Dihydrolipoyl Transacetylase of *Escherichia coli*. Formation of 8-*S*-Acetyldihydrolipoamide†

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ABSTRACT: The dihydrolipoyl transacetylase component (E_2) of the pyruvate dehydrogenase complex catalyzes the reaction of acetyl coenzyme A (acetyl-CoA) with dihydrolipoamide, producing coenzyme A and *S*-acetyldihydrolipoamide. The acetyl group is shown by experiments reported herein to be bonded to S^8 in the enzymatic product. ^1H NMR analysis of synthetic samples of both structural isomers of *S*-acetyl-*S*-(phenylmercurio)dihydrolipoamide enabled structural assignments to be made. Reaction of 8-*S*-acetyl-6-*S*-(phenylmercurio)dihydrolipoamide with 3-mercaptopropionic acid in chloroform produced 8-*S*-acetyldihydrolipoamide which contained a small amount (5%) of the 6-S isomer. Reaction of 6,8-di-*S*-acetyldihydrolipoamide with NH_2OH produced a 4:1 mixture of 6-*S*-acetyldihydrolipoamide and the 8-S isomer. These compounds did not isomerize at significant rates in chloroform but rapidly isomerized to the equilibrium mixture in aqueous solution (K_{eq} = 3.4). The second-order rate constants for the hydroxide-catalyzed isomerization were found to be $k_f = (1.15 \pm 0.07) \times 10^6$ $M^{-1}\cdot s^{-1}$ and $k_r = (3.36 \pm 0.20) \times 10^5$ $M^{-1}\cdot s^{-1}$ in the direction of the formation of the 8-S isomer. The enzymatic product was trapped by addition of phenylmercuric hydroxide within 15 s–30 min after starting the reaction. ^1H NMR analysis of the products obtained at various times showed that the enzymatic product was 8-*S*-acetyldihydrolipoamide, which underwent progressive isomerization to the mixture of isomers within a few minutes. In the reaction of acetyl-CoA with dihydrolipoamide, the latter substrate reacts in place of enzyme-bound dihydrolipoyl moieties. Therefore, acetylation occurs at the 8-S position of bound lipoyl groups.

The pyruvate dehydrogenase complex of *Escherichia coli* catalyzes the decarboxylation and dehydrogenation of pyruvate by the pathway:

$$H^+ + CH_3COCO_2^- + E_1\cdot TPP^1 \rightarrow E_1\text{–hydroxyethylidene-TPP} + CO_2 \quad (1)$$

$$E_1\cdot\text{hydroxyethylidene-TPP} + E_2\text{-Lip}S_2 \rightarrow E_1\cdot TPP + E_2\text{-Lip(SH)SCOCH}_3 \quad (2)$$

$$E_2\text{-Lip(SH)SCOCH}_3 + CoASH \rightarrow E_2\text{-Lip(SH)}_2 + CH_3COSCoA \quad (3)$$

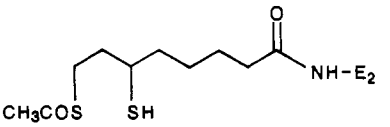
$$E_2\text{-Lip(SH)}_2 + E_3\cdot FAD \rightarrow E_2\text{-Lip}S_2 + \text{dihydro-}E_3\cdot FAD \quad (4)$$

$$\text{dihydro-}E_3\cdot FAD + NAD^+ \rightarrow E_3\cdot FAD + NADH + H^+ \quad (5)$$

$$\text{sum: } CH_3COCO_2^- + CoASH + NAD^+ \rightarrow CO_2 + CH_3COSCoA + NADH \quad (6)$$

The complex is composed of the following three enzymes: pyruvate dehydrogenase (E_1), the TPP-dependent component; dihydrolipoyl transacetylase (E_2), which contains covalently bound lipoic acid; and dihydrolipoyl dehydrogenase (E_3), a flavoprotein with noncovalently bound FAD. Equations 1–5 show that each enzyme catalyzes specific steps of the overall reaction and that E_2 serves also as the coupling factor for electron transfer from E_1·hydroxyethylidene-TPP to E_3·FAD and for acetyl group transfer from the same complex to CoASH.

A key intermediate in acetyl group transfer is the *S*-acetyldihydrolipoyl group in E_2-Lip(SH)$SCOCH_3$, which is produced in eq 2 and donates its acetyl group to CoASH in eq 3. This intermediate can in principle exist in two isomeric forms, as 8-*S*-acetyldihydrolipoyl-E_2 (**1**) or 6-*S*-acetyldihydrolipoyl-E_2 (**2**).



