PROPERTIES OF A NEWLY CHARACTERIZED PROTEIN OF THE BOVINE KIDNEY PYRUVATE DEHYDROGENASE COMPLEX

by

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ABBREVIATIONS

DTT	dithiotreitol
Elα	α subunit of pyruvate dehydrogenase component
Elβ	β subunit of pyruvate dehydrogenase component
E2	dihydrolipoyl transacetylase
E2-L	outer lipoyl bearing domain of the dihydrolipoyl transacetylase
E2-I	inner subunit binding domain of the dihydrolipoyl transacetylase
E3	dihydrolipoyl dehydrogenase
EDTA	ethylenediaminotetraacetic acid
HETPP	2-hydroxyethylthiamine pyrophosphate
K	catalytic subunit of the kinase
KGDC	α-ketoglutarate dehydrogenase complex
LTS	dihydrolipoyl transsuccinylase
Mops	(3-[N-Morpholino])propanesulfonic acid
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl flouride
TEMED	N,N,N'N',-tetramethylethylenediamine
TPP	thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminomethane
X	protein X
X' and X"	tryptic peptides derived from protein X

CHAPTER 1: INTRODUCTION

The pyruvate dehydrogenase complex is a multienzyme complex that catalyzes the overall reaction (1,2) pyruvate + CoA + NAD^+ → acetyl-CoA + CO_2 + NADH + H^+. The complex is composed of an aggregate of enzymes which are: pyruvate dehydrogenase (E1) containing the cofactor thiamine pyrophosphate (TPP); dihydrolipoyl transacetylase (E2) with covalently bound α-lipoic acid; dihydrolipoyl dehydrogenase (E3), a flavoprotein; and two regulatory enzymes, a kinase and a phosphatase (3-5). Most generally accepted for the oxidation of pyruvate is the following sequence (1,2).

1). H^+ + E1-TPP + CH_3COCOO^- → CO_2 + E1-HETPP

2). E1-HETPP + lipoyl-E2 → E1-TPP + S-acetyldihydrolipoyl-E2

3). S-acetyldihydrolipoyl-E2 + CoA ⇄ acetyl-CoA + dihydrolipoyl-E2

4). dihydrolipoyl-E2 + E3-FAD ⇄ lipoyl-E2 + dihydro-E3-FADH

5). dihydro-E3-FADH + NAD^+ ⇄ E3-FAD + NADH + H^+

The pyruvate dehydrogenase catalyzes reactions 1 and 2. Reaction 3 is catalyzed by the dihydrolipoyl transacetylase which also participates in reaction 2 (6,7) while reactions 4 and 5 are catalyzed by dihydrolipoyl dehydrogenase.

This large multienzyme complex has been shown to exhibit a number of differences in size and quaternary structure among various organisms (8,9). In eukaryotic organisms, the pyruvate dehydrogenase complex is found in the mitochondrial matrix (8). In addition, PDC isolated from E. coli appears to have a molecular weight of 4.6×10^6 while bovine kidney and heart have molecular weights of 7×10^6 and 8.5×10^6, respectively (10,11). Nevertheless, in all organisms, the E2 component

forms a core about which multiple copies of the E1, the E3, and the two regulatory enzymes are bound (8).

The core component (E2) in the bovine kidney PDC is arranged in a pentagonal dodecahedron and its design appears to be based on icosohedral symmetry. This pentagonal dodecahedron appears to be composed of 60 identical polypeptides (8) and has a molecular weight of 3.1×10^6. These subunits have a molecular weight of 52,000 and are arranged in a pattern of three subunits to each of the 20 corners of the pentagonal dodecahedron core. (12).

The nucleotide sequence of the ace F gene in E. coli K-12 which encodes E2 has been isolated and sequenced (13). In E. coli, each E2 polypeptide chain has three 100 amino acid repeating regions in the outer lipoyl domain of the subunit (13). Each of these repeating regions posseses a lipoyl binding site and a region rich in alanine and proline residues. Packman et al. (14) have shown that all three sites are lipoylated i.e. there are three bound lipoics per transacetylase chain. These three lipoylated segments of the E2 chain can be isolated as distinct functional domains after limited proteolysis. Moreover, in intact complex in the presence of substrate, each of these domains can become partly acetylated. Each of these lipoic acid residues are covalently bound in an amide linkage to the transacetylase subunit through the ε-amino group of a lysine. The remaining approximately 300 amino acids contain the capacity to bind subunits and to catalyze the transacetylation reaction.

The sequencing work of Stevens et al. clearly supports a two domain model of the E. coli E2 (7) in which a hinge region in the E2 component is highly sensitive to cleavage by trypsin. Thus upon

treatment with trypsin, the E2 component separates into two distinct, heterologous domains. In E. coli, the first domain is a compact, subunit binding, catalytic domain with an apparent molecular weight of about 26,000 (7). The other domain containing a lipoyl region has a molecular weight of about 28,600. This lipoyl-containing domain is a flexible extension and is connected to the compact core domain by a hinge region which contains two closely spaced trypsin-sensitive bonds.

Moreover, Bleile et al. (15) using electron micrographs, showed that the inner domain retains the full inner core structure. They additionally showed high resolution electron micrographs of E2 which indicated the presence of fiber-like extensions of the lipoyl domain i.e. the E2 loops surrounding the E2 octahedral core.

Work by Spencer et al. (16) on the nucleotide sequence of the dihydrolipoyl transsuccinylase component of α-ketoglutarate dehydrogenase, an analogous complex to the pyruvate dehydrogenase complex, shows a remarkable similarity in comparison to the dihydrolipoyl transacetylase of the pyruvate dehydrogenase complex. Both contain two analogous domains, a lipoyl domain linked to a catalytic and subunit binding domain. Moreover, both sequences contain segments containing regions rich in proline and alanine which could form flexible hinge-like regions. However, there is only a single lipoyl-containing domain in the dihydrolipoyl transsuccinylase (16).

Limited proteolysis of the mammalian E2 component also suggests that two domains are present. In the mammalian complex (6), the inner domain (M_r ~26,000) contains the intersubunit binding site of E2 and the catalytic site for transacetylation. Moreover, this domain is responsible for the quaternary structure (pentagonal dodecahedron) of

the core component as seen in electron micrographs (17). The lipoyl domain (M_r ~28,600) is acidic and contains a much higher porportion of proline residues than the lipoyl domain of the E. coli dihydrolipoyl transacetylase (6). As in E. coli core, there is an apparent trypsin sensitive hinge region between the two domains. This hinge region permits the lipoyl domain to move thus allowing the lipoyl moities to interact with the active centers of the three components of the complex. In short, this domain consists of large and flexible moities attached to large, mobile protein extensions so that the movement of both units can contribute to the transfer of intermediates between active sites.

NMR studies (18) have suggested that large segments of the polypeptide chains containing the lipoic acid exhibit a high degree of conformation mobility. Thus this greatly increases the effective radius of the lipoyl-lysyl swinging arms which carry substrates between the catalytic sites of the three enzymatic components and between the different E2 subunits.

In the mammalian complex more than 60 acetyl groups can be incorporated per core and this suggests that there are additional sites present that may undergo acetylation (19). Based on measuring the extent of acetylation of the transacetylase, it appears that two classes of sites may exist. This has been suggested because some acetyl groups are more rapidly incorporated while others are incoporated more slowly i.e. there is one class of sites that undergoes rapid acetylation presumably at lipoyl moities (~60) while other sites acetylate more slowly possibly at cysteines (19). Using isotopic dilution, White et al (20) presented evidence that there was one lipoic group per E2 subunit and additionally they observed as many as 2 acetyl groups being

incorporated per E2 subunit (data not shown). We have observed greater than 100 acetyl groups incorporated by 20 s at 30° (38). Those groups are incorporated into not only the core subunit but into a smaller subunit that will be characterized in this thesis and referred to as protein X.

In the mammalian system the fast movement of the lipoyl groups is a requisite step for acetylation. That is to say that in the complex the transacetylation and oxidation reactions proceed more rapidly than the rate limiting step which is reductive acetylation [Step #2] (21). Hence it had been proposed by Cate et al (21) that the observed acetylation of more than one site per subunit of the dihydrolipoyl transacetylase which occurs at the rate limiting step results from one of the following mechanisms. Either more than one lipoyl moiety has the capacity to service an active site region of the pyruvate dehydrogenase subunit or acetyl groups are rapidly shuttled to adjacent lipoyl moities with rapid reuse of a single lipoyl moiety. It is believed that the former may be more likely.

In summary, the E2 subunit differs between mammalian and E. coli in molecular weight and quaternary structure. In addition, information gleaned from the sequencing of the ace F gene in E. coli K-12 which encodes for E2 has greatly increased understanding of the mammalian E2 component. It appears that the E2 component is composed of two domains, a flexible lipoyl domain and an inner subunit binding domain that forms the core and catalyses the transacetylation reaction.

The pyruvate dehydrogenase component has a molecular weight of 154,000. Bovine pyruvate dehydrogenase is composed of two nonidentical subunits (α and β) and is arranged as a tetramer ($\alpha_2\beta_2$) (12).

Furthermore, kidney and heart bovine PDC differ in the number of pyruvate dehydrogenase bound to the core. Heart PDC has approximately 30 pyruvate dehydrogenase tetramers bound per core while kidney PDC contains approximately 20 tetramers per core. The pyruvate dehydrogenase tetramers based on symmetry arguments are thought to be arranged at the 2-fold positions along the edges to the transacetylase core.

The α sequence of the pyruvate dehydrogenase which is encoded by the ace F gene in E. coli K-12 has also been determined by Stephens et al. (22). There are 885 amino acids encoded by the ace F gene which corresponds to a polypeptide with a weight of 99,474. (This is in good agreement with published estimates of 90,000 - 100,000). However, in contrast to the mammalian pyruvate dehydrogenase, there are no sequences comparable in the E. coli to the phosphorylation sites of mammalian E1. This is not surprising since the E. coli PDC is not regulated by phosphorylation - dephosphorylation. It is interesting to note, that in contrast to the homology between dihydrolipoyl transacetylase and dihydrolipoyl transsuccinylase, there is no similiarity in sequences between pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (23). Electron micrographs reveal two domains within the E. coli E1 subunit which correspond to the above mentioned α and β subunits of the mammalian E1 component (17).

The third component in the mammalian system, the flavoprotein, has a molecular weight of 110,000 and is present as a dimer composed of two identical subunits (α_2) with one bound FAD per dimer. There are 6 dimers bound per each transacetylase core and these are thought to be bound at the faces of the dodecahedron.

In addition to the catalytic subunits there are two regulatory subunits associated with mammalian PDC. These are the pyruvate dehydrogenase$_a$ kinase and the pyruvate dehydrogenase$_b$ phosphatase (24, 25). Phosphorylation by the kinase inactivates the complex while dephosphorylation catalyzed by the phosphatase activates the complex.

Three kinase molecules bind tightly to the core (26) and in the resolution procedure copurify with the core. Kinase has been purified to homogeneity (27) and is thought to consist of two subunits (α,β) having molecular weights of (SDS-gel electrophoresis) of 48,000 and 45,000, respectively. Limited proteolysis of the α subunits by chymotrypsin resulted in selective modification and subsequent loss of kinase activity. On the other hand, the small subunit was selectively modified by trypsin with little or no change in kinase activity. Thus kinase activity appears to be associated with the larger subunit while the other subunit may possibly serve as a regulatory subunit.

The other regulatory component, the phosphatase is loosely bound by the core and requires Ca^{++} for binding (28). Phosphatase has a molecular weight of 146,000 and is composed of two nonidentical subunits (α,β) with a molecular weight of 89,000 for the α subunit and 49,000 for the β subunit (29,30). The small β subunit appears to contain the phosphatase activity and is sensitive to proteolysis (30). Additionally the phosphatase (α,β) contains 1.0 mol of FAD/mol of enzyme (α,β) and the FAD was found to be associated with the large α-subunit (28). The function of the α-subunit is as of yet unknown.

The pyruvate dehydrogenase complex is regulated through effects of the levels of its substrates and products on the overall reaction catalyzed by the complex and on the regulatory enzymes, the pyruvate

dehydrogenase kinase and the pyruvate dehydrogenase phophatase that, as noted above, catalyze inactivation and activation of the complex, respectively (31). An important feature for both modes of control is that the series of reactions catalyzed by the components of the complex result in the formation of steady-state levels of stable intermediates. Considerable evidence has been presented that acetylation of sites in the complex mediates an increase in the activity of the kinase (19,31-35). The pyruvate dehydrogenase component catalyzes both the decarboxylation of pyruvate as well as the reductive acetylation of oxidized lipoyl moieties covalently bound to the dihydrolipoyl transacetylase core (6,21,35). Reduced lipoyl moities can also be acetylated by acetyl-CoA through reverse of the transacetylation reaction.

