Jencks, W. P., & Regenstein, J. (1970) in *CRC Handbook of Biochemistry, Selected Data for Molecular Biology*, pp J187–J226, Chemical Rubber Co., Cleveland, OH.
Jencks, W. P., Cordes, S., & Carriuolo, J. (1960) *J. Biol. Chem. 235*, 3608.
Koike, M., & Reed, L. J. (1960) *J. Biol. Chem. 235*, 1931.
Maldonado, M. E., Oh, K., & Frey, P. A. (1972) *J. Biol. Chem. 247*, 2711.
O'Connor, T. P., Roche, T. E., & Paukstelis, J. V. (1982) *J. Biol. Chem. 257*, 3110.
Redcliffe, A. H. (1950) *R. Aust. Chem. Inst. J. Proc. 17*, 205.
Reed, L. J., & Willms, C. R. (1966) *Methods Enzymol. 9*, 247.
Reed, L. J., & Mukherjee, R. B. (1969) *Methods Enzymol. 13*, 55.
Sanner, T., & Pihl, A. (1962) *Biochim. Biophys. Acta 62*, 171.
Speckhard, D. C., & Frey, P. A. (1975) *Biochem. Biophys. Res. Commun. 62*, 614.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923.

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

Susan E. Hamilton,‡ Michael Recny,§ and Lowell P. Hager*
Roger Adams Laboratory, Department of Biochemistry, University of Illinois, Urbana, Illinois 61801
Received June 30, 1986; Revised Manuscript Received August 27, 1986

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

† This research was supported in part by a grant from the National Institutes of Health (GM 07768).
* Correspondence should be addressed to this author.
‡ On leave from the Department of Biochemistry, University of Queensland, St Lucia, Qld, Australia 4067.
§ Present address: Genetics Institute, Cambridge, MA 02140.

[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.

0006-2960/86/0425-8178$01.50/0

presence of pyruvate, TPP, and divalent metal ions, α-chymotrypsin catalyzes the cleavage of a 23-residue peptide (the "α-peptide") from the carboxyl terminus of each subunit (Recny & Hager, 1983; Recny et al., 1985). The increase in k_{cat} and decrease in K_m after removal of the α-peptide are quantitatively similar to the corresponding changes in these kinetic constants in the presence of lipid. The protease-activated enzyme binds lipids less effectively than the native enzyme and is not further activated by lipids. Further, the lipid-activated enzyme is much less susceptible to protease digestion at the α-peptide site than is the native enzyme (Russell et al., 1977b). The foregoing observations imply a close relationship between the mechanisms of activation of pyruvate oxidase by lipid and by protease digestion.

The aim of the present investigation has been to identify the lipid binding site associated with lipid activation of pyruvate oxidase. Previous work in this laboratory has established a procedure for the covalent attachment of a small number of molecules of [^{14}C]lauric acid to each pyruvate oxidase tetramer, using the water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Leisman et al., 1985). The covalently labeled enzyme is essentially "irreversibly" activated. In the present study, we have used this [^{14}C]lauric acid labeled enzyme to locate the lipid binding site associated with lipid activation.

Materials and Methods

Materials. TPP, sodium pyruvate, EDC, and bovine pancreatic trypsin were obtained from Sigma Chemical Co. SDS, trifluoroacetic acid, phenyl isothiocyanate, and constant-boiling HCl (all Sequanal grade) and PTH-amino acid standards were obtained from Pierce Chemical Co. Glycerol was obtained from Fisher Scientific. Sodium ferricyanide was obtained from ICN Pharmaceuticals, Inc. [^{14}C]Lauric acid (36 mCi/mmol, 2.75 mM, in hexane) and [^{14}C]toluene, for use as an internal standard, were obtained from Amersham. Unlabeled and carbon-14-labeled low molecular weight protein standards for polyacrylamide–SDS gel electrophoresis were obtained from Bethesda Research Laboratories, Inc. Other reagents for gel electrophoresis were obtained from Bio-Rad. Photographic film for fluorography (X-Omat RP) and GBX developer were Eastman Kodak products. Organic solvents for HPLC were Baker analyzed reagents, and HPLC-grade water was prepared by redistillation from alkaline potassium permanganate. All other reagents were analytical grade.

