Activating Effect of *p*-(Chloromercuri)benzoate on the Cytoplasmic Aldehyde Dehydrogenase from Sheep Liver†

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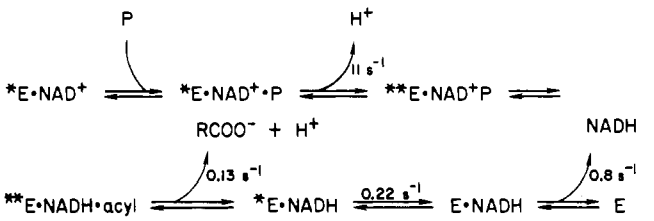
ABSTRACT: In the absence of NAD^+, up to 12 SH groups on aldehyde dehydrogenase (ALDH) reacted rapidly with *p*-(chloromercuri)benzoate (PCMB); a slow reaction with more than twice this number of SH groups then occurred. When PCMB was added to an assay mixture at low (<100 μM) concentrations of propionaldehyde, the steady-state rate of production of NADH increased with increasing PCMB concentration up to a maximum activity at a [PCMB]/[ALDH] ratio of 1.9 and then decreased as the [PCMB]/[ALDH] ratio increased further. Under some conditions, activation, or inhibition, showed hysteretic effects as the initial slope after mixing changed to a final linear steady state in a first-order manner, the rate constants for which were proportional to the concentration of free PCMB. Activating levels of PCMB had little effect on the NADH and proton burst amplitudes or rate constants and did not affect the rate of dissociation or association of NADH. However, when a 20-fold excess of PCMB concentration over enzyme concentration was premixed with the enzyme, neither a burst nor a steady-state turnover of substrate was observed. It is concluded that activation arises from the tight binding of PCMB with a single thiol group per subunit which is exposed after the binding of NAD^+ to the enzyme, followed by a slow conformational change which causes activation by altering the steady-state mechanism so that NADH dissociation becomes largely rate limiting. The combined effects of activating levels of PCMB and of disulfiram on the assay showed that PCMB and disulfiram react with different thiol groups, which are probably adjacent to each other in the P2 [Blackwell, L. F., Bennett, A. F., & Buckley, P. D. (1983) *Biochemistry 22*, 3784–3791] binding site. Inactivation of ALDH at higher [PCMB]/[ALDH] ratios arises from the binding of PCMB to a second SH with much lower affinity, followed by a slow conformational change leading to inactivation of the enzyme. At very high [PCMB]/[ALDH] ratios, inhibition arises from reaction of PCMB with a large number of thiol groups on the enzyme, leading to a progressive denaturation which cannot be reversed by the addition of 2-mercaptoethanol.

We have recently proposed (Bennett et al., 1982) a mechanism for the dehydrogenase activity of the cytoplasmic aldehyde dehydrogenase from sheep liver (EC 1.2.1.3) at low concentrations of propionaldehyde (<100 μM) which is shown in Scheme I. In Scheme I, 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·NAD^+·P ternary complex (accompanied by a proton loss) which controls the rate of both proton and NADH bursts (Bennett et al., 1982) and the formation of an **E·NADH·acyl ternary complex which undergoes a conformational rearrangement and is hydrolyzed to form the **E*·NADH binary complex in a step which, together with an isomerization of this binary complex, controls the steady-state rate. Studies on the rate of NADH dissociation from the enzyme have shown that this isomerization step has a rate constant of 0.22 s^{-1} (MacGibbon et al., 1977b). Therefore, since the hydride transfer step is fast, the rate constant for the hydrolysis of the **E·NADH·acyl 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·NADH·acyl in the steady-state phase of the reaction.

In common with other mammalian aldehyde dehydrogenases, sheep liver aldehyde dehydrogenase (ALDH)[1] possesses a large number of thiol groups [36 according to MacGibbon et al. (1979)], and it has been suggested that one of these thiol groups is catalytically essential (Jakoby, 1963),

Scheme I



being the site of acylation during the enzyme-catalyzed oxidation of aldehydes. However, although the enzyme is very sensitive to inhibition by the thiol reagent disulfiram (Kitson, 1978, 1982a,b), it has not been possible to reduce the enzyme activity to zero, even at very high disulfiram to enzyme concentration ratios and with highly purified enzyme samples (Dickinson et al., 1981). Such an observation suggests that the disulfiram is reacting to give a modified enzyme of much reduced activity rather than combining with the group on the enzyme which becomes acylated during aldehyde oxidation. Recently Kitson (1982a,b) has found that the thiol reagent 2,2′-dithiodipyridine activates the enzyme, presumably by reaction with the disulfiram-sensitive thiol group.

In order to gain further information on the role of thiol groups in the enzyme activity of aldehyde dehydrogenase, we have studied the effect of the thiol reagent *p*-(chloromercuri)benzoate (PCMB) on the dehydrogenase activity. We conclude that the PCMB-sensitive thiol groups are not directly

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[1] Abbreviations: ALDH, aldehyde dehydrogenase; PCMB, *p*-(chloromercuri)benzoate; PMB, *p*-mercuribenzoate; P1, catalytic low-K_m (1.1 μM) propionaldehyde binding domain; P2, high-K_m (3.5 mM) propionaldehyde binding domain as defined in Blackwell et al. (1983); DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PNPA, *p*-nitrophenyl acetate; SDS, sodium dodecyl sulfate.

