Kinetics and Specificity of Reductive Acylation of Lipoyl Domains from 2-Oxo Acid Dehydrogenase Multienzyme Complexes†

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ABSTRACT: Lipoamide and a peptide, Thr-Val-Glu-Gly-Asp-Lys-Ala-Ser-Met-Glu lipoylated on the N^6-amino group of the lysine residue, were tested as substrates for reductive acetylation by the pyruvate decarboxylase (E1p) component of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. The peptide has the same amino acid sequence as that surrounding the three lipoyllysine residues in the lipoate acetyltransferase (E2p) component of the native enzyme complex. Lipoamide was shown to be a very poor substrate, with a K_m much higher than 4 mM and a value of k_{cat}/K_m of 1.5 $M^{-1}\cdot s^{-1}$. Under similar conditions, the three E2p lipoyl domains, excised from the pyruvate dehydrogenase complex by treatment with *Staphylococcus aureus* V8 proteinase, could be reductively acetylated by E1p much more readily, with a typical K_m of ~26 μM and a typical k_{cat} of ~0.8 s^{-1}. The value of k_{cat}/K_m for the lipoyl domains, ~3.0×10^4 $M^{-1}\cdot s^{-1}$, is about 20 000 times higher than that for lipoamide as a substrate. This indicates the great improvement in the effectiveness of lipoic acid as a substrate for E1p that accompanies the attachment of the lipoyl group to a protein domain. The free E2o lipoyl domain was similarly found to be capable of being reductively succinylated by the 2-oxoglutarate decarboxylase (E1o) component of the 2-oxoglutarate dehydrogenase complex of *E. coli*. The 2-oxo acid dehydrogenase complexes are specific for their particular 2-oxo acid substrates. The specificity of the E1 components was found to extend also to the lipoyl domains. Thus, the lipoyl domain of E2o was virtually inactive as a substrate for reductive acetylation by E1p, and each of the three lipoyl domains of E2p was at best a poor substrate for reductive succinylation by E1o. This suggests that the lipoyl domain confers considerable specificity on its lipoyllysine residue in the reductive acylation reaction. The behavior of the lipoylated peptide as a substrate for reductive acetylation by E1p was similar to that of lipoamide, demonstrating that the primary structure around the lipoyllysine residue is insufficient to enhance the ability of the dithiolane ring to undergo reductive acetylation. All of these experiments are consistent with the view that some molecular recognition and interaction between a lipoyl domain and its cognate E1 component play a major part in the mechanism of oxidative decarboxylation by 2-oxo acid dehydrogenase multienzyme complexes.

The 2-oxo acid dehydrogenase multienzyme complexes catalyze the oxidative decarboxylation of 2-oxo acids to the corresponding acyl-CoA with the concomitant generation of NADH. The pyruvate dehydrogenase (PDH)[1] multienzyme complex of *Escherichia coli* comprises three enzymes: a structural core of dihydrolipoamide acetyltransferase (E2p; EC 2.3.1.12) consisting of 24 E2p polypeptide chains arranged with octahedral symmetry, to which are bound multiple copies of pyruvate decarboxylase (E1p; EC 1.2.4.1) and dihydrolipoamide dehydrogenase (E3; EC 1.8.1.4). The 2-oxoglutarate dehydrogenase (2OGDH) complex is similar: the structural core consists of 24 dihydrolipoamide succinyltransferase (E2o; EC 2.3.1.61) polypeptide chains to which are bound multiple copies of 2-oxoglutarate decarboxylase (E1o; EC 1.2.4.2) and the same dihydrolipoamide dehydrogenase [for reviews, see Reed (1974) and Perham et al. (1987)]. During catalysis the substrate is anchored in thioester linkage to a lipoyl group which is itself attached by an amide bond to the N^6-amino group of a lysine residue in the E2p or E2o polypeptide chains (Reed, 1974; Ambrose & Perham, 1976).

The E2p and E2o chains are highly segmented in structure. The E2p chain of *E. coli* comprises three highly homologous lipoyl domains of about 80 residues linked to each other in tandem array by long (about 25 residues) segments of polypeptide chain rich in alanine, proline, and charged amino acids (Stephens et al., 1983; Packman et al., 1984a). The innermost of these three lipoyl domains is attached by a similar segment of polypeptide chain to a domain of about 50 amino acid residues responsible for binding the E3 component, and this in turn is linked to a larger, and C-terminal, domain that is responsible for the aggregation and symmetry of the E2p core and for housing the acetyltransferase active site (Packman & Perham, 1986, 1987; Radford et al., 1987). On the basis of ^{1}H NMR spectroscopy of the intact PDH complex (Perham et al., 1981), of synthetic peptides (Radford et al., 1986) and of complexes genetically engineered in vitro (Miles et al., 1987; Radford et al., 1987; Texter et al., 1988), it is clear that the interdomain segments are conformationally flexible. It is likely that this flexibility allows for movement of the lipoyl domains, which facilitates active site coupling and promotes the passage of substrate through the successive steps catalyzed by three physically separate active sites in three different enzymes. The octahedral 2OGDH complex of *E. coli* (Perham & Roberts, 1981; Spencer et al., 1984; Packman & Perham, 1986), the icosahedral PDH complex of *Bacillus stearothermophilus* (Duckworth et al., 1982; Packman et al., 1984b, 1988), and

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[1] Abbreviations: PDH, pyruvate dehydrogenase; 2OGDH, 2-oxoglutarate dehydrogenase; TPP, thiamin pyrophosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); HPLC, high-performance liquid chromatography.

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the icosahedral branched-chain 2-oxo acid dehydrogenase complex of mammalian mitochondria (Griffin et al., 1988) are very similar in basic design, except that their respective E2 chains contain only one lipoyl domain. Analysis of the sequence of a cDNA encoding a human mitochondrial autoantigen (Gershwin et al., 1987), identified as the E2p chain of the pyruvate dehydrogenase complex (Yeaman et al., 1988), indicates that this E2p chain too has the same basic design but that it may carry two lipoyl domains.