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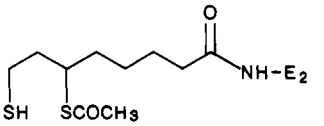

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It is impractical for a variety of reasons to determine whether enzyme-bound dihydrolipoyl moieties are acetylated at the 6- or 8-positions; however, the E_2 component of the complex catalyzes the direct transfer of the acetyl group from acetyl-CoA to free dihydrolipoamide (DHLP) according to eq 7. This reaction almost certainly models acetyl group

$$CH_3COSCoA + DHLP \rightarrow \text{acetyl-DHLP} + CoASH \quad (7)$$

transfer to enzyme-bound dihydrolipoyl moieties, and the

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[1] Abbreviations: TPP, thiamin pyrophosphate; HETPP, 2′-(1-hydroxyethylidene)thiamin pyrophosphate; FAD, flavin adenine dinucleotide; CoASH, coenzyme A; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; NEM, *N*-ethylmaleimide; Tris, tris(hydroxymethyl)aminomethane; PIPES, 1,4-piperazinebis(2-ethanesulfonic acid); DHLP, *dl*-dihydrolipoamide; Ac_2DHLP, diacetyldihydrolipoamide; 6-AcDHLP, 6-*S*-acetyldihydrolipoamide; 8-AcDHLP, 8-*S*-acetyldihydrolipoamide; 6-AcPMDHLP, 6-*S*-acetyl-8-*S*-(phenylmercurio)dihydrolipoamide; 8-AcPMDHLP, 8-*S*-acetyl-6-*S*-(phenylmercurio)dihydrolipoamide.

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structure of free acetyl-DHLP is much more easily determined than that of the enzyme-bound species.

It has long been thought, on the basis of the observations of Gunsalus et al. (1956), that the product of reaction 7 is 6-AcDHLP. However, O'Connor et al. (1982) have recently shown that both 6-AcDHLP and 8-AcDHLP are produced and that the two are interconverted on a very fast time scale.

In this paper we show that E_2 produces initially 8-AcDHLP and that this compound spontaneously isomerizes to the equilibrium mixture of 6-AcDHLP and 8-AcDHLP. The K_{eq} and rate constants for this process are also reported.

Materials and Methods

Enzymes. Phosphotransacetylase was purchased from Sigma. The pyruvate dehydrogenase complex was purified by the method of Reed and Mukherjee (1969) as modified and extended by Speckhard and Frey (1975) to include chromatography through a column of calcium phosphate gel–cellulose. The enzyme was stored at −70 °C after freezing in liquid N_2 at a concentration of about 20 mg/mL. The lipoyl moieties of the pyruvate dehydrogenase complex were reduced and alkylated (Danson et al., 1981) by incubation of 2–2.5 mg/mL of complex with TPP (0.2 mM), NADH (0.7 mM), NAD^+ (2 mM), and NEM (1 mM) in potassium phosphate buffer (0.1 M, pH 7) at room temperature. The pyruvate dehydrogenase complex activity (eq 6) after 10 min of incubation was about 2% of the initial activity. The dihydrolipoyl transacetylase activity (eq 7) was unaffected by alkylation. The NEM-alkylated enzyme was then dialyzed against the same buffer used for the subsequent enzymatic reaction. The E_1E_2 subcomplex derived from the pyruvate dehydrogenase complex was eluted from a calcium phosphate gel–cellulose column by 8% $(NH_4)_2SO_4$ after E_1 had been eluted by ethanolamine–phosphate buffer at pH 9.5 as described by Reed and Willms (1966). The solution containing E_2E_3 subcomplex was dialyzed against 0.1 M PIPES buffer at pH 6.5 and concentrated by ultrafiltration.

Assays. Pyruvate dehydrogenase complex was assayed according to the procedure of Maldonado et al. (1972). Dihydrolipoyl transacetylase was assayed as described by Reed and Willms (1966).

Substrates, Cofactors, and Chemicals. Thioctic acid amide (lipoamide), coenzyme A, TPP, NAD^+, NADH, sodium pyruvate, and acetyl phosphate were obtained from Sigma. Dihydrolipoamide was synthesized from lipoamide as described by Reed et al. (1958). All other chemicals were purchased commercially and used as supplied, with the exception of triethylamine, chloroform, and acetic anhydride, which were redistilled before use.

Spectroscopy. UV–vis spectroscopy was performed with a Cary 118c spectrophotometer. NMR spectra were obtained with a Brucker WH 270-MHz spectrometer, field frequency locked on the deuterium resonance of 99.8 atom % $CDCl_3$. Chemical shifts were referenced to 0.03% tetramethylsilane added as an internal standard.

Phenylmercuric Hydroxide Solution. Phenylmercuric hydroxide was used for synthesizing compounds prepared as NMR standards and for trapping acetyldihydrolipoamide in enzymatic reactions. The reagent solution was prepared as follows. Phenylmercuric hydroxide was partially dissolved in 95% ethanol (3–4.5 mg/mL). An equal volume of potassium phosphate buffer (0.1 M, pH 7) was added slowly with vigorous stirring until the solution became clear.