Analysis of the complex by SDS-gel electrophoresis reveals an additional component of pyruvate dehydrogenase complex. That component, as mentioned earlier, is referred to as protein X and undergoes rapid and specific acetylation. Immunological and ^{125}I-peptide mapping was used to determine if this component is structurally distinct from or derived from the dihydrolipoyl transacetylase core. Additionally, protein X shares with the pyruvate dehydrogenase kinase the property of being tightly bound with the transacetylase core. Further work has been done to distingush whether protein X is distinct from the catalytic subunit of the pyruvate dehydrogenase kinase. Other studies I have conducted are described in Abstracts (36,37) and in manuscripts (38,39) submitted for publication.

Chapter 2: EXPERIMENTAL PROCEDURES

Materials. Acetyl-CoA, CoA, NAD, NADH, Mops, and N-ethylmaleimide were purchased from Sigma. ATP, ADP, malonyl-CoA and [1-^{14}C]acetyl-CoA were from P-L Biochemicals, Inc. En^{3}Hance Spray, [2-^{14}C]pyruvate, [2-^{14}C]malonyl-CoA, and [^{125}I]goat antimouse IgG were obtained from New England Nuclear. [1-^{14}C]Propionyl-CoA, [1-^{14}C]butyryl-CoA, and Na^{125}I were from Amersham Corp. Ampholines were from LKB and o-phthaldialdehyde was from Eastman Kodak. Nitrocellulose sheets were purchased from Schleicher and Schuell, Inc. Goat anti-rabbit IgG-horseradish peroxidase conjugate was obtained from Bio-Rad. Other reagents and materials were the highest quality commercially available.

Purification of the pyruvate dehydrogenase complex. To obtain highly purified kidney pyruvate dehydrogenase complex (14-19 μmol NADH min^{-1} mg^{-1}), mitochondria from bovine kidney were initially isolated using minor modifications of a procedure by Pettit and Reed (40). The mitochondrial fraction was collected and washed through the various procedural steps by centrifugation in a JA-10 rotor in a Beckman JA 21B centrifuge at 8,000 rpm for 25 minutes. The mitochondria from the water wash were, however, collected at 9,000 rpm for 30 minutes. On the average, forty pounds of bovine kidneys were processed resulting in a yield of approximately 3 kg (wet weight) of washed mitochondrial paste . The mitochondria were resuspended in a minimal volume of 20.0 mM potassium phosphate, pH 6.5, (250 ml/500 g) containing 1 mg/ml leupeptin (Sigma) and 1 ml/liter of 0.10 M phenylmethylsulfonyl flouride (Sigma). The mitochondrial suspension was then shell-frozen in a dry ice/isoproponal bath and stored at -20°. Subsequently the pyruvate dehydrogenase complex was purified by a modification of the procedure of

Roche and Cate (41) from these frozen mitochondrial preparations. The latter were stored at -20° and were rapidly thawed under running tap water. The preparation was diluted with 20.0 mM potassium phosphate, pH 6.5, to a volume three times the original weight and 10 ml of 5.0 M NaCl and 10 ml of rabbit serum (Pel Freez) were added per liter. Moreover, as the preparations were thawed, additional protease inhibitors were added to the final levels as follows: 0.10 mM PMSF, 0.50 mM benzamidine, and 0.05% (v/v) aprotinin. This suspension, after being centrifuged for 25 minutes at 8,000 rpm in a JA-10 rotor, was then diluted to 9-12 mg protein/ml with 20 mM potassium phosphate, pH 6.5, (including 1% NaCl and 1% rabbit serum). To precipitate the enzyme, the pH was adjusted to 6.55 and 10 ml of 1.0 M $MgCl_2$ was added per liter. 50% PEG was added to 5-7% (v/v) or until all of the PDC and most of the KGDC precipitated. (Note these steps are all done on ice). The precipitated PDC and KGDC were collected by centrifugation in the JA-10 rotor at 8,000 rpm for twelve minutes. The pellet was homogenized in a minimal volume of 50 mM Mops-Na, pH 7.5, 0.20 mM EDTA, and 2.0 mM DTT (Buffer 1) which also contained 0.004 mg/ml leupeptin, 0.05% (v/v) aprotinin, and 0.50 mM benzamidine. The homogenized pellets were then kept on ice with an occasional stirring for two hours after which the solution was clearspun in the JA-20 rotor at 14,000 rpm for 10 minutes. This step removed a considerable amount of insoluble proteins without a subsequent loss of enzyme units. $MgCl_2$ was added to the resulting supernatent to bring the concentration to 1 mM $MgCl_2$ and the solution left on ice overnight.

The second day the supernatent was clarified in the JA-20 rotor at 14,000 rpm for 10 minutes and the supernatent was diluted to 6 mg/ml with fresh Buffer 1. The solution was adjusted to pH 6.55 on ice and as

a result, a large amount of protein precipitated. The solution was clarified as before, warmed to 25° and 50% PEG added to 4-5% (v/v). At this step, most of the KDGC precipitated from solution leaving less than 5% (units of KGDC/units of PDC) of the KDGC in solution. The solution was then centrifuged at 25° in the JA-20 rotor for 10 minutes at 14,000 rpm. The supernatent was then cooled to 4°, made 5.0 mM in $MgCl_2$, and 50% PEG added until all of the PDC precipitated. The solution was subsequently centrifuged in the same manner as before. Note that precise levels of enzyme were determined at each step by the minifuge technique described by Roche and Cate (41). The pellets were dissolved overnight in 50 mM Mops-Na, pH 7.5, 0.10 mM EDTA, and 0.50 mM DTT (Buffer 2) containing in addition the same levels of protease inhibitors as Buffer 1. On the third day, the enzyme solution was then carefully layered onto a stepwise sucrose gradient containing 15%, 10%, and 7.5% sucrose (w/v) which was then centrifuged in a Beckman SW 27 rotor for 4 hours at 26,000 rpm. The KGDC solution was centrifuged an additional hour under the same conditions. The resulting pellets were dissolved overnight in 50 mM Mops-Na, pH 7.5, 1.0 mM DTT, and 0.20 mM EDTA. The purified enzyme was then stored in aliquots at -70°. All protein determinations were done by the Biuret method (42).

A portion of the purified complex was separated from contaminating KGDC (2-10%) by gel filtration on a 2.5 x 90 cm Sepharose CL-4B column. About 60% of the first portion of the peak was free of KGDC. The fraction was subsequently pelleted at 38,000 rpm for 1.5 hours in a Beckman 50.2 Ti rotor. Analysis of the preparation by SDS-gel electrophoresis revealed no KGDC.

Resolution of the pyruvate dehydrogenase complex. The dihydrolipoyl

transacetylase-protein X-kinase subcomplex and pyruvate dehydrogenase were prepared with minor modifications by the procedures of Linn et al (43). After concentrating purified pyruvate dehydrogenase complex by centrifugation at 38,000 rpm in a Beckman 50.2 Ti rotor for 1.5 hours, the pellets were dissolved overnight in 50 mM Mops-Na, pH 7.5, 1.0 mM DTT, and 0.20 mM EDTA to give a final concentration of 30-35 mg protein/ml. The solution was brought to 0.10 M glycine, pH 9.0, 0.40 mM EDTA, 1.0 mM $MgCl_2$, and 1.0 mM dithiotreitol. Subsequently, the solution was made 1.0 M in NaCl by the gradual addition of ground NaCl into a continously stirring solution. After incubating for 20 minutes on ice, the protein solution was applied to a Sepharose CL-6B column (2.0 x 60 cm) which had been equilibrated in 0.10 M glycine, pH 9.0, 1.0 M NaCl, 0.40 mM $MgCl_2$, 0.10 mM EDTA, 0.5 mM benzamidine, and 0.1% (v/v) aprotinin. Prior to loading the column (at least ten hours), 20 ml of 10.0 mM DTT were loaded onto the column. The column flow rate was 6 drop/min. The interval between fractions was 20 minutes. The fractions were then read for protein concentration at 280 nm. A typical elution consisted of two large peaks, the first containing the dihydrolipoyl transacetylase-protein X-kinase subcomplex and the second the pyruvate dehydrogenase with the dihydrolipoyl dehydrogenase. The first peak containing the dihydrolipoyl transacetylase-protein X-kinase subcomplex was dialyzed for three hours against several liters of 50 mM potassium phosphate, pH 7.5, 0.10 mM $MgCl_2$, 0.10 mM EDTA, and 1.0 mM DTT. The solution was then centrifuged in a Beckman 50.2 Ti rotor for 2 hours at 38,000 rpm and the pellet was dissolved in 50 mM potassium phosphate, pH 7.5, 0.20 mM EDTA and 0.50 mM DTT. The fraction containing the pyruvate dehydrogenase-dihydrolipoyl dehydrogenase peak was made 5.0 mM with

respect to dithiotreitol, stirred on ice 10 minutes, and brought to 0.40 saturation with solid ammonium sulfate. After standing for 30 minutes on ice, the solution was clarified in the JA-20 rotor at 14,000 rpm for 10 min. The pellet, which contains the pyruvate dehydrogenase, was dissolved in 20.0 mM potassium phosphate, pH 7.5, 0.10 mM $MgCl_2$, 0.10 mM EDTA, and 1.0 mM DTT and dialyzed against several liters of this buffer overnight. The supernatent, in which could be found the dihydrolipoyl dehydrogenase, was brought to 60% of saturation with ammonium sulfate to precipitate the enzyme and treated as before.

After determination of the protein concentrations by the method of Lowry et al (44), the resolved components were assayed for specific activity using a reconstition assay. To determine the specific activity of the dihydrolipoyl transacetylase, the dihydrolipoyl transacetylase was titrated in varying amounts (e.g. 0, 2, 4, 6, 10 µg) against approximately 50 µg of pyruvate dehydrogenase. Pyruvate dehydrogenase was preincubated in a tube at 30° in 40 mM Mops-K, pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, and 10.0 mM $MgCl_2$. To this was added the appropriate amount of dihydrolipoyl transacetylase. The solution was vortexed, and then incubated for twelve minutes at 30°. At the end of the incubation, the solution was assayed for PDC activity and the specific activity determined from the slope using the following equation:

$$\text{Specific activity} = \frac{(\Delta A_{340})(0.44)}{6.22(\text{mg protein})}(\text{sample volume/total volume})$$

where 0.44 is the approximate percent of dihydrolipoyl transacetylase or pyruvate dehydrogenase in the whole complex. Thus the activities are related to the activity of the complex prior to resolution for easy comparison and do not reflect the purification achieved in the

resolution process. After determination of the protein concentrations and specific activities, the various components were aliquoted and stored at -70°.

To obtain a purified kinase fraction, resolved dihydrolipoyl transacetylase-kinase subcomplex was treated with p-hydroxymecuriphenyl sulfonic acid according to the procedures of Stepp et al. (27). In some cases the dihydrolipoyl transacetylase following the kinase preparation was retreated with the mercurial agent which resulted in a release of protein X and thus yielded a fraction containing purified protein X.

Quantitative analysis of level of acylation of complex and components. Complex or the resolved dihydrolipoyl transacetylase core was equilibrated in 50.0 mM Mops-K, pH 7.3, 0.05 mM DTT, 1.0 mM EDTA, and 2.0 mM $MgCl_2$ for at least 45 minutes at 4°. Acylation with [1-^{14}C]acetyl-CoA (80,000-120,000 cpm/nmol) or [2-^{14}C]pyruvate (40,000-50,000 cpm/nmol) was conducted in duplicate at 30° in the presence of: 50.0 mM Mops-K plus 20.0 mM potassium phosphate, pH 7.3, 60 mM KCl, 1.5 mM $MgCl_2$, 0.50 mM EDTA,and 0.10 mM DTT. The indicated level of substrate was added in the presence of 1.0 mg/ml complex (or resolved core) for the time indicated. The level of acylation of the complex was determined by application of portions of reaction mixtures to trichloroacetic acid-containing paper discs and protein-bound radioactivity was determined as previously described (19,31).

Slab gel electrophoresis. SDS-slab gel electrophoresis was routinely performed using the standard Laemmli system (45) except when acetylated samples were analyzed. In those cases, a modified Laemmli system (near neutral pH) was developed. The dimensions of the slab gels,

irrespective of the system used, were 25 cm wide, 1.5 mm thick with a 12.5 cm running gel with the electrophoresis being conducted at 4^{o} for 16 hours with a constant current of 10 mA/gel for the Laemmli system and for 30-40 hours at 35 mA/gel for the modified system. In both cases, the dimensions of the sample wells were 0.5 cm by 1.5 cm.

To prepare the gels, washed glass plates were extensively cleaned on both sides with 95% ethanol. Polyvinyl chloride (PVC) spacers, after cleaning with ethanol, were lightly greased with Vaseline. Care was taken to keep all areas of the glass plate free of grease and the grease film on the spacers was made no more than 1 cm wide. During assembly of the plates, in order to ensure no leakage would occur, the side spacers were checked to ensure that no gap existed between them and the bottom spacers. Following this, the glass plates were held together with 2 inch binding clips and checked with a level to keep the top of the gel perfectly horizontal.

