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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with a Bifunctional Arsenoxide: Selective Inactivation of Lipoamide Dehydrogenase†

S. Robert Adamson and Kenneth J. Stevenson*

ABSTRACT: The bifunctional reagent *p*-[(bromoacetyl)-amino]phenyl arsenoxide ($BrCH_2CONHPhAsO$) in the presence of excess reduced nicotinamide adenine dinucleotide has been shown to cause the irreversible active site directed inactivation of the lipoamide dehydrogenase (E3) component of the pyruvate dehydrogenase multienzyme (PD) complex from *Escherichia coli*. The ability of the lipoate acetyltransferase (E2) component to bind coenzyme A was decreased by about 50% in this system. In the presence of thiamine pyrophosphate, pyruvate, coenzyme A, and Mg^{2+}, E3 inactivation by $BrCH_2CONHPhAsO$ was selective (coenzyme A binding was unaffected) and stoichiometrically related to PD complex inactivation, indicating that a complement of E3 is necessary for full complex activity. The activity of the pyruvate dehydrogenase (E1) component was unaltered by $BrCH_2$-$CONHPhAsO$ in both systems. On inhibition of the PD complex with $BrCH_2CONHPhAsO$, the reagent mediated interchain cross-linking between E2 and about half of the E3 subunits. A marked change occurred in the quaternary structure of the PD complex, with some E1 and E3 subunits being dissociated from the E2 core. The mechanism outlined by Stevenson et al. [Stevenson, K. J., Hale, G., & Perham, R. N. (1978) *Biochemistry 17*, 2189] for the inhibition of the PD complex by $BrCH_2CONHPhAsO$ must be revised on the basis of these findings. E3 is only partially modified by delivery of the bromoacetyl moiety of the bifunctional reagent (covalently attached to lipoyl residues of E2 through dithioarsinite bonds) into the active site of bound E3. The inhibition of E3, dissociated from the PD complex during cross-linking, likely occurs via direct interaction of the free enzyme with $BrCH_2CONHPhAsO$ by initial dithioarsinite modification of the reduced active-site disulfide followed by alkylation of a nearby residue.

The pyruvate dehydrogenase multienzyme complex (PD complex)[1] from *Escherichia coli* catalyzes the overall reaction:

$$\text{pyruvate} + NAD^+ + CoA \rightarrow \text{acetyl-CoA} + NADH + H^+ + CO_2$$

The complex is composed of multiple copies of three different enzymes, which in order of participation are pyruvate dehydrogenase (lipoate) (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (E3 (NADH) (EC 1.6.4.3) (Reed, 1974; Hucho, 1975; Perham, 1975). A total of 24 apparently identical E2 chains form the structural core of the complex (Reed & Oliver, 1968; Reed, 1974). Recent evidence suggests that two (Danson & Perham, 1976; Brown & Perham, 1976; Speckhard et al., 1977; Collins & Reed, 1977; Bates et al., 1977) or possibly three (Hale & Perham, 1979) lipoyl residues are present per polypeptide chain of E2. The lipoyl residues seem to function in a series of transacetylation reactions within the E2 core of the complex (Bates et al., 1977; Collins & Reed, 1977), and these reactions are kinetically competent in the overall PD complex reaction (Danson et al., 1978).

For many years, trivalent arsenicals such as alkyldihaloarsines ($RAsX_2$) and alkyl arsenoxides (RAsO) have been known to react with dithiols such as reduced lipoic acid to form stable cyclic dithioarsinites (Whittaker, 1947; Stocken & Thompson, 1946).

We have recently begun to investigate the structure and function of the pyruvate dehydrogenase multienzyme complex from *E. coli* by using a new approach involving mono- and bifunctional arsenoxides (Stevenson et al., 1978). It is the purpose of the experiments reported here to clarify the mechanism of the inhibitory action of the bifunctional reagent

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[1] Abbreviations used: PD complex, pyruvate dehydrogenase multienzyme complex; $H_2NPhAsO$, *p*-aminophenyl arsenoxide; $BrCH_2CONHPhAsO$, *p*-[(bromoacetyl)amino]phenyl arsenoxide; $BrCH_2CONHPh$, *N*-phenyl-α-bromoacetamide; $NaDodSO_4$, sodium dodecyl sulfate; TPP, thiamine pyrophosphate; DHAT, dihydrolipoamide acetyltransferase; NADH, reduced nicotinamide adenine dinucleotide.

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p-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2CONHPhAsO$) on the PD complex and to establish if the inhibition of lipoamide dehydrogenase (E3) by this reagent is selective. The results indicate that E3 inhibition is selective under appropriate conditions and that a full complement of E3 is necessary for complete complex activity.

Experimental Procedures

Materials

[2-^{14}C]Pyruvate (sodium salt) was obtained from New England Nuclear (Boston, MA) and assayed spectrophotometrically as NADH produced in the presence of PD complex. *p*-Aminophenyl arsenoxide, *p*-[(bromoacetyl)amino]phenyl arsenoxide, and *N*-phenyl-α-bromoacetamide were synthesized as described by Stevenson et al. (1978). 2,3-Dithiopropanol, 2,3-dithiopropanesulfonic acid, and phosphotransacetylase were obtained from Sigma.