Lipoyl domains are evidently an important structural feature of the enzyme, presumably related to the catalytic mechanism. Although free lipoic acid and lipoamide can act as substrates in the reactions catalyzed by the E2p and E3 components of the PDH complex (Reed et al., 1958), direct reductive acetylation of these compounds by the E1p component does not seem to occur (Reed et al., 1958; Reed, 1966). However, lipoyl domains excised proteolytically from the complex are reductively acetylated quite readily by E1p in the presence of TPP-Mg^{2+} and pyruvate (Packman et al., 1984a), and similar results have been obtained with the lipoyl domains excised from the ox heart (Bleile et al., 1981) and *B. stearothermophilus* (Packman et al., 1984b) PDH complexes. Clearly, the domains which accommodate the lipoyl groups in the E2p chains are important to this first step of the overall reaction, perhaps through some form of protein–protein interaction required for the successful presentation of the dithiolane ring to the E1p active site. One possibility is an electrostatic attraction between one or more parts of the E1p active site and the region of E2p polypeptide that immediately surrounds the lipoyllysine residue, the amino acid sequence of which is rich in acidic residues and is conserved between domains.

In the present paper we describe experiments in which direct reductive acetylation of lipoamide by *E. coli* E1p has been detected. Similar experiments were carried out with a synthetic lipoyl decapeptide, the amino acid sequence of which is that of the cofactor-bearing sites in the E2p subunit. We also describe experiments in which purified lipoyl domains from the *E. coli* PDH and 2OGDH complexes were characterized as substrates for reductive acetylation by the E1p and E1o components, in terms of both specificity and steady-state kinetics. Taken together the results indicate that a folded lipoyl domain greatly boosts the catalytic activity and plays a major part in conferring specificity on the substrate in the reductive acylation reaction.

For kinetic experiments, the use of purified E1p was preferred to that of intact complex to avoid any risk of interference from endogenous lipoyl domains. Although endogenous acetylated lipoyl domains would probably not contribute to the reaction by permitting an indirect transfer of acetyl groups to an exogenous lipoyl moiety (Yang & Frey, 1986), it was thought possible that they might cause dead-end competitive inhibition. Excision of lipoyl domains from *E. coli* PDH complex by treatment with *Staphylococcus aureus* V8 proteinase yields a mixture of the three domains (designated LpV1, LpV2, and LpV3 from the N-terminus of the E2p chain) in approximately equimolar amounts (Packman & Perham, 1987). These domains are highly homologous, and in the complex, all are active as substrates for E1p (Packman et al., 1984a). It is likely that the three domains behave similarly, and they have been treated here as a single substrate, thereby greatly simplifying the kinetic equations.

The lipoamide tested for substrate activity was a racemic mixture, (6*RS*)-lipoamide, and (6*RS*)-lipoic acid was used to derivatize the synthetic decapeptide. Thus, all model compounds tested for substrate activity were mixtures of the two stereoisomers. In contrast, lipoyl domains isolated from the PDH complex contain only the naturally occurring 6*R* isomer (Mislow & Meluch, 1956; Parry & Trainor, 1978), and given that it is not yet known whether E1p discriminates between stereoisomers of the lipoyl group, this difference must be borne in mind. Furthermore, no attempt was made to distinguish between the formation of S^6- and S^8-acetyl isomers of the dihydrolipoyl group (Hale & Dixon, 1981; Yang & Frey, 1986).

Materials and Methods

Enzymes and Reagents. The PDH complex was purified according to Danson et al. (1979) from a mutant *E. coli* K12 constitutive for overproduction of the complex. The 2OGDH complex was purified by the same procedure from *E. coli* K12 (pGS110) described by Guest et al. (1985). Free E1p was obtained from the PDH complex as described by Coggins et al. (1976). E1o was not purified directly but used as the subcomplex remaining after treatment of the 2OGDH complex with *S. aureus* V8 proteinase. This causes release of the lipoyl domains, the E3-binding domains and their associated E3 subunits, and leaves the E1o component bound to a still-assembled inner core of truncated E2o chains (residues 157–404) (Packman & Perham, 1986). Sodium [2-^{14}C]pyruvate (specific radioactivity 10.4 Ci/mol) and sodium [5-^{14}C]-2-oxoglutarate (specific radioactivity 21.9 Ci/mol) were obtained from Amersham International plc, Amersham, U.K. Optiphase Safe and Optiscint Safe scintillation cocktails were obtained from LKB, Croydon, U.K. (6*RS*)-Lipoic acid was from Sigma Chemical Co. Ltd., Poole, U.K., and (6*RS*)-lipoamide was from R. Emanuel Ltd., Wembley, U.K. The synthetic decapeptide, Thr-Val-Glu-Gly-Asp-Lys-Ala-Ser-Met-Glu, was a gift from Dr. E. Appella (National Cancer Institute, NIH, Bethesda, MD). All other reagents used were of analytical grade.

Estimation of Peptides and Proteins. The concentrations of peptide and protein solutions were estimated from amino acid analyses (Perham, 1978), carried out using an LKB 4400 amino acid analyzer. The concentrations of enzyme complexes were usually calculated from the absorbance of the solutions at 280 nm, after correction had been made for light scattering (Danson et al., 1979). The concentration of E1p was normally determined by measuring the absorbance at 280 nm, taking the specific absorption coefficient A_{280} (1 mg/mL) as 1.34 cm^{-1} (P. N. Lowe, personal communication).

Thin-Layer Electrophoresis and Chromatography. Separations were performed on Polygram SilG thin-layer plates (Machery-Nagel & Co., Duren, West Germany). Ascending chromatography was carried out in a mixture of butan-1-ol, acetic acid, water and pyridine (15:3:12:10 by vol) as solvent. Electrophoresis was carried out at pH values of 6.5, 3.5, and 2.1 at 100 V/cm, as described by Perham (1978). Peptides were stained by spraying with fluorescamine or ninhydrin–cadmium reagent or by means of the chlorine stain (Perham, 1978). Sulfur-containing compounds were visualized by spraying with a solution of fluorescein mercuric acetate at high pH (Karush et al., 1964).