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involved in the catalytic activity, but reaction with some thiol groups can trigger an isomerization which activates the enzyme in such a way that the rate of loss of NADH alone becomes largely rate limiting.

Experimental Procedures

Materials. Cytoplasmic aldehyde dehydrogenase (ALDH) was prepared essentially as described by MacGibbon et al. (1979). The sulfhydryl reagent *p*-(chloromercuri)benzoate (PCMB), NAD^+ (grade III), and NADH (grade III) were Sigma (St. Louis, MO) chemicals and were used without further purification. Propionaldehyde (Koch-Light Laboratories, Colnbrook, Bucks, U.K.) was distilled under nitrogen before use, and all other chemicals were of the highest purity available.

Enzyme Assay. Aldehyde dehydrogenase activity was measured by monitoring the appearance of NADH at 340 nm with an Aminco DW-2a UV–visible spectrophotometer at 25 °C. The usual conditions for V_{max} assays were 25 mM NaH_2PO_4 buffer containing NAD^+ (1 mM) and propionaldehyde (20 mM), and a value of 0.25 s^{-1} (MacGibbon et al., 1977a) was used for k_{cat} to calculate the active-site concentrations as previously described (Bennett et al., 1983). Thus, all of our kinetic data are reported as rate constants per active site (NAD^+ or NADH binding site), and all concentrations are given as moles per liter of active sites unless stated otherwise.

Apart from the different aldehyde concentrations used, the same assay system was used for the kinetic experiments. The concentration of propionaldehyde was always kept low enough so that only the P1 binding domain was occupied. Assays in the presence of PCMB were carried out by adding the appropriate volume of a stock solution of PCMB (in 0.2 M glycylglycine, pH 7.6, buffer) in place of phosphate buffer. The concentration of the stock PCMB solution was determined by the absorbance at 232 nm using an extinction coefficient of 1.69×10^4 cm^{-1} (Boyer, 1954). Protein concentrations were determined by using a value of 11.3 for $A^{1\%}_{1cm}$ (Dickinson et al., 1981). The enzyme was always dialyzed overnight (2 × 3–4 L of 25 mM pH 7.6 phosphate buffer) to remove 2-mercaptoethanol.

Stopped-Flow Experiments. These were carried out on a Durrum-Gibson D110 stopped-flow spectrophotometer as described by Bennett et al. (1982) in either the fluorescence or the absorbance modes. The data were analyzed on a Cromemco Z2D minicomputer using computer programs written by Hardman (1983). 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).

NADH Titration. Binding of NADH in the presence and absence of PCMB was carried out by using nucleotide fluorescence essentially as described previously (MacGibbon et al., 1979). The data were analyzed by means of Scatchard plots of $R/([NADH] - RE_t)$ vs. R, where R is the ratio of the fluorescence change at a particular NADH concentration to the maximum fluorescence change when all the binding sites are saturated and E_t is the total enzyme concentration (in moles per liter of protein) as determined previously (Bennett et al., 1982).

Results

Titration of Thiol Groups with PCMB at pH 7.6. The initial fast increase in absorbance observed at 250 nm upon mercaptide formation when PCMB reacted with ALDH was measured during a titration of the enzyme (1.6 μM active sites; 1.6 μM protein) with aliquots of PCMB (1.48 μM per aliquot). The slope of plots of the change in absorbance at 250 nm on binding of PCMB against the total PCMB concentration gave an average value of 7940 M^{-1} cm^{-1} for the increase in the extinction coefficient. This value was not significantly different from the value of 7600 M^{-1} cm^{-1} determined by Boyer (1954), and thus, the literature value was used in all subsequent calculations. When the data were plotted in terms of the moles of mercaptide produced per mole of protein against the moles of PCMB added per mole of protein, an average of 12 SH groups per mol of protein reacted rapidly (i.e., within the time of mixing), and thereafter the titration curve showed significant departures from linearity.

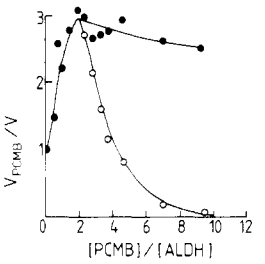

FIGURE 1: Titration of steady-state activity with PCMB at pH 7.6. PCMB was added to assays which contained ALDH (2.32 μM), NAD^+ (1 mM), and propionaldehyde (100 μM). The steady-state rate immediately following the addition of PCMB (●) and the final steady-state rate (○) are plotted against the [PCMB]/[ALDH] ratio.

