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Inhibition of Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli* with a Radiolabeled Bifunctional Arsenoxide: Evidence for an Essential Histidine Residue at the Active Site of Lipoamide Dehydrogenase†

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ABSTRACT: Incubation of pyruvate dehydrogenase multienzyme complex (PD complex) from *Escherichia coli* with thiamin pyrophosphate, pyruvate, coenzyme A, Mg^{2+}, and the radiolabeled bifunctional arsenoxide *p*-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2{}^{14}CONHPhAsO$) led to the irreversible loss of lipoamide dehydrogenase (E3) activity. The mode of inactivation occurred by initial "anchoring" of the reagent via its –AsO group to reduced lipoyl residues on lipoate acetyltransferase (E2) (generated by substrates) followed by the delivery of the $BrCH_2{}^{14}CO$– moiety into the active site of E3 where an irreversible alkylation ensued [Stevenson, K. J., Hale, G., & Perham, R. N. (1978) *Biochemistry 17*, 2189]. To account for nonspecific alkylations, not mediated by this delivery process, control experiments were conducted in which the radiolabeled bifunctional reagent was incubated with PD complex in the absence of substrates. E3 subunits were isolated from inhibited and control PD complexes by chromatography on hydroxylapatite in the presence of 8 M urea. Acid hydrolysis of the alkylated E3 and control E3 samples produced radiolabeled carboxymethylated amino acids that were identified and quantitated by high-voltage electrophoresis and amino acid/radiochemical analysis. The inhibited sample contained N^3-(carboxymethyl)histidine and a small amount of *S*-(carboxymethyl)cysteine. These residues were not present in significant amounts in the controls. The loss of 81% of E3 activity correlated with the alkylation of about 0.7 residue of histidine and 0.1 residue of cysteine per mol of E3.

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

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

The complex consists of three different enzymes, which in order of partipication are pyruvate dehydrogenase (lipoate) (E1) (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). A total of 24 apparently identical E2 chains form the structural core of the complex (Reed & Oliver, 1968; Reed, 1974). Evidence suggests that two lipoyl residues are present per polypeptide chain of E2 (Danson & Perham, 1976; Brown & Perham, 1976; Speckhard et al., 1977; Collins & Reed, 1977; Bates et al., 1977; White

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[1] Abbreviations: PD complex, pyruvate dehydrogenase multienzyme complex; TPP, thiamin pyrophosphate; HVE, high-voltage electrophoresis; $BrCH_2{}^{14}CONHPhAsO$, *p*-[(bromo[^{14}C]acetyl)amino]phenyl arsenoxide; 1-CM-His, N^1-(carboxymethyl)histidine; 3-CM-His, N^3-(carboxymethyl)histidine; 1,3-CM-His, N^1,N^3-bis(carboxymethyl)-histidine; N^ϵ-CM-Lys, N^ϵ-(carboxymethyl)lysine; S-CM-Cys, *S*-(carboxymethyl)cysteine; S-CM-Homocys, *S*-(carboxymethyl)homocysteine; $NaDodSO_4$, sodium dodecyl sulfate; BAL, 2,3-dithiopropanol (British Anti-Lewisite); NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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et al., 1980), only one of which is essential for PD complex activity (Frey et al., 1978; Bleile et al., 1979; Ambrose-Griffin et al., 1980; Danson et al., 1981). The mechanism by which one lipoyllysine "swinging arm" can span the distances between active sites in the *E. coli* PD complex has been clarified by limited proteolysis (Bleile et al., 1979; Hale & Perham, 1979) and proton NMR spectroscopy experiments (Perham et al., 1981). These authors showed that lipoyllysine residues on E2 are present on a flexible extension of polypeptide chain with mobility enough to increase the potential distance (2.8 nm) that can be spanned by a single lipoyllysine residue.

The interactions of mono- and bifunctional organoarsenoxides with PD complex from *E. coli* have been investigated (Stevenson et al., 1978; Adamson & Stevenson, 1981). The bifunctional reagent *p*-[(bromoacetyl)amino]phenyl arsenoxide ($BrCH_2CONHPhAsO$) in the presence of substrates (pyruvate, coenzyme A, TPP, and Mg^{2+}) irreversibly inhibits PD complex activity and the component activity of E3. The reagent initially forms a stable dithioarsinite complex with reduced lipoyl residues (the substrate of E3) on E2. The subsequent loss of E3 activity is consistent with the delivery of the anchored reagent into or near the active site of E3 where an irreversible alkylation takes place (Stevenson et al., 1978). This paper provides evidence for the alkylation of a histidine residue at or near the active site of E3 by the bifunctional radiolabeled reagent $BrCH_2{}^{14}CONHPhAsO$. This residue may be the putative base implicated in the mechanism of E3 (Matthews & Williams, 1976; Matthews et al., 1977). Some of the data in this paper have been published in a shortened format (Adamson & Stevenson, 1982).

