al. (1978). [c] MacGibbon et al. (1977a). [d] α is defined by Scheme I.

binding domain and the high-affinity propionaldehyde binding domain (P1) of the active site are shown as superscripts and subscripts, respectively, and a separate modifier site (which contains the P2 and PNPA binding domains) is shown as a second subscript, but in addition Scheme V includes enzyme forms which have PNPA binding at the modifier site. The kinetic constants which are applicable to Scheme V are summarized in Table II. All steps are assumed to be at equilibrium, except the catalytic steps shown by arrows (i.e., a rapid equilibrium model). In Scheme V the enzyme forms which show dehydrogenase activity are indicated by solid arrows, and the forms which show esterase activity are indicated by broken arrows. The normal dehydrogenase pathways (i.e., with and without the modifier site occupied by propionaldehyde) are indicated by solid lines. The competitive inhibition of the esterase activity (in the absence of NAD^+) by high concentrations of propionaldehyde is accounted for by the presence of both E_{PNPA} and E_{P2} species in the scheme. As required by the compulsory order kinetics (MacGibbon et al., 1977a), the model does not allow binding of propionaldehyde in the P1 binding domain of the active site until NAD^+ is bound to the enzyme. According to Scheme V the activation of the esterase activity by the presence of NAD^+ arises from ester hydrolysis via the $E^{NAD^+}_{PNPA}$ intermediate while the partial competitive inhibition of the esterase activity by low concentrations of propionaldehyde in the presence of NAD^+ arises because of the contribution of $E^{NAD^+}_{P1,PNPA}$ to ester hydrolysis. The complex inhibition of esterase activity by chloral hydrate arises by competition by chloral hydrate for the PNPA binding domain of the modifier site in the absence of NAD^+ (with a high K_i value of 287 μM), while in the presence of NAD^+ tight binding of chloral hydrate (K_i = 19 μM) to the P1 binding domain of the active site gives rise to the observed mixed inhibition pattern. The model presented gives satisfactory explanations which can be quantitatively reproduced by computer calculations by using the kinetic constants from Table II [see, for example, Bennett et al. (1983)] for a wide range of systematically gathered kinetic data. However, a knowledge of the precise relationship between the esterase and dehydrogenase sites on the surface of the protein (and their relative positions on the subunits) awaits the elucidation of the three-dimensional structure of the enzyme.

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Registry No. ALDH, 9028-86-8; NAD, 53-84-9; PNPA, 830-03-5; esterase, 9013-79-0; propionaldehyde, 123-38-6; chloral hydrate, 302-17-0.

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