Enzyme samples which had been denatured with SDS reacted rapidly with, on the average, 34 SH groups per mol of protein. When an excess of PCMB (54 μM) was added to native ALDH (1 μM; 1 μM protein) in a cuvette, there was an initial rapid increase in the absorbance at 250 nm (within the time of mixing) followed by a slower time-dependent increase over about 180 min. The amplitude of this initial "burst" in absorbance was very variable (probably as a result of the difficulty in accurately determining the base-line absorbance) but corresponded to approximately 11 SH groups per mol of protein. The total amplitude of the slow absorbance change was often significantly greater than that expected on the basis of the number of remaining thiols on the enzyme.

Effect of PCMB Concentration on Dehydrogenase Activity at Low Concentrations of Propionaldehyde and at pH 7.6. In Figure 1 are shown the results of a typical experiment where the steady-state production of NADH in the presence of PCMB (v_{PCMB}) was monitored on the addition (in separate experiments) of different amounts of PCMB to an assay mixture containing ALDH (2.32 μM), NAD^+ (1 mM), and propionaldehyde (100 μM). With low concentrations of PCMB (1.08–4.31 μM), there was an increase in the steady-state velocity as compared to the control value (v), with the velocity increasing with increasing PCMB concentration to reach a maximum stimulation of 3 when the [PCMB]/[ALDH] ratio was 1.9.

When higher concentrations of PCMB (5.43–21.6 μM) were added to the assay, hysteretic effects were observed with the initial rate of production of NADH after the addition of PCMB (measured as a tangent to the nonlinear absorbance increase at time zero; see Figure 2) remaining more or less constant at the maximum stimulation observed for PCMB of 3. However, the final steady-state rates (as measured from the linear part of the assay; Figure 2) decreased as a function of the PCMB to enzyme ratio until all stimulation was lost at a [PCMB]/[ALDH] ratio of 4.2, and thereafter, inhibition

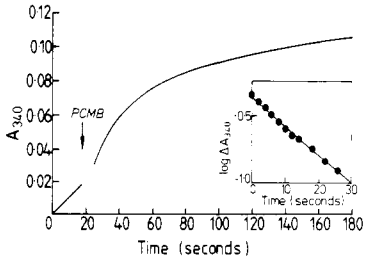

FIGURE 2: Hysteretic approach to the final steady-state rate at pH 7.6. The time-dependent decrease in the steady-state rate after the addition of more than activating levels of PCMB (16.2 μM) to an assay containing ALDH (2.32 μM), NAD^+ (1 mM), and propionaldehyde (100 μM) at pH 7.6 is shown. A value of 0.048 s^{-1} was obtained for k_{app} (see inset).

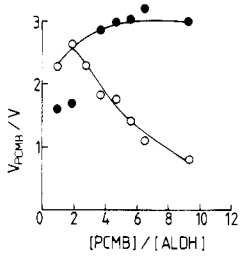

FIGURE 3: Titration of steady-state activity with PCMB at low ALDH concentrations. PCMB was added to assays which contained ALDH (0.23 μM), NAD^+ (1 mM), and propionaldehyde (100 μM) at pH 7.6. Initial rates (●) immediately following PCMB addition and final rates (○) are shown.

was observed with the steady-state rate decreased almost to zero at a ratio of 9.0.

When the enzyme concentration in the assay was reduced to 0.23 μM, keeping the NAD^+ and propionaldehyde concentrations unchanged, hysteretic effects were observed for all the assays after the addition of PCMB. At low [PCMB]/[ALDH] ratios, the rate of NADH production increased to a final linear activated level, while at high [PCMB]/[ALDH] ratios the rate of NADH production decreased from an initial activated level to a final lower value. Compared to the assays with 2.32 μM ALDH, there was much less effect on the initial rates of added PCMB at low [PCMB]/[ALDH] ratios, although a 3-fold stimulation was again observed but at a [PCMB]/[ALDH] ratio of 4 (Figure 3). When the final steady-state rates were plotted against the [PCMB]/[ALDH] ratio, a maximum stimulation (2.64) was again obtained at a [PCMB]/[ALDH] ratio of 1.9. However, at the lower enzyme concentration, a net stimulatory effect on the final linear part of the assay was observed until the [PCMB]/[ALDH] ratio reached 7.8, and the steady-state rate was not reduced to almost zero until the ratio reached 23.

The extent of the activation of the steady-state rate depended on the order of mixing of the substrates and PCMB in the steady-state assays. Generally the greatest extent of activation was obtained if PCMB was added last, and this mixing condition was used for the steady-state activation experiments reported in this paper unless stated otherwise. If PCMB was added either before propionaldehyde or simultaneously with propionaldehyde, there was marginally less activation for a given level of PCMB, but if activating levels of PCMB were mixed with ALDH before the addition of NAD^+, the activation was considerably less (1.4-fold compared with 2.3-fold for the other mixing conditions in control experiments).