Experimental Procedures

Materials

Homocysteine, N^{α}-acetyllysine, N^1- and N^3-(carboxymethyl)histidine, N^1,N^3-bis(carboxymethyl)histidine, and *S*-(carboxymethyl)cysteine were obtained from Calbiochem. Iodoacetic acid and 2,3-dithiopropanol were obtained from Sigma.

Methods

Syntheses. (*A*) *Dihydrolipoamide.* The synthesis of dihydrolipoamide was as described by Reed et al. (1958).

(*B*) N^{ϵ}-*(Carboxymethyl)lysine.* N^{α}-Acetyllysine (32.3 mg, 0.170 mmol) in 3 mL of 1 mM NaOH was added to iodoacetamide (74.7 mg, 0.403 mmol) in 0.5 mL of ethanol. The pH was adjusted to 10.5, and the mixture was incubated at 37 °C for 40 h. The solution was taken to dryness in vacuo and was hydrolyzed in 6 M HCl at 110 °C for 20 h. The HCl was removed in vacuo, and the brownish hydrolysate was dissolved in 2 mL of deionized water. Aliquots were applied to Whatman 3MM paper as 40-cm bands and subjected to HVE at pH 2.0, 3 kV for 40 min. Side strips stained with cadmium–ninhydrin solution indicated the position of N^{ϵ}-(carboxymethyl)lysine between alanine and valine. The remaining portion of the band was eluted with deionized water and lyophilized.

(*C*) *S-(Carboxymethyl)homocysteine.* Homocysteine (30 mg, 0.22 mmol) in 8 mL of deionized water was added to iodoacetic acid (40 mg, 4.21 mmol) in 0.8 mL of 2-propanol. The pH was adjusted to 8 with NaOH, and the solution was gassed thoroughly with N_2, covered, and incubated with stirring in the dark for 4 h. The solution was then frozen and lyophilized. The resulting brownish powder was dissolved in deionized water and applied to Whatman 3MM paper as 40-cm bands and subjected to HVE at pH 6.5, 3 kV for 1 h. Side strips stained with cadmium–ninhydrin solution indicated the position of *S*-(carboxymethyl)homocysteine just behind *S*-(carboxymethyl)cysteine. The remaining portion of the band was eluted and lyophilized.

(*D*) *p-[(Bromo[*^{14}*C]acetyl)amino]phenyl Arsenoxide.* Bromo[1-^{14}C]acetic acid (0.6 mg, 4.30 μmol, sp act. 57.0 mCi/mmol) in dichloromethane was added to cold recrystallized bromoacetic acid (10.0 mg, 0.072 mmol) dissolved in dichloromethane. This solution, in turn, was added to dicyclohexylcarbodiimide (16.5 mg, 0.083 mmol) also dissolved in dichloromethane (0.5 mL). The mixture was stirred for 5 min to promote the formation and precipitation of cyclohexylurea. The cyclohexylurea was removed by filtration through a glass wool plug in a Pasteur pipet. The filtrate containing bromoacetic anhydride was added to aminophenyl arsenoxide (7.5 mg, 0.041 mmol) dissolved in 20 mL of anhydrous acetone, and the mixture was stirred for 30 min. Unlabeled bromoacetyl bromide was added (5 μL, 11.5 mg, 0.052 mmol) and the mixture stirred for a further 15 min. Dilute ammonia (10%) was added, and the precipitate that formed was removed by filtration. The solvent was removed from the filtrate by flash evaporation, and the white solid obtained was dried under vacuum. The yield from the synthesis was 68%. The compound decomposed at 196 °C.

