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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with Mono- and Bifunctional Arsenoxides†

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ABSTRACT: Pyruvate dehydrogenase multienzyme complex (PD complex) from *Escherichia coli* is comprised of three enzymes: pyruvate dehydrogenase (E1), lipoate acetyltransferase (E2), and lipoamide dehydrogenase (E3). Incubation of PD complex with pyruvate, coenzyme A, and *p*-aminophenyl arsenoxide ($H_2NPhAsO$; 100 μM) lead to a loss of 92% of the PD complex activity within 25 min at 4 °C. Controls lacking pyruvate and/or coenzyme A, but containing $H_2NPhAsO$, retained nearly all their PD complex activity. The loss of activity associated specifically with E3 was only 5% in the presence or absence of $H_2NPhAsO$. The arsenoxide formed a stable cyclic dithiolarsinite with reduced lipoic acid on E2 which was generated by pyruvate and coenzyme A according to the accepted reaction sequence within the complex. PD complex activity could be recovered to 78% within 2 min following the addition of 2,3-dithiopropanol (350 μM). Replacing $H_2NPhAsO$ with the bifunctional reagent $BrCH_2CONHPhAsO$ (100 μM) in the presence of pyruvate and coenzyme A caused a 100% loss in PD complex activity within 15 min. The loss of E3 activity was found to lag a few minutes behind the loss of PD complex activity and reached a value of 90% within 20 min. The initial reaction of the bifunctional reagent occurred on E2 via the R-AsO moiety and resulted in the rapid loss in PD complex activity. The inactivation of E3 was attributed to the subsequent delivery of the reagent into the active-site of E3. This is in keeping with the "swinging-arm hypothesis" ascribed to the flexible lysinyl–lipoyl group on E2 and suggests that the loss of E3 activity occurs via an active-site directed irreversible alkylation by the bromoacetyl moiety of the bifunctional reagent. At this stage E2 and E3 were cross-linked. Addition of 2,3-dithiopropanol failed to regenerate PD complex activity and E3 activity indicating that, although the reduced lipoic acid group on E2 was regenerated, the alkylation occurring in E3 was not reversed. Experiments with bromoacetylaniline ($BrCH_2CONHPh$;100 μM) caused only a slight loss of PD complex activity (10%) and E3 activity (3%) under conditions where the bifunctional reagent ($BrCH_2CONHPhAsO$) led to nearly complete inactivation of both activities. Monofunctional and bifunctional arsenoxides offer a new approach to the studies of 2-oxoacid dehydrogenase multienzyme complexes.

Pyruvate dehydrogenase multienzyme complex of *Escherichia coli* is comprised of three different enzymes that catalyze the overall reaction:

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

The enzymes, in order of their participation, are: pyruvate dehydrogenase (lipoate) (E1)[1] (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12), and lipoamide dehydrogenase (NADH) (E3) (EC 1.6.4.3) (Reed, 1974; Hucho, 1975; Perham, 1975). E2 forms the core of the complex to which enzymes E1 and E3 bind in a noncovalent manner. The polypeptide chain stoichiometry of E1:E2:E3 approaches 2:1:1 (Bates et al., 1975, 1977; Perham & Hooper, 1977). A flexible "swinging-arm" of length 1.4 nm enables the reactive dithiolane ring of the covalently bound lipoic acid on E2 to interact with E1, coenzyme A, and E3 (Nawa et al., 1960; Koike et al., 1963; Green & Oda, 1961). Lipoic acid within the PD complex possesses a high degree of mobility (Ambrose & Perham, 1976; Grande et al., 1975). However, a single lipoic acid residue may not be able to span the distance of about 4.5 nm considered to exist between the active sites of E1 and E3 (Moe et al., 1974). Recent evidence (Danson & Perham, 1976; Brown & Perham,

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[1] Abbreviations used: PD complex, pyruvate dehydrogenase multienzyme complex; $H_2NPhAsO$, *p*-aminophenyl arsenoxide; $BrCH_2CONHPhAsO$, *p*-bromoacetylaminophenyl arsenoxide; $BrCH_2CONHPh$, bromoacetylaniline; E1, pyruvate dehydrogenase (lipoate) or pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating) (EC 1.2.4.1); E2, lipoate acetyltransferase (EC 2.3.1.12); E3, lipoamide dehydrogenase (NADH) (EC 1.6.4.3).