Methods

Enzyme Isolation. Pyruvate dehydrogenase complex was purified from a constitutive mutant of *E. coli* K12 (kindly provided by Professor H. L. Kornberg and Dr. R. N. Perham) essentially as described by Reed & Mukharjee (1969) with modifications suggested by Danson et al. (1979). Cells were grown to late log phase on 1 or 2% glycerol and harvested by using a refrigerated Sharples continuous centrifuge. The cell paste was suspended in 20 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA and passed continuously through an ice-cooled sonication cell (100–150-mL dead volume). Sonication was carried out at minimum intensity by using a Branson sonifier at a pulse efficiency of 50% and an intensity of 7. The cell extract was processed by the method of Reed & Mukharjee (1969) except that 2 mM EDTA and 0.2% sodium azide were used in all buffers, as recommended by Danson et al. (1979), to prevent proteolysis (Harrison, 1974) and bacterial growth, respectively.

Enzyme Assays. The activities of the PD complex and E3 were assayed spectrophotometrically in the direction of NAD^+ reduction by the method of Reed & Mukharjee (1969), as described by Danson & Perham (1976) at 25 °C. DL-Dihydrolipoamide was prepared from DL-lipoamide (Reed et al., 1958). E1 was assayed by a slight modification of the method outlined by Schwartz et al. (1968) by using 20 mM phosphate buffer, pH 7.5, instead of Tris–glycine buffer.

Protein Determinations. Protein concentrations were determined from A_{280} measurements and, where stated, by the method of Lowry et al. (1951) by using bovine serum albumin as a standard.

Radiochemical Analysis. Radioactivity was measured in a Nuclear-Chicago Isocap-300 liquid scintillation counter with Aquasol-2 as the scintillant.

Polyacrylamide Gel Electrophoresis. The discontinuous pH system of Ornstein (1964) and Davis (1964) as described by Laemmli (1970) was used. Samples of PD complex cross-linked with $BrCH_2CONHPhAsO$ were prepared for electrophoresis by incubation at 45–50 °C for 2 min or at room temperature for 5–15 min in the presence of 1% $NaDodSO_4$, 10% glycerol, and 7.5 mM Tris-HCl, pH 6.7. Cross-linked samples in which the cross-link was to be broken were prepared by boiling for 2 min as above but in the presence of 1% 2-mercaptoethanol. Gels were scanned at 550 nm on a Beckman Acta III spectrophotometer with a gel scanning attachment or a Transidyne General Corp. RFT-II scanning densitometer after staining with Coomassie Brilliant Blue R-250 or G-250.

Sedimentation Analysis. Analytical and preparative ultracentrifugations were carried out, as described in the text, in a Beckman Model E ultracentrifuge fitted with an An-H rotor and a Beckman L5-50 preparative ultracentrifuge fitted with a Beckman SW50 rotor, respectively.

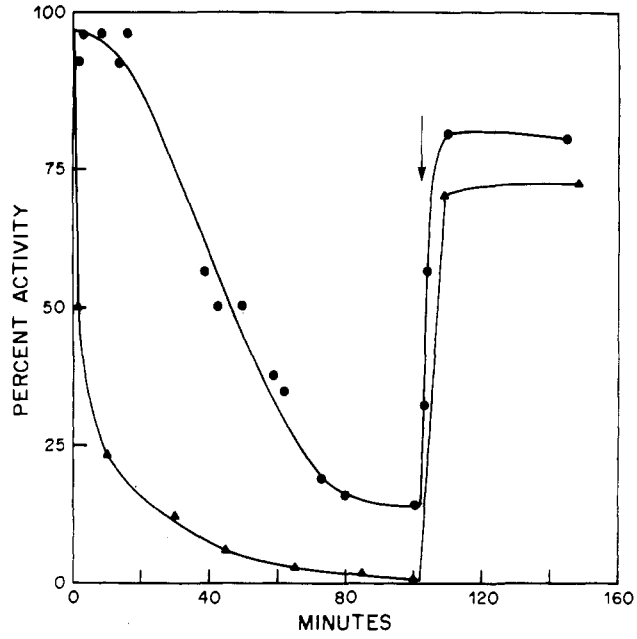

FIGURE 1: Inactivation of PD complex (1 mg/mL) with $H_2NPhAsO$ (2–4 mM) in 50 mM potassium phosphate buffer (pH 8) containing 2 mM NADH at 5 °C. Reagent was added as a powder prior to NADH addition. PD complex activity (▲); lipoamide dehydrogenase (E3) activity (●). 2,3-Dithiopropanol (10 mM) was introduced at the point shown by the arrow. The solution was bubbled with N_2 periodically during the incubation.

Results

Inhibition of the Complex by $H_2NPhAsO$. In order to establish that both PD complex and E3, inhibited with a monofunctional arsenical, could be effectively reactivated by a dithiol reagent, the following experiment was performed. PD complex was incubated with $H_2NPhAsO$ and an excess of NADH at 5 °C. A rapid loss of 50% of PD complex activity after 3 min was followed by a much slower loss of the remaining activity over 98 min (Figure 1). The loss of 85% PD complex activity takes place without an effect on E3; however, loss of E3 activity does proceed after a 20-min lag. In the presence of excess NADH, a direct reversal of the E3 reaction reduces the lipoyl residues on E2 (Brown & Perham, 1976), thus making them susceptible to dithioarsinite formation with arsenoxides.