Once the running gel solution was prepared, it was degassed for at least ten minutes with constant stirring, after which the SDS was added and the solution was allowed to degas for several more minutes. Immediately before pouring the gel, TEMED (Sigma) and ammonium persulfate were added and the solution poured into the glass plates to a level 2.5 cm below the top of the plate. Water was then carefully layered onto the solution and the gel was allowed to polymerize for at least 30 minutes at room temperature. The stacking gel was handled in the same manner as the running gel. After pouring the stacking gel, the well combs were inserted and checked with a ruler to make sure the bottoms were parallel to the top of the running gel. Polymerization proceeded for at least 2 hours.

Upon completion of the 2 hour polymerization, the combs were slowly and carefully withdrawn using electrode buffer. The wells were rinsed several times with the electrode buffer using a Pasteur pipette and then filled with buffer. Sample preparation was done in the presence of 2.0 mM N-ethylmaleimide without added thiol compounds. After dissolution into the sample buffer, the samples were underlaid into the sample wells with a syringe which had been prewashed in nitric acid.

The separating gel in the modified Laemmli system was composed of 375 mM Tris-Cl, pH 7.3, 0.1% SDS, 15% acrylamide (20:0.8 acrylamide:bis acrylamide ratio), 0.1% ammonium persulfate and 0.025% (v/v) TEMED. In the modified system the stacking gel was prepared with chloride ion rather than phosphate and with a higher level of acrylamide than the standard level. It consisted of 125 mM Tris-Cl, pH 6.8, 0.1% SDS, 5.0% acrylamide (20:0.8 acrylamide:bis acrylamide ratio), 0.1% ammonium persulfate, and 0.5% (v/v) TEMED. Degassed electrode buffer contained 5.0 mM Tris, 380 mM glycine, and 0.1% SDS. The pH was adjusted to 7.1 with acetic acid. Samples of acetylated proteins which had been trichloroacetic acid-precipitated were dissolved for a 16 hour period in 62.5 mM Tris-Cl, pH 6.8, 12.5% (v/v) glycerol, 2.5% SDS, 2.0 mM N-ethylmaleimide and 12.5 μg/ml bromophenol blue.

Two-dimensional gel electrophoresis. Two dimensional gel electrophoresis was conducted by a modification of the procedure of O'Farrell (46). In the first dimension, tubes with a length of 17 cm and an i.d. of 2.5 mm were used which had been soaked in chromic acid solution and extensively rinsed with deionized water prior to use.

The gel consisted of 3.0% acrylamide (17.85:1 acrylamide:bis acrylamide ratio), 55% (w/v) urea, 1.4% (v/v) ampholines, 0.03% (w/v)

ammonium persulfate and 0.15% (v/v) TEMED. Ampholines used were either pH 3.5-8.0 (LKB) or pH 3-10 (Serva). These gels were done in the absence of Nonidet P-40 in all cases, and in the case of acetylated samples, thiols were omitted.

After degassing the gel solution, the gels were poured using a syringe with a length of tubing attached into the tubes which were parafilmed on one end. The gels were poured to a height of 15 cm and care was taken to dislodge any bubbles trapped in the tubes. The gels were then allowed to polymerize for 1/2 to 1 hour after which sample buffer consisting of 57% urea, 2% ampholines, and in the case of nonacetylated samples, 4.5% (v/v) of 2-mercaptoethanol, was layered on the top. The gels then set for at least one hour. Nonacetylated samples were heated for one minute in a boiling water bath and then kept for one hour at room temperature. The tube gels were placed in the apparatus which contained degassed 10 mM phosphoric acid in the upper reservoir and 0.2 M NaOH in the lower reservoir. To each tube gel was added 50 µl of sample overlay buffer which consisted of 54% urea and 1% ampholines. The samples were then carefully underlayed into the tubes. Focusing was conducted with a constant potential of 1,000 volts for either three hours (nonequilibrium) or sixteen hours (equilibrium). Following focusing, the gels were equilibrated in two 20 ml volumes of 2.3% SDS, 63.2 mM Tris-Cl, pH 6.8, 10% (v/v) glycerol, and in the case of nonacetylated samples, 4.5% 2-mercaptoethanol for one hour each on a shaker. The second dimension was conducted either with the Laemmli system (45) or with the lower pH slab gel system described previously.

Analysis of acetylation of individual components. For analysis of the level of acylation of specific subunits, the acylation reaction was

terminated with an equal volume of 20% (w/v) trichloroacetic acid containing 2.0 mM N-ethylmaleimide. In a parallel sample the level of total acylation was determined as described above. After a ten minute incubation at 4^{o}, protein was pelleted by centrifugation for 4 min at 14,000 rpm (Eppendorf 3200 microfuge); the pellet washed with 200 µl of ether containing 2.0 mM N-ethylmaleimide; and protein dissolved in SDS-sample buffer. Aliquots of dissolved samples were removed and protein-bound acetyl groups estimated by precipitation on trichloroacetic acid soaked paper to monitor losses[1] in protein-bound acetyl groups during sample preparation and their retention during the subsequent steps associated with gel electrophoretic analyses.

Following gel electrophoresis with the neutral system described above, protein bands were stained with a solution containing 0.25% Coomassie Brilliant Blue, 45% (v/v) methanol, 10% acetic acid, 1% Triton X-100, and 2.4% (w/v) $AlCl_3$. The gels were stained for a minimun of three hours and then destained in 10% acetic acid and 25% ethanol (95%). Usually 1 liter of destain with several changes of solution was sufficent to destain the gel. Bands corresponding to the various components were cut out and solubilized by treatment with 30% H_2O_2 overnight at 50^{o} in a sealed scintillation vial. Scintillation fluids were added to the vials after freezing the samples. After adapting in the dark to avoid chemiluminescence, the sets of samples were repeatedly counted until constant levels of radioactivity were measured. Similar results were obtained with this approach when protein bands were blotted

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[1]This measurement could not be used to quantitate levels of acylation and only about 75% of protein was precipitated in trichloroacetic acid soaked papers when dissolved in SDS-sample buffer.

onto nitrocellulose, stained, cut out, and then counted. In either case, the relative acylation of subunits per mol of complex was corrected to the initial level of acylation and, therefore, it was assumed that there was an equivalent recovery of subunits and retention of acyl groups.

Where performic acid lability of acetyl groups linked to individual components was tested, bands were blotted onto nitrocellulose and then incubated in a dessicator over 95.0 ml 90% formic acid plus 5.0 ml 30% hydrogen peroxide. A slight retention of acetyl groups (presumably due to reaction of thioesters with nitrocellulose) was corrected for by spotting [1-^{14}C]acetyl-CoA and measuring the retention of label following performic acid treatment.

When autoradiography was conducted following gel electrophoresis, two approaches were used. In the first, after staining and destaining, gels were soaked in Enlightning (NEN) plus 10% glycerol with gentle shaking for 30 min. Gels were then dried onto Whatman 3MM paper with a BioRad slab gel dryer and autoradiographs developed with Kodak XAR-5 x-ray film. Alternatively, following electrophoresis, samples were transblotted onto nitrocelluose (0.45 μm, Schleicher and Schuell) at a constant current of 1 amp for 4 hours using a watercooled TE42 transphor electrophoresis cell [Hoefer] (47). Transfer was carried out in 24 mM Tris and 192 mM glycine with the pH adjusted to 7.1 with acetic acid. It was found that in the absence of methanol, complete or very nearly complete transfer of the protein bands was achieved. Furthermore, to eliminate distortion of the gels, the gels were presoaked in the transfer buffer for one hour prior to blotting. Following transfer, the protein bands were stained with 0.2% (w/v) Coomassie Brilliant Blue in

50% methanol plus 7.5% acetic acid for 20 minutes. Destaining was carried out in 90% methanol and 4% acetic acid with 4-5 changes (5-10 min) of destaining solution. The nitrocellulose sheets were dried onto Whatman 3 MM paper with a BioRad slab gel dryer (Model 224). Autoradiography was carried out with Kodak XAR-5 x-ray film after treating with En^3Hance Spray.

Peptide mapping studies. Approximately 10 µg of whole complex were dissolved in a solution containg 2% SDS amd 50 mM Tris-Cl, pH 7.5. To this solution 0.5 millicuries of carrier-free ^{125}Iodine (Amersham) were added, followed immediately by 20 µl of 1 mg/ml chloramine T (Sigma). The reaction was allowed to proceed for one minute at room temperature and then quenched by adding an equal volume of standard gel electrophoresis sample buffer containing 2% 2-mercaptoethanol. 20 µl of the iodinated complex were then loaded onto a standard SDS gel (Laemmli system [45]) and the sample was electrophoresed in same manner as described earlier. To remove the SDS at the completion of the electrophoresis, the gel was washed extensively in a solution of 25% isopropanol and 10% acetic acid for at least 24 hours with frequent changes. Following staining and destaining in the standard system, gels slices containing individual subunits were dried under vacuum and then rehydrated in 50.0 mM NH_4HCO_3, pH 7.8. After crushing the gel slices, digestion with 50 µg L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was conducted for 24 hours at 37°. The gel particles were removed by centrifugation and salt was removed from the supernatant by repeated lyophilization. The lyophilized samples were then dissolved in 25 µl of water and counted. Approximately 50,000 counts were applied 1.5 cm from each side of Kodak cellulose sheets

(Eastman Chromagram, 13255 Cellulose without fluorescent indicator). The plates were sprayed with electrophoresis buffer (acetic acid:formic acid:water at 3:1:16) and run at 1,000 volts until the pyronin-Y dye (Sigma) had migrated 12 cm. To avoid heating problems, the flat bed electrophoresis unit was cooled to 0° with a mixture of water and ethylene glycol. The plates were dried 2-3 hours in a warm oven at 35° and then developed at right angles to the electrophoresis dimension in ascending chromatography in butanol: pyridine:acetic acid:water (32.5:25:5:20) until the buffer reached the top of the plate. The plates were dried at room temperature, wrapped in plastic wrap, and exposed to film (Kodak XAR x-ray film) at -70° for about 72 hours using intensifying screens.

In preparing acetylated peptides of protein X and the transacetylase core, digestion of protein in crushed gel slices gave poor recoveries of acetylated peptides. Electroelution of protein X from gel slices was also inefficent. Two procedures were used for preparation of subunits from acetylated, N-ethylmaleimide-treated complex. The first involved collecting protein X and the transacetylase in dialysis bags attached at the appropriate time to the bottom of 5 cm SDS-tube gels (neutral system above). Movement of bands in the tube gel could be followed with the high level of protein loaded (40 µg) due to a visible interface. (Clear interfaces were not visible when the regular Laemmli system (45) was used.) Protein samples were precipitated and extensively washed with methanol-acetic acid (18:1) and then ether to remove SDS.

The alternative procedure, which avoided the use of SDS, involved separation of subunits by chromatography on DEAE-Sephadex in the

presence of 8.0 M urea. Following acetylation of 4.0 mg of complex in 2 ml with 100 μM [2-^{14}C]pyruvate, N-ethylmaleimide was added to a concentration of 5.0 mM and incubation continued for 60 s at 30°. Then the protein components were denatured in 8.0 M urea and reacted with an additional 40 μmol N-ethylmaleimide in 100 mM imidazole-HCl, pH 7.1, in a final volume of 8.0 ml. Chromatography was conducted in the presence of 8.0 M urea with the following sequence of buffers: 100 mM imidazole, pH 7.1, 100 mM MES, pH 6.0, 20.0 mM formic acid, 20.0 mM trifluoroacetic acid. Protein X eluted before the transacetylse component but after the other components. Fractions were dialyzed against 100 mM trifluoroacetic acid and lyophilized. Samples of aceylated subunits (<50 μg) prepared by either of the above procedures were digested with 25 μg of trypsin in 0.5 ml 50 mM NH_4HCO_3 buffer (adjusted to pH 7.1 with dry ice) for 3 h in a sealed container at 37°. Peptide mapping was conducted as described above. Approximately 5,000 counts were applied to the origins and prior to exposure to Kodak XAR-5 x-ray film the plates were sprayed with En^{3}Hance Spray.

Immunological studies. Rabbit immunoglobin G directed against the bovine dihydrolipoyl transacetylase was supplied by Mulchand Patel (Case Western Reserve). We further purified the rabbit IgG fraction by reacting it with transacetylase separated from the other components by SDS-slab gel electrophoresis (200 μg of KGDC-free complex in an 8 cm strip) and blotted onto nitrocellulose following SDS-slab gel electrophoresis. Following blocking with 10% (w/v) bovine serum albumin in Tris-buffered saline, 2.0 mg of IgG was reacted with the nitrocellulose strip containing the transacetylase subunit for 2 h at 22°. The nitrocellulose strip was repeatedly washed with Tris-buffered

saline, followed by incubation in 2.5 ml 0.1 M glycine-HCl, pH 2.5, 20 mM $MgCl_2$, and 50 mM KCl for 30 min at 22°. The solution, containing affinity purified rabbit anti-transacetylase IgG, was immediately adjusted to pH 7.5 and dialyzed against 50.0 mM Na-borate, pH 8.0, and 0.20 M NaCl overnight. The product was aliquoted and stored at -20°.