When PCMB (2.9 μM) was added to an assay containing ALDH (2 μM), NAD^+ (1 mM), and propionaldehyde (100 μM), the steady-state rate was activated (2.2-fold), and when

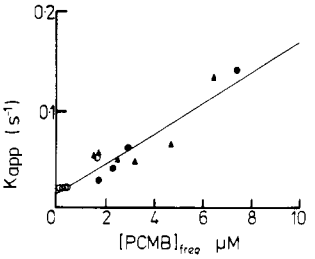

FIGURE 4: Plot of k_{app} against the free PCMB concentration at pH 7.6. k_{app} was plotted against the free PCMB concentration estimated from the PCMB titration curve for ALDH concentrations of 0.23 (○), 1.6 (●), and 2.32 μM (▲).

excess 2-mercaptoethanol was added to the assay mixture, the activating effect of PCMB was completely removed. If ALDH, NAD^+, and PCMB were premixed for 10 min prior to the addition of propionaldehyde, an activated steady state was still obtained, and the effect of PCMB was still entirely removed on addition of excess 2-mercaptoethanol. The effect of high concentrations of PCMB (20–40-fold excess) on the enzymic activity could be reversed by the addition of 2-mercaptoethanol within 2–5 min, but after 45 min, only a partial reactivation of the enzyme activity by 2-mercaptoethanol could be achieved.

When acetaldehyde was used as a substrate, an activation experiment in which ALDH (0.83 μM) was treated with a 3.7-fold excess of PCMB also showed a 2-fold increase in the steady-state activity over an acetaldehyde concentration range from 100 μM to 2 mM in the assay, thus demonstrating that the activation is a general property of the enzyme.

Time-Dependent Changes in Steady-State Activity in the Presence of PCMB at pH 7.6 and Low Concentrations of Propionaldehyde. Once the PCMB to ALDH concentration ratio was 2 or greater, a time-dependent decrease in the activated steady-state rate was observed (Figure 2). A log plot of the differences between the actual absorbances and the values extrapolated from the linear portion of the curve as a function of time give the apparent first-order rate constant (k_{app}) for the approach to the final steady-state rate. Even though the concentration of free PCMB (as determined from the titration curve) never approached the 8–10-fold excess that should be required for a pseudo-first-order condition to apply, a plot of k_{app} against $[PCMB]_{free}$ was nevertheless linear (Figure 4), and the data for the three different enzyme concentrations (0.23, 1.61, and 2.32 μM) lay on the same line within experimental error.

At the lowest enzyme concentration which was used in these experiments (0.23 μM), for the first two concentrations of PCMB (0.216 and 0.432 μM; Figure 3) there was a time-dependent increase in the steady-state rate until the final (activated) steady state was established. The increase became faster as the PCMB concentration was increased. The first two points in Figure 3 appear to be partly activated immediately after the addition of PCMB to the assay, but they could not be determined until 5–10 s had elapsed. As the apparent half-life for the lag was about 14 s, the initial steady-state rates could have been nearly 50% activated before measurements were taken.

Effect of Activating Levels of PCMB on NADH Titration at pH 7.6. The stepwise addition of aliquots (0.01 cm^3) of NADH (124 μM) to ALDH (0.79 μM) in a fluorometer cuvette gave a smooth titration, the Scatchard replots of which resulted in a K_D value of 2.0 μM and a value of 1.24 for the number of NADH binding sites per tetramer. When PCMB (3.8 μM) was added to the titration mixture, there was no

Table I: Summary of Effect of PCMB on Burst Rate Constants and Amplitudes

[PCMB]/[ALDH]	mixing conditions	k_b/k_b^{0} [b]	A_b/A_b^{0} [b]	burst type
4.13[a]	E, PCMB/NAD$^+$, Prop[e]	0.62	0.23	NADH
1.91[a]	E, NAD$^+$/PCMB, Prop	1.1	0.94	NADH
1.91[a]	E, NAD$^+$, PCMB/Prop	0.92	0.63	NADH
4.0[a]	E, NAD$^+$, PCMB/Prop	0.9	0.66	proton
6.0[c]	E, NAD$^+$/PCMB, Prop	1.0	0.77	NADH
20.0	E, NAD$^+$/PCMB, Prop	0.67	1.1[d]	NADH
20.0	E, NAD$^+$, PCMB/Prop	[f]	[f]	NADH
20.0	E, PCMB/NAD$^+$, Prop	[f]	[f]	NADH

[a] Using the maximum activating levels of PCMB as determined at the beginning of each set of experiments. [b] Burst rate constants (k_b) and amplitudes (A_b) are expressed relative to the corresponding values in the absence of PCMB (k_b^0 and A_b^0) as 1.00. The control values were in good agreement with those reported by MacGibbon et al. (1977c) and Bennett et al. (1982). [c] Using a [PCMB]/[ALDH] ratio which in the steady-state turnover of propionaldehyde gave initial activation followed by hysteresis to a final slower linear steady-state rate. [d] The burst was followed by a displacement of about 70% of the enzyme-bound NADH (see Results). [e] Prop is propionaldehyde. [f] No burst or steady state was observed.

significant increase in the fluorescence of enzyme-bound NADH, and a Scatchard plot of the titration data gave a K_D value of 2.7 μM and a value of 1.46 for the number of NADH binding sites per tetramer, values which were similar to those obtained for the control titration.