Specific Activity Determination of p-[(Bromo[^{14}*C]-acetyl)amino]phenyl Arsenoxide.* The specific activity of $BrCH_2{}^{14}CONHPhAsO$ was determined by liquid scintillation counting of known amounts of *S*-([^{14}C]carboxymethyl)cysteine formed from reaction of L-cysteine with $BrCH_2{}^{14}CONHPhAsO$. L-Cysteine hydrochloride (7 mg, 40 nmol) was dissolved in 200 μL of 95% ethanol by heating at 50 °C for 5 min. The solution was transferred to a hydrolysis tube containing 20 μL of a 10 mM stock solution of $BrCH_2{}^{14}CONHPhAsO$ in 95% ethanol (20 nmol). The solution was bubbled with N_2, made weakly alkaline with NaOH, covered with Parafilm, and allowed to stand at room temperature for 1 h. The solution was then dried in vacuo and hydrolyzed in 6 M HCl at 110 °C for 26 h. HCl was removed in vacuo, and the hydrolysate was dissolved in 0.2 N sodium citrate buffer, pH 3.2, prior to amino acid and radiochemical analysis. The radioactivity of *S*-(carboxymethyl)cysteine so formed was determined by liquid scintillation counting of the peak corresponding to *S*-(carboxymethyl)cysteine (elution time ~23 min; elution time of Asp ~26 min). The amount of *S*-(carboxymethyl)cysteine in the peak was determined from integration of the peak area. To determine the color constant of *S*-(carboxymethyl)cysteine, equimolar amounts of pure *S*-(carboxymethyl)cysteine and aspartic acid were carefully weighed out and dissolved in citrate buffer, pH 3.2. Approximately 200 pmol of both amino acids was applied to the column, and the color constant of *S*-(carboxymethyl)cysteine was calculated as 88% relative to aspartic acid (three determinations). The specific radioactivity of $BrCH_2{}^{14}CONHPhAsO$ determined in this manner was 8165 dpm/nmol. Counting efficiencies were calculated by employing the channel ratio technique.

High-Voltage Electrophoresis of E3 Hydrolysates. High-voltage electrophoresis (HVE) of acid hydrolysates utilized the vertical strip high-voltage apparatus similar to that described by Michl (1951) as modified by Ryle et al. (1955). The buffer systems and coolants were as described by Smillie & Hartley (1966). Amino acids were detected by staining the ionograms with cadmium–ninhydrin dip reagent (Heilman et al., 1957) and were developed for 20 min at approximately 60 °C. Acid hydrolysates of purified, radiolabeled E3, in 0.2 N sodium citrate buffer, pH 3.2, were applied to Whatman

3MM paper at the same spot on the origin as the carboxymethylated standards: N^{ϵ}-(carboxymethyl)lysine, N^1-(carboxymethyl)histidine, N^3-(carboxymethyl)histidine, N^1,N^3-bis(carboxymethyl)histidine, *S*-(carboxymethyl)homocysteine, and *S*-(carboxymethyl)cysteine. The presence of salt from the sodium citrate buffer retarded the acidic residues but did not interfere with resolution of amino acids or the carboxymethylated standards. After electrophoresis, ionograms were stained with cadmium–ninhydrin, the location of the standards was noted, and the band was cut into 0.5-in. sections. The sections were counted for radioactivity in 10 mL of Aquasol 2 on an LKB 1215 Rackbeta liquid scintillation counter.

Amino Acid Analysis. The determination of the amino acid composition of unmodified E3 was performed (after strong acid hydrolysis for 24, 42, and 72 h) on a Beckman 121 automatic amino acid analyzer by the method of Spackman et al. (1958). The determination of cystine and cysteine as cysteic acid and of methionine as methionine sulfone was performed essentially by the procedure of Hirs (1956). Cysteic acid values were corrected for 90% recovery (Hirs, 1967).

Amino Acid and Radiochemical Analysis of E3. Samples of E3 were hydrolyzed in 6 M HCl in evacuated, sealed, glass tubes for 24 h at 110 °C. At the completion of hydrolysis, HCl was removed in vacuo. The desiccated hydrolysates were dissolved in citrate buffer, pH 3.2, and run on a Beckman 121 M amino acid analyzer essentially as described by Schultz et al. (1981). The elution profile was monitored by a Spectra Physics SP4100 integrator/printer/plotter programmed to advance a fraction collector at the beginning and end of each peak and between fused peaks. The fraction collector was advanced after a calibrated time delay so as to coordinate peak collection with detection. Fractions were subjected to liquid scintillation counting in scintillation cocktail containing 0.4% Omnifluor, 80% toluene (scintillant grade), 15% Beckman Biosolve, and 5% water. Determinations of cpm were made on a Beckman LS-250 liquid scintillation counter and corrected for counting efficiencies of 74%. The amounts of protein under analysis were determined by quantitating tyrosine and phenylalanine, both of which were consistently on-scale.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ was carried out as described by McKay & Stevenson (1979).