Estimation of Disulfide and Thiol Groups. Disulfide compounds, dissolved in a total volume of 0.1 mL of 20 mM sodium phosphate buffer, pH 7.0, were first reduced by adding 30 μL of sodium borohydride solution (114 mg/mL). After 15 min at room temperature, 10 μL of glacial acetic acid was added to destroy excess borohydride. After a further 10 min at room temperature, 0.9 mL of an ice-cold solution of Nbs_2 (0.4 mg/mL) in 0.5 M Tris-HCl buffer, pH 8.8, was added, and the absorbance at 412 nm was measured (Ellman, 1959).

The molar absorbtion coefficient for the thionitrobenzoate anion was taken to be 12800 M^{-1} cm^{-1}. Portions of a standard glutathione solution were analyzed with every set of samples to check the efficiency of reduction, which was usually greater than 90%. The reduction and acidification steps were omitted when thiol compounds were being measured.

Synthesis of the Lipoyl Peptide. The *N*-hydroxysuccinimide ester of lipoic acid was synthesized from 5 mmol of lipoic acid and *N*-hydroxysuccinimide according to the method of Gorecki and Patchornik (1979). The product was obtained in approximately 50% yield and had a melting point of 90–91 °C.

The synthetic decapeptide Thr-Val-Glu-Gly-Asp-Lys-Ala-Ser-Met-Glu was shown to have the correct sequence by amino acid analysis and dansyl-Edman degradation (Perham, 1978) and was then selectively lipoylated on the N^6 position of the lysine residue by treatment with the *N*-hydroxysuccinimide ester of lipoic acid. Protection of the N^2-amino group by involving it in a copper complex (Nawa et al., 1960) was found to be unnecessary. A sample of the peptide (74 μmol) was dissolved in 0.88 mL of 0.1 M $NaHCO_3$. A portion (300 μmol) of the *N*-hydroxysuccinimide ester of lipoic acid [0.18 mL of concentrated solution (500 mg/mL) in dimethylformamide, prepared freshly before use] was introduced; much of the ester precipitated from solution as it was not very soluble in water. The solution was brought to pH 11.0 with 7 M NaOH. The reaction mixture was vortexed for 1 h at room temperature, keeping the pH near to 11 by further additions of 7 M NaOH every 15 min. At the end of this time, the pH was adjusted to 7.0 with 12 M HCl.

The lipoylated peptide was purified as follows. An addition (0.5 mL) of 3.0 M sodium borohydride solution was made to the reaction mixture, and after 15 min at room temperature, excess borohydride was destroyed by acidification with 0.2 mL of glacial acetic acid. The gel which formed at this step was solubilized by raising the pH to about 12 with 7 M NaOH, and the solution was clarified by centrifuging in an Eppendorf minifuge for 2 min. The supernatant was applied to a column of Sephadex G-25 (30 × 1 cm, flow rate 0.4 mL/min, room temperature) equilibrated with 70 mM triethanolamine solution (aqueous) containing 5 mM dithiothreitol. A sample of each fraction was analyzed by thin-layer chromatography and electrophoresis. The monolipoylated peptide, which stained with fluorescamine, fluorescein mercuric acetate, and chlorine, was the only peptide species detected. Fractions containing the lipoyl peptide were pooled and freeze-dried. The residue was redissolved in 1–2 mL of 10 mM ammonium acetate solution and brought to a final pH value of 5.0 with glacial acetic acid. The sample was then applied to a column of Whatman DE52 (38 × 1.25 cm, flow rate 25 mL/h, room temperature) equilibrated with 10 mM ammonium acetate buffer, pH 5.0. The column was washed with several volumes of this buffer, and the lipoylated peptide was eluted by developing the column with 370 mM ammonium acetate buffer, pH 5.0. Since the ion-exchange chromatography was performed in the absence of dithiothreitol, all of the modified peptide reverted spontaneously to the oxidized form. To complete the purification, the fractions containing the lipoyl peptide were pooled, freeze-dried, and applied to a C-18 reverse-phase HPLC column (Varian, MCH-5-n-CAP, 15 × 0.4 cm, flow rate 1.0 mL/min, room temperature) equilibrated with 10 mM ammonium acetate buffer, pH 5.0, and the lipoyl peptide was eluted isocratically with 15% acetonitrile. The overall yield of lipoyl peptide was 11%, and the peptide was pure as judged by thin-layer electrophoresis and chromatography and by HPLC under various conditions.

Characterization of the Lipoyl Peptide. Confirmation of the expected structure of the product was obtained by various methods. When chromatograms and electrophoretograms of the lipoylated and unmodified peptides were stained with ninhydrin–cadmium reagent, both species generated a yellow color, characteristic of an N-terminal threonine or glycine residue (Offord, 1969). In the case of the unmodified peptide, the N^6-amino group of the internal lysine residue also reacted slowly with the ninhydrin reagent to give a red color, and the spots turned from yellow to orange; with the lipoylated peptide, however, the spot remained yellow. When the peptides were dansylated and hydrolyzed (Perham, 1978), both yielded dansylthreonine from the N-terminus, but only the unmodified peptide yielded N^6-dansyllysine in addition. The modified peptide was no longer susceptible to cleavage by trypsin, as judged by its unchanged electrophoretic mobility, which also indicated a derivatized lysine side chain. Comparison of the amount of thiol group present after borohydride reduction of the lipoylated peptide (measured with Nbs_2) with the amount of peptide present (determined by amino acid analysis) gave a molar ratio of 2.0 (±0.2):1. The M_r of the lipoyl peptide was determined to be that expected from theory (M_r 1253) by fast atom bombardment mass spectrometry (Williams et al., 1982).

Preparation of Lipoyl Domains. Lipoyl domains LpV1, LpV2, and LpV3 (representing residues 1–92, 93–192, and 193–292, respectively) of E2p were prepared from a limited digest of the PDH complex with *S. aureus* V8 proteinase as described by Packman & Perham (1987). Similar digestion of the 2OGDH complex with trypsin generated lipoyl domains LoT1 (residues 1–100 of E2o), LoT2 (residues 1–93), and LoT3 (residues 1–89) (Packman & Perham, 1987). Lipoyl domain LoT1 was used in these studies.