Preparation of Labeled Enzyme. Pyruvate oxidase was purified from an *E. coli* strain (CG3) harboring a plasmid carrying the oxidase gene of *E. coli* using established procedures (Recny & Hager, 1982; Recny et al., 1985). The active-site concentration of the purified enzyme was determined by measuring the decrease in absorbance at 438 nm, the λ_{max} of the oxidized flavin (Williams & Hager, 1966), upon flavin reduction, using $\Delta\epsilon_{438} = 10\,844\ M^{-1}\ cm^{-1}$. [^{14}C]Lauric acid labeled enzyme was prepared by a slight modification of the previously reported method (Leisman et al., 1985). Pyruvate oxidase was reduced by dilution of the enzyme into Pipes buffer, pH 6.0, containing TPP, Mn^{2+}, pyruvate, and glycerol at the following final concentrations: enzyme, 4 μM; Pipes, 0.1 M; pyruvate, 0.2 M; TPP, 0.01 M; Mn^{2+}, 0.2 mM; glycerol, 20% (v/v). The reduced enzyme was equilibrated for 5 min with [^{14}C]lauric acid (26 mCi/mmol, in Me_2SO) at the following final concentrations: lauric acid, 0.29 mM; Me_2SO, 8% (v/v). After 5 min, the solution was noticeably turbid due to aggregation of the lipid-activated enzyme. An aliquot of freshly prepared EDC in water was added to give a final EDC concentration of 4.0 mM, and aliquots (10 μL) of the reaction were withdrawn at 1–2-min intervals for assays of enzyme activity and incorporated radioactivity. After a 3-min equilibration of enzyme, lauric acid, and EDC, an aliquot of freshly prepared hydroxylamine solution, pH 6.2 (final concentration 0.2 M), was added to stop further reaction. Enzyme activity was assayed in the *absence* of SDS as previously described (Blake et al., 1978). In these assays, the free lauric acid concentration was only ~1% of the concentration required to fully activate the enzyme through noncovalent binding. Incorporated radioactivity was quantified by precipitation of labeled protein with 5% (w/v) trichloroacetic acid followed by filtration on a 0.45-μm membrane (type HAWP, from Millipore Corp.). Radioactivity was quantified by liquid scintillation counting using [^{14}C]toluene as an internal standard.

This preparation of labeled enzyme was used for mild tryptic digestion without further purification but was subjected to gel filtration in SDS prior to thermolysin digestion.

Mild Tryptic Digestion of Labeled Enzyme. Previous studies have shown that when the oxidized form of pyruvate oxidase is subjected to mild tryptic digestion in the *absence* of TPP, a peptide containing 100 amino acids (the "β-peptide") is removed from the carboxyl terminus of the protein, and the enzyme is inactivated (Russell et al., 1977a). In the present work, the lauric acid labeled enzyme was therefore first subjected to mild tryptic digestion in the absence of TPP to ascertain whether the bound lipid was contained within this β-peptide region of the protein. To facilitate the removal of noncovalently bound TPP, an aliquot of EDTA (final concentration 0.02 M) was added to the preparation to chelate Mn^{2+} ions which are essential for the binding of TPP to the apoenzyme. Trypsin (50 μg/mL) was then added to the labeled enzyme (0.15 mg/mL) and the digest equilibrated at 25 °C. Samples were removed 30 and 60 min thereafter and subjected to electrophoresis (12 h, 100 V, 20 °C) in 15% polyacrylamide gels containing 6 M urea and 0.1% (w/v) SDS (Bethesda Research Laboratories, 1981; Shapiro et al., 1967). Gels were stained for protein with Coomassie Blue or subjected to fluorography essentially as described previously (Chamberlain, 1979).

The unlabeled β-peptide was isolated by HPLC from a mild tryptic digest of pyruvate oxidase. The digest was chromatographed first on a TSK 3000 SW column (Altex) to remove trypsin from the digested enzyme. The β-peptide remained associated with the 51 000-dalton fragment of the protein under the nondenaturing conditions used for this chromatography. The digested protein was then chromatographed on a reverse-phase C18 column. [Solvent A was 5% formic acid in H_2O; solvent B was 5% formic acid in ethanol. A gradient from 0% to 80% solvent B and a flow rate of 1 mL/min were used.] Under these conditions, the inactive 51 000-dalton fragment and the β-peptide were cleanly separated. The β-peptide fraction was identified by SDS–PAGE.

Thermolysin Digestion of Labeled Enzyme. The labeled enzyme preparation was solubilized by the addition of 1% (w/v) SDS. The preparation was purified by HPLC on a TSK 3000 SW column equilibrated at room temperature with 0.1 M phosphate buffer, pH 5.80, containing 0.1% (w/v) SDS and 5% (w/v) glycerol, at a flow rate of 0.75 mL/min. Pooled fractions containing the monomeric labeled enzyme were concentrated to 0.5 mg/mL at 15 °C in a Centricon-30 microconcentrator (Amicon Corp.). Prior to exhaustive thermolysin digestion, the enzyme was dialyzed into 0.01 M Tris buffer, pH 8.0, containing 0.1% (w/v) SDS and 5% (w/v) glycerol. Virtually quantitative recovery (≥90%) of labeled