Effect of Activating Levels of PCMB on the NADH and Proton Bursts at pH 7.6 and under Different Mixing Conditions. Since it has been shown that different orders of mixing of substrates and PCMB have different effects on the steady-state rates, the effect of activating levels of PCMB on the NADH and proton bursts was studied at pH 7.6 under different mixing conditions, and the results are summarized in Table I. The steady-state rates immediately following the burst were activated except for the first mixing condition given in Table I when no activation was observed.

Effect of Activating Levels of PCMB on NADH Displacement and Binding at pH 7.6. The effect of activating levels of PCMB on the rates of binding of NADH to ALDH and displacement of NADH from the binary E·NADH complex by NAD$^+$ was determined, and the results are summarized in Table II.

Effect of High Concentrations of PCMB on the NADH Burst at pH 7.6. The effect of high concentrations of PCMB (higher than the activating level) on the NADH burst was also determined. The results are included in Table I. At a PCMB to ALDH concentration ratio of 20:1 (when PCMB was added to the propionaldehyde syringe), the amplitude of the burst (Figure 5A) was unchanged within experimental error when compared with a control burst under identical conditions in the absence of PCMB. The NADH fluorescence decreased after the burst (Figure 5B) with a time constant of 0.23 s^{-1}, and then a small steady-state increase in NADH production

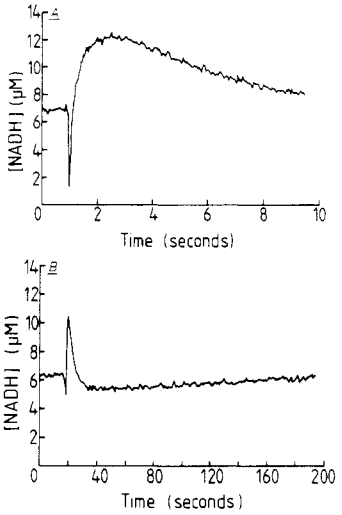

FIGURE 5: Effect of excess PCMB on the NADH burst at pH 7.6. ALDH (5.6 μM) and NAD$^+$ (2 mM) from one syringe were rapidly mixed with propionaldehyde (400 μM) and PCMB (104 μM) from the second syringe. (A) The burst rate constant was 5.3 ± 0.7 s^{-1}, and a displacement of NADH followed the burst. (B) On a longer time scale, the displacement of NADH is more obvious. The decay constant was 0.23 s^{-1}.

was observed with an apparent k_{cat} of 0.002 s^{-1}.

Effect of Activating Levels of PCMB in the Presence of Disulfiram. When PCMB (4.68 μM) was added to an assay containing ALDH (1.2 μM), NAD$^+$ (1 mM), and propionaldehyde (100 μM), the k_{cat} value was increased from 0.073 to 0.219 s^{-1} (an increase of 302%). When disulfiram (5.2 μM) was added to the activated mixture, there was a virtually instantaneous reduction in the k_{cat} value to 0.027 s^{-1}. However, this k_{cat} value decreased with time to give a final k_{cat} value of 0.01 s^{-1}, a value which was similar to that obtained (0.014 s^{-1}) in the control experiment carried out in the absence of PCMB. If disulfiram was added to the enzyme assay before PCMB, no activation of the disulfiram-modified enzyme was observed, the final k_{cat} (0.014 s^{-1}) value being similar to that obtained in the control experiment.

Discussion

Evidence for a Thiol Group Which Causes Activation of the Enzyme Activity at pH 7.6. Organic mercurials such as PCMB react specifically and usually rapidly with available thiol groups of proteins (Means & Feeney, 1971) to give thiol–mercurial complexes with dissociation constants that are much lower than those for any other common protein group. That PCMB is reacting specifically with the available thiol groups of ALDH is confirmed by the fact that the change in extinction coefficient at 250 nm on binding of PCMB agrees within experimental error with the value given by Boyer (1954) for mercaptide formation with cysteine. Furthermore, the

Table II: Effect of Activating Levels of PCMB on NADH Displacement and Binding at pH 7.6

expt	[ALDH][a] (μM)	[PCMB]/[ALDH]	[NADH][a] (μM)	λ_F (s^{-1})	λ_S (s^{-1})	A_b/A_b^{0} [b]
displacement[c]	3.0	0	33	0.93 ± 0.02	0.23 ± 0.06	1.0
displacement[d]	3.0	3.0	33	1.4 ± 0.03	0.18 ± 0.01	1.0
binding[e]	5.8	0	84	7.9 ± 0.1	1.05 ± 0.01	1.0
binding[d]	5.8	4.14	84	8.9	1.08	0.84[f]

[a] All concentrations are those prior to rapid mixing. The enzyme concentration is the active-site concentration. [b] Amplitudes are expressed relative to the value in the absence of PCMB as 1.0. A_b^0 was always 85–90% of the active-site concentration. [c] ALDH and NADH from one syringe were rapidly mixed with NAD$^+$ (3.6 mM) from the other syringe (MacGibbon et al., 1977c). [d] PCMB was added to the ALDH syringe in both experiments. [e] ALDH from one syringe was rapidly mixed with NADH from the other syringe. [f] The decrease in amplitude resulted almost entirely from a decrease in the amplitude of the slow process (0.4 μM in the presence of PCMB vs. 1.0 μM in its absence).

changes in absorbance at 250 nm indicated that when ALDH was treated with PCMB at pH 7.6 in the absence of cofactors or substrates, 12 thiol groups per mol of protein (3 per subunit) reacted within 15 s. Further reaction then occurred slowly over a period of 3 h.