Kinetics of Reductive Acetylation of E2p Lipoyl Domains by E1p. Incubation mixtures consisted of 0.3 mL of 20 mM sodium phosphate buffer, pH 7.0, containing E1p (1.2 μg; subunit concentration 41 nM), TPP (0.2 mM), $MgCl_2$ (1.0 mM), sodium [2-^{14}C]pyruvate (0.35 mM), bovine serum albumin (0.3 mg/mL) as a carrier protein, and various concentrations of lipoyl domains. Incubations, which were performed at room temperature (20–25 °C), were started by adding the radioactive pyruvate. Samples (50 μL) were withdrawn at regular intervals, typically 20 s, and added to 1–2 mL of ice-cold 10% (w/v) trichloroacetic acid, and mixed immediately. Precipitated proteins were collected on Whatman GF/C filters under suction, dried under vacuum, and counted for radioactivity in 3.5 mL of Optiscint Safe scintillation cocktail. Any protein remaining adhered to the walls of the incubation tubes was solubilized in 400 μL of 1% (w/v) sodium dodecyl sulfate and counted in 3.5 mL of Optiphase Safe scintillation cocktail. Protein present in the detergent wash generally represented less than 5% of the total protein recovered.

Specificity of Reductive Acylation of Lipoyl Domains by E1 and 2-Oxo Acid. Lipoyl domains (0.4-0.5 nmol) from E2p were reductively acetylated with [2-^{14}C]pyruvate (0.5 mM) and E1p (10 μg) by incubation for 20 min at room temperature (25 °C) in a final volume of about 50 μL of 100 mM sodium phosphate buffer, pH 7.0, containing TPP (0.2 mM), $MgCl_2$ (1 mM), and bovine serum albumin (3.0 mg/mL). The reaction was quenched by adding 100 μL of 0.15% (w/v) sodium deoxycholate followed immediately by 1 mL of ice-cold 12% (w/v) trichloroacetic acid. The precipitated proteins were collected as described above. Reductive succinylation of the lipoyl domain (LoT1, 0.4–0.5 nmol) from E2o by E1o (50 μg

of the subcomplex, containing 20 μg of E1o) and [5-^{14}C]-2-oxoglutarate (0.5 mM) was carried out under identical conditions.

In cross-acylation experiments, lipoyl domains from E2p were treated with E1o and [5-^{14}C]-2-oxoglutarate. Conversely, the lipoyl domain from E2o was exposed to E1p and [2-^{14}C]pyruvate. A series of control incubations lacked each protein in turn, providing the necessary background counts for each test incubation.

Results

Reductive Acetylation of Lipoamide. Lipoamide (0.15 mM), sodium [2-^{14}C]pyruvate (0.14 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) were incubated with *E. coli* E1p (30 μg/mL; 0.30 μM in subunits) for 2 h at 20 °C, in 20 mM sodium phosphate buffer, pH 7.0 (total volume 0.1 mL). The mixture was extracted with 0.5 mL of butyl acetate, which removed the bulk of the lipoyl compounds but not the pyruvate, and the extract was analyzed by thin-layer chromatography. A single radioactive species was detected unless lipoamide, pyruvate, or enzyme was omitted from the incubation. The radioactive species migrated with lipoamide under the chromatographic conditions described under Materials and Methods but did not do so when other mobile phases, such as acetonitrile, were used. The radioactivity was released from the enzymic product by performic acid oxidation (Perham, 1978), as expected of a thioester.

If a hydrophobic maleimide, such as *N*-phenylmaleimide or *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide, was added to the reaction mixture after the 2-h incubation period, the mobility of the radioactive spot in acetonitrile was very much increased; this is consistent with the enzymic product containing a thiol group free to react with the aromatic maleimide. All these results suggest that the enzymic product was *S*-[1-^{14}C]acetyldihydrolipoamide.

When the radioactive product was eluted from the appropriate area of the chromatogram and quantified by means of liquid scintillation counting, its formation was found to be linear with time during the period (2 h) investigated. With lipoamide at 0.5 mM and pyruvate at 0.26 mM, thioester formation occurred at a rate of 4.8×10^{-5} mol·s^{-1} per mole of E1p subunit at 20 °C. However, this method of measuring the formation of product was tedious, and other assays were therefore developed. Spectrophotometric determination of *S*-acetyldihydrolipoamide was possible, provided high concentrations of lipoamide and sodium pyruvate were used and relatively long incubation times were employed. Lipoamide (5.0 mM), sodium pyruvate (20 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) were incubated with E1p (1.4 μM in subunits) in 20 mM sodium phosphate buffer, pH 7.0, at 21 °C under N_2. After 4 h, the amount of thioester formed was measured by the ferric hydroxylamine method (Stadtman, 1957; Wainfan & Van Bruggen, 1957), and the amount of thiol was measured by treatment with Nbs_2 reagent as described under Materials and Methods. Thioester and thiol were formed at rates estimated as 6.2×10^{-3} and 9.8×10^{-3} mol·s^{-1} per mole of E1p subunit, respectively. The rate of formation of thiol groups was expected to be higher because of hydrolysis of the thioester occurring at the high pH (8.8) of the thiol assay with Nbs_2; to allow for this, the observed rate of thiol formation should be halved.

The Nbs_2 assay proved much simpler and more sensitive than that with ferric hydroxylamine. It was therefore used to investigate the dependence of the reaction rate upon lipoamide concentration. This relationship was found to be linear over the range investigated (Figure 1), indicating that the concentrations of lipoamide tested were low relative to the K_m for this substrate. Under these conditions, the Michaelis–Menten equation describing initial rates reduces to the linear relationship $v/[E]_{total} = k_{cat}[S]/K_m$, and so the slope of the line in Figure 1, 1.5 $M^{-1}\cdot s^{-1}$, is the value of k_{cat}/K_m.