In the immunological studies, SDS-slab gel electrophoresis was carried out by the method of Laemmli (45) as described previously and the gel blotted as also discussed earlier. Following blotting, the nitrocellulose was blocked by treatment with 10% bovine serum albumin in the presence of 20.0 mM Tris-Cl, pH 7.5, and 50 mM NaCl for 30 minutes. The subsequent steps were conducted in Tris buffered saline which contained 50 mM Tris-Cl, 150 mM NaCl, and 0.05% sodium azide. The rinsed blots were then blocked by treatments with 5% (v/v) fetal bovine serum (KC Biologicals) for 1 hour, followed by rinsing and treatment for 30 minutes with 10% bovine serum. The blots were subsequently incubated with anti-transacetylase rabbit IgG in buffer containing 1% bovine serum albumin for 14 hours. Following incubation, the blots were repeatedly and briefly rinsed with water, then with Tris-buffered saline plus 0.05% Tween 20 (BioRad) for 10 minutes, and followed by a 10 minute wash in buffer containing 1% ovalbumin. The blots were then incubated for 1 hour in a buffer containing 15 µl of goat-antirabbit IgG-horseradish peroxidase conjugate (BioRad) in buffer containing 1% bovine serum albumin. Color development was conducted in a 5-minute period using BioRad HRP color development reagent and the reaction was terminated by rinsing in deionized water.

Polyclonal antibody against protein X was prepared in BALB/c mice by subcutaneous injection in Freund's complete adjuvent. A booster was

given twice by injection in Freund's complete adjuvent after intervals of 4 weeks. A final injection was done one week before collecting blood from the animal. Protein X was prepared by the procedure described previously involving treatment of the transacetylase core with p-hydroxymecuriphenyl sulfonate. Prior to incubation with mouse serum, the nitrocellulose was blocked by three 30 minute treatments with 3% ovalbumin (w/v) in Tris-buffered saline containing 0.05% Tween 20 with intervening washes with buffer lacking ovalbumin for 30 minutes. Antiserum, diluted 1:20,000 in blocking buffer containing 3% ovalbumin, was allowed to react for 2 hours at 22°. The nitrocellulose was washed six times for 30 minutes each with Tris-buffered saline containing 0.05% Tween 20 and then incubated with ^{125}I goat anti-mouse antibody (70,000 cpm/ml) for 2 hours at 22°. This was subsequently followed by six 60 minute washes with Tris-buffered saline containing 0.05% Tween 20 and the nitrocellulose was subsequently exposed to Kodak XAR-5 x-ray film.

Proportion of X in the kidney complex. Protein X was purified by chromatography on a SynChropak RP-P larger pore (300A) column using a 0.1% triflouroacetic acid (v/v)-acetonitrile gradient elution. That preparation gave a single band corresponding to protein X in SDS-slab gel electrophoresis. Folowing lyophilization, the isolated protein X and a sample of complex were dissolved in SDS-sample buffer lacking 2-mercaptoethanol and bromophenol blue. The protein concentrations of these samples was determined by the procedure of Fried et al. (48) which involves reacting the dissociated protein with O-phthaldialdehyde followed by separation of the O-phthaldialdehyde-derivatized protein from interfering fluorescent signals with a TSK guard column equilibrated with 0.1% SDS. The protein which elutes at the void volume

was detected with a Turner Designs fluorometer (Model 10-000) equipped with a 40 µl flow cell. The assay was linear in the range of 30-600 ng protein. Crystalline bovine serum was used as a standard.

Several aliquots of the above preparation of protein X (range 7.0 to 28 ng) and of the sample of complex (range 30.6 to 612 ng) were subjected to SDS-slab gel electrophoresis (regular Laemmli [45]) and silver stained by the method of Oakley et al. (49). In the silver staining procedure it was found that in order to reduce the background it was necessary to wash the gels extensively in 50% methanol and 10% acetic acid. Additionally if the gel was transferred to another tray containing development solution as soon as the protein bands became visible, the background was also significantly reduced. Overstained gels were cleared using the destain described by Marshall (50) in a 1/3 dilution and stopping the reaction at the appropriate time with concentrated Kodak hypoclearing agent. Densinometric traces of the stained gels were performed with a Kontes Fiber Optic Scanner operated in the transmittance mode.

Pyruvate dehydrogenase kinase assay. Kinase activity was determined as the initial rate of incorporation of ^{32}P-phosphoryl groups incorporated into protein from [γ-^{32}P]ATP (70,000-100,000 cpm/nmole) (27,31). Within intact complex endogenous pyruvate dehydrogenase component served as the substrate. With resolved components purified dehydrogenase component was added at 0.80-0.85 mg/ml (unless otherwise indicated). Except as indicated, resolved kinase was assayed in the presence of the dihydrolipoyl transacetylase (at the indicated level). For measurement of maximal kinase activity, assays were conducted at least in duplicate in the buffer system (referred to as buffer A) described by Stepp et al.

(27) which contained in the 20 mM potassium phosphate, pH 7.0, 1.0 mM $MgCl_2$, 0.1 mM EDTA, and 2.0 mM dithiothreitol. The source of kinase (complex, transacetylase-protein X-kinase subcomplex, or resolved kinase) was added to the buffer equilibrated at 30^{o} to give a final volume of 45 µl and after 30 s, 5.0 µl of a 10x concentrate of [γ-^{32}P]ATP was added. In most assays, the final ATP concentration was 100 µM ATP, but in some assays 500 µM ATP was used. A 35 µl aliquot was applied at 20 s (unless otherwise indicated) to a dry paper disc (2.2 cm) pre-soaked in 10% (w/v) trichloroacetic acid and 10 mM pyrophosphoric acid. The paper discs were washed and ^{32}P incorporation determined as previously described (31). Some assays were conducted in a more physiological (higher isotonic strength) buffer system (31) that is referred to as buffer B. Buffer B contained in the final reaction mixture: 50.0 mM Mops-K, pH 7.3, 60 mM KCL, 2.0 mM dithiothreitol, 1.5 mM $MgCl_2$, and 0.5 mM EDTA. Units of kinase activity were nmol ^{32}P incorporated into the phruvate dehydrogenase component per min. Protein was determined by the Biuret (42) or Lowry (44) methods with crystalline serum albumin as the standard.

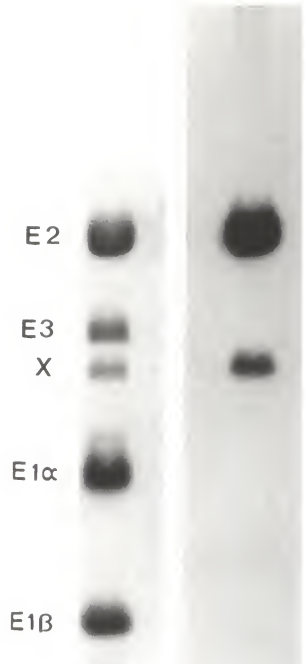
Chapter 3: RESULTS

Purification and resolution of complex. Purification of the complex by using the procedure outlined in the methods section yielded on the average about 2500 units (μmol NADH min^{-1}) with specific activities ranging from 14-19 μmol NADH min^{-1} mg^{-1}. KGDC contamination ranged from 2-10% (units of KGDC/units of PDC). A portion of the complex was further purified on a Sepharose CL-4B column as described earlier in Experimental Procedures to yield a PDC fraction containing less than 0.1% KGDC contamination (units of KGDC/units of PDC). About 80-120 mg of complex were utilized in a typical resolution yielding about 18-35 mg of dihydrolipoyl transacetylase and 15-50 mg of pyruvate dehydrogenase with specific activities of 9-15 and 4-12 μmol NADH min^{-1} mg^{-1}, respectively. 10-20 mg of resolved transacetylase-protein X-kinase subcomplex were titrated with the p-hydroxymercuriphenyl sulfonic acid to yield about 1.2-2.0 mg of a kinase fraction containing dihydrolipoyl dehydrogenase, protein X, and the catalytic component of the kinase. Approximately 10-15 mg of transacetylase-protein X subcomplex were also recovered. Initial specific activities of the kinase fraction (measured in the presence of excess transacetylase-protein X with 0.1 mM [γ-^{32}P]ATP) ranged from 140 to 178 nmol ^{32}P incorporated $min^{-1}mg^{-1}$ at 30° but kinase activities dropped after a few days or when immediately frozen to 80 to 110 nmol $min^{-1}mg^{-1}$.

SDS-gel electrophoretic systems and acylation of components. The left side of Fig 1 shows a typical gel electrophoresis pattern for the dissociated subunits of the complex. In addition to the standard subunits, an additional subunit of unknown function was also observed which is designated as protein X. Protein X was present in preparations

FIGURE 1

SDS-gel(Laemmli system) of acetylated bovine kidney pyruvate dehydrogenase complex and autoradiograph showing acetylated subunits. Samples of the complex were acetylated with 20 μM [1-^{14}C]acetyl-CoA in the presence of 250 μM NADH for 20 s. Other reaction conditions and conditions for preparation of samples for gel electrophoresis were as described in Experimental Procedures. The lane on the left shows the pattern for subunits of acetylated complex (25 μg) separated by the procedure of Laemmli (45) and stained with Coomassie blue. Beside it is shown the autoradiograph made from the gel. Subunits are identified as follows: Dihydrolipoyl transacetylase as E2, dihydrolipoyl dehydrogenase as E3, protein X as X, α subunit of the pyruvate dehydrogenase component as Elα, and β subunit of the pyruvate dehydrogenase as Elβ.


E2
E3
X
E1α
E1β

of bovine kidney or bovine heart complex analyzed by the standard SDS gel electrophoresis system of Laemmli (45) during the last five years in this laboratory. Moreover, only when the preparations were rigorously freed of α-ketoglutarate dehydrogenase complex, was it possible to establish unambiguously that protein X is distinct from the dihydrolipoyl transsuccinylase component which migrates at about the same position. Moreover, many earlier gel electrophoresis studies (12,41,43) were conducted with the neutral Weber-Osborne system (51). In this system as well as in the neutral system described previously, protein X tended to migrate with dihydrolipoyl dehydrogenase component. Fig. 2 shows two gel electrophoresis patterns in which subunits were separated by the neutral system. In the gel on the left, protein X is directly under the dihydrolipoyl dehydrogenase (E3) and in the gel on the right, protein X has a mobility slightly slower than the dihydrolipoyl dehydrogenase. When referenced against standards of known molecular weight, the shift in the relative positions of those two proteins were found to result primarily from a higher mobility of the dihydrolipoyl dehydrogenase in the neutral pH system.

The samples of complex shown in Figs. 1 and 2 were treated with 20 μM [1-^{14}C]acetyl-CoA in the presence of 250 μM NADH for 20 s at 30°. Autoradiographs are shown for the sample separated by the regular Laemmli system (Fig. 1) and for the left sample run in the neutral system (Fig. 2). Both the transacetylase core component and protein X were acetylated by that treatment.

Figures 3 and 4 (left panels) show a two-dimensional gel electrophoresis pattern for complex and resolved dihydrolipoyl transacetylase-kinase subcomplex, respectively. Isoelectric focusing

FIGURE 2

SDS-gels (Neutral system) of acetylated bovine kidney pyruvate dehydrogenase complex and autoradiographs showing acetylated subunits. Samples of complex were acetylated with 20 μM [1-^{14}C]acetyl-CoA in the presence of a 250 μM NADH for 20 s. Other reaction conditions and conditions for preparation of samples for gel electrophoresis were as described in Experimental Procedures. The first and third lanes of Fig. 2 show patterns for samples of acetylated complex (20 μg) when separation was done with the lower pH system described in Experimental Procedures. The middle lane shows an autoradiograph made from the gel to the left. Subunits are identified as described in the legend to Fig. 1.

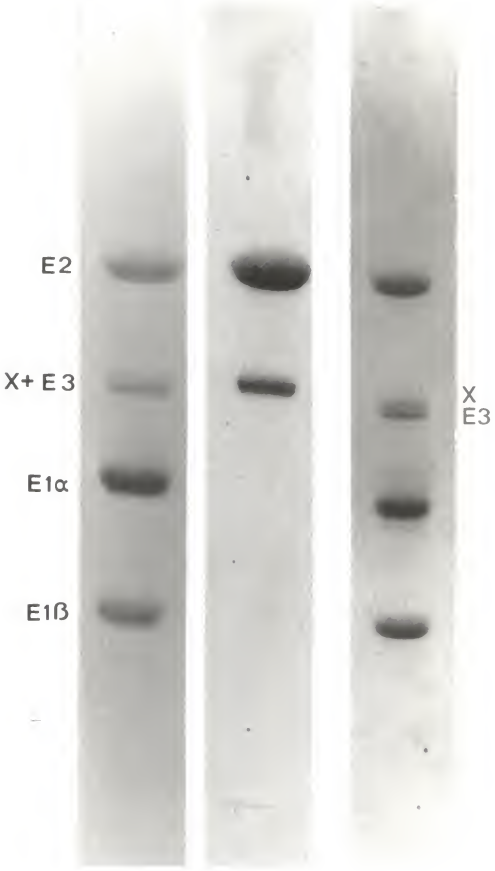

E2
X+ E 3
E1α
E1ß
X
E3

FIGURE 3

Two-dimensional gel electrophoresis pattern and autoradiograph for pyruvate dehydrogenase complex lightly acylated by treatment with [2-^{14}C]malonyl-CoA. Kidney complex (50 μg) was treated with 20.0 μM [2-^{14}C]malonyl CoA plus 250 μM NADH for 20 s at 30°. Following electrofocusing and SDS-gel electrophoresis with the neutral system, the proteins were blotted onto nitrocellulose, stained, and autoradiography conducted as described in Experimental Procedures. Subunits are identified as described in the legend to Fig. 1.