Enzyme Assays. The activity of PD complex was assayed by the method of Reed & Mukharjee (1969) as described by Danson & Perham (1976) at 25 °C. E3 was assayed essentially by the method of Reed & Mukharjee (1969) by measuring the formation of NADH spectrophotometrically at 340 nm from NAD^+ (2.5 mM) and DL-dihydrolipoamide (0.3 mM) in potassium phosphate buffer (50 mM, pH 8.0) at 25 °C.

Enzyme Isolation. PD complex was purified from a constitutive mutant of *E. coli* K12 by the method of Reed & Mukharjee (1969) as described by Adamson & Stevenson (1981).

Inhibition of PD Complex by $BrCH_2{}^{14}CONHPhAsO$ in the Presence of TPP, Pyruvate, Coenzyme A, and Mg^{2+} (E1-Mediated Reduction System). PD complex (2 mL, ~10 mg/mL) in 20 mM potassium phosphate buffer, pH 7, containing 2 mM EDTA and 0.02% sodium azide was flushed with nitrogen. Bifunctional reagent was added from a stock solution in 95% ethanol to 0.1 mM. Substrates were added from a stock solution in deionized water to a final concentration of 0.5 mM TPP, 2 mM pyruvate, 0.13 mM coenzyme A, and 10 mM $MgCl_2$. A further addition of substrates was made where indicated on Figure 1 (X). The solution was flushed with nitrogen at regular intervals, covered with Parafilm, and incubated at 0 °C. Control A was treated identically except that equal volumes of water were added instead of substrate stock solution. 2,3-Dithiopropanol (BAL) was added from a stock solution in 95% ethanol to give an 8–10-fold excess over reagent. In control B, the bifunctional reagent was added to 0.05 mM at the outset of the experiment, and after 40 min, another addition made the final reagent concentration 0.1 mM. BAL was added at 200 min. Both sample and controls were dialyzed overnight against two changes (2 L) of 20 mM sodium phosphate buffer, pH 7 at 4 °C.

Isolation of E3 from Inhibited PD Complex and Control. The sample and control A were both made 10 μg/mL with trypsin and incubated at room temperature for 75 min to degrade the E2 subunit. Lima bean trypsin inhibitor was added (30 μg/mL), and both solutions were incubated for a further 5 min. These solutions along with control B were made 8 M in urea, stirred at 25 °C for 1 h, and applied to a hydroxylapatite column that had been preequilibrated with 10 mM sodium phosphate buffer, pH 7, containing 8 M urea. The columns were washed with 50, 100, and 500 mM sodium phosphate buffers, pH 7, containing 8 M urea. In the case of control B, a 75 mM buffer was also employed. The columns were washed with the buffer employed at each step until the absorbance at 280 nm was near zero. Fraction volumes were approximately 1.4 mL, and the initial flow rate was 2.8 mL/h. The flow rate decreased at higher concentrations. E3 was eluted in the 500 mM buffer in both sample and control. Fractions containing pure E3 were pooled, dialyzed exhaustively against deionized water, and lyophilized. Samples of E3 used for amino acid composition determinations were isolated from PD complex, which had not been subjected to trypsin digestion, essentially as described except that E3 was eluted in 300 mM sodium phosphate buffer, pH 7, containing 8 M urea.

Results

Purity of Isolated E3. The amino acid composition of E3 isolated from PD complex by adsorption chromatography on hydroxylapatite in the presence of 8 M urea shows good agreement with that of other authors (Williams, 1976), although the values of lysine are lower. This may be due to the carbamylation of lysine side chains by cyanate ion formed from urea (Stark, 1967). The quantities of leucine and isoleucine are also lower than expected. Isolated E3 produced a single sharp protein band on 7.5% polyacrylamide gels in the presence of $NaDodSO_4$.

Inhibition of E3 by $BrCH_2{}^{14}CONHPhAsO$. The irreversible inhibition of E3 in the PD complex by $BrCH_2{}^{14}CONHPhAsO$ in the presence of TPP, pyruvate, coenzyme A, and Mg^{2+} is shown in Figure 1. The inhibition was terminated by the addition of 2,3-dithiopropanol (Stevenson et al., 1978), which caused a reactivation of PD complex to about 22% of its original activity. E3 activity remained unchanged at near 19% of its original activity. The controls contained the same final concentration of reagent but no substrates were present. Both samples and controls were dialyzed overnight to remove unreacted reagent.