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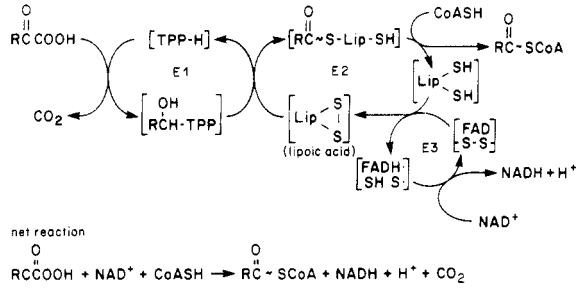

FIGURE 1: Reaction mechanism of the pyruvate dehydrogenase multienzyme complexes of *E. coli*. E1 refers to pyruvate dehydrogenase (a decarboxylase), E2 is lipoate acetyltransferase, and E3 is lipoamide dehydrogenase. TPP is thiamin pyrophosphate.

1976; Bates et al., 1977) has indicated that at least two lipoyl residues are present per polypeptide chain of E2 (assumed mol wt, 80 000) rather than the earlier value of one lipoyl residue per polypeptide chain of E2 (assumed mol wt 70 000; Eley et al., 1972). The two lipoyl residues may function in "transacetylation" reactions within the complex (Bates et al., 1977).

The presently accepted mechanism for the PD complex is shown in Figure 1 (Reed & Oliver, 1968). E2 thus assumes a central role within the complex both mechanistically and structurally (Reed, 1974; Hucho, 1975).

The toxic effects of trivalent arsenicals on organisms and, more specifically, on proteins possessing thiol groups have been known for many years (Ehrlich, 1909; Cohen et al., 1931; Gordon & Quastel, 1948; Stocken & Thompson, 1949; Webb, 1966). Trivalent arsenicals used to modify proteins are of two types (Lotspeich & Peters, 1951). The first group, alkyldihaloarsines ($RAsX_2$) and alkyl arsenoxides (RAsO), react with dithiols, such as reduced lipoic acid, to form stable cyclic dithioarsinites (Whittaker, 1947; Stocken & Thompson, 1946a). Although these arsenicals do react with single cysteine residues in proteins, the product (RAs(OH)S-cysteine) is unstable. The decomposition of cyclic dithioarsinites was effectively obtained by compounds possessing vicinal dithiols like 2,3-dithiopropanol (British AntiLewisite) (Peters et al., 1945; Stocken & Thompson, 1949; Stocken & Thompson, 1946b,c; Whittaker, 1947). At similar concentrations monothiols were much inferior. The second group, dialkylhaloarsines (R_1R_2AsX), yields stable derivatives only with cysteine. Reversal of the reaction is effected by monothiols.

In this paper aromatic arsenoxides have been reinvestigated. The inactivation of the PD complex from *E. coli* by $H_2NPhAsO$ in the presence of pyruvate and coenzyme A rapidly occurs through the formation of a cyclic dithioarsinite between the -AsO group of the reagent and reduced lipoic acid on lipoate acetyltransferase (E2). Reactivation is obtained by introducing 2,3-dithiopropanol. A new bifunctional arsenoxide ($BrCH_2CONHPhAsO$) has been synthesized and found to inactivate PD complex by reaction of the -AsO moiety with the reduced lipoic acid on E2 followed by the alkylation of E3 by the $BrCH_2CONH$ moiety of the reagent. Exposure to 2,3-dithiopropanol does not reactivate the complex. These reagents provide a novel approach to the study of 2-oxoacid dehydrogenase multienzyme complexes.

Experimental Procedures

Materials

Bromoacetyl bromide and 2,3-dithiopropanol were obtained from Ralph N. Emanuel Ltd. (Aldrich). Lipoamide and sulfur dioxide were obtained from British Drug Houses. Arsanilic acid and thiamin pyrophosphate were from Koch-Light. NAD^+ (free acid, grade 2), NADH (disodium salt, grade 2), and coenzyme A (grade 1) were from C. F. Boehringer und Soehne, Mannheim, West Germany.

Methods

DL-Dihydrolipoamide Synthesis. DL-Dihydrolipoamide was synthesized from lipoamide as described by Gunsalus et al. (1956).