E2
X
E3
E1α
E1β
E2
X
Electrophoresis →
Electrofocusing →

separated protein X and the dihydrolipoyl dehydrogenase, establishing the comigration of these subunits in the neutral pH system.

As can be seen in Fig. 4, protein X is retained with the transacetylase core following the resolution procedure (43) that removed all of the pyruvate dehydrogenase component from the core and nearly all of the dihydrolipoyl dehydrogenase component. Thus, protein X appears to be similar to kinase in that it binds tightly to the transacetylase core (4,12,41). Gel electrophoresis patterns showing the position of migration of kinase subunits will be presented below.

Both the samples of complex and resolved transacetylase core shown in Figs. 3 and 4 were treated with 20.0 μM [2-^{14}C]malonyl-CoA for 20 s prior to conducting 2-D gel electrophoresis. That treatment allowed the level of acylation of sites in the complex to be easily controlled since it depends on enzyme-catalyzed decarboxylation of malonyl-CoA (34). Furthermore, malonyl groups are rapidly incorporated into the dihydrolipoyl transsuccinylase component (34) of the α-ketoglutarate deydrogenase complex allowing trace levels of that component to be detected by autoradiography. The spot below and to the left of protein X in the autoradiographs on the right side of Figs. 3 and 4 is due to acylated transsuccinylase. Only about 3 acetyl groups were incorporated per mol of pyruvate dehydrogenase complex or resolved core under the conditions used; thus the autoradiographs established that, even at low levels of incorporation, protein X was acylated (see below). Both the transacetylase core and protein X were rapidly acylated additionally by low levels of pyruvate, propionyl-CoA, and higher levels of butyryl-CoA (cf. Tables II and III below). As in the case of acetyl-CoA, specific effects of these ligands on kinase activity result from acylation of

FIGURE 4

Two-dimensional gel electrophoresis pattern and autoradiograph for resolved dihydrolipoyl transacetylase lightly acylated by treatment with [2-^{14}C]malonyl-CoA. Resolved transacetylase (35 μg), which had a low level of malonyl-CoA decarboxylase activity (34) and sufficient dihydrolipoyl dehydrogenase for reduction of lipoyl moieties, was acylated by treatment for 20 s at 30° with 20 μM [2-^{14}C]malonyl-CoA in the presence of 250 μM NADH. Other procedures were as described in the legend to Fig. 3 and in Experimental Procedures. Subunits are identified as described in the legend to Fig. 1.

E2
X
E2
X
Electrophoresis →
Electrofocusing →

sites in the complex as described elsewhere (19,31-34).

Performic acid treatment. Following electrophoretic separation of acetylated subunits of protein X and the transacetylase core and transblotting these proteins to nitrocellulose, treatment with performic acid vapors for 24 h released greater than 95% of the acetyl groups from these protein components.

SDS systems and distribution of kinase activities. Figs. 5 and 6 show a series of gel electrophoresis patterns for the same preparation of complex, the transacetylase-protein X-kinase subcomplex (E2-K), the kinase fraction (K), and the dihydrolipoyl transacetylase depleted of kinase. Fig. 5 shows the pattern observed when electrophoresis was performed with the standard Laemmli system (45) and the bands detected with Coomassie blue. Fig. 6 was prepared with 20-fold smaller samples and the SDS-sample buffer was supplemented with 30 mM dithiotreitol and protein bands located by silver staining (49).

As noted earlier, protein X was retained with the transacetylase fraction after removal of the pyruvate dehydrogenase component and most of the dihydrolipoyl dehydrogenase component. Besides protein X and low levels of the dihydrolipoyl dehydrogenase component, the transacetylase-kinase subcomplex (E2-K) also contained a faster migrating band (designated K) which was difficult to detect in the intact complex both because it stains poorly with Coomassie blue and because it migrated in the region just above the α-subunit of the pyruvate dehydrogenase component. The region is labeled K for the catalytic subunit of the kinase based on several results presented below. As shown in Fig. 6, the greater sensitivity of the silver stain allowed the K band region to be more easily detected. It should be noted that the staining intensity

FIGURE 5

SDS gel electrophoresis of kidney pyruvate dehydrogenase complex and resolved components. This series was stained with Coomassie blue. The pyruvate dehydrogenase complex (PDC), transacetylase-protein X-kinase subcomplex (E2-K), K fraction (K), and kinase depleted transacetylase fraction (E2) were loaded respectively at 20 µg, 10 µg, 5 µg, and 10 µg. Specific bands are labeled as in Fig. 1.

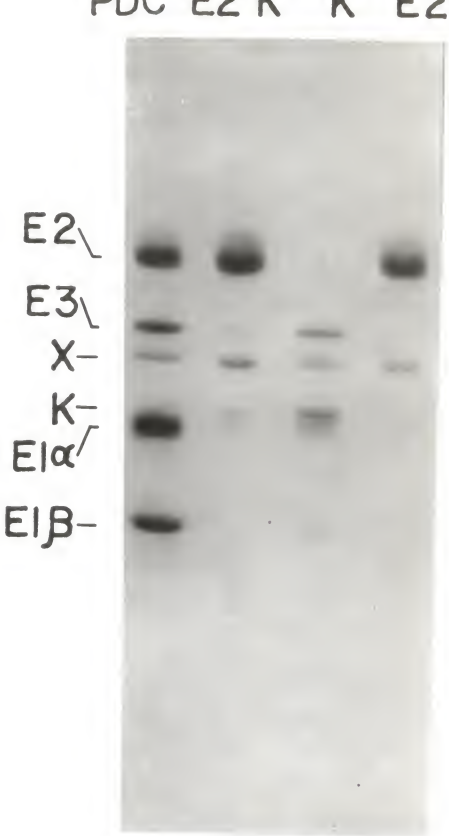
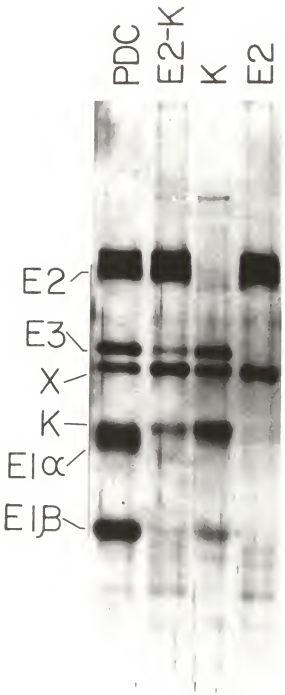

PDC
E2-K
K
E2
E2
E3
X
K
E1α
E1β

FIGURE 6

SDS-gel electrophoresis of kidney pyruvate dehydrogenase complex and resolved components. This series was stained by the silver stain procedure of Oakley et al. (49) and 1.0 µg of complex and 0.5 µg of the other components were loaded. Specific bands are labeled as in Fig. 1.


PDC
E2-K
K
E2
E2
E3
X
K
E1α
E1β

relative to other components for protein X and the transacetylase core subunit was higher when a silver stain rather than Coomassie blue was used.

The K fraction was prepared by reaction of reduced transacetylase-protein X-kinase subcomplex with p-hydroxymercuriphenyl sulfonate under conditions of high salt and a basic pH (27). Essentially, all the K band and residual dihydrolipoyl dehydrogenase were released by that treatment; however, while a portion of protein X was released into the K fraction, a significant portion of protein X was retained with the transacetylase core (Figs. 5 and 6). That result was observed in 8 preparations of the K fraction from different preparations of complex. Thus, since virtually all the kinase activity was released from the core (see Table I), these results establish that protein X is not the catalytic subunit of the kinase.

Estimation of protein X in the complex. Using the approaches described in the Experimental Procedures, the proportion of protein X in the complex was estimated in silver-stained gels (Protein X stained much stronger with silver stain than with Coomassie blue [39]). Using a standard curve of complex ranging from about 600 ng to 30 ng and purified protein X ranging 28 to 7 ng, densitometric traces were obtained. Densitometric traces were nonlinear at higher protein concentrations but approached linearity at lower concentrations such that the heights of protein X bands could be closely matched.

Using that approach, the number of protein X subunits per mole of complex was estimated to be in the range of 4.8 to 5.3. A molecular weight of 50,000 was used for protein X based on its relative mobility in SDS-slab gel electrophoresis.

TABLE I

Pyruvate Dehydrogenase Kinase Specific Activity in Various Fractions of Purified Enzymes.

Fraction	Specific Activity (nmol/min/mg)
Pyruvate dehydrogenase complex	9.65
Transacetylase-protein X-kinase subcomplex	17.8
Transacetylase-protein X subcomplex	0.34
K fraction	90.1

Kinase activites were measured with 100 μM [γ-^{32}P]ATP in buffer A with 0.84 mg/ml resolved pyruvate dehydrogenase component added except in assays of the intact complex where endogenous pyruvate dehydrogenase component served as substrate. Kinase activity in the K fraction (0.56 μg) was determined in the presence of 21.0 μg of transacetylase-protein X subcomplex. Activities were corrected as appropriate for low levels of kinase activity present in other fractions. Other conditions for assaying kinsase activity were as described in Experimental Procedures.

Peptide maps. Peptide mapping studies were conducted to evaluate whether protein X was derived from or structually related to the transacetylase core. In addition, these studies compared protein X to the dihydrolipoyl dehydrogenase component with which it comigrates in neutral gels as well as the dihydrolipoyl transsuccinylase which moves only slightly ahead of protein X in the Laemmli system. Figure 7 shows tryptic peptide maps of ^{125}I-labled peptides of these proteins as well as mixtures of protein X and the other three proteins. The maps of protein X were reproducible and distinct from those of other components. One indication that the mapping procedure was informative and reproducible is that the maps of the other three proteins corresponded in number of peptides and their relative positions to similar maps of those proteins prepared two years before in another laboratory.[2]

Tryptic maps were also conducted on the transacetylase core and protein X after acetylation with [1-^{14}C]acetyl-CoA or [2-^{14}C]pyruvate. Initially maps were analyzed of [1^{14}C]acetylated peptides from protein X and the transacetylase which were prepared by tube gel electrophoresis as described in Experimental Procedures. Both proteins gave a pattern of multiple acetylated peptides, in which the labeled peptides were aligned on peptide maps in horizontal and vertical directions. Because residual binding of SDS might contribute to that result, an alternative approach was developed involving separation of polypeptides in the presence of urea. Similar patterns were found on autoradiographs (Fig. 8) but there was less smearing of acetylated peptides.

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[2]Maps of components of the pyruvate (other than protein X) and α-ketoglutarate dehydrogenase complexes were performed by Jeff Hanson in the laboratory of Dr. L. Takemoto (Division of Biology, KSU).

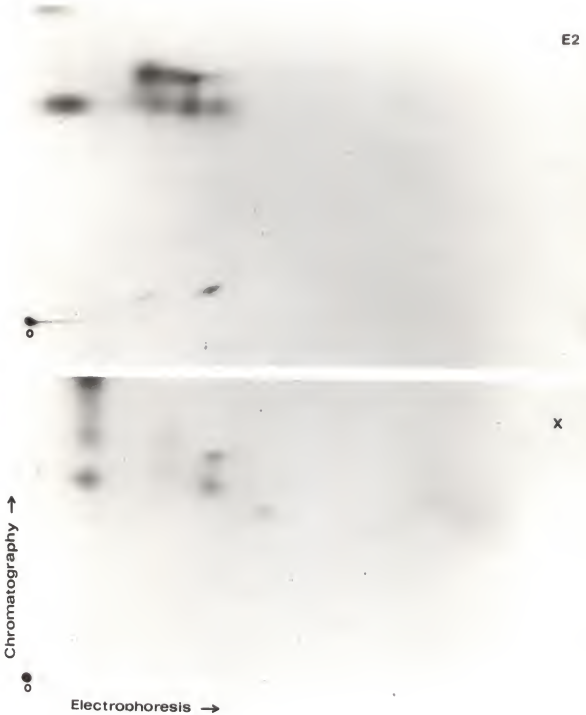
FIGURE 7

Maps of ^{125}I-tryptic peptides. Peptide maps are shown for the dihydrolipoyl transacetylase (E2), the dihydrolipoyl dehydrogenase (E3) and the dihydrolipoyl transsuccinylase (LTS), protein X as well as for mixtures of peptides of protein X with peptides of the transacetylase (E2+X). Iodination, preparation of subunits by SDS-slab gel electrophoresis, and tryptic digestion were conducted as described in Experimental Procedures.

E2
E3
LTS
Chromatography
Electrophoresis

FIGURE 8

Mapping of [1-^{14}C]acetyl-tryptic-peptides of protein X and the dihydrolipoyl transacetylase. Acetylated peptides were prepared as described in Experimental Procedures by DEAE-chromatography in the presence of 8 M urea. Peptide mapping was conducted by the same procedure detailed in Experimental Procedures.