Isolation of E3 from Inhibited Sample and Controls. In a preliminary experiment, E3 was isolated from an inhibited sample of PD complex and its respective control (control B in Figure 1). These samples were pretreated with 8 M urea at room temperature followed by chromatography on hydroxylapatite, and in both cases E3 was eluted in 500 mM phosphate buffer containing 8 M urea. $NaDodSO_4$–polyacrylamide gel electrophoresis revealed that the control eluate

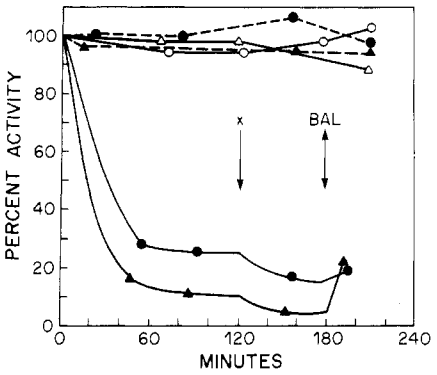

FIGURE 1: Inactivation of PD complex (20 mg) with $BrCH_2{}^{14}CONHPhAsO$ (0.1 mM) in 2 mL of 20 mM potassium phosphate buffer, pH 7, containing 2 mM EDTA, 0.02% sodium azide, 0.5 mM TPP, 2 mM pyruvate, 0.13 mM coenzyme A, and 10 mM $MgCl_2$ at 0–4 °C. PD complex activity in the inhibited sample, control A, and control B are denoted by (▲—▲), (△), and (▲--▲), respectively. E3 activity in the inhibited sample, control A, and control B are denoted by (●—●), (○), and (●--●), respectively. 2,3-Dithiopropanol (BAL, 0.8–1 mM) was introduced at the point shown by the arrow, and a further addition of substrates was made where indicated (X).

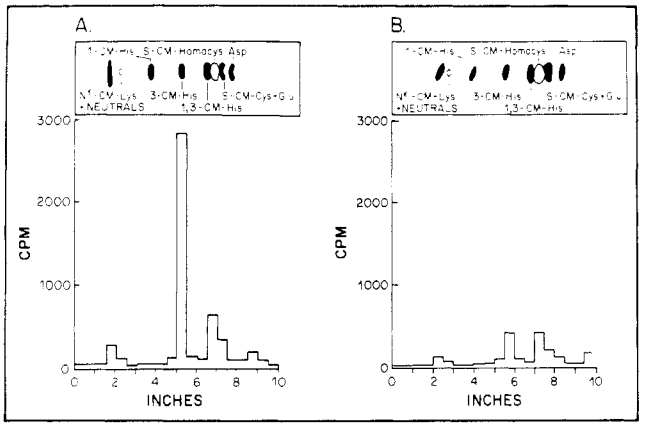

FIGURE 2: High-voltage electrophoresis of hydrolysates of inhibited and control E3 (control A) at pH 6.5. (A) Ionogram of inhibited E3 (inhibition shown in Figure 1). (B) Ionogram of E3 control A (Figure 1). See footnote 1 and Experimental Procedures.

contained pure E3 whereas in the inhibited sample a considerable amount of E2 coeluted with E3. In a subsequent experiment (inhibition data shown in Figure 1, sample and control A), trypsin digestion (1 h, 25 °C) of control and inhibited samples fragmented E2 and resulted in the elution of pure E3 from both sample and control with 500 mM phosphate buffer containing 8 M urea. The isolation procedure for inhibited E3 and controls A and B is given under Experimental Procedures. The yield of alkylated E3 from the inhibited PD complex was about 58% of its respective control (A).