Synthesis of p-Aminophenyl Arsenoxide. The synthesis was carried out as described by Blicke & Smith (1929). *p*-Arsanilic acid (10.9 g) was added to a solution containing 30 mL of methanol, 24 mL of HCl, and 100 mg of potassium iodide. Sulfur dioxide was bubbled through the stirred solution for 30 min during which time the color changed from orange to pale yellow and precipitation of *p*-aminophenyldichloroarsine·HCl ensued. The solution was cooled in ice and the precipitate was collected and washed with diethyl ether. The dichloroarsine was dissolved in 200 mL of 10% ammonium hydroxide. After stirring for 15 min, *p*-aminophenyl arsenoxide began to precipitate and the solution was cooled in ice. It was collected by filtration, washed with diethyl ether and dried under vacuum over NaOH: yield, 6 g; melting range, 56–58 °C. Anal. Calcd for $C_8H_9AsN_2O_2 \cdot 2H_2O$: C, 33.22; H, 4.81; N, 6.28. Found: C, 33.15; H, 4.66; N, 6.42 (Dr. F. B. Strauss, Microanalytical Laboratory, Oxford, U.K.).

Synthesis of p-Bromoacetylaminophenyl Arsenoxide. *p*-Aminophenyl arsenoxide (1 g, 3.44 mmol) in 20 mL of acetone was added to an equal volume of acetone containing bromoacetyl bromide (550 μL, 8 mmol) and the solution was stirred for 1 h. Diethyl ether was added to precipitate the product which was collected by filtration: yield, 600 mg; melting range, 64–67 °C. Anal. Calcd for $C_8H_7AsBrNO_2$: C, 31.61; H, 2.31; Br, 26.29; N, 4.61. Found: C, 31.82; H, 2.40; Br, 25.85; N, 4.71.

Synthesis of p-Bromoacetylaniline. Bromoacetyl bromide (500 μL, 7.2 mmol) was slowly added to 10 mL of acetone containing 670 mg of aniline (7.2 mmol) at 0 °C. A precipitate formed immediately but dissolved upon stirring for 30 min. The product was reprecipitated by the addition of diethyl ether and was collected by filtration. Recrystallization from ethanol yielded 170 mg of *p*-bromoacetylaniline: melting range, 127–128 °C. Anal. Calcd for C_8H_8BrNO: C, 44.88; H, 3.77; Br, 37.33; N, 6.54. Found: C, 44.93; H, 3.99; Br, 37.25; N, 6.62.

Enzyme Isolation. Pyruvate dehydrogenase complex of *E. coli* was purified as described by Reed & Mukherjee (1969). The complex was prepared from a mutant of *E. coli* K12 constitutive for pyruvate dehydrogenase kindly provided by Professor H. L. Kornberg and had a specific activity of about 15 units/mg in the pyruvate dehydrogenase assay and about 30 units/mg in the lipoamide dehydrogenase assay.

Enzyme Assays. The overall pyruvate dehydrogenase complex activity and the activity of lipoamide dehydrogenase (E3) were assayed by a modification of the methods of Reed & Mukherjee (1969) as outlined by Brown & Perham (1976) and Danson & Perham (1976).

Inhibition of Pyruvate Dehydrogenase Multienzyme Complex. PD complex (100 μg) was added to 500 μL of 50 mM sodium phosphate buffer, pH 7, containing 0.5 mM thiamin pyrophosphate, 2 mM pyruvate, 0.13 mM coenzyme A, and 5 mM $MgCl_2$. The solution was bubbled with N_2 and chilled to 0 °C. $H_2NPhAsO$, $BrCH_2CONHPhAsO$, or $BrCH_2CONHPh$ was introduced as 5-μL aliquots of 10 mM solutions in ethanol to yield a final concentration in the reaction mixture of about 100 μM. Aliquots were removed over a period of 45 min and assayed for PD complex activity and lipoamide

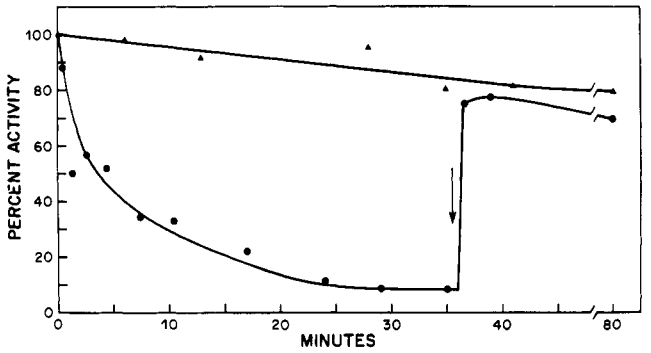

FIGURE 2: The inactivation of PD complex with $H_2NPhAsO$ (102 μM) in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM pyruvate, 0.5 mM thiamin pyrophosphate, 5 mM $MgCl_2$, and 0.13 mM coenzyme A at 0 °C. PD complex activity is denoted by ●—● whereas lipoamide dehydrogenase (E3) activity is shown by ▲—▲. 2,3-Dithiopropanol (370 μM) was introduced at the point shown by the arrow.