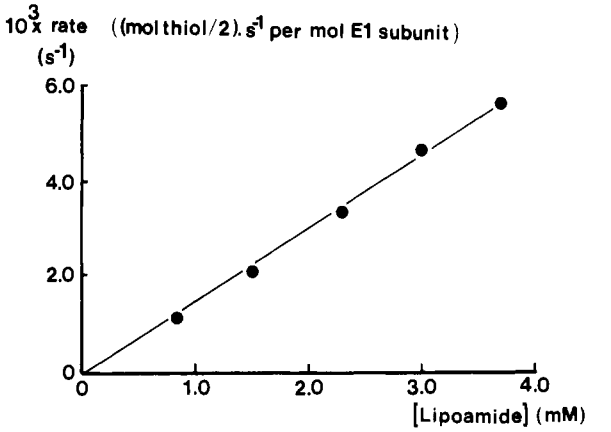

FIGURE 1: Kinetics of reductive acetylation of lipoamide by E1p. Lipoamide was incubated with sodium pyruvate (20 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) in the presence of E1p (1.4 μM in subunits) in 20 mM sodium phosphate buffer, pH 7.0, at 21 °C under N_2, as described in the text. The generation of thiol was measured by treating 0.1-mL samples with Nbs_2 and in each case was found to be linear with respect to time within the 90-min assay period. The rate of reaction was tested as a function of lipoamide concentration, up to the solubility limit of this compound.

Reductive Acetylation of the Lipoyl Peptide. A trial experiment revealed that the lipoyl peptide was substantially retarded in its passage through SP-Sephadex at pH 2. Lipoyl peptide (10.0 μM), sodium [2-^{14}C]pyruvate (0.13 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) were incubated with E1p (0.30 μM in subunits) for 20 min at 20 °C, in 20 mM sodium phosphate buffer, pH 7.0. The reaction was stopped by the addition of formic acid to a final concentration of 1.0 M, and the incubation mixture was applied to a small column (0.5 cm × 3.5 cm) of SP-Sephadex which had been equilibrated with a mixture of acetic acid, formic acid, and water (8:2:98 by vol), pH 2. The column was washed with the same buffer and was then developed with 1.8 M NH_4OH to release any bound peptide species. No radioactivity was eluted from the column by this treatment, indicating that no [1-^{14}C]acetyl derivative of the lipoyl peptide had formed to any appreciable extent. However, when lipoyl domains (1.0 μM) were used in place of (or in addition to) the lipoyl peptide, a substantial amount of radioactivity was eluted by the NH_4OH treatment.

To investigate whether slow reductive acetylation of the lipoyl peptide might occur in the presence of E1p, it was necessary to employ more favorable reaction conditions and a more powerful method of separating the components in the reaction mixture. Thus, lipoyl peptide (0.5 mM), sodium [2-^{14}C]pyruvate (0.26 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) were incubated with E1p (0.30 μM in subunits) for 2 h at 20 °C, in 20 mM sodium phosphate buffer, pH 7.0, and the mixture was analyzed by reverse-phase HPLC. A small radioactive peak was observed close to the elution position of the lipoyl peptide; this was not seen in chromatograms from control incubations which lacked either lipoyl peptide or E1p. When the incubations were repeated in the presence of *N*-phenylmaleimide (0.1 mM), the elution of radioactivity in the control chromatograms was unaltered, whereas the peak thought to represent the product of reductive acetylation was both larger and more strongly retarded (Figure 2a). This was consistent with the radioactive product being *S*-[1-^{14}C]-

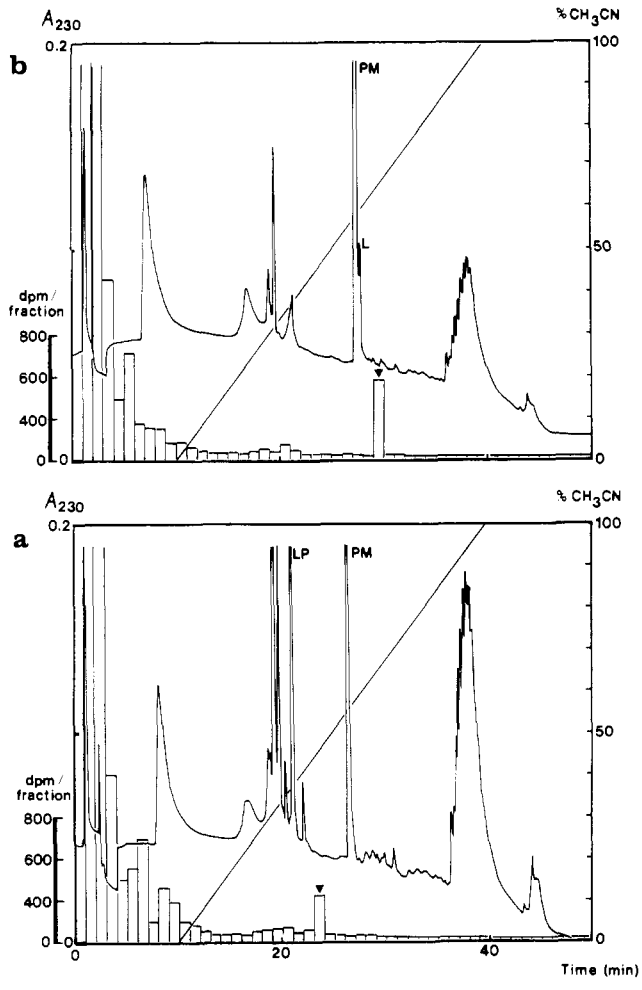

FIGURE 2: Analysis of reductive acetylation by reverse-phase HPLC. Reaction mixtures containing sodium [2-^{14}C]pyruvate (0.26 mM), TPP (0.20 mM), and $MgCl_2$ (1.0 mM) were incubated with E1p (0.30 μM in subunits), *N*-phenylmaleimide (0.1 mM), and either (a) lipoyl peptide (0.50 mM) or (b) lipoamide (0.50 mM), as described in the text. After incubation, the mixtures were applied to a reverse-phase HPLC column (Varian, MCH-5-n-CAP, 15 × 0.4 cm, flow rate 1.0 mL/min, fraction volume 1.0 mL, 20 °C) equilibrated with 10 mM ammonium acetate buffer, pH 5.0. The column was developed in an acetonitrile gradient, and the radioactivity in each fraction of effluent was measured by scintillation counting of a 0.5-mL sample. LP, lipoyl peptide; PM, *N*-phenylmaleimide; L, lipoamide. The radioactive peak attributed in each case to the *S*-acetyldihydrolipoyl product is marked (▼). The small radioactive peak at $t_R \sim 20$ min was also found in control incubations lacking lipoyl peptide and lipoamide, and was presumed to be acetoin or acetolactate produced by the action of E1p on the pyruvate (Sanadi, 1963).

acetyldihydrolipoyl peptide, which would react with the maleimide to form a more hydrophobic adduct. This adduct would be more stable than the underivatized thioester, owing to its inability to undergo ring closure.