Synthesis of 6,8-Di-S-acetyldihydrolipoamide (Ac_2DHLP). DHLP (9.6 mmol) was partially dissolved in 25 mL of $CHCl_3$, and triethylamine (38 mmol, 5.1 mL) and acetic anhydride (38 mmol, 3.4 mL) were then added with stirring. The solution immediately became clear and was stirred at 0 °C for 20 min. The mixture was extracted with 20 mL of 0.1 M HCl 5 times and with 30 mL of water twice to remove triethylamine and acetic anhydride. The $CHCl_3$ was removed by rotary evaporation to give a quantitative yield of crude Ac_2DHLP. The crude product was purified by flash chromatography according to the procedure described by Still et al. (1978). A dry-packed column of silica gel (2 cm × 14 cm) was equilibrated with ethanol–$CHCl_3$ (1:15) at a flow rate of 16 mL/min regulated by the application of pressure. The crude product (200 mg) dissolved in the same solvent was chromatographed through the column at a pressure-regulated flow rate of 16 mL/min. The purified product was detected in the column effluent by thin-layer chromatography and detection with I_2 vapor. Solvent was removed from the purified product by rotary evaporation. The yield was 90%. ^1H NMR (chemical shift in ppm, multiplicity, integration, assignment, coupling constant if available) 1.3–2.0 (m, 8 H, C3, C4, C5, and C7), 2.21 (t, 2 H, C2, $J_{2,3}$ = 7.3 Hz), 2.33 (s, 6 H, acetyl), 2.91 (m, 2 H, C8), 3.56 (m, 1 H, C6), 5.5 (s, 2 H, amide).

Synthesis of 8-S-Acetyl-6-S-(phenylmercurio)dihydrolipoamide (8-AcPMDHLP). Ethanolic solutions of DHLP (2.6 mmol in 10 mL of 95% ethanol) and KOH (2.6 mmol in 5 mL of 95% ethanol) were combined and stirred at room temperature for 3 min. Acetic anhydride (2.6 mmol, 0.25 mL) was added and the mixture stirred for 3 min at room temperature. Phenylmercuric hydroxide (2.6 mmol in 170 mL of ethanol–phosphate buffer) was then added, resulting in the production of 6-AcPMDHLP, Ac_2DHLP, and 8-AcPMDHLP in the ratio 0.3:1:1, respectively, as determined by ^1H NMR. The product mixture was filtered to remove a precipitate, and the filtrate was concentrated by rotary evaporation until a new precipitate appeared. This was collected by filtration and consisted of a 1:10 mixture of 6-AcPMDHLP and 8-AcPMDHLP as determined by ^1H NMR, while Ac_2DHLP remained in solution. The AcPMDHLP mixture was purified by flash chromatography through a silica gel column as described above for Ac_2DHLP. Solvent was removed from the eluted product by rotary evaporation, and the residue was dried in vacuo over P_2O_5. The product was 8-AcPMDHLP as determined by ^1H NMR. The yield was 20%, based on DHLP. ^1H NMR (chemical shift in ppm, multiplicity, integration, assignment, coupling constant if available) 1.5–2.1 (m, 8 H, C3, C4, C5, and C7), 2.24 (t, 2 H, C2, $J_{2,3}$ = 7.3 Hz), 2.31 (s, 3 H, acetyl), 3.19 (m, 2 H, C8), 3.47 (m, 1 H, C6), 5.5 (2 H, amide), 7.4 (m, 5 H, phenyl).

Synthesis of 80% 6-S-Acetyl-8-S-(phenylmercurio)dihydrolipoamide (6-AcPMDHLP). Ethanolic solutions of Ac_2DHLP (0.75 mmol in 5 mL of 95% ethanol + 5 mL of 0.1 M potassium phosphate buffer, pH 7) and hydroxylamine (1.1 mmol in 4 mL of the same ethanol–phosphate buffer) were mixed and stirred at room temperature for 20 min. Phenylmercuric hydroxide solution (1.1 mmol in 80 mL of ethanol–phosphate buffer) was added. The precipitate was removed by filtration, and the filtrate was concentrated by rotary evaporation until a second precipitate was deposited on the wall of the flask. The remaining solution was decanted and the viscous residue dissolved in $CHCl_3$. The $CHCl_3$-insoluble material was removed by low-speed centrifugation. Chloroform was removed from the decantate by rotary evaporation, leaving a mixture of 6- and 8-AcPMDHLP in a ratio of 3:2. The mixture was dissolved in 1 mL of 95% ethanol, and 1 mL of water was then added. The resulting precipitate was collected by centrifugation and dissolved in 2 mL of 95% ethanol.

After centrifugation to remove insoluble material the solvent was removed by rotary evaporation. The residue was further dried in vacuo over P_2O_5. The product consisted of a 4:1 mixture of 6-AcPMDHLP and 8-AcPMDHLP, respectively (0.08 mmol total), as determined by ^1H NMR. ^1H NMR of 6-AcPMDHLP (chemical shift in ppm, multiplicity, integration, assignment, coupling constant if available) 1.3–2.0 (m, 8 H, C3, C4, C5, and C7), 2.20, (t, 2 H, C2, $J_{2,3}$ = 7.3 Hz), 2.27 (s, 3 H, acetyl), 3.1 (m, 2 H, C8), 3.79 (m, 1 H, C6), 5.5 (2 H, amide), 7.4 (m, 5 H, phenyl).