Identification of the Chemically Modified Residues of E3. High-voltage paper ionograms of the strong-acid hydrolysates of native (control A) and inhibited E3 are illustrated in Figure 2. The location of maximum radioactivity present in the hydrolysate of inhibited E3 coincided with the position of N^3-(carboxymethyl)histidine. Radioactivity, albeit in lower amounts, is also seen at three other positions on the ionogram: the neutral region, a region between N^1,N^3-bis(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine, and in advance of aspartic acid. In the acid hydrolysate of the control E3, the pattern of radioactivity is very similar to the inhibited E3 except for a notable decrease in the level of N^3-(carboxymethyl)histidine. These data indicate that the alkylation of a histidine residue at the nitrogen-3 position of the imidazole

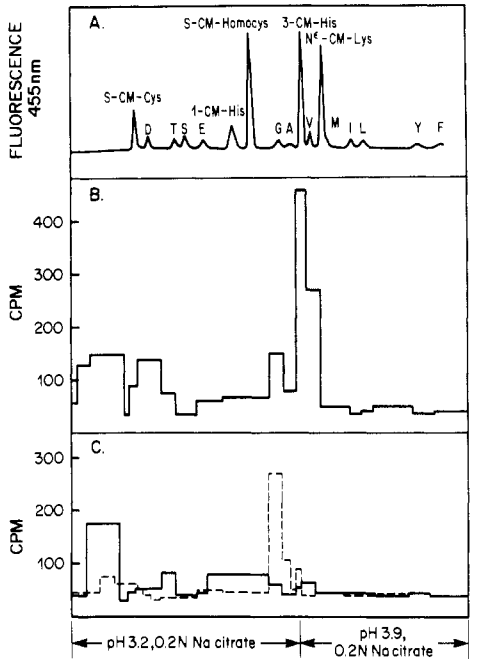

FIGURE 3: Amino acid and radiochemical analysis of inhibited and control samples of E3. (A) Elution profile of standard carboxymethylated and common amino acids (single letter code). (B) Occurrence of radioactivity in the elution profile of strong-acid hydrolysate of 0.19 nmol of inhibited E3 (inhibition shown in Figure 1). (C) Occurrence of radioactivity in the elution profiles of strong-acid hydrolysates of 0.13 nmol of E3 control A (—) and 0.35 nmol of control B (--) Figure 1).

ring is associated with the loss of biological activity.

Further evidence for the alkylation of a histidine residue was gained from quantitative amino acid analysis of inhibited and control E3 coupled with liquid scintillation counting of the eluents from the amino acid analyzer. The elution positions of a number of standard carboxymethylated amino acids and common amino acids (Figure 3A) are compared directly with the occurrence of radioactivity in the elution profile of acid hydrolysates of both inhibited and control E3 (panels B and C of Figure 3, respectively). Fractions corresponding to amino acid peaks were collected and subjected to liquid scintillation counting. The majority of radioactivity in the inhibited sample again was eluted at a position corresponding to N^3-(carboxymethyl)histidine. A significant amount of radioactivity eluted well in front of aspartic acid. This cannot be accounted for by the occurrence of radiolabeled *S*-(carboxymethyl)cysteine, which elutes just prior to aspartic acid. Furthermore, it is unlikely to be due to acidic decomposition products of methionine sulfonium salts since radiolabeled *S*-(carboxymethyl)homocysteine, which is diagnostic of methionine sulfonium salt formation, was not formed. The observed radioactivity may be due to the presence of glycolic acid resulting from the hydrolysis of a small amount of insoluble reagent—2,3-dithiopropanol adduct that is not removed by dialysis. Figure 3C shows the superimposed radiochemical analysis of the control samples A and B from both experiments in which E3 activities were unaffected. Unidentified ^{14}C-labeled derivatives observed eluting with glycine and ahead of aspartic acid were also seen in the inhibited sample; however, since no loss of E3 activity was apparent in the controls, the occurrence of these derivatives is unlikely to be associated with any loss of E3 activity in the inhibited sample. Significantly, radioactivity corresponding to N^3-(carboxymethyl)histidine and *S*-(carboxymethyl)cysteine was absent in the controls. These findings are in good agreement with the HVE data and in-

dicate that the alkylation of histidine and a small amount of cysteine results in the inactivation of E3 when PD complex is exposed to $BrCH_2{}^{14}CONHPhAsO$ in the E1-mediated lipoyl reduction system.