E2
X
Chromatography →
Electroohoresis →

In marked contrast to the patterns for ^{125}I-tryptic peptides, [^{14}C]acetylated tryptic peptides were similar for the transacetylase core and protein X; however, some apparent differences were observed (Fig. 8). Movement of the peptides suggested relatively acidic and hydrophobic peptides were involved.

Immunological properties of protein X and the transacetylase. Affinity purified rabbit IgG prepared against the dihydrolipoyl transacetylase core reacted with the transacetylase and trace levels of transacetylase present in a preparation of the α-ketogluterate dehydrogenase complex as shown in Fig. 9. There was no reaction of the affinity purified rabbit IgG with protein X or with the dihydrolipoyl transsuccinylase component. The transacetylase was converted by trypsin treatment into the outer lipoyl-bearing domain (E2-L) and the inner domain (E2-I) which is the intersubunit binding region of the transacetylase and contains the active site for the transacetylation reaction (6). (E2-L stains poorly with Coomassie brilliant blue but well with silver stains as shown in (39). The anti-transacetylase rabbit IgG reacted with both domains (Fig. 9) although to a much lesser extent than it reacted with the intact transacetylase. That difference was very large since trace levels of intact transacetylase reacted to a much greater extent than higher levels of either subdomain. The observed specificity suggests that much of the IgG reacts preferentially with the hinge region connecting E2-I and E2-L segments and that portions of the proline-rich domain (22) are removed upon proteolytic treatment. That is consistent with recent work showing antibodies preferentially react with the more mobile regions of antigens (52,53).

Mouse antiserum to protein X reacted preferentially with protein

FIGURE 9

Reaction of affinity-purified anti-transacetylase rabbit IgG with the transacetylase and its trypsin-derived subdomains. The middle of the figure shows a stain of protein bands with Coomassie Brilliant blue following blotting of samples from SDS-slab gels onto nitrocellulose. The left and right sides of the figure show immunoblotting patterns for protein samples blotted from the same slab gel. From left to right electrophoresis was conducted on: 1) the transacetylase-kinase-protein X subcomplex (10 μg); 2) trypsin-derived lipoyl-bearing domain of the transacetylase core (E2-L) and inner-intersubunit binding domain of the transacetylase (E2-I) from 10 μg of transacetylase; 3) the bovine kidney α-ketoglutarate dehydrogenase complex (20 μg); and 4) the kidney pyruvate dehydrogenase complex (20 μg). In the blotting pattern of the right side, the kidney complex was not included in the series. For preparation of subdomains of the transacetylase (6), 1.0 mg of resolved core was treated for 60 min with 1 μg of trypsin at 4^{o} in 0.05 M Na-phosphate buffer, pH 6.5. Visualization of the antibody was done with the procedures outlined in Experimental Procedures.

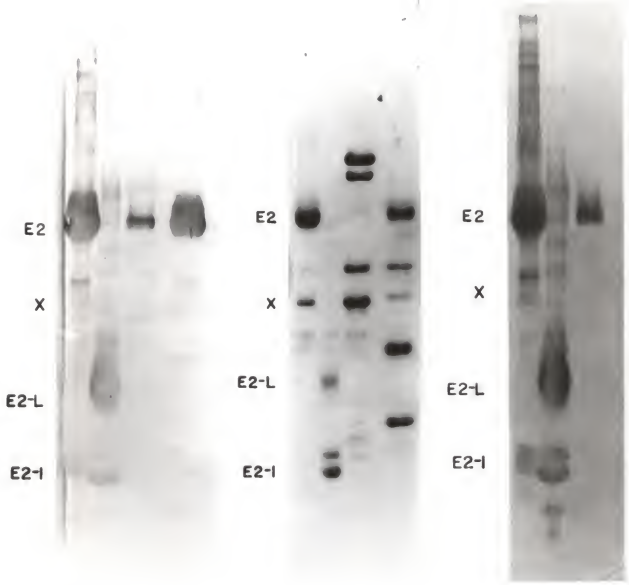
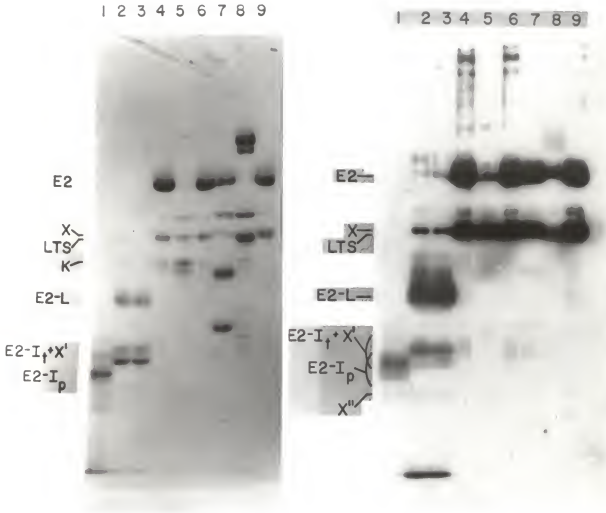

E2
X
E2-L
E2-I
E2
X
E2-L
E2-I
E2
X
E2-L
E2-I

FIGURE 10

Reaction of mouse antiserum to protein X with subunits from various fraction and polypeptides derived from the kidney and heart pyruvate dehydrogenase complex. The left side of the figure shows a stain of protein bands with Coomassie blue following blotting of samples from SDS-slab gels onto nitrocellulose. The right side of the figure shows immunodetection of antigenic polypeptides by autoradiography for an identical set of protein samples blotted from the same slab gel. Subunits are designated as in Fig. 1 with additional components identified as follows: the catalytic subunit of the kinase (27,39) as K and limit ploypeptides derived by trypsin treatment from protein X as X' and X". From left to right electrophoresis was conducted on: sucrose gradient purified inner domain of transacetylase (20 μg) generated by papain treatment (lane 1); limit polypeptides (20 μg) from 30 and 60 min treatment with trypsin (see the legend to Fig. 9) of the transacetylase (lanes 2 and 3, respectively); the kidney transacetylase-kinase subcomplex (10 μg) (lane 4), the kidney kinase fraction (10 μg) prepared by the procedure of Stepp, et. al.(27) (lane 3), heart transacetylase-kinase subcomplex (10 μg) (lane 6); kidney pyruvate dehydrogenase complex (20 μg) (lane 7); the kidney α-ketogluterate dehydrogenase complex (20 μg) (lane 8) and the kidney transacetylase core (10 μg) (lane 9) from which all the kinase but not all protein X has been removed (39). Immunoblotting and detection was carried out as described in Experimental Procedures. Autoradiography was conducted for 14 h.


1 2 3 4 5 6 7 8 9
E2
X
LTS
K
E2-L
E2-I$_f$+X'
E2-I$_p$
1 2 3 4 5 6 7 8 9
E2
X
LTS
E2-L
E2-I$_f$+X'
E2-I$_p$
X''

X, but also with the transacetylase core as shown in Fig. 10. Fig 10 also shows that this antiserum reacted with the two domains of the transacetylase core (lanes 1-3), the transsuccinylase component (lane 8) as well as reacting with protein X associated with the resolved bovine heart transacetylase core (lane 6) and resolved kinase fraction (lane 5). Interestingly, in the region of the two tryptic derived peptides coming from the inner domain of the transacetylase core (lanes 2 and 3) and in the region of the smaller polypeptide for the inner domain of the core generated by papain treament [3] (lane 1), the most intense reactivity was at a region between the tryptic peptides and above the papain derived inner domain. That region and a lower polypeptide are designated as X' and X'' based on results below which suggest that they are derived from protein X.

To ascertain whether reaction of mouse antibody with the transacetylase core resulted from contamination of core in the original antigen or reflected reaction with antigenic sites in protein X that were also present in core, the antiserum was affinity purified using strips of nitrocellulose containing the core and protein X derived from SDS-slab gel electrophoresis of 200 µg of kidney complex.

Fig. 11 shows the reactivity of both affinity purified mouse anti-protein X antibody (left side) and mouse anti-transacetylase (right side) with the pattern for stained bands in the middle. From left to right in each series the samples were tryptic-treated

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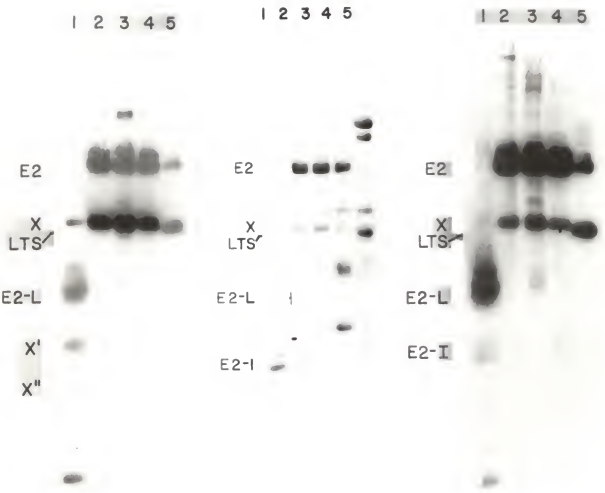
[3] The inner domain of papain treated core was perpared by the procedure of Kresze et al. (54) followed by pelleting the large inner domain ($M_r \sim 1.6 \times 10^6$) through a sucrose pad consisting of three layers containing 7.5% (1.0 ml), 10% (1.0 ml), and 15% sucrose (1.5 ml) by centrifugation at 26,000 rpm for 4 h in a Beckman SW 27 rotor at 4°.

FIGURE 11

Reaction of affinity purified mouse anti-protein X and mouse anti-transacetylase antibodies with the subunits and polypeptides prepared from the kidney or heart pyruvate dehydrogenase complex and with the kidney α-ketoglutarate dehydrogenase complex. The left side shows the pattern from reaction of affinity purified anti-protein X mouse antibody and the right side shows anti-transacetylase mouse antibody. The pattern of proteins stained with Coomassie blue is in the middle. In each pattern from left to right the lanes contained: 1) the limit polypeptides (10 μg) from trypsin treatment of the transacetylase core (see Fig. 9); 2) kidney transacetylase kinase subcomplex (10 μg), 3) heart transacetylase kinase cubcomplex (10 μg), 4) the kidney pyruvate dehydrogenase complex (20 μg). Bands are identified using symbols given in Fig. 1. Antibodies were prepared as described in Experimental Procedures. Immunoblotting and detection were conducted using one fifteenth of the affinity purified antibodies diluted into 6 ml by the procedure described in Experimental Procedures. Autoradiography was conducted for 24 h.


1 2 3 4 5
E2
X
LTS
E2-L
X'
X"
1 2 3 4 5
E2
X
LTS
E2-L
E2-I
1 2 3 4 5
E2
X
LTS
E2-L
E2-I

transacetylase-kinse subcomplex, intact transacetylase-kinase from kidney (lane 2) and heart (lane 3), the intact kidney complex, and the kidney α-ketoglutarate dehydrogenase complex. The results are involved but informative. Affinity purified anti-protein X antibody preferentially reacted with protein X and affinity purified anti-transacetylase antibody preferentially reacted with the transacetylase. However, both antibodies reacted to some extent with the other subunit and with the transsuccinylase core subunit. That might, in part, reflect reaction of the antibody with lipoyl-containing domains. Both antibodies reacted with the lipoyl-containing domain of the transacetylase. In contrast, in the region of the tryptic-derived inner core, the anti-protein X antiserum reacted only with a polypeptide that moves between the two polypeptides generated from the transacetylase core and with another polypeptide below that region. These are designated X' and X", respectively. Furthermore, the anti-transacetylase antibody failed to react with the X' or X" regions and reacted only with the polypeptides derived from the transacetylase core.

Relative acylation of the transacetylase and protein X. Since the above results indicate that protein X is a distinct protein, the relative incorporation of acyl groups into the transacetylase core and protein X under a variety of conditions was evaluated. All procedures following acylation were done in the absence of free thiols and the presence of the N-ethylmaleimide. Loss of protein-bound [^{14}C]acyl groups during preparation of samples, electrophoresis, and staining was corrected for as described in Experimental procedures.

As shown in Table II, about 10 acetyl groups were incorporated

TABLE II

Extent of acylation of the dihydolipoyl transacetylase core (E2) and protein X.