Synthesis of 8-S-Acetyldihydrolipoamide (8-AcDHLP). 3-Mercaptopropionic acid (6 mmol, 0.5 mL) was added to 8-AcPMDHLP (0.2 mmol) partially dissolved in 1 mL of $CHCl_3$. The product mixture in the resulting clear solution was separated by silica gel column chromatography as described above. The 8-AcDHLP-containing fractions were extracted with water to remove a small amount of 3-mercaptopropionic acid, which overloaded the column. The solvent was removed by rotary evaporation and the residue dried in vacuo over P_2O_5. The yield was 0.18 mmol of 8-AcDHLP contaminated with about 5% 6-AcDHLP, which may have been introduced by isomerization of the product during purification and workup. ^1H NMR (chemical shift in ppm, multiplicity, integration, assignment, coupling constant if available) 1.39 (d, 1 H, SH, $J_{6,6'}$ = 7.6 Hz), 1.4–2.0 (m, 8 H, C3, C4, C5, and C7), 2.25 (t, 2 H, C2, $J_{2,3}$ = 7.3 Hz), 2.34 (s, 3 H, acetyl), 2.81 (m, 1 H, C6), 3.05 (m, 2 H, C8), 5.5 (2 H, amide).

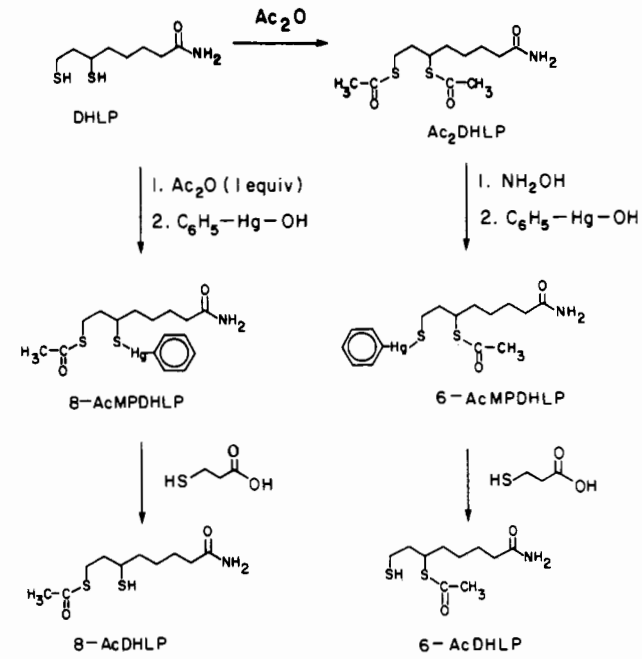
Synthesis of 70% 6-S-Acetyldihydrolipoamide (6-AcDHLP). The procedure was the same as that described above for 8-AcDHLP, except that the starting material was the 4:1 mixture of 6-AcPMDHLP and 8-AcPMDHLP described above and the product was a 3:1 mixture of 6-AcDHLP and 8-AcDHLP. ^1H NMR of 6-AcDHLP (chemical shift in ppm, multiplicity, integration, assignment, coupling constant if available) 1.45 (t, 1 H, SH, $J_{8,8'}$ = 8 Hz), 1.4–2.0 (m, 8 H, C3, C4, C5, and C7), 2.23 (t, 2 H, C2, $J_{2,3}$ = 7.3 Hz), 2.34 (s, 3 H, acetyl), 2.57 (m, 2 H, C8), 3.64 (m, 1 H, C6), 5.5 (2 H, amide).

Results

Synthesis of Acetyldihydrolipoamide Derivatives. Since the isomers of acetyldihydrolipoamide were known to undergo rapid interconversion (O'Connor et al., 1982), we planned to trap the enzymatic product in a chemically stable form and identify the derivative. A logical trapping reagent would be an organomercurial, since they are selective for sulfhydryl groups and *p*-(chloromercuri)benzoate has been reported to react with a second-order rate constant of 6×10^6 $M^{-1} \cdot s^{-1}$ at pH 6.8 and 30 °C (Hasinoff et al., 1971). Thio esters have been found to resist cleavage by organomercurials (Sanner & Pihl, 1962). Therefore, addition of an organomercurial to an enzymatic reaction mixture a few seconds after generating acetyldihydrolipoamide could be expected to stabilize the product as a derivative whose structure could be assigned by ^1H NMR spectroscopy. Preliminary studies indicated that phenylmercuric hydroxide would be a suitable reagent for use in these experiments.

To facilitate structural analysis of the enzymatic product, authentic samples of 6-AcPMDHLP and 8-AcPMDHLP were synthesized and their ^1H NMR signals assigned. These compounds proved to be useful as precursors for 6-AcDHLP and 8-AcDHLP, which were used to measure the isomerization rate constants in water. The ^1H NMR spectra of the latter compounds also confirmed the structures assigned to the precursor molecules.

Scheme I



Scheme I illustrates the synthetic routes by which the above-described compounds were prepared. Reaction of DHLP with 4 equiv of acetic anhydride and triethylamine in $CHCl_3$ produces Ac_2DHLP in high yield. Similar reaction of DHLP first with 1 equiv of acetic anhydride and KOH and then with phenylmercuric hydroxide produces 8-*S*-AcPMDHLP in about 20% isolated yield. 6-*S*-AcPMDHLP is also produced in substantial amounts but can be removed by differential solubilization. The latter isomer can be prepared as the principal component of a mixture by carrying out a partial deacetylation of Ac_2DHLP using NH_2OH and then adding phenylmercuric hydroxide. Reaction of 6-AcPMDHLP or 8-AcPMDHLP with 3-mercaptopropionic acid in chloroform produces 6-AcDHLP or 8-AcDHLP, respectively. These compounds can be prepared in and isolated from chloroform because they do not undergo isomerization in that solvent.