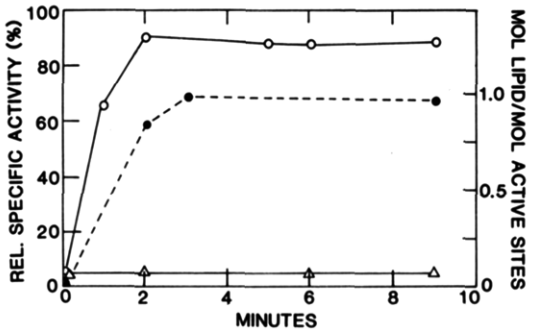

FIGURE 1: Incorporation of [^{14}C]lauric acid into the lipid activation site of pyruvate oxidase. Pyruvate oxidase was equilibrated with [^{14}C]lauric acid and EDC or with lauric acid alone as described under Materials and Methods. Aliquots (10 μL) were withdrawn and assayed for enzyme activity at the times indicated. Specific activities are expressed as a percentage of the specific activity of the unmodified enzyme assayed in the presence of 20 μM SDS: (○) specific activity of the enzyme equilibrated with lauric acid and EDC; (△) specific activity of the enzyme equilibrated with lauric acid alone. Incorporated radioactivity was determined at the times indicated and was corrected for the low background radioactivity incorporated in the absence of EDC. The concentration of active enzyme was determined prior to reaction with lauric acid and EDC, as described under Materials and Methods; (●) moles of lauric acid bound per mole of active sites.

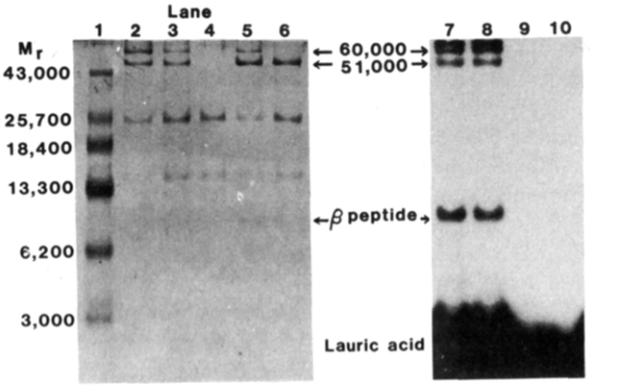

FIGURE 2: Polyacrylamide–SDS gel electrophoresis of mild tryptic digest of lauric acid labeled and unlabeled pyruvate oxidase. Electrophoresis was carried out as described under Materials and Methods. Lane 1, low molecular weight protein standards; lanes 2 and 7, 30-min digest of pyruvate oxidase equilibrated with [^{14}C]lauric acid and EDC; lanes 3 and 8, 90-min digest of pyruvate oxidase equilibrated with [^{14}C]lauric acid and EDC; lane 4, trypsin alone; lanes 5 and 9, 30-min digest of pyruvate oxidase equilibrated with [^{14}C]lauric acid (no EDC); lanes 6 and 10, 90-min digest of pyruvate oxidase equilibrated with [^{14}C]lauric acid (no EDC). Lanes 1–6 were stained with Coomassie Blue; lanes 7–10 were subjected to fluorography. The band of radioactivity of lowest mobility in lanes 7 and 8 corresponds to the top of the gel.

protein was obtained during all of the above procedures. Thermolysin (final concentration 40 μg/mL) and calcium acetate (final concentration 2 mM) were added to the concentrated, labeled protein, and the digestion was allowed to proceed for 20 min at 25 °C (mild digestion) or for 16 h at 37 °C (exhaustive digestion). Digests were chromatographed on a 10-μm Vydac C4 reverse-phase HPLC column (4.6 × 250 mm) at a flow rate of 0.75 mL/min [solvent A, 0.1% (v/v) trifluoroacetic acid in water; solvent B, 0.1% (v/v) trifluoroacetic acid in acetonitrile/2-propanol (2:1 v/v)]. A linear gradient from 10% solvent B to 75% solvent B over 75 min was employed. Fractions containing carbon-14 were rechromatographed twice on a 10-μm Vydac C4 column (4.6 × 150 mm) at 0.75 mL/min. Solvents A and B were as above. A linear gradient from 35% solvent B to 45% solvent B over 30 min was employed.