Consistent with the findings of Massey & Veeger (1960) and Searls et al. (1961) by using pig heart lipoamide dehydrogenase and arsenite, E3 in the presence of excess NADH is inactivated by $H_2NPhAsO$ to 88% of its original activity after 100 min (Figure 1). The visible spectrum of the PD complex in the presence of NADH (2 mM) is consistent with the four-electron reduced form of E3 which possesses $FADH_2$ and the reduced active-site disulfide (S. R. Adamson and K. J. Stevenson, unpublished experiments). The inhibition of E3 is due to cyclic dithioarsinite formation between the arsenoxide and the vicinal thiol groups formed from the NADH reduction of the disulfide bond in the active site (Williams, 1976). The sedimentation coefficient of the PD complex decreased from 56.4 to 50.4 S on inhibition of PD complex and E3 component activities. Accordingly, the lag in E3 inhibition may be due to the dissociation of E3 from the PD complex, resulting in "free" E3 in which the vicinal thiols are more susceptible to modification by the reagent than in the native PD complex. The addition of 2,3-dithiopropanol to the inhibited PD complex

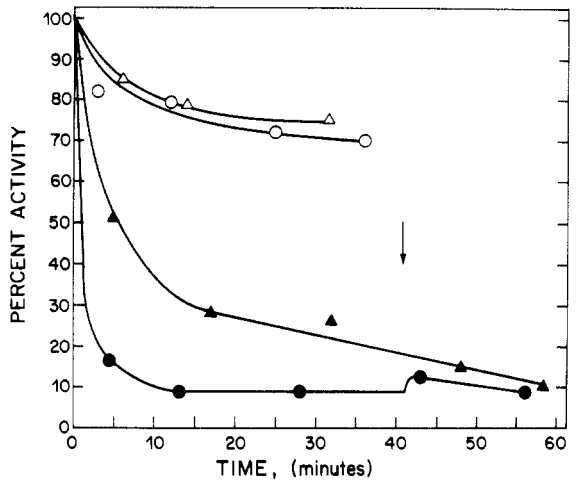

FIGURE 2: Inactivation of PD complex (0.5 mg/mL) with $BrCH_2CONHPhAsO$ (0.5 mM) and $BrCH_2CONHPh$ (0.5 mM) in 50 mM potassium phosphate buffer (pH 8) containing 2 mM NADH at 0 °C. Reagents were added from 10 mM stock solutions in 95% ethanol prior to NADH addition. PD complex (●) and E3 (▲) activities in the presence of $BrCH_2CONHPhAsO$; PD complex (○) and E3 (△) activities in the presence of $BrCH_2CONHPh$. 2,3-Dithiopropanol (10 mM) was introduced at the point shown by the arrow.

results in the effective reactivation of both PD complex and E3 activities (Figure 1). This reagent competes most favorably for $H_2NPhAsO$, covalently bound to E2 and E3, through formation of a very stable five-membered cyclic dithioarsinite (Whittaker, 1947; Stocken & Thompson, 1946, 1949). These studies indicate that monofunctional arsenoxides are effective *reversible* inhibitors of both E2 and E3.

Inhibition of the PD Complex by $BrCH_2CONHPhAsO$. The inactivation of the PD complex by the bifunctional arsenoxide, $BrCH_2CONHPhAsO$, in the presence of NADH at 0 °C is shown in Figure 2. A rapid loss of 83% of PD complex activity occurred within 4 min followed by a loss of E3 activity. The addition of 2,3-dithiopropanol (after 41 min) had no effect on E3 activity and regenerated the PD complex only to the level of residual E3 activity. The ability of 2,3-dithiopropanol to effectively reverse dithioarsinite inhibtion of both E2 and E3 by $H_2NPhAsO$ was previously shown in Figure 1. Thus, the irreversible inactivation of E3 by $BrCH_2CONHPhAsO$ was attributed to the alkylation of a residue at or near the active site of E3 by the bromoacetyl moiety ($BrCH_2CO$–) of the bifunctional reagent. The inactivation of the PD complex and E3 in the presence of NADH and $BrCH_2CONHPhAsO$ was more rapid at 25 °C. The PD complex was completely inactivated and E3 was 90% inactivated within 15 min under the same conditions as described above. Complex activity was again regenerated only to the level of E3 inhibition after 2,3-dithiopropanol treatment. Inactivation of PD complex and E3 by *N*-phenyl-α-bromoacetamide ($BrCH_2CONHPh$) in the presence of NADH at 0 °C was notably slower than in the presence of $BrCH_2CONHPhAsO$ and NADH (Figure 2). Similar comparisons were also obtained at 25 °C. The a priori "anchoring" of the bifunctional reagent via the arsenoxide moiety to vicinal thiol groups on E2 (reduced lipoyl residues) or E3 (reduced active site disulfide bond) was crucial in leading to the efficient irreversible inactivation of E3.

In view of the reactivity of the bromoacetyl moiety of the bifunctional reagent, it was possible that alkylations could take place at sites other than the active site of E3. In order to establish if PD complex activity was affected by alkylations at alternate sites, component activities within the complex (other than E3) were monitored in both the E1- and E3-mediated lipoyl reduction systems. No inactivation of E1 was apparent on incubation of the PD complex (1 mg/mL) with $BrCH_2CONHPhAsO$ (1 mM), TPP (0.5 mM), pyruvate (2 mM), coenzyme A (0.13 mM), and $MgCl_2$ (5 mM) or in the presence of NADH (2 mM) at 0 °C in 50 mM potassium phosphate, pH 8, for periods in excess of those required for complete E3 and PD complex inactivation. In this experiment, controls lacked the bifunctional reagent.

