Interactions of lipoyl domains with the E1p subunits of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli*

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Equilibrium binding experiments were carried out with lipoyl domains and the pyruvate decarboxylase [pyruvate dehydrogenase (lipoamide), E1p, EC 1.2.4.1)] component of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. The dissociation constant (K_s) was estimated to be not less than 0.3 mM, exceeding the K_m value (33 μM) for reductive acetylation of the domains by an order of magnitude. Thus, the lipoyl domain, which is required to promote reductive acetylation of the lipoyl group, does not appear to do this simply by enhancing the binding to E1p. The difference between K_s and K_m suggests that the formation and release of reductively acetylated lipoyl domains from the enzyme may be a relatively rapid step in the mechanism.

Lipoyl domain; Pyruvate dehydrogenase; Multienzyme complex; Equilibrium binding; Thiamin pyrophosphate

1. INTRODUCTION

The pyruvate dehydrogenase complex of *Escherichia coli* ($M_r \sim 5 \times 10^6$) contains three different types of enzyme which function successively to catalyse the oxidative decarboxylation of pyruvate to acetyl-CoA. The functional core of the complex is composed of 24 copies of dihydrolipoamide acetyltransferase (E2p, EC 2.3.1.12), assembled with octahedral symmetry. To this core are bound multiple copies of pyruvate decarboxylase (E1p, EC 1.2.4.1), which uses thiamin pyrophosphate (TPP) as a cofactor, and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4), a flavoprotein [1,2]. The primary structure of the E2p chain is distinctly segmental; in particular, the N-terminal half of the polypeptide chain comprises three highly homologous repeats of approx. 100 amino acids [3]. Each repeat contains a potential lipoyl-lysine residue, and can be isolated as an intact functional lipoyl domain after limited proteolysis [4]. Each repeat also includes a C-terminal region of 20–30 amino acids rich in alanine, proline and charged residues. These inter-domain regions appear to enjoy substantial conformational mobility [2] and are thought to promote active site coupling in the complex by allowing movement of the lipoyl domains between the catalytic centres of the three different enzymes [2,5,6]. Studies on the lipoyl domain isolated from the *Bacillus stearothermophilus* complex suggest that the remainder of each lipoyl domain is tightly folded [7].

We have demonstrated recently that purified lipoyl domains are very much better substrates for reductive acetylation by E1p than is free lipoamide [8]. In the present paper, an attempt is made to establish what part is played by the protein moiety of a lipoyl domain in binding and catalysis. It is not known how the protein domain enhances the ability of the lipoyl group to undergo reductive acetylation, but clearly the effectiveness of this group as a substrate for E1p would be greatly increased if the protein component of the domain were to bind tightly to the active site of this subunit [8]. It is possible that binding of a lipoyl domain to the E1p active site might occur only if TPP were already bound there; moreover, since binding in the physiological reaction occurs when the cofactor is present as hydroxyethylidene- or acetyl-TPP [9], it is conceivable that the form of the TPP molecule – or the subunit conformation associated with it – might be relevant to domain binding. This paper describes binding studies done in the presence of TPP and in the presence of the transition-state analogue thiamin thiothiazolone pyrophosphate (TTTPP) [10].

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Abbreviations: TPP, thiamin pyrophosphate; TTTPP, thiamin thiothiazolone pyrophosphate; DCPIP, 2,6-dichlorophenolindophenol

2. MATERIALS AND METHODS

2.1. *Materials*

Sodium [2-^{14}C]pyruvate was obtained from NEN Research Pro-

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ducts, Boston, USA. Methyl [^{3}H]acetimidate was synthesized as described in [11], and TTTPP was made according to [10]. Pyruvate dehydrogenase complex and free E1p were obtained as described elsewhere [8].

2.2. *Determination of proteins and enzymes*

The concentrations of protein solutions were measured as described in [8]. The activity of E1p with 2,6-dichlorophenolindophenol (DCPIP) was measured by monitoring spectrophotometrically the reduction of the coloured dye [12–14]. The activity of E1p with purified lipoyl domains as substrate was measured as described previously [8].

2.3. *Preparation and radiolabelling of lipoyl domains*

Pyruvate dehydrogenase complex (20 mg/ml) was incubated at room temperature with 0.05% (w/w) *Staphylococcus aureus* V8 proteinase in 20 mM sodium phosphate buffer, pH 7.0, containing 2.7 mM EDTA and 3.0 mM sodium azide, in a total volume of 13.6 ml. The overall complex activity decreased to 10% of the starting value in 2 h, and the reaction was then stopped by adding 27.1 mg of α_2-macroglobulin and incubating for a further 10 min at room temperature. The lipoyl domains were purified by applying the digestion mixture to a column of Sephadex 6B (92 × 3 cm, flow rate 30 ml/h, 4°C) equilibrated with the same buffer. Fractions (8 ml) were analysed by polyacrylamide gel electrophoresis, freeze-dried, and pooled appropriately. Further purification was obtained by applying the sample to a column of Sephadex G75 (Superfine) (54 × 1.5 cm, flow rate 7.9 ml/h, 4°C). Lipoyl domains (total concentration 4.33 mM) were incubated on ice with methyl [^{3}H]acetimidate (1.67 mM, about 40 Ci/mol) in 1 M *N*-ethylmorpholine/acetic acid buffer, pH 8.5, in a total volume of 0.3 ml. The incubation was stopped after 60 min by adding 1 μl of aqueous methylamine solution (300 ml/l) and, 10 min later, sufficient glacial acetic acid to lower the pH of the solution to 6.0. After 10 min on ice, the pH was readjusted to 7.0 with concentrated sodium hydroxide solution and applied to a Sephadex G75 (Superfine) column (54 × 1.5 cm, flow rate 9 ml/h, 4°C) equilibrated with 50 mM sodium phosphate buffer, pH 7.0.