Structural assignments to the compounds in Scheme I were made by ^1H NMR spectroscopy. Chemical shifts assigned to C6 and C8 protons of Ac_2DHLP were 3.56 (1 H) and 2.91 (2 H) ppm, respectively. The shifts for these protons in DHLP were found to be 2.9 (1 H) and 2.7 (2 H) ppm, respectively. These shifts in one isomer of AcDHLP were 3.64 (1 H) and 2.57 (2 H) ppm, and this isomer was assigned as 6-AcDHLP. The assignment was confirmed by the fact that the signal for the free SH group was a triplet at 1.45 ppm, consistent with the 8-SH group. The corresponding chemical shifts for the other isomer were 2.81 (1 H) and 3.05 (2 H) ppm, respectively, and this was assigned as 8-AcDHLP. The shift for the 6-SH group in this compound was a doublet at 1.39 ppm. The structures of these compounds were the principal basis for the structural assignments to their precursor compounds, 6-AcPMDHLP and 8-AcPMDHLP; however, the chemical shift values for the C6 and C8 protons in these latter compounds, when compared with the values for Ac_2DHLP, were also consistent with the assigned structures.

Interconversion of 6-AcDHLP with 8-AcDHLP. Although these compounds are sufficiently stable in chloroform to permit their preparation and spectroscopic characterization, they undergo rapid isomerization in aqueous solutions to an equilibrium mixture of the two compounds. This is illustrated in Figure 1, where it is shown that each of the two is spontaneously converted to the equilibrium mixture within 1 min

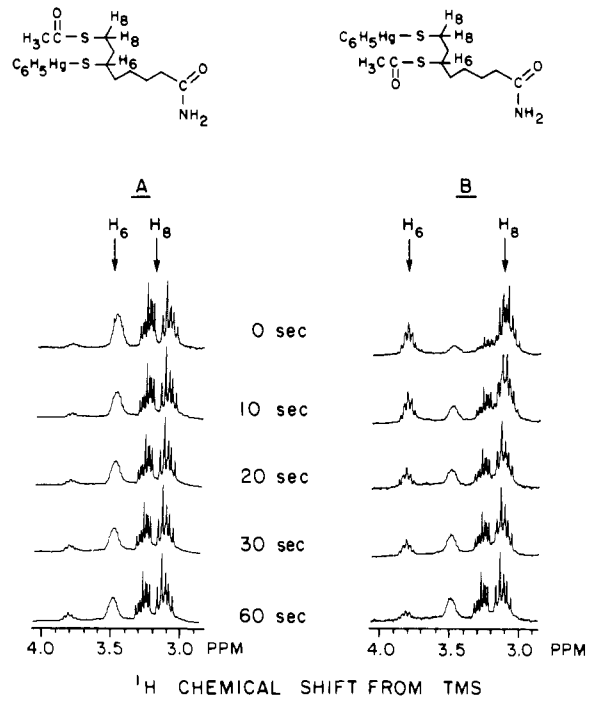


FIGURE 1: Isomerization of acetyldihydrolipoamide at pH 6.5. 8-AcDHLP (part A) or 6-AcDHLP (part B) was dissolved in ethanol (4 mM) and added to 0.1 M PIPES buffer, pH 6.5, to start the isomerization. The final concentration of acetyldihydrolipoamides was 0.2 mM. Phenylmercuric hydroxide solution was added to stop the isomerization at the desired time. The acetyl(phenylmercurio)-dihydrolipoamide was collected by chloroform extraction followed by rotary evaporation and desiccation in vacuo in the presence of P_2O_5 to remove the solvent. Samples were dissolved in $CDCl_3$ for ^{1}H NMR analysis.

at pH 6.5. The K_{eq} for this process is straightforwardly measured by allowing the reaction to reach equilibrium from either direction and then adding phenylmercuric hydroxide. The ratio 8-AcPMDHLP/6-AcPMDHLP is then measured by integrating the C6 signals for these compounds at 3.47 and 3.79 ppm, respectively, and computing the ratio. The K_{eq} in the direction of 8-AcDHLP formation is 3.43 ± 0.14 (mean of 16 independent measurements ± SD).

The approach to equilibrium follows a first-order rate law in which $k_{obsd} = k_f + k_r$, where k_f and k_r are the forward and reverse rate constants (Frost & Pearson, 1953) and k_{obsd} is proportional to [OH^-]. Since $K_{eq} = k_f/k_r$, the rate constants can be calculated from experimental values of k_{obsd}, K_{eq}, and [OH^-] at any pH. The pH-independent second-order rate constants measured in both directions at pH 6.5 (0.1 M PIPES buffer) and in the forward direction at pH 6.0 and 7.0 (0.1 M potassium phosphate buffers) are $k_{2f} = (1.15 \pm 0.07) \times 10^6$ $M^{-1}\cdot s^{-1}$ and $k_{2r} = (3.36 \pm 0.20) \times 10^5$ $M^{-1}\cdot s^{-1}$ at 25 °C. These are mean values ± SD from four independent determinations and refer to the conversion of 6-AcDHLP to 8-AcDHLP as the forward direction.