The dihydrolipoamide acetyltransferase (DHAT) assay (Reed & Willms, 1965) is a model for the physiological reaction and does not require covalently bound lipoyl residues. The acetyl group of acetyl coenzyme A is transferred to exogenous reduced lipoamide in the presence of E2. This assay was used to establish if the substrate binding ability of E2 was altered due to irreversible alkylations possibly occurring on E2. PD complex (1 mg/mL) was incubated with $BrCH_2CONHPhAsO$ as outlined above for E1 for 15 min at room temperature (25 °C) or for 2 h at 0 °C. The reaction was quenched by adding 2,3-dithiopropanol (10 mM), thereby breaking dithioarsinite bonds within the PD complex. Controls lacked the bifunctional reagent or contained $H_2NPhAsO$ instead of $BrCH_2CONHPhAsO$. No decrease in DHAT activity with $BrCH_2CONHPhAsO$ was apparent in the E1-mediated lipoyl reduction system. However, 45% ± 6.5% of the original DHAT activity was lost in the E3-mediated lipoyl reduction system. This loss of activity was attributed to an alkylation of a residue (or residues) at or near the active site of E2. Such an alkylation is consistent with the commonly accepted mechanism in which *S*-acetyllipoamide residues (covalently bound to E2) interact with coenzyme A at the active site of E2. The presence of coenzyme A in the E1-mediated system very likely protected the active site of E2 from covalent modification. Thus, the ability of E2 to bind coenzyme A remained unaltered on incubation of $BrCH_2CONHPhAsO$ with the PD complex, in the presence of pyruvate, coenzyme A, TPP, and $MgCl_2$.

In view of the high degree of interaction between lipoyl residues within the E2 core of the PD complex (Bates et al., 1977; Collins & Reed, 1977), it was necessary to establish if irreversible alkylations of lipoyl residues on E2 were rate determining in terms of overall complex activity. PD complex (10 mg/mL) was incubated in 20 mM potassium phosphate buffer, pH 7, containing 2 mM NADH or pyruvate, coenzyme A, TPP, and $MgCl_2$ and inhibited by $BrCH_2CONHPhAsO$ (0.5 mM) at 25 °C until both PD complex and E3 activities were less than 10% of a control which did not contain the bifunctional reagent (1 h). The reaction was quenched with 2,3-dithiopropanol (10 mM), and these solutions plus a control were dialyzed overnight at 4 °C against several changes of 20 mM phosphate buffer, pH 7. The degree of lipoyl modification was established by measuring the [1-^{14}C]acetyl incorporation from [2-^{14}C]pyruvate in the presence of TPP (0.4 mM), $MgCl_2$ (2 mM), and NAD^+ (0.5 mM) essentially by the method of Speckhard et al. (1977). The acetylated complex was isolated by gel filtration through Sephadex G-25. The control incorporated 1-^{14}C acetyl groups to the level of 5.2 ± 0.17 nmol/mg of total protein whereas the E3- and E1-mediated inhibited samples incorporated 4.0 ± 0.26 and 4.1 ± 0.10 nmol/mg of total protein, respectively. These values, in comparison to the control, correspond to the alkylation of 23% and 22% of lipoyl residues, respectively. Blielle et al. (1979) have removed 50% of lipoyl residues from the complex by limited proteolysis with the loss of only 6% of PD complex activity. Thus, the irreversible loss of complex activity observed with $BrCH_2CONHPhAsO$ would appear not to be due to the alkylation of lipoyl residues on E2. The value of 5.2 nmol/mg of protein

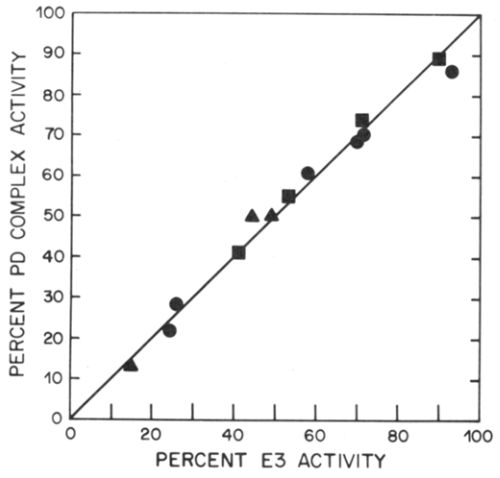

FIGURE 3: Selective inactivation of E3 by $BrCH_2CONHPhAsO$ in the presence of TPP (0.5 mM), pyruvate (2 mM), coenzyme A (0.13 mM), and $MgCl_2$ (5 mM). PD complex (1 mg/mL) was incubated at 0 °C with varying amounts of $BrCH_2CONHPhAsO$ in 50 mM potassium phosphate buffer, pH 7 or 8, in the presence of TPP, pyruvate, coenzyme A, and $MgCl_2$. Aliquots (100 μL) were removed at various times and made 10 mM with respect to 2,3-dithiopropanol. Points on the graph comprise E3 and PD complex activities from the same 2,3-dithiopropanol-treated aliquot. Reagent concentrations and pH are as follows: (●) 0.1 mM reagent, pH 8; (■) 0.06 mM reagent, pH 7; (▲) 0.25 mM reagent, pH 8. Controls lacked the reagent.

of incorporated acetyl moieties is lower than the value of 6.5 nmol/mg of protein originally recorded by Danson & Perham (1976) by using PD complex purified from the same constitutive mutant of *E. coli* K12. Protein concentrations were directly determined from Folin assays without correlation to the dry weight of the complex. This and possible variations in subunit composition are sufficient to account for the discrepancy.