Conditions				Mol acyl group incorp. / Mol complex (or subcomplex) into		% incorp. into protein X
Enzyme Source	CoA Ester	Time (s)	Other Additions	E2	protein X[a]	
Dihydrolipoyl transacetylase kinase subcomplex	acetyl-CoA	60	---	44.3	4.30	9.7
Pyruvate dehydrogenase complex	acetyl-CoA	60	---	42.5	3.55	8.4
"	acetyl-CoA	60	250 μM CoA	7.2	0.95	13.2
"	acetyl-CoA	20	---	31.7	3.20	10.1
"	acetyl-CoA	600	---	45.1	4.55	10.1
"	acetyl-CoA	20	20 μM TPP	38.0	3.75	9.9
"	acetyl-CoA	600	20 μM TPP	19.9	2.15	10.8
"	propionyl-CoA	20	---	28.4	2.40	8.5
"	butyryl-CoA	60	---	56.0	4.15	7.4

[a] rounded to nearest 0.05

Acylation reactions were carried out in the presence of 20.0 μM [1-^{14}C]acetyl-CoA, 20.0 μM [1-^{14}C]propionyl-CoA, or 50.0 μM [1-^{14}C]butyryl-CoA at 30° in the presence of 250 μM NADH. Other conditions were as described in the Table or Experimental Procedures.

into the transacetylase core for each group incorporated into protein X when acetylation of either the intact complex or resolved subcomplex was allowed to proceed for 60 s at 30^{o}. The similar level of acetylation of protein X associated with the resolved core, in comparison to intact core, indicates that virtually all protein X was retained with the transacetylase core during resolution. CoA at 250 μM reduced the level of acylation about six-fold for both the transacetylase core and protein X. Thus deacylation of protein X involves transfer to CoA which may be direct or indirect (i.e. indirect could involve initial transfer to lipoyl moities on the transacetylase core). There was little change in the relative acetylation of the transacetylase core and protein X with incubation times ranging form 20-600 s (selected data from the extreme times are shown in Table II). Both propionyl-CoA and butyryl-CoA, which have been shown to be fast and relatively slow substrates, respectively, for acylating sites in the complex (31), gave a similar but slightly higher ratio for acylation of the transacetylase core relative to protein X.

TPP at 20.0 μM had little effect on acetylation by acetyl-CoA at short times (20 s) but at longer times 20.0 μM TPP significantly decreased the extent of acylation and caused a proportional reduction in the acetylation of the transacetylase core and protein X. Since acetylated lipoyl moieties can slowly react with TPP bound to the pyruvate dehydrogenase component to form 2-hydroxyethylthiamine pyrophosphate (and oxidized lipoyl moities), it would appear that acetyl groups linked to protein X are available as substrates for this reaction. That is consistent with the rapid acylation of protein X by pyruvate (below).

TABLE III

Extent of acetylation of the dihydrolipoyl transacetylase (E2) and protein X by [2-^{14}C]-pyruvate.

Conditions			Mol incorp/mol complex into		% incorp into
Pyruvate (μM)	TPP (μM)	Time (s)	E2	protein X[a]	protein X
20	-	20	8.8	0.90	10.2
20	-	600	71.0	7.75	10.9
20	20	20	65.5	6.70	10.2
20	20	600	35.0	4.80	13.7
100	2	20	60.8	6.40	10.5
100	2	600	97.3	9.50	9.8

[a] Rounded to nearest 0.05

Complex was incubated at 30° with the indicated level of pyruvate. Other conditions were as described in the Table or Experimental Procedures.

Table III shows the relative acetylation of the transacetylase core and protein X when pyruvate was used as the acylating agent in the presence of TPP (2.0 or 20.0 μM) or absence of added TPP. The preparation of complex used had only 3% of its maximal activity when assayed in the absence of added TPP. Low levels of TPP enhanced acetylation but fairly high levels of acetylation were achieved in the absence of added TPP at longer times (e.g. 600 s). At 600 s there was a decrease in acetylation when a lower pyruvate level (20.0 μM vs. 100.0 μM) but higher TPP level (20.0 μM vs. 2.0 μM) was used. That decrease was presumably aided by accumulation of side products (acetoin or acetolactate) which contributes to the turnover of enzyme-bound 2-hydroxyethylthiamin pyrophosphate.

With [2-^{14}C]pyruvate as a substrate (Table III), about 10 acetyl groups were incorporated into the transacetylase core for each group incorporated into protein X. Higher levels of acetylation were observed than with 20 μM acetyl-CoA; however, when 200 μM [1-^{14}C]acetyl-CoA was used, levels of acetylation of protein components approached that achieved with pyruvate. While the ratio for the relative acetylation of core subunits and protein X was very close to that observed with acetyl-CoA, there appeared to be a small time-dependent variation in the relative acetylation of the transacetylase core to protein X with pyruvate. In five experiments, a 23 ±9% higher ratio for acetylation of protein X relative to acetylation of the core was observed at 300 or 600 s as compared to 20 s. On the other hand, even when acetylation was conducted at 5° in the presence of 20.0 μM TPP and 100 μM pyruvate, there was rapid acetylation (by 10 s) to nearly 60 acetyl groups/mol complex with nearly 10% of the acetyl groups incorporated into protein X

(data not shown).

The above series of results (Tables II and III) indicate that the acetylation of protein X relative to transacetylase subunits is close to the apparent molar ratio of 1.0 protein X subunit to 12 transacetylase subunits per molecule of complex. Thus the results strongly suggest either that there is direct acylation of protein X or that there is a rapid transfer of acyl groups from the lipoyl moieties on the transacetylase core to a thiol acceptor on protein X. For the former to occur, it would seem most likely that a lipoyl-containing domain on protein X would compete with the lipoyl-containing domains of the transacetylase at the active sites catalyzing the reductive acetylation and transacetylation reactions.

CHAPTER 4: DISCUSSION

The polypeptide chains present in purified pyruvate dehydrogenase complex were separated with both the system of Laemmli (45) and with a modified system that was closer to neutral pH. Because this study characterised the acylation of protein components, the lower pH system was used to minimize hydrolysis of the thioester linkages. Additionally following acylation, acid precipitated proteins were maintained in the presence of N-ethylmaleimide and in the absence of free thiols. Under such conditions, the transfer of acetyl groups to free thiols or to nonspecific sites on other protein components was eliminated. Moreover, those conditions also prevented the formation of interchain disulfides resulting in the same band pattern as that observed when gel electrophoresis was performed in the presence of thiols. If incubation mixtures were not immediately terminated by acid-precipitation, the addition of N-ethylmaleimide did not immediately stop the reaction. Thus some transfer of acetyl groups (presumably nonspecific) to the thiol rich area of α-subunits of the pyruvate dehydrogenase was occasionally observed. If either the higher pH (standard) system was used or thiols were present in the SDS-gel sample buffer, the level of acylation was significantly reduced. However the qualitative pattern of which subunits underwent acylation did not change.

As shown in the results section, protein X along with the transacetylase component, underwent rapid and specific acetylation. As noted above, addition of N-ethylmaleimide eliminated nonspecific acetylation. Thus the acetylation of protein X is probably not an artifact.

The number of protein X subunits per mol of complex was calculated to be in the range of 4.8 to 5.3. As shown earlier, a

molecular weight of 50,000 based on gel electrophoresis was used. If as is true with the transacetylase component (6), protein X is transported slower than standard proteins of the same molecular weight in SDS-gel electrophoresis, the above estimate could be low. Thus the maximun number of protein X per core could approach six. It is interesting to speculate that if six faces of the dodecahedron core are occupied by flavoprotein, the remaining six could be occupied by protein X. If protein X has an unusually high or low content of lysine relative to bovine serum albumin, that would also cause error in this determination since protein levels were determined by the method of Fried et al. (48) in which protein derivitization depends primarily on the lysine content. Fried et al. (48) maximally found a 30% difference in the slopes of calibration curves with various proteins; it seems unlikely that more than a 10% error in our estimate of the amount of protein X would result from the protein measurement since bovine serum albumin gave an intermediate slope.

The performic acid studies demonstrate acetylation (>95%) is at thiol residues which is consistent with protein X containing a lipoyl region. This is confirmed by the acetylated peptide work which further implies that these regions may be similar. Both the protein X and the transaceylase showed similar peptides although differences could be found (Fig. 8). The position of the peptides suggested that relatively acidic and hydrophobic peptides are involved. That is characteristic of the lipoyl-containing region of the transacetylase core component and suggests a similar lipoyl-containing region may be present in protein X. It is unclear whether multiple peptides are due in part to more than one acetylation site, but the incorporation of at least two acetyl

groups per polypeptide chain was indicated by the maximal levels of acetylation, as will be discussed below. In further studies we have established the presence of lipoyl moieties (39) and are extending those studies to quantitate the number of lipoyl moieties in the dihydrolipoyl transacetylase component and protein X.

One can achieve levels of acetylation with pyruvate of the bovine kidney complex in the range of 100-120 acetyl groups incorporated per mol of complex. Assuming that there were equal losses from protein X and the transacetylase in subsequent treatments, data with [2-^{14}C]pyruvate suggest that ∿10 acetyl groups can be incorporated by subunits of protein X per molecule of complex. Based on estimates of the number of subunits of protein X, that suggests there are two acetylation sites per molecule of protein X.

Since there are about 60 transacetylase subunits (12), it would appear that there would have to be more than one site per subunit of the transacetylase that can be acetylated. The lipoyl domain of the transacetylase core from E. coli K12 contains three lipoyl-containing regions per subunit in three highly-conserved regions (13). Comparison of the amino acid composition (6) of the bovine kidney transacetylase lipoyl-bearing domain to that of the lipoyl domain from the E. coli transacetylase suggests[4] that two lipoyl-containing regions may be present in subunits of the mammalian transacetylase.

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[4]The lipoyl-bearing domain of Echerichia coli K12 contains three lipoyl-containing regions (∿100 amino acids each) of known sequence (26). The amino acid composition of the lipoyl domain of the bovine heart transacetylase has been reported (13). Comparison of that compostion to the average compostion for two-thirds of the lipoyl domain of the E. coli K12, (2/3 of 1-316 in the amino acid sequence) yields enough similarity to suggest a double repeat in the mammalian lipoyl

^{125}I-peptide mapping firmly established that protein X is structurally distinct from and not derived from the transacetylase core. Moreover, despite the fact that the complex was essentially free of KGDC contamination, the possibility that protein X was merely the dihydrolipoyl transsuccinylase was eliminated with this result. A comparison of the protein X and LTS maps show very little similarity between the two proteins. It is worth noting that although the protein X is distinct from the transacetylase and the flavoprotein, some peptides appear to be common between protein X and the transacetylase and between protein X and the flavoprotein. An interesting possibility to keep in mind in further work is that protein X is a "hybrid" composed in part of sequences originally derived from the transaceylase and the flavoprotein. The fact that E. coli PDC does not contain protein X could reflect the possibility the protein X arose in the evolution of PDC. That E. coli PDC is not regulated by phosphorylation/ dephosphorylation (8) whereas mammalian PDC is, leads one to think that there may be a link between the function of protein X and the regulation

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domain. Thus one finds in the comparison of the composition of the E. coli K12, to the bovine heart the following: gly: 16.7 vs 15.5; ser: 8.7 vs 13; glu, gln, asp, asn: 44.7 vs 50.7; lys, arg: 19.3 vs 21.4; and ala, val, ileu, leu, thr, met, phe: 107 vs 112. Interestingly, three amino acids not present in the E. coli lipoyl domain were pesent at a level of 2 per lipoyl domain in the mammalian (i.e. his, cys, trp). Despite the fact that the molecular weights are closer for the entire E. coli lipoyl domain and the lipoyl domain for the heart transacetylase, the above comparisons are not nearly as close when calculated based on the entire lipoyl domain of the E. coli core component. There was much more pro (47.7 vs 14.7) and tyr (5 vs 0) in the lipoyl domain of the heart enzyme which accounts for ~4000 daltons out of ~7000 daltons higher molecular weight required for the mammalian lipoyl domain than for two-thirds of the E. coli lipoyl domain. The rest is accounted for by the excesses of the various amino acids listed above. In the transacetylase core of the E. coli complex, prolines were in the hinge regions between subdomains and between the innermost of those subdomains and the inner-domain of the transacetylase core.

of the complex.

The immunological results with the anti-transacetylase rabbit IgG also strongly support the conclusion that protein X cannot be derived by proteolytic cleavage of the transacetylase component since antibody still reacted with both the domains derived from the transacetylase without reacting with protein X which is larger than either subdomain. Although evidence points out that a lipoyl-containing domain is present in protein X, the lack of reactivity suggests both that this lipoyl-containing region must be structurally distinct in part from that in the transacetylase core. Therefore, if it is connected to an inner region of protein X by a hinge region, that hinge region must be different from the hinge region in the transacetylase since that hinge region appears to be the major site at which the rabbit IgG reacted with the intact transacetylase.

These results do suggest that while protein X posesses regions similar to the transacetylase (as shown by the acetylated peptide mapping data), protein X also posesses regions very different from the transacetylase. This further supports the idea that protein X may have evolved from the transacetylase and another protein i.e. at some point a gene duplication might have occured from part of the transacetylase sequence (perhaps the lipoyl region) and part of the sequence of another protein, possibly the flavoprotein.

The work with the mouse antiserum is consistent with a transacetylase-like outer domain in protein X and a distinct inner domain in which a high degree of antibody specificity is achieved. The results strongly support the conclusion that protein X is not derived by proteolytic clipping from the transacetylase core. However, further

studies will be required to determine whether the crossreactivity of the affinity purified antibodies, apparently reflecting reaction with the outer domain, reflects, in part, related epitope (or epitopes) in the transacetylase core and protein X or incomplete purification of the two antibodies. Because of the large number of washes used in the purification procedure, it is more likely that there is a common antigenic region, possibly a broadly related feature of lipoyl-containing regions. This is consistent with the similarity of the acetylated-tryptic peptides derived from core and protein X and reaction of affinity purified antibodies with the transuccinylase. Thus my work has established that protein X is not identical to the dihydrolipoyl transacetylase but apperars to contain similar structural features.