If the enzyme was first denatured with SDS, an average of 34 thiol groups per mol of protein reacted very rapidly with PCMB in agreement, within experimental error, with the value of 36 SH groups per 212000 g of protein expected on the basis of the amino acid analysis and from the reaction of DTNB with the SDS-denatured enzyme reported by MacGibbon et al. (1979). The absorbance changes after reaction of denatured enzyme with PCMB remained unchanged over 10–20 min. It should be noted, however, that when a 50–100-fold excess of PCMB over enzyme concentration was allowed to react for several hours with native enzyme, the total absorbance change corresponded to more than 36 SH groups per mol of protein, indicating that PCMB was reacting with other protein groups after all the thiol groups had been modified, as suggested previously (Webb, 1966).

Most thiol reagents cause inhibition of the steady-state initial velocity of ALDH, and it was expected that reaction with PCMB would also cause inhibition. However, at concentrations of propionaldehyde which were just sufficient to saturate the P1 binding domain (Blackwell et al., 1983), when PCMB was added last, the steady-state rate was activated. With enzyme from different preparations, the maximum activation did not always occur at [PCMB]/[ALDH] ratios of 1.9, as in Figure 1, but ranged from 1.0 to 4.0 depending on the sample and its age.[2] As reported previously, the number of active sites per tetramer for the pure [see Agnew et al. (1981)] enzyme varies from sample to sample (Bennett et al., 1983). In this work, a maximum [PCMB]/[ALDH] activation ratio of 1 was obtained for the few enzyme samples which had four active sites (as determined by NADH titration) per tetramer, while the maximum activation ratio of 4 was obtained for enzyme samples with one active site per tetramer.[3] Thus, the number of thiols required to be modified to produce the maximum activation was clearly one per subunit irrespective of the number of active sites per tetramer.

When the ratio of [PCMB] to [ALDH] was increased above that required for maximum activation, further SH groups were modified by PCMB, but the extra PMB groups had no immediate effect on the activity of the enzyme (see the initial rates shown in Figures 1 and 3). This is not entirely surprising since it would be expected that reactive SH groups must be easily accessible to PCMB and hence addition of the relatively bulky PMB group need not cause too much disruption of the protein structure.

Since the addition of PCMB at maximum activating levels to the enzyme in the absence of NAD^+ did not cause much activation of the steady-state rate in conventional mixing experiments and no activation at all of the steady-state rate immediately following the burst under similar conditions during stopped-flow experiments, either PCMB does not bind as well with the activating thiol group (or groups) in the absence of NAD^+ or, alternatively, the activating thiol group (or groups) is only completely exposed to PCMB after exposure of the enzyme to NAD^+ as suggested by MacGibbon et al. (1977b).

[2] The most recent published data for the sheep liver ALDH have given a value of 1–1.5 binding sites per tetramer (Hart & Dickinson, 1983).

[3] Although the number of binding sites reported here varied from 1 to 4, the enzyme samples did not contain extraneous protein bands on isoelectric focusing (Agnew et al., 1981).

Scheme II

$$*E\cdot NAD^+ + PCMB \underset{k_{-1}\ \text{fast}}{\overset{k_1}{\rightleftharpoons}} E\cdot NAD^+\text{-}PMB \underset{k_{-2}\ \text{slow}}{\overset{k_2}{\rightleftharpoons}} **E\cdot NAD^+\text{-}PMB$$

Mechanism for Activation of the Steady-State Activity of ALDH by PCMB at pH 7.6. As long as PCMB was reacting with the $E\cdot NAD^+$ complex, there was no significant effect on the burst rate constants or amplitudes for either the production of NADH or the release of protons, but the steady-state rates immediately following the burst were activated as in the conventional mixing experiments. Modification of ALDH with activating levels of PCMB under these conditions therefore had no effect on the substrate binding steps and did not cause a dissociation of the enzyme into dimers as proposed by Takahashi et al. (1981) to account for the increase in the activity of the horse liver enzyme in the presence of magnesium ions.

The rate constants for the binding of NADH to the enzyme, or for the displacement of NADH from the E·NADH complex, were also unaffected in the presence of activating levels of PCMB, observations which were confirmed by the fact that the K_D value for the E·NADH complex and the number of binding sites per tetramer were also unaffected. Since the fluorescence enhancement on binding of NADH to ALDH was unaltered, it is clear that the conformational change which controls the rate of release of NADH from the E·NADH complex (MacGibbon et al., 1977c) is not affected by the presence of activating levels of PCMB either.