From the above studies, the irreversible inhibition of the PD complex by $BrCH_2CONHPhAsO$ in the presence of pyruvate, coenzyme A, TPP, and $MgCl_2$ and after dithioarsinite breakdown by 2,3-dithiopropanol is due wholly to the active site directed inhibition of E3. This may not be the case in the E3-mediated lipoyl reduction system since the substrate binding ability of E2 was decreased.

Selective Inhibition of E3. Correlation of E1 depletion (Bates et al., 1977; Danson et al., 1978) and selective inactivation of E1 (Gutowski & Lienhard, 1976; Collins & Reed, 1977; Angelides & Hammes, 1978) have proved invaluable in elucidating the enzyme–enzyme interactions within the PD complex. From our studies, a method was available through the use of $BrCH_2CONHPhAsO$, in the presence of TPP, pyruvate, coenzyme A, and Mg^{2+}, to monitor the effect of selective E3 inactivation on overall complex activity. PD complex was incubated with TPP, pyruvate, coenzyme A, and $MgCl_2$ for 2 min at 0 °C at both pH 7 and pH 8. $BrCH_2CONHPhAsO$ was added, and at suitable intervals aliquots were removed and further incubated with 2,3-dithiopropanol (10 mM) for 1 min at 0 °C. PD complex and E3 NAD^+ reduction assays were performed on these solutions. In Figure 3, PD complex activity is plotted against E3 activity. Each point constitutes those activities from the same aliquot treated with 2,3-dithiopropanol. A stoichiometric relationship exists between E3 and PD complex inactivation. Since it was previously shown that the rate of irreversible inactivation of PD complex was determined by the active site directed alkylation of E3, these data suggest that a single E3 molecule participates in each catalytic cycle and that a full complement of E3 is necessary for maximum PD complex activity.

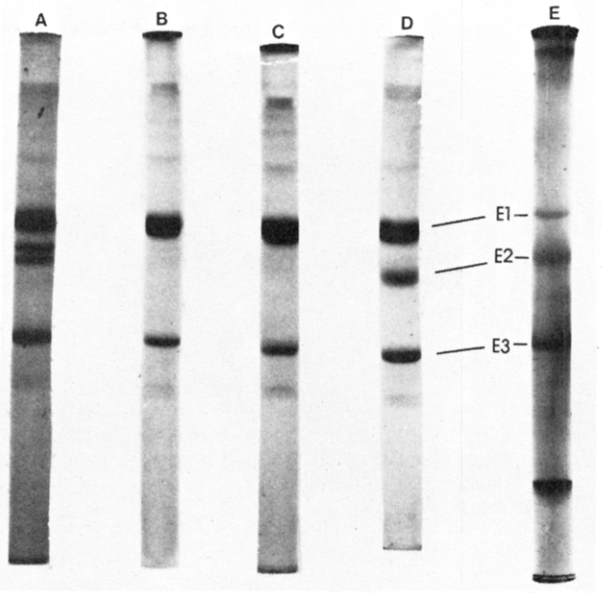

FIGURE 4: $NaDodSO_4$–polyacrylamide (7.5%) gel electrophoresis of PD complex incubated with $BrCH_2CONHPhAsO$ for 20 h. (A) PD complex in the absence of $BrCH_2CONHPhAsO$ (see Methods). (B) PD complex incubated with $BrCH_2CONHPhAsO$ in the presence of 2 mM NADH at room temperature until negligible complex and E3 activities remained, followed by a 20-h incubation at 5 °C. Reagent was added from a stock solution in 95% ethanol prior to NADH addition. (C) Duplicate of (B). (D) As outlined for (B) and (C) but followed by treatment with 10 mM 2,3-dithiopropanol. (E) The polymerized material from the top of four gels [cf. (B) and (C)] was extracted, before staining, by macerating the gel slices followed by shaking at 42 °C in a solution of 0.5% ammonium bicarbonate and 0.1% $NaDodSO_4$ for 12–16 h. The extract was freeze-dried and dissolved in a sample preparation solution containing 2-mercaptoethanol (see Methods) and 10 mM 2,3-dithiopropanesulfonic acid and boiled for 2 min.

Cross-Linking of the PD Complex by $BrCH_2CONHPhAsO$. The proposed scheme for E3 inhibition by $BrCH_2CONHPhAsO$ as outlined by Stevenson et al. (1978) involved the initial "anchoring" of the reagent to reduced lipoyl residues of E2 via cyclic dithioarsinite formation followed by delivery of the bromoacetyl group of the reagent into the active site of E3 where an irreversible alkylation takes place. This scheme would necessarily involve the cross-linking of E2 to E3. $NaDodSO_4$–polyacrylamide gel electrophoresis was used (see Methods) in order to investigate this cross-linking phenomenon.