Besides complications (described above) in establishing in gel electrophoresis systems that protein X was a distinct component, another reason that protein X was not detected in the early studies might have been due to its sensitivity to proteolytic degredation. The use of protease inhibitors in the preparation of the complex (particularly serum [41]) led to protection of protein X. Associated with the use of protease inhibitors, there was at least a two-fold increase in the kinase activity in preparations of the bovine kidney complex. In further studies (39) we have established that protein X is distinct from the catalytic subunit of the kinase which is also difficult to detect in gel electrophoresis studies; i.e., high kinase activity was associated with the complex and resolved core in Figs. 3 and 4 but the band corresponding to the kinase was not readily observable. In addition to the kidney complex, protein X was present in previously published gel

patterns of complex isolated from bovine heart (29, 55) and its resolved core (6) and is present in the porcine liver complex (Roche, unpublished). Protein X has been detected in immunoblotting patterns of crude subcellular extracts of rat liver and bovine kidney cell lines using antibodies prepared against the bovine heart complex (56).

Besides the potential role of protein X in regulating kinase activity, protein X may have a role in the association of the kinase and/or phosphatase with the transacetylase core. Each transacetylase subunit binds at separate interaction sites to three other transacetylase subunits, to two catalytic components of the complex, and to protein X. Additionally the transacetylase catalyzes one reaction and participates in two other catalytic steps. It would seem surprising if the transacetylase subunits were also capable of binding both regulatory enzymes. Data presented elsewhere (39) are consistent with an association between the kinase and protein X.

In conclusion, a distinct protein component, tightly bound to the transacetylase core, undergoes rapid acylation probably on a lipoyl-containing region. It would appear to have more than one acetylation site and its acetylation tends to occur at a constant ratio of about 1:10 relative to the acetylation of the transacetylase core.

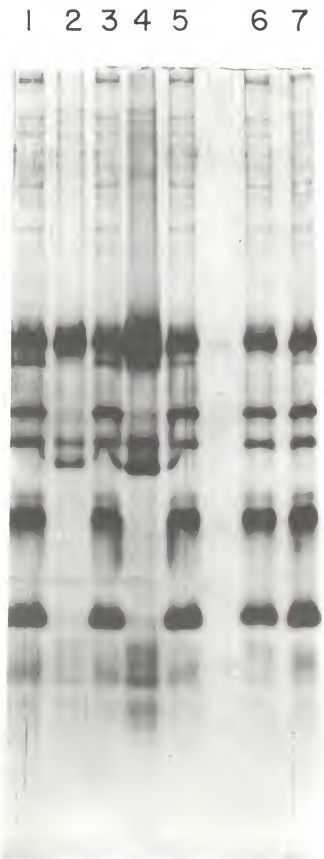
CHAPTER 5: APPENDIX

When the resolved kinase (K fraction) and dihydrolipoyl transacetylase-protein X were prepared as described by Stepp et al. (27), there were major changes in gel electrophoresis patterns due to the presence of p-hydroxymercuriphenyl sulfonate. The transacetylase precipitated by treatment with a mercurial agent was dissolved in 0.05 M phosphate buffer, pH 7.5, containing 30.0 mM dithiothreitol, 0.10 mM $MgCl_2$, and 0.01 mM EDTA. Figure 12 shows silver-stained gel patterns revealing the effect of a 2.0 µg sample of resolved core (lane 5) on adjacent samples (1.0 µg) of complex. Shifts are noted in the bands for the transacetylase, protein X, and the α-subunit of pyruvate dehydrogenase component. When the transacetylase-kinase subcomplex was used, the K band was seen to smear (see Fig.12, lanes 1-5). Also shown in Fig. 12 is the effect of a 1.0 µg sample of resolved transacetylase (lane 2) that was dialyzed over a 24 h period against several changes of the above phosphate buffer but containing 2.0 mM dithiothreitol. Even that treatment and the use of a smaller sample did not remove effects on adjacent samples of complex. Lane 7 shows a sample of complex which 30 mM dithiothreitol was included in the sample buffer. The bands are not shifted in this sample; note that the use of 30.0 mM dithiothreitol in the sample buffer did not completely prevent a broadening effect of the mercurial agent on the transacetylase but the position of protein X and the kinase appear the same as samples of complex where there is no effect of the mercurial agent (i.e., lane 7, Fig. 12). The extra bands are not due to less purity of the complex used in this gel. (It is the same preparation as was used in Figure 5.)

The effects of the mecurial agent can also be seen in Fig. 12.

FIGURE 12

Effect of diffusion of p-hydroxymecuriphenyl sulfonate on gel electrophoresis patterns at low levels of loading of proteins. In lanes 1,3,5,6, and 7, 1.0 µg of kidney complex was loaded. With the sample in lane 6, 30 mM dithitreitol was included in the sample buffer. Lane 4 contained 3.0 µg of transacetylase-protein X subcomplex from which the kinase was removed by mecurial treatment and the pellet dissolved in buffer containing 30 mM dithiotreitol as described by Stepp et al. (27). Lane 2 contained a 1.0 µg sample of subcomplex that had been exhaustively dialyzed in phosphate buffer (27) containing 2 mM dithiotreitol. All sample buffers contained 2.0% (w/v) 2-mercaptoethanol and bands were detected by silver staining (49).


1 2 3 4 5 6 7

In lane 1 1.0 μg of whole complex was loaded with no addition of the mecurial agent. In lanes 2 and 3, 0.5 and 50 mM p-hydroxymecuriphenyl sulfonate was included with the sample. Land 4 included in addition to the mecurial agent, 26 mM dithiotreitol. Dithiotreitol was only partially successful in overcoming this effect.

In Fig. 13, the addition of 50 mM thioglycolate prevented the increased mobility and smearing caused by the mercurial compound. Presumably, reaction of the negatively-charged thioglycolate with the negatively-charged mercurial agent produced a species that migrated at or near the ion front.

To our surprise and independant of the presence of any mercurial agent (i.e., when the subunits of the transacetylase-protein X-kinase subcomplex were separated on gels lacking any mercurial agent containing smaples in other lanes), thioglycolate had the additional effect of causing the K band to migrate with a slower mobility and the band designated K_r polypeptide was retained with the transacetylase-kinase core during gel filtration under chaotropic conditions (i.e., during the resolution procedure (43) used to remove the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase components), it seems possible that it could be a specific subunit of the complex. Furthermore, it would appear to correspond to the trypsin-sensitive subunit of the kinase described by Stepp et al. (27) which lacked catalytic activity and for which a regulatory role was suggested. When development of the silver stain was allowed to proceed for a long time, or when staining was done with Coomassie blue following electrophoresis in the presence of thioglycolate, the relative intensities of the K and K_r bands appeared similar. Both bands stained very lightly with Coomassie blue.

FIGURE 13

Effect of different levels of p-hydroxymecuriphenyl sulfonate and dithiotreitol on gel electrophoresis patterns. All lanes were loaded with 1.0 µg of kidney complex with the following levels of mecurial agent: lane 1 (0), lane 2 (0.5 mM), and lanes 3 and 4 (50 mM). In addition, lane 4 contained 26 mM dithiotreitol. The gel was silver-stained by the method of Oakley et al. (49).

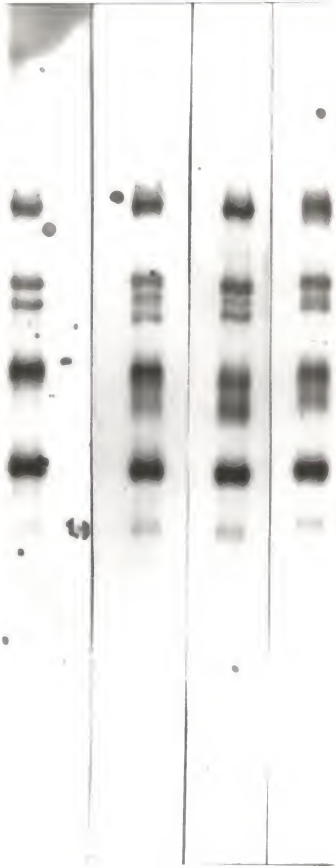
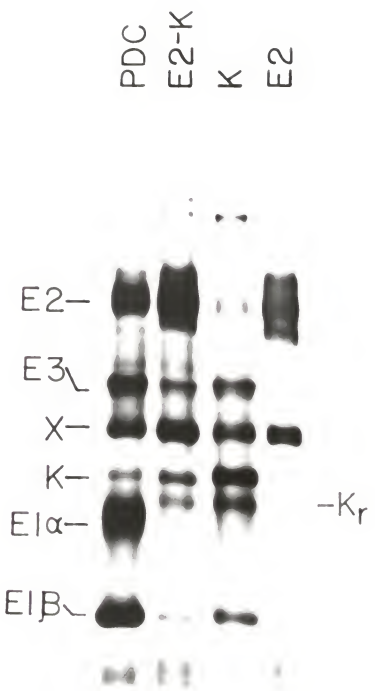


FIGURE 14

Effect of 50 mM thioglycolate on overcoming pattern shifts caused by p-hydroymecuriphenyl sulfonate. Lane 1 contains 1.0 µg of whole kidney complex. Lane 2 contains 0.5 µg of resolved kidney transacetylase-protein X-kinase subcomplex while lane 3 contains 1.0 µg of isolated kinase-protein X fraction (27). Lane 4 contains 0.5 µg of transacetylase-protein X depleted of kinase. All samples contained 50 mM thioglycolate.


PDC
E2-K
K
E2
E2—
E3
X—
K—
E1α—
E1β
—Kr

Following intense staining with the silver stain, reduction of staining intensity by the procedure described by Marshall (50) gave much stronger staining of the K band than of the K_r band.

However, as of yet, we have been unsuccessful in distingushing the K_r band from the pyruvate dehydrogenase band. Two-dimensional mapping of the complex and the resolved transacetylase-protein X-kinase subcomplex did not reveal a K_r band in the presence of 50 mM thioglycolate. More work will be needed to distingush between the two.

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PROPERTIES OF A NEWLY CHARACTERIZED PROTEIN OF THE BOVINE KIDNEY PYRUVATE DEHYDROGENASE COMPLEX

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ABSTRACT

The dihydrolipoyl transacetylase component, which serves as the structural core of mammalian pyruvate dehydrogenase complexes, accumulates in the acetylated form when treated with either pyruvate or with acetyl-CoA in the presence of NADH. Besides the dihydrolipoyl transacetylase component, it has been found that another protein, referred to as protein X, is rapidly acetylated at thiol residues. Protein X remains fully bound to the transacetylase core under conditions that remove the pyruvate dehydrogenase component and dihydrolipoyl dehydrogenase components. Besides protein X, the pyruvate dehydrogenase kinase also remains tightly bound to the transacetylase core. Even after an addditional treatment that removed virtually all the kinase activity, a significant portion of protein X was retained bound to the transacetylase core indicating that protein X is distinct from the catalytic subunit of the kinase. Mapping of ^{125}I-tryptic peptides indicated that the transacetylase subunits and protein X are structurally distinct; however, there is considerable similarity in the mobility of acetylated peptides derived from protein X and the transacetylase core, suggesting protein X may also contain lipoyl moieties. Affinity purified rabbit immunoglobulin G prepared against the dihydrolipoyl transacetylase core reacted exclusively with the transacetylase and with both its tryptic-derived inner domain and outer lipoyl-bearing domain. Since the rabbit antibody did not react with protein X which is larger than the subdomains of the transacetylase, protein X is probably not derived from the transacetylase component. Affinity purified mouse antibody to protein X reacted in a highly preferential manner with protein X, reacted selectively with large tryptic polypeptides derived from protein

X and did not react with the inner domain of the transacetylase. However, the affinity purified mouse anti-protein X antibody reacted to some extent with the intact transacetylase subunit, the lipoyl bearing domain of the transacetylase, and weakly with the transsuccinylase core of the α-ketoglutarate dehydrogenase complex. It is suggested that this cross reactivity reflects specificity of a portion of the polyclonal antibodies for a broadly related structural region in these proteins, possibly a similar lipoyl bearing region. There approximately 5 moles of protein X per mole of the kidney pyruvate dehydrogenase complex. About 10 acyl groups can be incorporated into protein X per mole of complex suggesting that protein X can undergo acylation at more than one site. Under a variety of conditions that result in a wide range of levels of acety- lation of sites in the complex, about one acetyl group is incorporated into protein X per 10 acetyl groups incorporated into the transacetylase subunits per mol of complex. That ratio is close to the ratio of protein X subunits to transacetylase subunits in the complex. Thus, the constancy of the ratio indicates either that a lipoyl containing region of protein X can undergo direct acetylation at active sites in the complex or that there is a rapid equilibrium for transfer of acetyl groups from lipoyl moieties on the transacetylase core to protein X.