Analysis of Purified Peptides. Amino acid analysis was performed by using the Waters Pico-Tag system. PTC-amino acids were analyzed on a 5-μm Altex Ultrasphere–ODS column (4.6 × 250 mm) at 45 °C, at a flow rate of 1 mL/min [solvent A, 12 mM sodium acetate, pH 7.0; solvent B, 15% (v/v) acetonitrile in methanol]. The following gradient was employed: 5% B at 0 min, to 20% B at 1 min, to 27% B at 5 min, to 34% B at 9 min, to 44% B at 12 min, to 54% B at 15 min. Sequencing of ~1 nmol of the unlabeled β-peptide and 100–300 pmol of the ^{14}C-labeled thermolysin peptides was performed by using an Applied Biosystems gas-phase sequenator. PTH–amino acids were analyzed on a 5-μm Altex Ultrasphere–ODS column (4.6 × 250 mm) at 45 °C at a flow rate of 1.5 mL/min. Solvents A and B were the same as those used for PTC-amino acid analysis. The following gradient was employed: 10% B at 0 min, to 18% B at 0.5 min, to 27% B at 4.5 min, to 34% B at 8.5 min, to 44% B at 9.5 min, to 55% B at 24.5 min. In the analysis of the radioactive peptides, each of the PTH derivatives released in the sequencing reaction was separated into two fractions: one fraction was subjected to PTH–amino acid analysis, and the other was assayed for radioactivity.

RESULTS

Preparation of the Labeled Enzyme. When substrate-reduced pyruvate oxidase was equilibrated with [^{14}C]lauric acid in the presence of EDC, the specific activity of the enzyme increased within 3 min to a value which was 90% of the specific activity of the unmodified enzyme assayed in the presence of 20 μM SDS. When either lauric acid or EDC was omitted from the reaction, the specific activity remained the same as the specific activity of the unactivated enzyme (Figure 1). In the presence of lauric acid and EDC, the radioactivity incorporated into protein increased within 3 min to a value corresponding closely to 1 mol of lauric acid per mole of active sites (Figure 1).

Mild Tryptic Digestion of the Labeled Enzyme. As noted above (Materials and Methods), a preliminary experiment was performed to determine whether covalently bound lauric acid was located within the β-peptide region of pyruvate oxidase. The labeled enzyme was subjected to mild tryptic digestion in the absence of bound TPP. SDS–polyacrylamide gel electrophoresis of the digest (Figure 2) showed that the labeled enzyme was substantially degraded within 90 min to a 51 000-dalton fragment and the β-peptide (lanes 2 and 3). The unlabeled enzyme was similarly degraded (lanes 5 and 6). (The β-peptide in both cases is barely visible in photographs of Coomassie Blue stained gels but was clearly visible in the original.) The major fraction of covalently bound [^{14}C]lauric acid was located within the β-peptide rather than the 51 000-dalton fragment (lanes 7 and 8), and, as expected, there was no label associated with any protein or peptide fraction in digests of enzyme which had been equilibrated with [^{14}C]lauric acid in the absence of EDC (lanes 9 and 10). The band of radioactivity just above the 60 000-dalton band corresponds to the top of the gel and is reasonably attributed to a fraction of enzyme which is cross-linked by EDC to form high molecular weight aggregates. Evidence for the presence of such aggregates was also obtained upon HPLC gel filtration of the modified enzyme in the presence of SDS (see Figure 4).

Isolation and Sequencing of the β-Peptide. The unlabeled β-peptide was readily purified by reverse-phase HPLC. The first 25 residues of the peptide were sequenced as a contiguous stretch. The complete sequence of pyruvate oxidase has been determined elsewhere from the DNA sequence of the pyruvate oxidase gene (Grabau & Cronan, 1986). There was complete

```
416          420
LEU GLY ALA GLN ALA THR GLU PRO GLU ARG GLN VAL VAL ALA MET CYS GLY ASP GLY GLY PHE SER MET LEU MET GLY ASP PHE LEU SER
                                                                                                    472
VAL VAL GLN MET LYS LEU PRO VAL LYS ILE VAL VAL PHE ASN ASN SER VAL LEU GLY PHE VAL ALA MET GLU MET LYS↑ALA GLY GLY TYR
                                                                                  496
LEU THR ASP GLY THR GLU LEU HIS ASP THR ASN PHE ALA ARG ILE ALA GLU ALA CYS GLY ILE THR GLY ILE ARG VAL GLU LYS ALA SER

GLU VAL ASP GLU ALA LEU GLN ARG ALA PHE SER ILE ASP GLY PRO VAL LEU VAL ASP VAL VAL VAL ALA LYS GLU GLU LEU ALA ILE PRO
                              543 544                    550            554
PRO GLN ILE LYS LEU GLU GLN↑ALA LYS* GLY PHE SER LEU TYR↑MET LEU ARG ALA ILE↓ILE SER GLY ARG GLY ASP GLU VAL ILE GLU LEU
                          572
ALA LYS THR ASN TRP LEU ARG
```

FIGURE 3: Amino acid sequence of the carboxyl-terminal domain of pyruvate oxidase. The primary sequence of pyruvate oxidase was determined by Grabau and Cronan (1986). The first 25 residues of the tryptic β-peptide (Ala-472–Ile-496) and the first 5 residues of the 15 000-dalton thermolysin peptide (Leu-416–Ala-420) were determined as described in the present work. The start of the chymotryptic α-peptide (Met-550) was determined previously (Recny et al., 1985). The residues underlined (Ala-543–Ile-554) correspond to peptide A from the exhaustive thermolysin digest of the lauric acid labeled enzyme. Lys-544 (marked with an asterisk) is the residue which is labeled with lauric acid in peptide B.