8-AcDHLP as the Enzymatic Product. Dihydrolipoyl transacetylase in the pyruvate dehydrogenase complex and the E_2E_3 subcomplex catalyzes acetyl group transfer from acetyl-CoA to DHLP (eq 7). The structure of the acetyldihydrolipoamide produced can be determined by chemically trapping it as the stable phenylmercury derivative and determining its structure by ^{1}H NMR spectroscopic analysis.

In a series of experiments we incubated the acetyl-CoA-generating system acetyl phosphate, CoASH, and phosphotransacetylase with DHLP and dihydrolipoyl transacetylase and added phenylmercuric hydroxide at times varying from

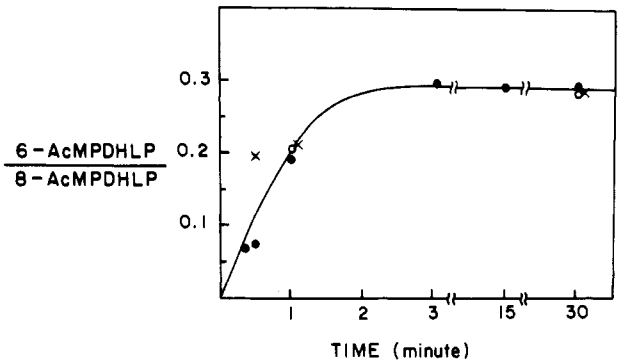


FIGURE 2: Enzymatic formation of 8-AcDHLP. The pyruvate dehydrogenase complex (closed circles), the NEM-inactivated complex (×), or the E_2E_3 subcomplex (open circles), each at 1.3–11.3 mg, was incubated with the following components in a volume of 4 mL: acetyl phosphate (10 mM), phosphotransacetylase (20–40 units), CoASH (0.1 mM), DHLP (10 mM), and PIPES buffer (0.1 M, pH 6.5). The mixture minus DHLP was stirred at room temperature for 5 min. DHLP dissolved in 95% ethanol (200 mL) was added to the above mixture to start the reaction. Phenylmercuric hydroxide solution was added to stop the reaction. The product was isolated by chloroform extraction and prepared for ^{1}H NMR analysis as described in Figure 1.

15 s to 30 min after starting the reaction. 8-AcPMDHLP and 6-AcPMDHLP were isolated from the reaction mixtures by extraction with chloroform and subjected to ^{1}H NMR analysis to determine the product composition. Experiments at pH 6.5 and 7.2 using pyruvate dehydrogenase complex, E_2E_3 subcomplex, and the pyruvate dehydrogenase complex in which dihydrolipoyl moieties were alkylated by NEM as sources of dihydrolipoyl transacetylase gave similar results. In all experiments the major product at early times was 8-AcPMDHLP, with much smaller amounts of 6-AcPMDHLP. The ratio of 6-AcPMDHLP to 8-AcPMDHLP increased with time to the equilibrium value within 3 min.

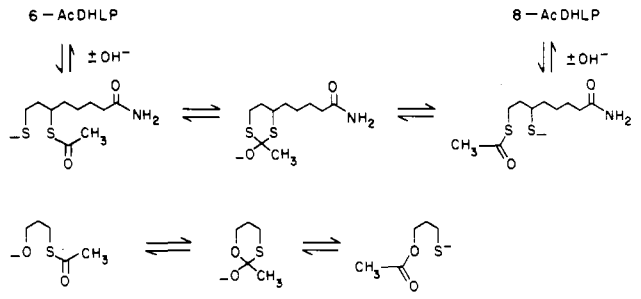
The ratios 6-AcPMDHLP/8-AcPMDHLP obtained at pH 6.5 are plotted in Figure 2 as a function of the time at which the reactions were quenched and products trapped by addition of phenylmercuric hydroxide. The figure clearly shows that the major product at early times is 8-AcPMDHLP and that this would be the only product at zero time. The observed ratio at 3 min corresponds to the equilibrium ratio of 6-AcDHLP and 8-AcDHLP. Inasmuch as the k_{obsd} for nonenzymatic isomerization to the equilibrium mixture of 6- and 8-AcDHLP is 0.045 s^{-1} at pH 6.5, corresponding to a half-time of 15 s, data such as those in Figure 2 must be expected even though the enzymatic product is 8-AcDHLP. The time course for the appearance of 6-AcDHLP and the approach to equilibrium is quantitatively in accord with the enzymatic product being 8-AcDHLP, which undergoes nonenzymatic isomerization to the equilibrium mixture of 8- and 6-AcDHLP.

Formation of Ac_2DHLP. In prolonged reactions, either enzymatic or nonenzymatic, at pH 7 or 8 Ac_2DHLP was produced from the equilibrium mixture of 8- and 6-AcDHLP. Under the conditions of Figures 1 and 2, Ac_2DHLP appeared within 30–60 min at pH 7 and within 10–15 min at pH 8.