The activating effect of PCMB on the steady-state rate must therefore be due to an increase in the rate constant for the step down in Scheme I as hydrolysis of the **E·NADH·acyl complex. The time dependence of this activation at low ALDH concentrations either could be due to a relatively slow rate of binding of PCMB to the enzyme or could be a consequence of a slow isomerization of the PCMB-modified enzyme as shown in Scheme II to give the more active $**E\cdot NAD^+$–PMB complex.

In Scheme II, PCMB binds rapidly with the enzyme (at the activating SH group) to form a modified enzyme which has the same k_{cat} value (0.082 s^{-1}) as the native enzyme, but this modified enzyme subsequently undergoes a conformational change which results in the more active form (k_{cat} = 0.25 s^{-1}) in which isomerization of E·NADH complexes is now largely rate limiting (see Scheme I). For Scheme II where the rate constants for the first step are both much larger than those for the second step, there will be a decay constant (eq 1) which just involves k_1 and k_{-1}

$$k_f = k_{-1} + k_1[PCMB] \quad (1)$$

and a second decay constant (eq 2) which also involves the rate constants for the slower isomerization step

$$k_s = k_{-2} + k_2[PCMB]/([PCMB] + K_S) \quad (2)$$

where $K_S = k_{-1}/k_1$ (Fersht, 1977). If k_1 is of the order 10^7 M^{-1} s^{-1}, then, even at concentrations of PCMB of 0.23 μM, k_f will be too fast to be observed with conventional mixing techniques. Thus, the time-dependent increase in the steady-state rate would be expected to be described by the slow decay constant k_s, and providing that [PCMB] is less than K_S, this decay constant would increase with increasing PCMB concentration as observed experimentally. To accommodate the experimental data, a K_S value of 1–10 μM is required which is in the range normally found for the binding of PCMB with proteins (Webb, 1966).

The alternative model involving slow binding of PCMB is excluded by the data since in this case (Fersht, 1977) an inverse relationship between the slow decay constant and the PCMB concentration would be expected which is clearly contrary to experiment.

The activation of ALDH by PCMB can therefore be explained by a model in which PCMB reacts rapidly with a thiol group which is exposed after binding of NAD^+ in the coenzyme binding site or by the subsequent steps in Scheme I, and this is followed by a conformational change which results in a more rapid hydrolysis of the **E·NADH·acyl complex so that NADH dissociation is now almost completely rate determining.

Location of the Activating Group. The observation that the addition of disulfiram immediately reduced the activity of the PCMB-activated ALDH to 10% of its initial activity, but which was still more than twice that obtained by adding the same amounts of disulfiram to the native ALDH, strongly suggests that PCMB and disulfiram are reacting with different thiol groups. However, it is obvious that there is some interaction between the disulfiram-modified thiol and the PCMB-modified thiol. When the enzyme was treated with activating levels of PCMB followed by inhibitory levels of disulfiram, although the disulfiram-inhibited steady state was initially activated as compared with controls which contained disulfiram only, the activating effect of PCMB was slowly lost. Also, if the enzyme was treated first with disulfiram, PCMB exerted no activation on the disulfiram-inhibited steady-state rate.

Since it has been shown that treatment of ALDH with disulfiram eventually results in the formation of an enzyme disulfide bridge (Vallari & Pietruszko, 1982; Kitson, 1983), a second SH group must be available to allow a disulfide exchange reaction to occur. Thus, it is reasonable to assume that PCMB and disulfiram both exert their effects in the same region of the protein. We have recently shown (Blackwell et al., 1983) that *p*-nitrophenyl acetate (PNPA) binds in, or near, the P2 binding domain (Blackwell et al., 1983), and since Kitson (1982b) has demonstrated that PNPA also effectively protects the enzyme against inhibition by disulfiram, we conclude that the disulfiram-sensitive thiol group binds in, or near, the P2 binding domain. This conclusion therefore suggests that the activating SH group, with respect to PCMB, also lies in, or near, the P2 binding domain.

The similarity in the 3-fold activation of the steady-state rate of ALDH caused by high concentrations (20 mM) of propionaldehyde, and also by treatment of the enzyme with activating levels of PCMB, raises the intriguing possibility that both substances act in the same manner. We have suggested (Blackwell et al., 1983) that propionaldehyde, at concentrations of about 20 mM, might bind to a thiol anion in the P2 binding domain to form a thiohemiacetal derivative. If this putative thiol anion were involved in maintaining the structural integrity of the protein, modification by either propionaldehyde (forming the thiohemiacetal) or PCMB (forming the mercaptide) may result in the same conformational change which leads to NADH dissociation becoming mainly rate limiting in the steady-state phase of the reaction.

Although a k_{cat} value for acetaldehyde is not well established, Kitson (1982b) has shown that 2,2′-dithiodipyridine and diethylstilbestrol both cause a 2-fold activation of the steady-state rate when acetaldehyde is the substrate, and it was interesting to note that addition of activating levels of PCMB to an assay which contained acetaldehyde also caused a 2-fold activation in the present work. Since Kitson (1982b) has also shown that diethylstilbestrol inhibits the esterase activity of ALDH, it must also be binding in, or near, the P2 binding domain. Thus, the binding of any one of a number of species in the P2 binding domain near the putative thiol anion (or chemically reacting with it) can cause activation of the steady-state rate.