Due to the nature of the presumed cross-linking in this study, it was necessary to exclude mercaptoethanol from the sample preparation. Monothiols are capable of dissociating dithioarsinites although they are much inferior to reagents with vicinal thiols such as 2,3-dithiopropanol (Whittaker, 1947). Under the conditions employed (see Methods), the only alteration in mobility of the three components of the complex was in E2, which tended to migrate as a doublet or as a single band of lower than normal apparent molecular weight (Figure 4A,D). PD complex was incubated with $BrCH_2CONHPhAsO$ and NADH at 25 °C until both complex and E3 activities were negligible (25 min). The samples were further incubated for 20 h at 5 °C and were analyzed by electrophoresis in 7.5% (w/v) polyacrylamide gels in the presence of $NaDodSO_4$. Complete interchain cross-linking of E2 subunits and partial cross-linking of E3 with E2 subunits occurred with the concomitant production of a high molecular weight polymer (or polymers) which only partially entered the gel (Figure 4B,C). Interchain cross-linking involving E1 was not apparent in these experiments. Samples of PD complex were also cross-linked in the E3-mediated lipoyl reduction

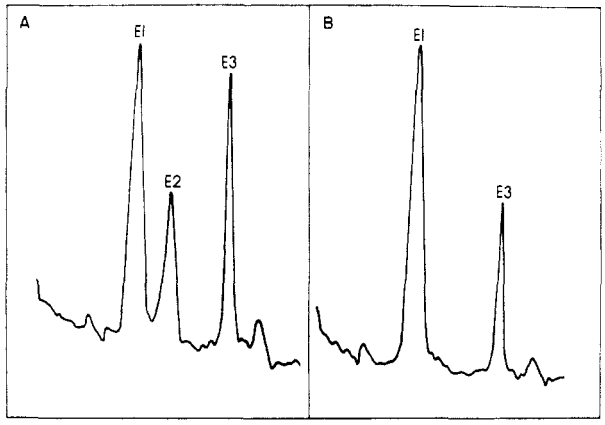

FIGURE 5: Densitometry scans of $NaDodSO_4$–polyacrylamide gels of PD complex cross-linked by $BrCH_2CONHPhAsO$ in excess NADH (B) and PD complex cross-linked by $BrCH_2CONHPhAsO$ in excess NADH but treated with 10 mM 2,3-dithiopropanol (A) to decompose cross-links.

system for 30 and 90 min at 25 °C (E3 and PD complex activities were negligible). These samples were applied directly to polyacrylamide gels without incubation at 5 °C, and the results obtained were similar to those shown in Figure 4B,C. The presumed E2/E3 composition of the polymeric material which only partially enters the gel was substantiated by extraction of cross-linked material from four gels (cf. Figure 4B,C). The extracted material was lyophilized, treated with 2,3-dithiopropanesulfonic acid (which is more soluble than 2,3-dithiopropanol), and rerun on polyacrylamide gels in the presence of $NaDodSO_4$ (Figure 4E). Approximately 5% of E1 was involved in cross-linking as estimated by scanning densitometry. Similar quantitation of a number of gels of cross-linked PD complex, prepared as outlined above, revealed that, after complete inactivation of PD complex and E3, 51% ± 9% of E3 was cross-linked. Profiles of two gels containing equal applications of PD complex cross-linked in the presence of excess NADH and incubated with and without 2,3-dithiopropanol are shown in Figure 5. In this case, 48% of E3 was cross-linked. Similar cross-linking experiments carried out at 25 °C for 30 min, 90 min, and 24 h by using E1-mediated lipoyl reduction in the presence of $BrCH_2CONHPhAsO$ revealed that less extensive cross-linking took place. After complete inactivation of the PD complex and E3, E2 was cross-linked to the extent of 70–80% whereas 38% ± 9% of E3 was cross-linked. To account for variations in sample applications, all cross-linked samples were normalized with respect to E1 content. Since a small proportion of E1 (~5%) apparently becomes cross-linked in this reaction, these values are slight underestimates.

For further investigation of the quaternary structure of PD complex modified by the bifunctional reagent, a number of sedimentation velocity measurements were performed. The sedimentation coefficient of native PD complex (3 mg/mL) when measured in 20 mM potassium phosphate buffer, pH 7, at 20 °C was 58.7 ± 0.7 S (four measurements). PD complex inhibited by $BrCH_2CONHPhAsO$ (0.4 mM) in the E3- and E1-mediated lipoyl reduction systems, until both PD complex and E3 activities were negligible, was 44.5 and 48.5 S, respectively. These values infer a marked change in the quaternary structure of the complex on inhibition by $BrCH_2CONHPhAsO$. In a separate experiment, PD complex (3 mg/mL) was inhibited by $BrCH_2CONHPhAsO$ (0.4 mM) in both lipoyl reduction systems. After a 15-min incubation in 20 mM potassium phosphate buffer, pH 7, at room temperature (25 °C), 2,3-dithiopropanol was added to break the interchain cross-links. The sedimentation coefficients of inhibited PD complex were 50.6 ± 0.0 S in the E-3 mediated lipoyl reduction system and 57.0 ± 1.6 S in the E1-mediated system (results from duplicate experiments). The change in the quaternary structure of the PD complex caused presumably by interchain cross-linking is apparently fully reversible in the E1-mediated lipoyl reduction system (cf. Figure 3). This is not the case in the E3-mediated system. The less extensive degree of cross-linking and, accordingly, the less marked decrease in the sedimentation coefficient seen in the E1- compared to the E3-mediated lipoyl reduction system may reflect the active site of E2 being protected by coenzyme A in the former system, and, thus, a potential site of alkylation is not available.