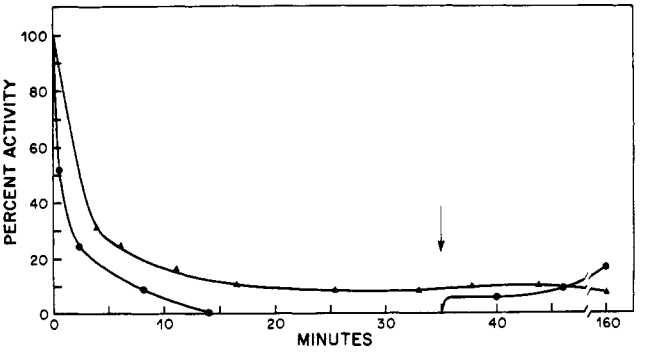

FIGURE 3: The inactivation of PD complex with $BrCH_2CONHPhAsO$ (104 μM) in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM pyruvate and 0.13 mM coenzyme A at 0 °C. PD complex activity is denoted by ●—●, whereas lipoamide dehydrogenase (E3) activity is shown by ▲—▲. 2,3-Dithiopropanol (350 μM) was introduced at the point shown by the arrow.

dehydrogenase (E3) activity. 2,3-Dithiopropanol was introduced as a 5-μL aliquot of a 20 mM solution in ethanol to yield a final concentration of about 350 μM. Aliquots were taken as before for enzyme assays.

Control experiments were performed with PD complex in the presence of $H_2NPhAsO$ and $BrCH_2CONHPhAsO$ but lacking pyruvate or coenzyme A.

Results and Discussion

A consideration of the reaction mechanism of pyruvate dehydrogenase multienzyme complex of *E. coli* (Figure 1) indicates that the production of reduced lipoic acid covalently attached to E2 requires the involvement of pyruvate and coenzyme A. Provided NAD^+ is absent, the lipoic acid moiety will remain in its reduced form. Under these conditions, the introduction of a monofunctional arsenoxide ($H_2NPhAsO$) should lead to the formation of a stable six-membered cyclic dithiolarsinite with the reduced lipoic acid resulting in the loss of biological activity of the PD complex. The inhibition of the PD complex with 100 μM $H_2NPhAsO$ (Figure 2) shows a rapid loss of 50% of the activity within 1 min followed by a more gradual loss of activity (up to 92%) over a period of 30 min. A plot of the log of percent activity remaining vs. time revealed two distinct rates of inactivation by $H_2NPhAsO$ (k_1 rate being 6.1 times the faster. This finding and recent observations that at least two lipoyl residues exist on each chain of E2 (Danson & Perham, 1976; Bates et al., 1977) suggests that the lipoic acid residues may have distinctly different rates of reaction with an arsenoxide. It is also possible that the initial rapid rate of inactivation is caused by the direct reaction of the arsenoxide with one residue of reduced lipoic acid and that the subsequent slower rate is a result of a transfer of the arsenoxide to a second residue of lipoic acid on the polypeptide chain of E2. Such a process is in keeping with "transacetylation" proposed by Bates et al. (1977).

Control experiments containing PD complex and $H_2NPhAsO$ but lacking either pyruvate, coenzyme A, or both did not show inhibition of the complex since loss of complex activity was no greater than when the PD complex was incubated with buffer alone. The observed loss of E3 activity under the conditions of the experiment shown in Figure 2 is no different from the loss of E3 activity observed in the absence of pyruvate, coenzyme A, or $H_2NPhAsO$.