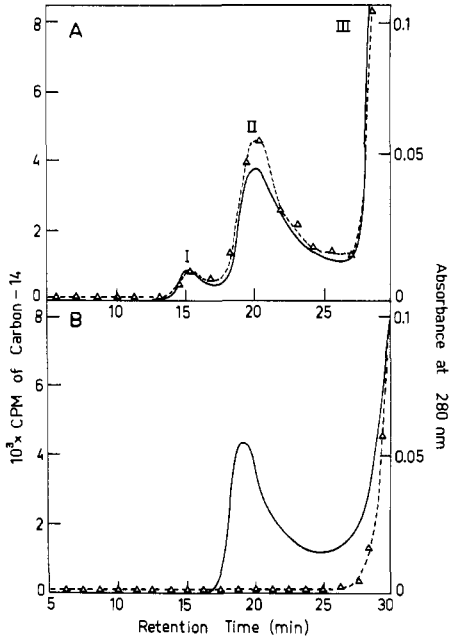

FIGURE 4: Purification of lauric acid labeled pyruvate oxidase. Enzyme was equilibrated with [^{14}C]lauric acid in the presence or absence of EDC for 3 min. The reaction was quenched with hydroxylamine, and the labeled preparation was solubilized by addition of 1% (w/v) SDS. The preparation was immediately chromatographed on a TSK 3000 SW Spherogel column in the presence of 0.1% (w/v) SDS. Aliquots (20 μL) of each fraction (750 μL) were assayed for radioactivity. (—) A_{280}; (▲) radioactivity. (A) Enzyme equilibrated with lauric acid and EDC; (B) enzyme equilibrated with lauric acid (no EDC). The peaks were identified as follows: peak I, labeled pyruvate oxidase cross-linked by reaction with EDC; peak II, labeled pyruvate oxidase monomer; peak III, free lauric acid and TPP.

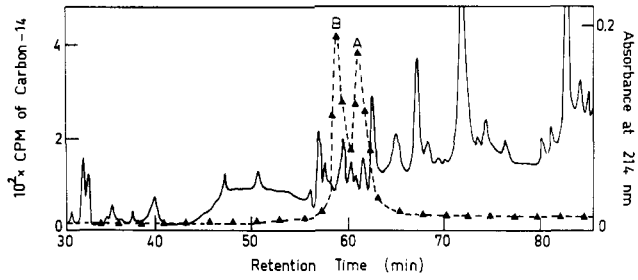

FIGURE 5: Purification of peptides from the exhaustive thermolysin digest of labeled pyruvate oxidase. Each digest contained 200 μg of purified, labeled enzyme and 20 μg of thermolysin. Chromatography of the digest on a C4 reverse-phase column (4.5 × 250 mm) employed a linear gradient in acetonitrile and 2-propanol (2:1 v/v). Radioactivity was recovered only in peaks A and B. (—) A_{214}; (▲–▲) radioactivity.

agreement between the 25-residue amino acid sequence determined in the present work and residues 472–496 of the complete sequence of Grabau and Cronan (Figure 3). The start of the β-peptide was thus unequivocally identified as Ala-472.

Location of Bound Lauric Acid within the β-Peptide Region. Whereas the unlabeled β-peptide was readily purified by HPLC in reasonable yield (~60%), the labeled peptide was recovered in very low yield (≤10%) from both C4 and C18 reverse-phase columns. The undigested labeled protein was therefore first subjected to gel filtration in the presence of 0.1% (w/v) SDS to remove free lauric acid (Figure 4). Detergent was necessary to solubilize the labeled enzyme preparation which was highly aggregated. The labeled protein (peaks I and II, Figure 4A) was cleanly resolved from free lauric acid and TPP (peak III). Peak I, which was variable in amount but always less than 20% of the total protein (A_{280}) in peaks I and II, is attributed to protein which has been cross-linked with EDC. This peak did not appear in the chromatogram of the control reaction (EDC omitted) (Figure 4B). As expected, no radioactivity coeluted with the single protein peak in the chromatogram of the control reaction containing no EDC (Figure 4B). Peak II was used for subsequent analysis of the position of the lauric acid label.