Similar results were obtained when lipoamide (0.5 mM) was used in place of the lipoyl peptide: a radioactive product absent from control incubations was found to elute close to lipoamide. It too was present in greater quantity and eluted at a higher concentration of acetonitrile when *N*-phenylmaleimide was added to the incubation mixture, and was thus presumed to be *S*-[1-^{14}C]acetyldihydrolipoamide (Figure 2b).

Comparison of the size of the radioactive peak attributed to the maleimide adduct of *S*-[1-^{14}C]acetyldihydrolipoyl peptide with that attributed to the same adduct of *S*-[1-^{14}C]acetyldihydrolipoamide indicated that reductive acetylation of the lipoyl peptide was occurring at a rate comparable with that of lipoamide. The formation of product was calculated

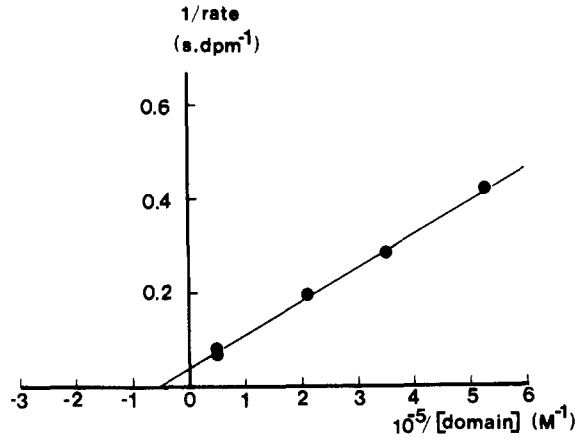

FIGURE 3: Dependence of the rate of reductive acetylation of E2p lipoyl domain mixture upon total domain concentration. Reaction mixtures containing E1p, TPP, $MgCl_2$, sodium [2-^{14}C]pyruvate, and various concentrations of lipoyl domain mixture were incubated as described under Materials and Methods. At each domain concentration tested, the rate of reductive acetylation was linear at least up to 100 s. A double-reciprocal plot of the results is shown. Best estimates of K_m and k_{cat} were determined by a direct linear plot (not shown).

to occur at rates of 8.2×10^{-5} and 14.3×10^{-5} mol·s^{-1} per mole of E1p subunit for the lipoyl peptide and for lipoamide, respectively.

Lipoyl Peptide as a Substrate for E3. Lipoamide and dihydrolipoamide are substrates for E3 and can be coupled with the oxidation of NADH and the reduction of NAD^+, respectively, under standard assay conditions (Danson & Perham, 1976). Similar experiments in which the concentration of NAD^+ was monitored at 340 nm revealed that reduction of the lipoyl peptide was catalyzed by E3 in the presence of NADH, and that oxidation of the dihydrolipoyl peptide was catalyzed by the same enzyme when NAD^+ was used. In each case, the result was similar to that obtained with the oxidized or reduced forms of lipoamide (data not shown).

Reductive Acetylation of Lipoyl Domains by E1p and E1o. Reductive acetylation of the mixture of E2p lipoyl domains by E1p was studied by measuring the kinetics of incorporation of radioactivity from sodium [2-^{14}C]pyruvate. The dependence of the reductive acetylation upon total domain concentration was investigated and was found to conform to the Michaelis–Menten model (Figure 3). Many preparations of lipoyl domain mixture, some showing evidence of further degradation by the proteinase, were characterized. When the kinetic constants were estimated from direct linear plots (Eisenthal & Cornish-Bowden, 1974; Wharton & Eisenthal, 1981), the apparent values of K_m were in the range 10–130 μM, and those for k_{cat} were between 0.7 and 5.8 s^{-1}. The constants derived from the results shown in Figure 3 (K_m = 26 μM, k_{cat} = 0.8 s^{-1}) may, however, be considered typical of undegraded domain mixtures. Kinetic determinations made at lower concentrations of sodium [2-^{14}C]pyruvate yielded results identical with those shown in Figure 3, and it was found that concentrations of this substrate as low as 0.14 mM were still saturating. In consequence, the values of k_{cat} and K_m derived from Figure 3 represent true values for the kinetic constants. These results lead to a value for k_{cat}/K_m of 3.0×10^4 M^{-1} s^{-1}.

Similar experiments were also carried out with the lipoyl domain of the E2o chain of the *E. coli* 2OGDH complex. It was found to be capable of being reductively acylated by the E1o component of the parent complex in the presence of 2-oxoglutarate (Table I). Thus, it is clear that reductive acylation in the presence of the relevant 2-oxo acid is a general

Table I: Cross-Acylation of Lipoyl Domains from Each 2-Oxo Acid Dehydrogenase Complex by the E1 Component of the Complementary Complex[a]

incubation (20 min at 25 °C)	mol of [^{14}C]acyl group/mol of lipoyl domain
E1p, LpV1, pyr	0.66
E1p, LpV2, pyr	0.57
E1p, LpV3, pyr	0.81
E1p, LoT1, pyr	0.01
E1o, LoT1, 2-oxog	0.69
E1o, LpV1, 2-oxog	0.08
E1o, LpV2, 2-oxog	0.05
E1o, LpV3, 2-oxog	0.08

[a] pyr, sodium [2-^{14}C]pyruvate; 2-oxog, sodium [5-^{14}C]-2-oxoglutarate.

property of the excised lipoyl domains of 2-oxo acid dehydrogenase multienzyme complexes.