Discussion

The principal conclusion from the present research is that dihydrolipoyl transacetylase catalyzes acetylation of DHLP by acetyl-CoA at S^8 and not at S^6. The same result is obtained when the source of dihydrolipoyl transacetylase is the intact complex, E_2E_3 subcomplex, or complex having the lipoyl groups reductively alkylated by NEM. Inasmuch as the bound lipoyl moieties can be removed without affecting the transacetylase activity in eq 7 (Koike & Reed, 1960) and reductive

Scheme II



alkylation of lipoyl moieties by the suicide substrate 3-bromopyruvate does not reduce transacetylase activity (Maldonado et al., 1972; Apfel et al., 1984), the bound lipoyl groups are unlikely to be involved in mediating acetyl group transfer. Bound lipoyl groups cannot be involved in our experiments utilizing reductively alkylated pyruvate dehydrogenase complex, with all lipoyl groups alkylated by NEM, yet Figure 2 shows the same result with that complex as with the intact complex and E_2E_3 subcomplex. DHLP apparently occupies the transacetylation active site of dihydrolipoyl transacetylase in place of bound dihydrolipoyl groups and accepts the acetyl group from acetyl-CoA. Production of 8-AcDHLP is, therefore, a model for the acetylation of enzyme-bound dihydrolipoyl groups, which would presumably also be acetylated at S^8.

Isomerization of 8-AcDHLP to 6-AcDHLP is catalyzed by OH^-. Since Ac_2DHLP does not appear until well after isomerization has reached equilibrium, it is not a compulsory intermediate. Isomerization is probably an intramolecular process proceeding according to the mechanism in Scheme II. Being intramolecular, this mechanism might be expected to lead to isomerization of enzyme-bound 8-acetyldihydrolipoyl groups. However, isomerization does not occur during normal catalytic turnover because the isomerization rate constant is 0.045 s^{-1} at pH 6.5, whereas the enzymatic turnover number is 1.6×10^2 s^{-1}.

Gunsalus et al. (1956) applied a different approach to the determination of which sulfhydryl group is acetylated in enzymatically generated acetyldihyrolipoic acid and concluded that acetylation was on S^6. They synthesized methyl 8-*S*-acetyldihydrolipoate and verified that this compound would not react with diphenyl *N*-propionylketimine, a reagent that had been shown to undergo an addition reaction in organic solvents with primary but not secondary sulfhydryl groups to form crystalline derivatives (Redcliffe, 1950). They then generated acetyldihydrolipoate enzymatically, isolated it as the free acid, prepared the methyl ester, subjected it to reaction with diphenyl *N*-propionylketimine in benzene, and isolated a crystalline derivative that analyzed correctly for the product expected from a methyl acetyldihydrolipoate. Since methyl 8-*S*-acetyldihydrolipoate had been shown not to produce such a derivative under comparable conditions, Gunsalus et al. (1956) concluded that the enzymatic product must have been 6-*S*-acetyldihydrolipoate.

Our results confirm that 6-*S*-acetyldihydrolipoate would be produced under the conditions employed by Gunsalus et al. (1956) in their experiments. This product could well have arisen, however, from the isomerization of the primary product 8-*S*-acetyldihydrolipoate under the conditions of their enzymatic reaction, which proceeded for 90 min at pH 7. Our measurements of the isomerization rate at pH 7 show that k_{obsd} is 0.17 s^{-1} (half-time = 4 s) for the conversion of 8-AcDHLP to the equilibrium mixture of 6- and 8-AcDHLP. Although our data refer to the acetyldihydrolipoamides, the rates should be comparable within less than 1 order of magnitude for the acetyldihydrolipoates. Therefore, by the time the enzymatic product was transferred to an organic medium, where isomerization does not occur, it should have been an equilibrium mixture of 6- and 8-*S*-acetyldihydrolipoic acids. After conversion to the methyl esters, reaction with diphenyl *N*-propionylketimine would have produced the crystalline addition compound derived from methyl 6-*S*-acetyldihydrolipoate in a yield that reflected its contribution to the equilibrium mixture. This interpretation is consistent with the fact that the diphenyl *N*-propionylketimine derivative of the enzymatic product was isolated in only 13% yield, whereas the yield from methyl dihydrolipoate was 67% (Gunsalus et al., 1956). In the case of the acetyldihydrolipoamides, 6-AcDHLP is the minor component (30%). This would account for the low yield of the diphenyl *N*-propionylketimine derivative in the work of Gunsalus et al. (1956).

Comparison of the isomerization rates measured in this work with available data on related isomerizations shows that our results are reasonable and essentially as should be expected for a reaction proceeding as outlined in Scheme II. According to the results of Jencks et al. (1960) the second-order rate constant for hydroxide-catalyzed isomerization of *S*-acetyl-3-mercaptopropanol at 39 °C is 2.9×10^3 $M^{-1}\cdot min^{-1}$. The corresponding values from the present work, expressed in the same units, are 7.2×10^7 $M^{-1}\cdot min^{-1}$ and 2.1×10^7 $M^{-1}\cdot min^{-1}$ for transfer of the acetyl group from S^6 to S^8 and S^8 to S^6, respectively, in acetyldihydrolipoamides. The difference in rate constants for acetyl group transfer from S to O in *S*-acetyl-3-mercaptopropanol compared with S to S in acetyldihydrolipoamide reflects the difference in pK_a values for the alcohol and thiol acceptor groups in these molecules. Rate-limiting attack by the alkoxide and thiolate species can be expected in the mechanism of Scheme II at pHs near neutrality. Typical pK_a values for simple alcohols such as methanol and ethanol are 15.5–16, whereas those for typical 1,3-, 1,4-, and 1,5-dimercaptoalkanes are 11.1–11.8 (Jencks, 1970). Therefore, at a given pH the fraction of acetyldihydrolipoamide in the reactive thiolate form in Scheme II will be greater than 10^4 times the fraction of *S*-acetyl-3-mercaptopropanol in the reactive alkoxide form. This is nearly the difference in isomerization rates. Thiolate ions are less than 10 times more reactive toward electrophilic carbon than hydroxide (Hine, 1962), and this could not alone account for the rate difference. The reactivity difference is probably a contributing factor, but the pK_a difference is dominant.