Neither trypsin nor α-chymotrypsin effectively digested the purified, labeled enzyme in the presence of 0.1% SDS. Effective digestion was achieved by thermolysin in the presence of the detergent. Mild digestion (20 min, 25 °C) resulted in the degradation of the 60 000-dalton monomer to a ~45 000- and a ~15 000-dalton fragment (results not shown). Fluorography of the corresponding SDS–polyacrylamide gel showed that lauric acid was located predominantly (>80%) on the smaller fragment. Sequence analysis of the unlabeled 15 000-dalton fragment, which was again readily isolated by reverse-phase HPLC, showed the N-terminal sequence to be Leu-Gly-Ala-Gln-Ala. Comparison of this sequence with that of the entire monomer identified the start of the thermolysin peptide as Leu-416 (Figure 3). The results support the previous finding (Figure 2) that lauric acid was bound predominantly in the carboxyl-terminal region of the protein.

The labeled 15 000-dalton peptide, like the labeled β-peptide, could not be isolated in reasonable yield by reverse-phase HPLC. However, an exhaustive thermolysin digestion of the labeled enzyme (16 h, 37 °C) produced two and only two smaller labeled peptides in approximately equal amounts, which were recovered in >90% yield from a C4 reverse-phase column (Figure 5). Radioactivity coeluted with A_{214} peaks at 59 and 61 min (peptides B and A, respectively). Free lauric acid was eluted at 66 min under the same chromatographic conditions. Rechromatography of the peak fractions twice on a smaller C4 column achieved complete separation of the two fractions.

Analysis of Thermolysin Peptides A and B. The amino acid compositions of the isolated peptides A and B are shown in Table I. A careful comparison of the analysis of peptide A (which is seen from these data to be cleaner than peptide B) with the sequence of the entire protein shows that only one

Table I: Amino Acid Composition of Peptides A and B

amino acid	residues/mol A	residues/mol B	residues/mol (Ala-543–Ile-554)[a]
Asp + Asn	0	0	0
Glu + Gln	0	0	0
Thr	0	0	0
Ser	0.96	1.0	1
Pro	0.11	0.40	0
Gly	1.29	2.40	1
Ala	2.00	2.20	2
Val	0	0	0
Met	1.14	1.50	1
Ile	0.95	0.60	1
Leu	2.15	1.60	2
Phe	0.86	1.00	1
Tyr	0.53	0.50	1
Trp	nd[b]	nd	0
Lys	0.96	1.00	1
His	0	0	0
Arg	0.63	0.81	1
Cys	nd	nd	0

[a] Calculated from the sequence shown in Figure 3. [b] Not determined.

sequence, from Ala-543 to Ile-554, is consistent with the analysis. Except for the low recoveries of Arg and Tyr, the data are in excellent agreement. Peptide A is thus located within the β-peptide sequence, as anticipated from the results of the preliminary experiments. This peptide constituted 40–60% of the total radioactivity in the original thermolysin digest. The location of peptide B within the protein sequence is more equivocal because the isolated peptide was not pure. It is nevertheless clear from the data of Table I that A and B are very similar peptides. We conclude from a detailed examination of the sequence of pyruvate oxidase that peptide B probably corresponds to a peptide which is one or two residues shorter than A at the carboxyl end. Thermolysin preferentially hydrolyzes peptide bonds to the left of Ala, Leu, Ile, and Phe, which would account for the loss of Ile-554 or Ala-553 and Ile-554 (Matsubara & Sasaki, 1968). There are no other sequences within the protein which are reasonable alternatives for peptide B, given the restricted range of amino acids in the composition of the peptide. The SDS–PAGE analysis of the tryptic digest of labeled enzyme also indicated some minor labeling of pyruvate oxidase outside the β-peptide region (Figure 2). Since no corresponding peptides were seen in the thermolysin digest, we conclude that this label is distributed nonspecifically within the 51 000-dalton fragment.

Attempts to sequence peptides A and B were severely hampered by the hydrophobic nature of the peptides, which led to extensive washout from the filter of the sequenator cartridge. In separate experiments, peptide A (100 and 200 pmol), quantified by amino acid analysis and by liquid scintillation counting, yielded only ~3% recovery of PTH–Ala in the first cycle. Peptide B (140 pmol) yielded 14 pmol of PTH–Ala in the first cycle and 9 pmol of an unidentified radioactively labeled residue in the second cycle. No further residues could be identified. The results nevertheless provide valuable support for the assignment of residue 1 in both peptides A and B as Ala-543 and suggest that in peptide B, lauric acid is bound via an amide linkage to the side chain of Lys-544. Although the position of the label in peptide A was not identified, Lys-544 appears to be the only reasonable labeling site.