The inability to cross-link all of E3 to the E2 core, as indicated by polyacrylamide gel electrophoresis (Figures 4 and 5) and the decrease in the apparent S value of the cross-linked sample, could have arisen from the dissociation of E3 from the complex during cross-linking. This possibility was investigated by subjecting inhibited PD complex to preparative ultracentrifugation followed by $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of the fast sedimenting material and the supernatant. PD complex (2 mg/mL) was inhibited in both E1- and E3-mediated lipoyl reduction systems in 20 mM sodium phosphate buffer, pH 7, at 25 °C until complex and E3 activities were negligible. These samples and a control containing only PD complex were centrifuged at 20 °C for 90 min at 45 000 rpm in a Beckman SW50 rotor. The pellets obtained were suspended in 1 mL of phosphate buffer and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under conditions which break the cross-links (see Methods). Scanning densitometry of these gels showed that about 45% of E1 and 27% of E3 became dissociated from the inhibited complex in the E3-mediated system, and 30% of E1 and 23% of E3 became dissociated in the E1-mediated system. The supernatants from these ultracentrifugations were similarly analyzed. In both lipoyl reduction systems, the supernatants contained notably increased amounts of both E1 and E3 in comparison to the control. However, scanning densitometry showed that less E1 was apparently dissociated from the PD complex, in both lipoyl systems, than previously indicated. Nonetheless, these results do indicate that E3, as well as E1, becomes dissociated from the complex on inhibition and cross-linking by $BrCH_2CONHPhAsO$.

Since complete inhibition of E3 was obtained in these systems, it is likely that dissociated E3 subsequently becomes modified by the bifunctional reagent. Isolated E3 from *E. coli*, in the presence of excess NADH, was irreversibly inhibited by $BrCH_2CONHPhAsO$ at a rate considerably faster than by $BrCH_2CONHPh$ when compared at the same concentrations and under the same conditions (S. R. Adamson and K. J. Stevenson, unpublished experiments). We envisage that the initial modification of the reduced disulfide bond of E3 by dithioarsinite formation facilitates an irreversible intramolecular alkylation of a residue near the active site of E3 by the bromoacetyl moiety of the "anchored" reagent. This mode of inactivation of E3 by $BrCH_2CONHPhAsO$ and NADH has been suggested from experiments with pig heart lipoamide dehydrogenase (Stevenson & Robinson, 1980). The visible spectra of the PD complex in both the E1 (Frey et al., 1978) and E3 lipoyl reduction systems (data not shown) are both consistent with the presence of the four-electron reduced form of E3 (Williams, 1976) in which the active site disulfide is reduced. Therefore, in both systems, modification of free E3 by this mechanism is likely.

Discussion

The experiments described were performed to clarify the mechanism of inhibition of the PD complex by the bifunctional reagent $BrCH_2CONHPhAsO$ and to establish if the E3 inactivation by this reagent, first described by Stevenson et al. (1978), was selective. This work has shown that the rate of irreversible inactivation of the PD complex (after dithiol treatment) in the presence of TPP, pyruvate, coenzyme A, and Mg^{2+} was determined by the active site directed alkylation of E3. Under these conditions, the activity of E1 and the substrate binding capacity of E2 are both unaffected although some alkylations of reduced lipoyl residues on E2 likely take place (22% when PD complex and E3 activities are zero). The conclusion that E3 inactivation is rate determining for PD complex activity, under the above conditions, depends on whether or not this degree of lipoyl alkylation is deleterious to overall complex activity. Recent evidence indicates that the rate-limiting step in the overall reaction does not involve the network of interacting lipoyl moieties (Danson et al., 1978). Indeed, a considerable amount of evidence indicates that a substantial loss of lipoyl residues from the core of the PD complex can occur without affecting the rate of the overall reaction (Frey et al., 1978; Angelides & Hammes, 1978; Blielle et al., 1979). Blielle et al. (1979) have removed approximately 50% of the lipoyl residues from the complex with the loss of only 6% PD complex activity. This experiment was carried out by using PD complex in which lipoyl residues had been intrinsically labeled with ^3H. In the presence of $BrCH_2CONHPhAsO$ in the E1-mediated lipoyl reduction system and after dithiol treatment, PD complex and E3 were almost completely inactivated, whereas 22% of lipoyl residues capable of being reductively acetylated were alkylated by the bifunctional reagent. The degree of lipoyl modification in this case is well within the range outlined by Blielle et al. (1979) in which complete complex activity is maintained. However, it should be noted that our results reflect the percent of lipoyl residues which can be reductively acetylated by exogenous radiolabeled pyruvate [cf. Hale & Perham (1979)]. Angelides & Hammes (1978) have made a direct correlation between the percent PD complex activity and the fraction of modified lipoyl residues on E2 by using *N*-[^3H]ethylmaleimide in the presence of TPP and pyruvate. The modification of 22% of lipoyl residues, in their study, corresponded to approximately 80% complex activity. Apparently, the alkylation of lipoyl residues by $BrCH_2CONHPhAsO$ is not rate limiting in terms of overall complex activity. The close agreement between the rate of E3 modification and complex inactivation, apparent in Figure 3, further implies that E3 modification determines the resulting overall complex activity. Since the loss of E3 activity is directly proportional to the loss in the overall enzyme activity, a single E3 molecule must be involved in each catalytic cycle, and a full complement of E3 is necessary for maximum complex activity. Angelides & Hammes (1978) showed that only half of the flavin content of the native enzyme is necessary for full complex activity (assuming a binomial distribution of reassociated FAD on E3). They suggest that extensive reoxidation can take place among lipoyl residues with a single FAD bound to E3 able to "service" at least eight lipoyl residues on E2. Our results do not support this proposal but rather infer that an E3 molecule interacts with a limited number of E2 chains such that during active site directed inactivation of an E3 molecule by $BrCH_2CONHPhAsO$ one particular catalytic cycle within the complex is destroyed. However, the possibility that the disruptive nature of PD complex modification in our experiments may have a deleterious effect on complex activity, even though the apparent native structure is regained after breakage of interchain cross-links, cannot be discounted.