Cross-Acylation of Lipoyl Domains. The lipoyllysine residue that becomes reductively acylated is of course common to all lipoyl domains, and it was therefore of interest to investigate the specificity of reductive acylation. This was achieved by measuring the reductive acetylation of the E2o domain by E1p in the presence of [2-^{14}C]pyruvate, and the reductive succinylation of the E2p domains by E1o in the presence of [5-^{14}C]-2-oxoglutarate.

The results of these cross-acylation experiments are also shown in Table I. Reductive acylation of each lipoyl domain by its cognate E1 component and natural substrate had reached its limit within 20 min. When the lipoyl domain of E2o was presented to E1p, acetylation by [2-^{14}C]pyruvate was barely detectable within 20 min, reaching a level of only 1–2% of that achieved when succinylation occurred by incubation with E1o and [5-^{14}C]-2-oxoglutarate for the same time. Similar results were obtained when a shorter version of the E2o lipoyl domain, LoVb—representing residues 1–85 of the E2o chain (Packman & Perham, 1987)—was substituted for LoT1 or when intact 2OGDH complex was substituted for the E1o–E2o(truncated) subcomplex. On the other hand, the lipoyl domains of E2p were found to be substrates, albeit poor ones, for E1o and [5-^{14}C]-2-oxoglutarate, reaching a level of acylation in 20 min equivalent to 9–12% of that observed when the same domains were treated with E1p and [2-^{14}C]pyruvate for the same time. Thus the lipoyl domains of E2p are somewhat better substrates for E1o than is the lipoyl domain of E2o for E1p, although none is particularly effective in the heterologous reaction. This is consistent with the ability of pyruvate to act as a poor substrate for a hybrid 2OGDH complex containing some E1p component (Steginsky et al., 1985).

Discussion

The experiments described in this paper show that free lipoamide can in fact be reductively acetylated by purified E1p, although the reaction is slow. Reaction rates of 4.8×10^{-5} to 14.3×10^{-5} mol·s^{-1} per mole of E1p subunit were obtained with lipoamide at 0.5 mM and pyruvate at 0.26 mM. On the other hand, the lipoyl domains excised from E2p by limited proteolysis appear to be much better substrates. Thus, with a mixture of the three lipoyl domains in place of lipoamide under the same conditions, reductive acetylation proceeded at about 0.8 mol·s^{-1} per mole of E1p subunit. Under these conditions, therefore, lipoamide becomes reductively acetylated about 8000 times more slowly than a free lipoyl domain. No saturation of the enzymic reaction was observed at concentrations of lipoamide up to 3.7 mM (Figure 1), close to the solubility limit of this compound, indicating that the K_m for free lipoamide must be much larger than this. In contrast, a typical K_m value for purified lipoyl domains was much lower, about 26 μM (Figure 3); the typical value of k_{cat} was about 0.8 s^{-1}.

The apparent K_m reported by Bleile et al. (1981) for the lipoyl domain from ox heart PDH complex is 18.4 μM, and the apparent V_{max} corresponds to a k_{cat} value of 4.3 s^{-1} for the E1$\alpha\beta$ subunit pair. Likewise, Packman et al. (1984b) obtained an apparent K_m of 4.3 μM for the lipoyl domains from *B. stearothermophilus* PDH complex. The apparent V_{max} reported in the latter case corresponds to an apparent k_{cat} of 5.5×10^{-2} s^{-1} for the E1$\alpha\beta$ subunit pair, but this low value probably reflects the fact that the incubation temperature used (23 °C) was well below the optimum (60 °C) observed for this enzyme from a thermophilic bacterium (Stanley et al., 1981). From these values, the ratio k_{cat}/K_m can be calculated to be 2.3×10^5 M^{-1} s^{-1} for the lipoyl domains from ox heart PDH complex and 1.3×10^4 M^{-1} s^{-1} for the lipoyl domain from the *B. stearothermophilus* complex. As described above, a typical value of k_{cat}/K_m for lipoyl domains from the *E. coli* PDH complex acting as substrates for pure E1p is 3.0×10^4 M^{-1} s^{-1}, from which it may be concluded that each type of lipoyl domain is comparable in its effectiveness as a substrate for the E1 enzyme with which it is found associated naturally in a PDH complex.

These values of k_{cat}/K_m should be compared with that of 1.5 M^{-1} s^{-1} measured for free lipoamide acting as a substrate for *E. coli* E1p. The very large increase (~20 000-fold) in k_{cat}/K_m when the lipoyl group is incorporated into the lipoyl domains of the *E. coli* PDH complex indicates the great improvement that ensues in its effectiveness as a substrate for the E1p component, a fact that must be taken into account in any consideration of the mechanism of oxidative decarboxylation. It is probable that lipoyl domains have a greater affinity for E1p than does lipoamide, but it is by no means certain that this will prove to be a complete explanation.

As a substrate for E1p, the lipoylated synthetic decapeptide corresponding to the lipoyl-bearing region of the E2 subunit behaved as poorly as lipoamide itself. Evidently the amino acid sequence immediately surrounding the lipoyl-bearing residue is insufficient to generate proper interaction with the E1p active site. Thus, a simple local electrostatic attraction is probably not the main feature of whatever interaction enables E1p to catalyze efficient reductive acetylation of intact lipoyl domains. However, a decapeptide is not large enough to support much secondary structure in aqueous solution (Schellman & Schellman, 1964), and so the lipoyl decapeptide could well differ in conformation from the corresponding sequence within an intact lipoyl domain. The most likely explanation remains that a structural interaction of some kind between the enzyme and the lipoyl domain is responsible for the improvement in catalysis. A detailed study of the structure of the lipoyl domains and of their interaction with E1p will be necessary to solve this question.

The E1 subunits of the 2-oxo acid dehydrogenase complexes are specific for their particular 2-oxo acid substrates, with the exception of the PDH complex of *Bacillus* spp, which appears to serve also as a branched-chain 2-oxo acid dehydrogenase complex (Lowe et al., 1983). As shown in Table I, this specificity extends also to the lipoyl domains, which act as second substrates for the E1 subunits. Thus, the protein domain in which the lipoyllysine residue is housed confers considerable specificity on the lipoyl group as a substrate for the cognate

E1 component, despite the sequence homology between the E2p and E2o lipoyl domains (Stephens et al., 1983; Spencer et al., 1984). These observations are also consistent with the idea that a specific molecular interaction between the lipoyl domain and the E1 component is a necessary part of the mechanism of reductive acylation.