Registry No. (±)-DHLP, 4265-09-2; (±)-Ac_2DHLP, 105229-73-0; (±)-8-AcPMDHLP, 105229-74-1; (±)-6-AcPMDHLP, 105229-75-2; (±)-8-AcDHLP, 105229-76-3; (±)-6-AcDHLP, 105229-77-4; HS-$(CH_2)_2CO_2H$, 107-96-0; E_1, 9014-20-4; E_2, 9032-29-5.

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Identification of the High-Affinity Lipid Binding Site in *Escherichia coli* Pyruvate Oxidase†

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ABSTRACT: Pyruvate oxidase from *Escherichia coli* is a peripheral membrane associated enzyme which is activated by lipids. We have investigated the high-affinity lipid binding site associated with lipid activation of pyruvate oxidase by covalent attachment of [^{14}C]lauric acid to the enzyme. Lauric acid is bound stoichiometrically (1 mol/mol of active sites), and the enzyme is essentially irreversibly activated. Mild tryptic digestion of the modified enzyme shows that the lauric acid is bound within the last 100 residues of the 572-residue monomer. Digestion with thermolysin releases two closely related peptides, A and B, in approximately equal amounts. Comparison of the amino acid composition of peptide A with the entire sequence of the protein shows that peptide A corresponds to the sequence from Ala-543 to Ile-554. The analysis of peptide B is very similar to that of A. Limited sequence analysis of peptide B shows that residue 1 is Ala and residue 2 is labeled. These results support the assignment of residue 1 in peptide B as Ala-543 and indicate that lauric acid is bound to Lys-544. Previous work in this laboratory has shown that pyruvate oxidase may be activated independently of lipids by mild protease digestion. Proteolytic activation is accompanied by the release of a small peptide (residues 550–572) from the carboxyl terminus of the protein. The present work locates the lipid binding site very close to this peptide. The significance of these results for the mechanism of activation of pyruvate oxidase and other lipid-activated systems is discussed.

Pyruvate oxidase (pyruvate:cytochrome b_1 oxidoreductase, EC 1.2.2.2) from *Escherichia coli* is a flavoprotein which catalyzes the oxidative decarboxylation of pyruvate to acetate (Hager, 1957). The enzyme has a molecular weight of 240 000 and is comprised of four identical subunits (O'Brien et al., 1976; Raj et al., 1977). It requires thiamin pyrophosphate (TPP)[1] and a divalent metal ion for activity (Williams & Hager, 1966; O'Brien et al., 1977). The reduced enzyme is reoxidized by the *E. coli* membrane-associated electron-transport system and also by artificial electron acceptors (Cunningham & Hager, 1975).

A remarkable property of pyruvate oxidase is its ability to be activated by amphiphilic lipids and detergents. In the presence of 20 μM SDS, the catalytic activity (k_{cat}/K_m) is increased approximately 450-fold—k_{cat} is increased by ~30-fold, and the K_m for pyruvate is decreased by ~15-fold. Reduction of the flavin prosthetic group in the presence of pyruvate, TPP, and divalent metal ions facilitates this activation process (Cunningham & Hager, 1971; Blake et al., 1978). The extents of activation by amphiphiles of widely differing structure and charge are quantitatively similar. Data obtained for the noncovalent binding of detergents to the enzyme and for the covalent attachment of lauric acid clearly suggest that activation is achieved through the binding of a small number of molecules of lipid to a discrete site or sites on the reduced enzyme (Schrock & Gennis, 1977; Leisman et al., 1985). Pyruvate oxidase is one of the very few soluble proteins to exhibit such well-defined lipid binding characteristics, and for this reason, it constitutes a rewarding system for studying lipid activation of enzyme activity and lipid–protein interactions in general.

A further property of the enzyme which is of particular relevance for the present work is that it may be activated by mild protease digestion under the same conditions as are required for lipid activation (Russell et al., 1977a). In the

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[1] Abbreviations: SDS, sodium dodecyl sulfate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HPLC, high-performance liquid chromatography; Me_2SO, dimethyl sulfoxide; PTC, phenylthiocarbamyl; PITC, phenyl isothiocyanate; PTH, 3-phenyl-2-thiohydantoin; PAGE, polyacrylamide gel electrophoresis; TPP, thiamin pyrophosphate; Pipes, 1,4-piperazinediethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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