Quantitation of Alkylation of Inhibited E3 by $BrCH_2{}^{14}CONHPhAsO$. Although the major modification involved in E3 inactivation is associated with a histidine residue, the task remained to correlate the extent of chemical modification with loss of the biological activity. In order to estimate the degree of alkylation of E3, it was necessary to accurately determine the specific activity of the bifunctional reagent and to quantitate the amount of E3 under analysis (see Experimental Procedures). The results from duplicate amino acid and radiochemical analysis of inhibited E3 were 0.57 and 0.78 residue of N^3-(carboxymethyl)histidine. The content of N^3-(carboxymethyl)histidine in the inhibited E3 sample was calculated as 0.68 ± 0.15 residue/mol. Cysteine also appears to be modified in this experiment to the extent of 0.1 residue/mol of E3. This estimate must be regarded as an approximation since the amount of radioactivity in *S*-(carboxymethyl)cysteine is small relative to background radioactivity. Thus, the active site directed inactivation of E3 to 19% residual activity by $BrCH_2{}^{14}CONHPhAsO$ in the presence of TPP, pyruvate, coenzyme A, and Mg^{2+} is associated with the alkylation of about 0.7 residue of histidine (at the nitrogen-3 position) and about 0.1 residue of cysteine.

Discussion

The yield of E3 from PD complex inhibited with the bifunctional reagent was considerably lower than E3 from the control PD complex. As a result of the presence of pyruvate, TPP, and coenzyme A, when forming the inhibited PD complex, the redox disulfide of E3 was reduced. This was not the case in the control PD complex samples where substrates were not present. Studies by Massey (1960) have shown that reduction of the redox disulfide of E3 led to a decrease in E3 activity in the presence of high concentrations of urea. In addition, de Kok et al. (1981) have shown that oxidized E3 from *Azotobacter vinlandii* is stable to proteolytic degradation by trypsin whereas, following reduction of the redox disulfide by NADH, the enzyme rapidly lost activity when exposed to trypsin. The compact structure of the oxidized enzyme is apparently changed to a more open structure upon reduction of the redox disulfide.

The decreased yield of E3 from PD complex inhibited with the bifunctional reagent may well arise from the persistence of the reduced form of E3 up to the stages where digestion of the PD complex with trypsin and incubation with 8 M urea occur. These treatments could well lead to loss of intact E3 and account for lower yields of enzyme obtained from chromatography on hydroxylapatite. This suggestion has received support from recent experiments (C. F. B. Holmes, S. R. Adamson, and K. J. Stevenson, unpublished results) where E3 was isolated from two different samples of PD complex: one reduced by NADH and inhibited with $BrCH_2{}^{14}CONHPhAsO$; the other in which the S^6-acetyllipoyl moieties on E2 were blocked with *N*-ethylmaleimide (NEM) followed by treatment of the PD complex with NADH and $BrCH_2CONHPhAsO$. In the former sample, E3 was inhibited by active site directed alkylation (Adamson & Stevenson, 1981; S. R. Adamson, C. F. B. Holmes, and K. J. Stevenson, unpublished results); however, in the latter sample, blockage of the lipoyl residues by NEM prevented delivery of the reagent into the active site of E3, and no inhibition of E3 resulted. The yield of E3 in these cases was 75 and 65%, respectively, relative to that of E3 isolated from native PD complex. This suggests that chemical modification of E3 makes a relatively minor contribution to the decrease in yield of inhibited E3 and that the change in tertiary structure following reduction of the redox disulfide is the key reason for reduced yields.

To minimize nonspecific alkylations in these studies, the concentration ratio of reagent to protein was 50-fold less than that described by Stevenson et al. (1978); however, the same mechanism for the inhibition is envisaged, and loss of E3 activity correlated well with the alkylation of a histidine residue (and possibly a small amount of cysteine). In previous experiments, where relatively high concentration ratios of reagent to protein were employed, chemical cross-linking of the E2 core of PD complex took place (Adamson & Stevenson, 1981). This caused a marked alteration in the quaternary structure of PD complex in which both E1 and E3 subunits became dissociated. Labeling studies using the ^{14}C-labeled bifunctional reagent at similar reagent to protein concentration ratios as used in this paper have shown that cross-linking of the E2 core is minimized and labeling of E3 is quite selective (S. R. Adamson, C. F. B. Holmes, and K. J. Stevenson, unpublished results). It is likely that the quaternary structure of PD complex is less perturbed under the conditions described here than at higher ratios of reagent to protein.

Lipoamide dehydrogenase (E3) and glutathione reductase are mechanistically very similar as they catalyze electron transfer between a pyridine nucleotide and an active site disulfide and contain a putative base that plays a crucial role in catalysis (Williams, 1976; Matthews & Williams, 1976). The three-dimensional structure of glutathione reductase has been elucidated (Schulz et al., 1978), and a histidine side chain from residue 467 (Untucht-Grau et al., 1979; Pai & Schulz, 1983) is situated close to the active site disulfide and the disulfide of enzyme-bound glutathione. Chemical-modification studies using diethyl pyrocarbonate have implicated an essential histidine residue in glutathione reductase (Boggram & Mannervick, 1978). To date, no chemical evidence has been presented that identifies a histidine residue as an essential component of E3. The strong implication from our findings is that the alkylated histidine residue is the essential base in E3 catalysis.