The efficient reactivation of the PD complex (Figure 2) upon addition of 2,3-dithiopropanol supports the suggestion that the site of modification by $H_2NPhAsO$ was the reduced lipoic acid groups on E2. Similar observations have been obtained in earlier studies of the inhibition by arsenoxides or dichloroarsines (Reiss, 1958; Reiss & Hellerman, 1958; Stocken & Thompson, 1949) of the pyruvate oxidation activity in brain extracts. 2,3-Dithiopropanol competes favorably for the arsenical bound to E2 since it forms a more stable five-membered cyclic dithiolarsinite with $H_2NPhAsO$ than the existing six-membered ring derivative between the arsenoxide and reduced lipoic acid (Whittaker, 1947; Stocken & Thompson, 1949). Monothiols, such as mercaptoethanol, are much inferior to reagents with vicinal dithiols even when compared at twice the concentration (Whittaker, 1947).

The activity of E3 in the PD complex inhibited with $H_2NPhAsO$ remains unchanged following the addition of 2,3-dithiopropanol (Figure 2). This supports the view that the arsenoxide specifically reacted with E2 under the conditions of the experiment. The recovery of only about 80% of the PD complex activity does not reflect the incomplete reactivation by 2,3-dithiopropanol but rather suggests that spontaneous oxidation of E3 limits the attainment of full complex activity.

The central role of E2, both structurally and mechanistically, in the PD complex (Figure 1) (Reed, 1974), necessitates the contact of the flexible lysinyl–lipoyl arms of E2 with the active sites of E1 and E3. The inactivation of the PD complex with the bifunctional arsenical $BrCH_2CONHPhAsO$ (100 μM) in the presence of pyruvate and coenzyme A is shown in Figure 3. A very rapid loss of complex activity to 50% was again observed within 1 min followed by a more gradual loss of all activity within 15 min. The first phase of the inhibition was attributed to the rapid formation of the cyclic derivative between reduced lipoic acid on E2 and the -AsO moiety of the bifunctional reagent. Significantly, the inhibition of E3 followed the loss of complex activity by a few minutes and led to a 92% reduction of the E3 activity within 25 min. This inactivation has been attributed most simply to the irreversible alkylation of a residue at or near the active site of E3 by the bromoacetylamido moiety ($BrCH_2CONH$-) of the bifunctional reagent. At this stage it is likely that E2 and E3 are cross-linked, as might be expected from the "swinging-arm hypothesis" (Green & Oda, 1961; Koike et al., 1963). The addition of 2,3-dithiopropanol to the E2-E3 cross-linked PD complex would regenerate reduced lipoic acid on E2 but would

have no effect, however, on the irreversible alkylation most likely occurring on E3. Thus, no regeneration of complex activity occurs since E3 remains inhibited.

Although two chemically reactive groups are present on the bifunctional reagent, the formation of the cyclic dithiolarsinite between the -AsO moiety and the reduced lipoic acid on E2 proceeds at the greatest rate. The alkylation of E3 is, in effect, an active-site directed irreversible modification since the flexible lysinyl-lipoyl arm can deliver the bromoacetylamido group into the active site of E3 in a manner suggested by the proposed mechanism of the PD complex (Figure 1). Support for the initial reaction of the bifunctional reagent occurring via the arsenoxide group was provided by incubating the PD complex with ʼromoacetylaniline ($BrCH_2CONHPh$, 115 μM) in the presence of pyruvate and coenzyme A. A loss of only 10% of the complex activity and about 3% of the E3 activity was observed over a 30-min period at 0 °C. Over the same period the bifunctional reagent inhibited all complex activity and about 90% of the E3 activity. Clearly, the "anchoring" of the bifunctional reagent to the reduced lipoic acid was the crucial step in leading to the inactivation of E3.

The site of alkylation on E3 has not been determined but a cysteine residue is suggested by studies employing *N*-ethylmaleimide (Brown & Perham, 1974, 1976). Considering the structure of the bifunctional reagent, a carboxymethylated amino acid will be released from the inhibited complex following acid hydrolysis. Work is in progress to elucidate and quantitate the alkylation occurring with E3 and to confirm the cross-linking of E2-E3 (S. R. Adamson & K. J. Stevenson, unpublished data).

Enzymes possessing closely associated cysteine residues within their active sites are susceptible to inhibition by arsenoxides, although the rate of inhibition is not rapid nor seldom complete (Webb, 1966). The application of arsenoxides to the study of selected "thiol" enzymes offers an interesting approach to the study of their structure and function.

Acknowledgments

The authors wish to thank Sir Rudolph Peters and Dr. H. B. F. Dixon for helpful discussions and Mr. A. R. Whittaker, Microbiological Research Establishment, Porton Down, for growing the bacteria.

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