The mechanism outlined by Stevenson et al. (1978) for the inhibition of the E3 component of the PD complex by $BrCH_2CONHPhAsO$ involved delivery of the bromoacetyl moiety of the bifunctional reagent (covalently attached to lipoyl residues of E2 through dithioarsinite bonds) into the active site of bound E3. Approximately 50% of E3 is apparently modified in this manner. The inhibition of E3, dissociated from the PD complex during cross-linking, seems to occur via direct interaction of the free enzyme with $BrCH_2CONHPhAsO$ by the initial modification of the reduced disulfide bond of E3 by dithioarsinite formation followed by an irreversible intramolecular alkylation of a residue near the active site of E3 by the bromoacetyl moiety of the reagent. The remaining E3, which is neither cross-linked nor dissociated, may also be inhibited in this manner. Both these routes involve irreversible alkylation of E3 and appear to be active site directed (cf. Figure 2). Treatment of inhibited cross-linked PD complex with a dithiol reagent results in cleavage of the dithioarsinite bonds and the re-formation of the apparent native quaternary structure of the complex (in E1-mediated inhibition); however, E3 remains irreversibly inhibited.

Work is in progress to elucidate the site or sites of alkylation on E3 and to correlate the extent of active site directed alkylations with the loss of PD complex activity. Because of the presence of the amide bond in the bifunctional reagent, a carboxymethylated residue will be released from alkylated E3 following acid hydrolysis. The use of $BrCH_2[^{14}C]$-$CONHPhAsO$ will further facilitate these studies (Stevenson & Robinson, 1980).

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Metal Ion Substitution at the Catalytic Site of Horse-Liver Alcohol Dehydrogenase: Results from Solvent Magnetic Relaxation Studies. 1. Copper(II) and Cobalt(II) Ions†

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ABSTRACT: The influence of paramagnetic Cu^{2+} and Co^{2+} ions, substituted for Zn^{2+} ions at the catalytic sites of native alcohol dehydrogenase from horse liver (EC 1.1.1.1), on the nuclear magnetic spin–lattice relaxation rates of solvent water and substrate (CH_3OD) protons was studied as a function of magnetic field strength. For the Cu^{2+} ions, the data can best be fit to a model in which the resulting "blue copper" center (type I) of the enzyme is characterized by inner sphere coordinated water or substrate, both more strongly bound in the binary complex of protein with coenzyme and displaced from the ternary complex with pyrazole. In the binary complex with pyrazole, a pentacoordinated species is indicated; thus, the coordination number is reduced upon formation of the enzyme–pyrazole–coenzyme ternary complex. Although the Cu^{2+}–enzyme is able to bind coenzyme, thereby distorting its metal-binding site, it cannot discriminate significantly between alcohol substrates and water. The resulting relatively weak binding of alcohol is sufficient to explain the observed absence of enzymatic activity of the Cu^{2+}-substituted protein under the usual experimental conditions. This is the first example of a blue copper protein for which the Cu^{2+} ion is accessible to solvent. The correlation times for the paramagnetic dipolar interaction between the solvent protons and the Cu^{2+} ion are unusually short, presumably due to a strong spin–orbit interaction of the electronic spins of the Cu^{2+} ions with their thiol-sulfur ligands. The magnetic spin–lattice relaxation rates of both solvent water protons and solvent methanol methyl protons were also measured for solutions of the native enzyme, the enzyme with Zn^{2+} ions removed from the catalytic sites, and with Co^{2+} ions specifically substituted for Zn^{2+} ions at the catalytic sites. We could detect no paramagnetic contribution from the Co^{2+} ions to the magnetic relaxation rate of the solvent water and methanol protons, despite attempts to enhance the detection of paramagnetic effects by altering a variety of experimental parameters, including temperature and ionic content of the solvent, and by the addition of coenzyme and inhibitors. There are small differences in the diamagnetic contributions to the relaxation rates of the native, demetalized, and Co^{2+}-substituted enzymes that change sign with magnetic field; these small variations can readily be mistaken for true paramagnetic effects when analysis of the relaxation data is limited to the high values of magnetic field strength usually used for measurements of relaxation enhancement. As a result, previous high-field data require reinterpretation: any paramagnetic effects that may be present are small and not easily separable from a variety of small diamagnetic effects that depend on solvent composition. The reason seems to be an unusually short correlation time for the interaction between solvent protons and the Co^{2+} ions, due to a strong spin–orbit interaction of the electronic spins of the Co^{2+} ions, as was found for the Cu^{2+}-substituted enzyme.

Native horse-liver alcohol dehydrogenase (EC 1.1.1.1; ADH),[1] a dimeric enzyme of 80000 daltons, contains two Zn^{2+} ions per identical monomeric subunit, one ion at the catalytic site and a second ion at a site ~20 Å from the first [for reviews, cf. Brändén et al. (1975) and Brändén & Eklund (1978)]. Recently, techniques have been developed by which various divalent ions can be substituted specifically for the Zn^{2+}

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[1] Abbreviations used: ADH, horse-liver alcohol dehydrogenase; NMRD, nuclear magnetic relaxation dispersion; NADH and NAD^+, respectively, reduced and oxidized nicotinamide adenine dinucleotide; A(c)B(n)-ADH, enzyme with divalent metal ions of type A in the catalytic sites and divalent metal ions of type B in the noncatalytic sites; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid.

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