Inhibition of the Steady-State Rate by PCMB at pH 7.6 and Low Concentrations of Propionaldehyde. When the [PCMB]/[ALDH] ratio was significantly greater than that required for maximum activation, the enzyme activity decreased with time from the initial activated level to a final linear steady-state rate in a simple first-order manner (Figures 1–3). The extent of the inhibition of the final steady state increased as the [PCMB]/[ALDH] ratio increased in a manner which suggested that the PCMB was binding reversibly to the enzyme. Reversible binding is consistent with the observation that higher [PCMB]/[ALDH] ratios were required to produce the same final inhibition of the steady-state rate when the total enzyme active-site concentration was less (Figures 1 and 3).

The subsequent inhibition of the activated enzyme at high [PCMB]/[ALDH] ratios (Figures 1 and 3) can be explained if PCMB again reacts rapidly and reversibly with the enzyme but now with a different SH group and one for which it has a lower affinity. When reaction occurs with this second SH group, a different slow conformational change occurs which inactivates or severely inhibits the E·NAD^+·PMB forms of Scheme II. Although two decay constants are again expected for the inhibition, if the PCMB binding steps are fast then only a single exponential corresponding to the slow decay constant would be observed. Thus, provided the free PCMB concentration is much less than K_S (for the PCMB binding step), then the observed slow burst should be first order and depend linearly on the PCMB concentration (see eq 2) as was observed experimentally (Figure 4).

In burst experiments where a [PCMB]/[ALDH] ratio around the maximum activating level was used, when PCMB was premixed with enzyme alone the burst amplitudes were less than for all other mixing conditions in which NAD^+ was present with the enzyme (Table I). In the absence of NAD^+, therefore, if the activating SH is not readily accessible (as suggested by the steady-state results), a considerable fraction of the enzyme would be converted to the inhibited form before rapid mixing occurred. Since the burst amplitude is reduced, it appears that the inhibited form of the enzyme cannot pass through the kinetic pathway shown in Scheme I.

When PCMB was not premixed with the enzyme, the burst rate constants and amplitudes were not greatly affected; however, a significant fraction of the enzyme was inactivated by the end of the burst since there was an immediate decrease in fluorescence (Figure 5A,B). The rate of this process (first-order decay constant of 0.23 s^{-1}) was similar to the slower of the two NADH displacement rates (MacGibbon et al., 1977c) which suggests that the decrease is due to the displacement of NADH from the enzyme by NAD^+ (indicating that NAD^+ may still be able to bind to the inhibited enzyme). The fact that there is very little subsequent steady-state turnover after the displacement is further evidence that the inhibited enzyme is unable to proceed through the pathway in Scheme I beyond the E·NAD^+ stage.

It seems probable that at high [PCMB]/[ALDH] ratios, such as 20 or more, some of the inhibition may arise from progressive and irreversible denaturation of the enzyme due to the binding of PCMB at some of the other large number of reactive sites on the enzyme. When these higher levels of

PCMB were premixed with the enzyme in either the presence or the absence of NAD^+, the enzyme appeared to be totally inactivated, showing no burst and no steady-state rate.

Conclusions. The identity of the functional group on the enzyme which is acylated by aldehydes during their oxidation has not yet been established, but it has commonly been assumbed that a thiol group fills this role. However, the fact that even in the presence of NAD^+ none of the rapidly reacting SH groups immediately inhibited the enzyme activity, while the modification of one SH group per subunit, probably in the P2 binding domain, caused a 3-fold activation of the steady state, excludes the possibility that any of these SH groups could be catalytically essential. Since the disulfiram-sensitive thiol groups also do not appear to be the sites of acylation (Kitson, 1982a,b), the conclusion must be drawn that none of the rapidly reacting thiol groups is directly involved in the catalytic activity.

This conclusion carries with it a warning that the mere fact that a protein contains a number of rapidly reacting thiol groups does not necessarily constitute evidence that these thiol groups are essential to the catalytic mechanism. It is of course still possible that a thiol group is acylated during the oxidation of aldehydes, but no direct evidence for this idea is currently available, and hence the identity of the functional group which is actually involved remains an open question. Despite this conclusion, an important finding from the present work is that modification of two of the rapidly reacting thiols per tetramer with PCMB leads to important changes (activation and inhibition) in the steady-state activity of ALDH. It is interesting that similar effects of modification with PCMB have been found for the allosteric enzyme fructose 1,6-diphosphatase (Little et al., 1969), and thus, it may be that the activating and inhibiting thiol groups revealed during the present work are important in vivo for the control of the enzymic activity of aldehyde dehydrogenase.

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Registry No. ALDH, 9028-86-8; PMB, 59-85-8; disulfuram, 97-77-8.

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