It is clear from Table I that full reductive acylation of the E2p or E2o lipoyl domains could not be achieved despite the prolonged incubation with the appropriate E1 component and substrate. All three E2p lipoyl domains (LpV1, LpV2, and LpV3) were active when incubated separately with E1p and [2-^{14}C]pyruvate, but their acylation, like that of the E2o lipoyl domain, was restricted to ca. 0.6–0.8 mol of acyl group/mol of domain. This is strikingly reminiscent of the results obtained with the intact *E. coli* PDH complex. Although there are three lipoyl domains per E2p chain in this complex, a total of only 1.7–2.0 acetyl groups per E2p chain can be incorporated when the complex is incubated with [2-^{14}C]pyruvate in the absence of CoA [reviewed by Packman et al. (1984a)]. The reason for this discrepancy is unknown, but it will clearly repay further study.

It is interesting to compare the kinetics of the overall reaction catalyzed by *E. coli* PDH complex with those observed for reductive acetylation of the isolated lipoyl domains by E1p. If a chain ratio (E1p:E2p:E3) of 1.3–1.5:1.0:0.5–0.7 is assumed (Packman et al., 1984a), a specific catalytic activity of ~30 μmol of NAD^+ min^{-1}·(mg of complex)$^{-1}$ (Reed, 1974; Danson et al., 1979) corresponds to a catalytic-center activity of ca. 80–100 s^{-1} for reductive acetylation of the lipoyl domains by E1p in the complex. This is known to be the rate-limiting step in the mechanism (Danson et al., 1978; Akiyama & Hammes, 1980). The apparent K_m value for pyruvate measured in the same reaction is about 0.4 mM (Danson et al., 1978; Akiyama & Hammes, 1980; Bisswanger, 1981). In contrast, reductive acetylation of the proteolytically excised lipoyl domains by purified E1p proceeds much more slowly, with a k_{cat} value of ~0.8 s^{-1} (see above). Moreover, pyruvate at 0.14 mM was sufficient to saturate E1p as a substrate for this reaction, indicating a K_m value much lower than this.

The experimental conditions used to measure the two reactions are of course not quite the same: CoA, NAD^+, and cysteine were of necessity included in the assay of overall complex activity, and there was a difference of 10 °C in assay temperature. However, assuming that the proteolysis of the complex required to excise the lipoyl domains has not substantially damaged them as potential substrates, it would appear that some fundamental rate constants for the E1p-catalyzed step in the overall complex reaction are different from their counterparts in the reductive acetylation of free lipoyl domains by isolated E1p. This may well be due to tethering the lipoyl domains in the vicinity of complex-bound E1p, given that studies with artificial electron acceptors have failed to detect differences between free and complex-bound E1p in terms of activity (Papadakis & Hammes, 1977) or the dependence of the reaction on substrate concentration, temperature, and pH (Saumweber et al., 1981). Similarly, experiments involving the reductive acetylation of free lipoyl domains by purified and by complex-bound E1p suggest that the kinetics of the reaction are not affected by the binding of E1 to core (L. D. Graham and R. N. Perham, unpublished results). The importance of the tethering of the lipoyl domains is vividly clear from the loss of PDH complex activity which accompanies release of the lipoyl domains by proteolysis and which occurs without damage to the three part-reactions catalyzed by the pheripheral subunits and residual E2p core (Packman & Perham, 1986; Radford et al., 1987).

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Registry No. PDH, 9014-20-4; 2OGDH, 9031-02-1; E2p, 9032-29-5; E2o, 9032-28-4; E1p, 9001-04-1; E1o, 37205-42-8; lipoamide, 940-69-2; lipoylated decapeptide, 118375-52-3; decapeptide, 118355-84-3.

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Fluorinated and Deoxygenated Substrates as Probes of Transition-State Structure in Glycogen Phosphorylase†

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ABSTRACT: A series of deoxyfluoro- and deoxy-α-D-glucopyranosyl phosphates have been tested as substrates of rabbit muscle glycogen phosphorylase *b*. All are found to be utilized by the enzyme, but at substantially reduced rates. Values of V_m/K_m for these analogues range from 10^2 to 10^5 times lower than that for the parent substrate. The large rate reductions are suggested to arise from a combination of intrinsic electronic effects and poorer binding of these substrates at the transition state. The data provide substantial evidence for an oxocarbonium-ion-like transition state. They also provide estimates of the strengths of hydrogen bonds to individual sugar hydroxyls at the transition state of the reaction. Further, comparison of such data with those obtained for glucose analogues binding as inhibitors to T-state phosphorylase suggests that these two glucose subsites are essentially identical; thus, the glucose pocket remains intact during the conformational transition associated with activation of the enzyme.

Glycogen phosphorylase (EC 2.4.1.1) catalyzes the reversible phosphorolysis of glycogen, producing glucose-1-P[1] (Graves & Wang, 1972; Fletterick & Madsen, 1980). The activity of this enzyme is modulated by, among many other effectors, glucose. The binding of glucose stabilizes an inactive T-state conformation of the enzyme that has been shown by a variety of techniques (Helmreich et al., 1967; Wang et al., 1965; Withers et al., 1979) to be different from the active R-state conformation induced by substrates and activators. The structure of the enzyme in this T-state conformation and its interactions with glucose are well understood, as the three-dimensional structure of the phosphorylase *a*–glucose complex has been determined by X-ray crystallography (Sprang & Fletterick, 1979; Sprang et al., 1982). The

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[1] Abbrevations: glucose-1-P, α-D-glucopyranosyl phosphate; deoxyglucose-1-P, deoxy-α-D-glucopyranosyl phosphate; fluoroglucose-1-P, deoxyfluoro-α-D-glucopyranosyl phosphate; DTT, dithiothreitol.

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