Normally, haloacetates react with sulfhydryl groups of proteins considerably more rapidly than imidazole groups (Means & Feeney, 1971). Therefore, in a situation where thiol and imidazole side chains are present in the same environment (presumably the active site of E3), the former side chain, regardless of its ionization state, would be expected to be the more reactive entity toward electrophiles such as the α-carbon of $BrCH_2{}^{14}CONHPhAsO$. The finding that a histidine residue is almost uniquely modified suggests that the bromoacetyl moiety of the bifunctional reagent (bound to dihydrolipoyl residues on E2) selectively encounters the histidine residue rather than the cysteines of the reduced redox disulfide. In the proposed mechanism for E3 (Williams, 1976), the primary encounter of the dihydrolipoyl residue is with a base, to deprotonate the dihydrolipoyl residue so as to generate a thiolate anion that in turn can attack the redox disulfide of E3. The geometry of the active site of E3 may well position the histidine so as to make immediate encounter with dihydrolipoyl residues favorable. Since only the N^3-(carboxymethyl)histidine derivative was detected [i.e., N^1-(carboxymethyl)histidine was absent] in acid hydrolysates of inhibited E3, these results suggest that the putative essential histidine residue is oriented with its nitrogen-3 toward the incoming substrate. A recent high-resolution X-ray diffraction analysis of glutathione reductase (Pai & Schulz, 1983) has shown that the catalytic

histidine residue of this enzyme (His-467) is fixed by a strong hydrogen bond between the nitrogen-1 of histidine and a glutamic acid residue (Glu-472). Our results indicate that the same situation occurs in E3.

The redox state of E3 under the conditions of inhibition is unclear. At the outset of the experiments, the samples were apparently bleached by the addition of TPP, Mg^{2+}, pyruvate, and coenzyme A. However, as the experiment progressed, the samples became progressively more oxidized. Frey et al. (1978) have shown that in the presence of TPP, Mg^{2+}, pyruvate, and coenzyme A under anaerobic conditions, E3 is four-electron reduced (EH_4). Akiyama & Hammes (1981) have shown that under other than strictly anaerobic conditions the predominant form of E3 is two-electron reduced (EH_2). We must conclude that under the conditions of our experiments, during which strict anaerobicity was not adhered to, we have a mixture of EH_2 and EH_4. If the modified histidine residue is indeed the putative base proposed in the model of Matthews & Williams (1976), then in the EH_4 form of the enzyme (in which the redox disulfide is reduced and the base is unprotonated) it will be nucleophilic and therefore reactive toward the α-carbon of $BrCH_2{}^{14}CONHPhAsO$. The EH_2 form of pig heart lipoamide dehydrogenase has been shown to involve a thiolate anion base pair between a thiolate anion from the redox disulfide and the putative base ($-S^{-}\cdots{}^{+}HB-$) (Matthews & Williams, 1976). In this form, the base would be unreactive. However, in the *E. coli* enzyme (Wilkinson & Williams, 1979) EH_2 appears to be a mixture of three species at neutral pH, only one of which involves a thiolate anion base pair. The remaining two forms may contain an unprotonated base. Thus, the fact that the two-electron-reduced enzyme is present by no means precludes the modified histidine from being the putative base. Significantly, a C-terminal peptide containing the radiolabeled histidine has been isolated from modified E3 (C. F. B. Holmes and K. J. Stevenson, unpublished results). The sequence of this peptide is homologous with that around the active site histidine in glutathione reductase (Untucht-Grau et al., 1979) and pig heart lipoamide dehydrogenase (Williams et al., 1982).

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Registry No. PD, 9014-20-4; E3, 9001-18-7; L-His, 71-00-1; N^{ϵ}-CM-Lys, 5746-04-3; S-CM-Homocys, 7374-02-9; N^{α}-Ac-Lys, 1946-82-3; Homocys, 6027-13-0; $BrCH_2{}^{14}CONHPhAsO$, 88610-25-7; $BrCH_2{}^{14}COOH$, 4561-21-1.

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