Discussion

The analysis of the high-affinity lipid binding site of pyruvate oxidase has employed a modified form of the enzyme to which lauric acid is covalently and stoichiometrically attached. That lauric acid is bound to the site associated with lipid activation of the enzyme is demonstrated by the observation that the modified enzyme is fully activated in the absence of other added lipid. The solubility properties of the modified enzyme resemble those of pyruvate oxidase which has been activated through the noncovalent binding of lipids. Both are visibly aggregated even at low protein concentrations (≥0.1 mg/mL). The labeled fragments derived from the modified enzyme by mild trypsin or thermolysin digestion are apparently more hydrophobic than the corresponding unlabeled peptides, as evidenced by their failure to elute from reverse-phase HPLC columns. The small peptides isolated from the exhaustive thermolysin digest were not retained on the filter of the sequenator cartridge, a fact which is also attributable to their hydrophobicity.

The combined results of amino acid analysis and sequence analysis provide good evidence that the location of the major site of covalent attachment of lipid to pyruvate oxidase is within residues 543–554 of the protein. We conclude that this sequence forms a part of the noncovalent lipid binding site which is associated with lipid activation of the enzyme. Although the sequence of the carboxyl-terminus region of pyruvate oxidase does not exhibit the obvious hydrophobic character of some membrane-associated proteins, it is clear from model studies that amino acid sequences containing an equal distribution of hydrophobic and charged residues may bind lipids with moderate to high affinity providing the sequences are folded in an appropriate conformation (Kaiser & Kezdy, 1984).

To our knowledge, this constitutes the first report of the identification of the lipid binding site in a lipid-activated enzyme. A preliminary investigation of the phospholipid binding site of β-hydroxybutyrate dehydrogenase was reported recently (Burnett et al., 1985). This enzyme is specifically activated by phosphatidylcholine, and labeling was achieved by using a photoactivatable phospholipid which contained the carbene precursor phenyldiazirine in the head group of the phospholipid. Enzymatic activity was fully reconstituted by the synthetic phospholipid prior to photolysis, and approximately 0.15 molecule of phospholipid was incorporated per molecule of protein after photolysis. The position of the bound label has not yet been reported. The application of photoactivatable lipids to the study of lipid binding proteins has provided valuable information in a number of other systems including cytochrome b_5 (Takagaki et al., 1983), bacteriorhodopsin (Huang et al., 1982), and phospholipid-transfer protein (Westerman et al., 1983). The procedure described in the present work may provide a useful alternative method for labeling some lipid binding proteins, viz., those which are able to bind fatty acids with moderate to high affinity. The method may offer some advantages over photoaffinity labeling, particularly considering its simplicity. The present study also shows that the procedure can yield stoichiometric labeling of a protein, and in this instance at least, the covalently modified protein is activated to the same extent as it is through noncovalent binding of lipid.

The peptide identified as the major site of labeling in pyruvate oxidase (residues 543–554) overlaps the α-peptide cleavage site (between residues 549 and 550) which is hydrolyzed when the enzyme is activated by mild protease digestion. The results thus demonstrate that the activation of pyruvate oxidase by lipids and that by mild protease digestion are, as anticipated from other observations, closely related processes. We can now propose a reasonable pathway of events

leading to the activation and inactivation of pyruvate oxidase. It is apparent that both processes are associated with modifications which occur in the carboxyl-terminal domain of the enzyme. We postulate that the carboxyl-terminal tail of the enzyme covers the active site in the native oxidized form of the enzyme. The optical absorption spectrum of this form of the enzyme indicates that the active-site FAD is in a hydrophobic environment (Recny & Hager, 1983). The binding of substrate ligands and subsequent reduction of the enzyme effect a conformational change which exposes a hydrophobic region in the vicinity of the carboxyl terminus of the protein. The reduced enzyme now aggregates at relatively low protein concentrations because of the exposure of this hydrophobic site. The conformational change which occurs upon reduction also facilitates both the binding of lipids to and the proteolytic cleavage of the carboxyl tail of the enzyme. Both processes produce the same result; that is, they prevent reassociation of the tail region with the remainder of the molecule. In this form, the enzyme is activated. The optical absorption spectrum of the protease-activated form of the enzyme indicates that the FAD prosthetic group now occupies an aqueous environment. The reason for the dramatic increase in catalytic activity remains to be established, although preliminary results suggest that it is the rate of the TPP-dependent decarboxylation step which is enhanced in the activated enzyme.[2] Finally, if limited proteolysis of pyruvate oxidase is carried out in absence of TPP, a new and different enzyme conformation promotes nicking at an alternate site, and a much larger peptide, the β-peptide, is produced from the carboxyl-terminal tail. Cleavage of the β-peptide produces an inactive enzyme.

It is of interest to note that at least two other enzymes exhibit very similar activation behavior to that of pyruvate oxidase. Both phenylalanine hydroxylase and protein kinase C may be activated independently by lipid or by mild protease digestion (Abita et al., 1984; Takai et al., 1979a,b). The lipid binding site has not been located in either enzyme. Lipid activation of protein kinase C is of particular interest because it is an important event in the transmission of signals at cell membranes in a variety of systems. We hazard a prediction that in both enzymes, the fragment which is removed upon proteolytic activation contains or is very close to the lipid binding site associated with lipid activation. Some tentative evidence supporting this view has already been obtained for protein kinase C (Kikkawa et al., 1982).

ACKNOWLEDGMENTS

We are grateful to Dr. John Cronan and Charlotte Grabau for their generosity in providing the sequence data for the β-peptide of pyruvate oxidase prior to publication. We are also indebted to Saw Kyin for skillful technical assistance with sequencing.

Registry No. L-Lys, 56-87-1; pyruvate oxidase, 9079-84-9; lauric acid, 143-07-7.

REFERENCES

Abita, J.-P., Parniak, M., & Kaufman, S. (1984) *J. Biol. Chem. 259*, 14560–14566.

Bethesda Research Laboratories (1981) *Biologue 1*, 6–7.

Blake, R., Hager, L. P., & Gennis, R. B. (1978) *J. Biol. Chem. 253*, 1963–1971.

Burnett, B. K., Robson, R. J., Takagaki, Y., Radharkrishnan, R., & Khorana, H. G. (1985) *Biochim. Biophys. Acta 815*, 57–67.

Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132–135.

Cunningham, C. C., & Hager, L. P. (1971) *J. Biol. Chem. 246*, 1575–1582.

Cunningham, C. C., & Hager, L. P. (1975) *J. Biol. Chem. 250*, 7139–7146.

Grabau, C., & Cronan, J. (1986) *Nucleic Acids Res. 14*, 5549–5566.

Hager, L. P. (1957) *J. Biol. Chem. 229*, 251–263.

Huang, K.-S., Radhakrishnan, R., Bayley, H., & Khorana, H. G. (1982) *J. Biol. Chem. 257*, 13616–13623.

Kaiser, E., & Kezdy, F. (1984) *Science (Washington, D.C.) 223*, 249–257.

Kikkawa, V., Takai, Y., Minakuchi, R., Inohara, S., & Nishizuka, Y. (1982) *J. Biol. Chem. 257*, 13341–13348.

Leisman, G., Recny, M. A., White, J. S., & Hager, L. P. (1985) in *The Pharmacological Effect of Lipids II* (Kabara, J. J., Ed.) pp 111–123, American Oil Chemists Society, Champaign, IL.

Matusbara, H., & Sasaki, R. M. (1968) *J. Biol. Chem. 243*, 1732–1757.

O'Brien, T. A., Schrock, H. L., Russell, P., Blake, R., & Gennis, R. B. (1976) *Biochim. Biophys. Acta 452*, 13–29.

O'Brien, T. A., Blake, R., & Gennis, R. B. (1977) *Biochemistry 16*, 3105–3109.

Raj, T., Russell, P., Flygare, W. H., & Gennis, R. B. (1977) *Biochim. Biophys. Acta 481*, 42–49.

Recny, M. A., & Hager, L. P. (1982) *J. Biol. Chem. 257*, 12878–12886.

Recny, M. A., & Hager, L. P. (1983) *J. Biol. Chem. 258*, 5189–5195.

Recny, M. A., Grabau, C., Cronan, J. E., Jr., & Hager, L. P. (1985) *J. Biol. Chem. 260*, 14287–14291.

Russell, P., Hager, L. P., & Gennis, R. B. (1977a) *J. Biol. Chem. 252*, 7877–7882.

Russell, P., Schrock, H. L., & Gennis, R. B. (1977b) *J. Biol. Chem. 252*, 7883–7887.

Schrock, H. L., & Gennis, R. B. (1977) *J. Biol. Chem. 252*, 5990–5995.

Shapiro, A. L., Vinuela, E., & Maizei, J. B. (1967) *Biochem. Biophys. Res. Commun. 28*, 815–820.

Takagaki, Y., Radhakrishnan, R., Wirtz, K. W. A., & Khorana, H. G. (1983) *J. Biol. Chem. 258*, 9136–9142.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., Nichizuka, Y., Tamura, A., & Fujii, T. (1979a) *J. Biochem. (Tokyo) 86*, 575–578.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., & Nishizuka, Y. (1979b) *J. Biol. Chem. 254*, 3692–3695.

Westerman, J., Wirtz, K. W. A., Berkhout, T., Van Deenan, L. M. M., Radhakrishnan, R., & Khorana, H. G. (1983) *Eur. J. Biochem. 132*, 441–449.

Williams, F. R., & Hager, L. P. (1966) *Arch. Biochem. Biophys. 116*, 168–176.

[2] R. Blake and L. P. Hager, unpublished results.