2.4. *Equilibrium binding experiments*

To investigate the binding of radiolabelled lipoyl domains to E1p, the sample of E1p was first incubated in column buffer (see below) to which had been added a saturating amount of either TPP or TTTPP (with magnesium chloride), as specified. It was then applied to a column of Sephadex G100 (Superfine) (25.5 × 0.7 cm, flow rate approx. 0.7 ml/h, fraction volume approx. 0.5 ml, 19–21°C) that was equilibrated with 30 mM sodium phosphate buffer, pH 7.0, containing 3.0 mM sodium azide, radiolabelled lipoyl domains (16.4 μM), and a supplement of either TPP or TTTPP (with magnesium chloride) at saturating concentrations, as specified. The volumes of fractions were determined individually because of the inaccuracy inherent in maintaining such a low flow rate, and in order to take any effect of evaporation into account. E1p, present as a dimer with an apparent M_r of 190 000 [15], was totally excluded from the gel filtration matrix, whereas the lipoyl domains, having apparent M_r values around 27 000 under such conditions [4], had K_{av} values of about 0.7. The elution of radiolabelled lipoyl domains was measured by liquid scintillation counting of a 0.4 ml sample of each fraction; measurement of the absorbance at 280 nm allowed the elution of protein and of cofactors to be monitored. When the concentration of E1p in each fraction was calculated using A_{280} [1 mg/ml] = 1.34 (P.N. Lowe, unpublished result), almost all (>90%) of the E1p applied to the column could be accounted for.

3. RESULTS

Lipoyl domains were excised proteolytically from a sample (272 mg) of *E.coli* pyruvate dehydrogenase complex, and were subsequently radiolabelled to a limited extent under non-denaturing conditions by treatment with methyl[^{3}H]acetimidate. Polyacrylamide gel electrophoresis in the presence and absense of SDS revealed no cross-linking and indicated that the domains were pure and almost completely undegraded; high voltage thin layer electrophoresis developed by fluorography [16] showed that the preparation was free of radioactivity not bound to protein. The amount of radiolabelled domain was determined to be 1.2 μmol, a yield of 38% relative to the amount calculated to have been present in the original sample of pyruvate dehydrogenase complex. The extent of radiolabelling corresponded to amidination of about 0.1% of the total amino groups present. Reductive acetylation of equal amounts of amidinated and unamidinated domains by E1p in the presence of sodium [2-^{14}C]pyruvate resulted in incorporation of equal amounts of ^{14}C radioactivity at equilibrium. Amidinated and unamidinated samples were characterized kinetically as substrates for E1p as described previously [8] and were found to be indistinguishable; both preparations displayed a true K_m value of 33 μM when the direct linear plot was used to evaluate kinetic constants.

The use of purified E1p was preferred to complete pyruvate dehydrogenase complex in all equilibrium binding experiments so that the possibility of interference from the complex-bound lipoyl domains, and from other binding sites for lipoyl domains in the complex, was avoided. Equilibrium dialysis was the first method used to investigate the behaviour of radioamidinated lipoyl domains as ligands for E1p, but the attempts were unsuccessful because passage of the domains through semipermeable membranes did not occur readily. As an alternative to equilibrium dialysis, the equilibrium binding method of Hummel and Dreyer [17] was used. In the event of equilibrium binding, a radioactive peak coincident with the absorbance peak derived from E1p should be seen at the exclusion volume, and a radioactive trough, equal in size to the peak, should appear with a K_{av} value around 0.7. In practice, the results shown in fig.1 were obtained. There was no clearly defined peak in the radioactivity elution profile when either TPP or TTTPP was present at saturating concentrations in the buffer, and no trough in either case. When the slightly increased amounts of radioactivity in E1p-containing fractions were taken to represent a peak derived from equilibrium binding, the estimates for K_s from these two experiments, about 0.4 mM, were in close agreement.

Proteolytic excision of lipoyl domains from wild-type complex by *S. aureus* V8 proteinase yields a mixture of the three domains (LpV1,LpV2,LpV3) in approximately equimolar amounts [4], and so this K_s value must be an average of the K_s values for the different domain forms present in the mixture [18]. Assuming a partial specific volume for E1p of 0.75

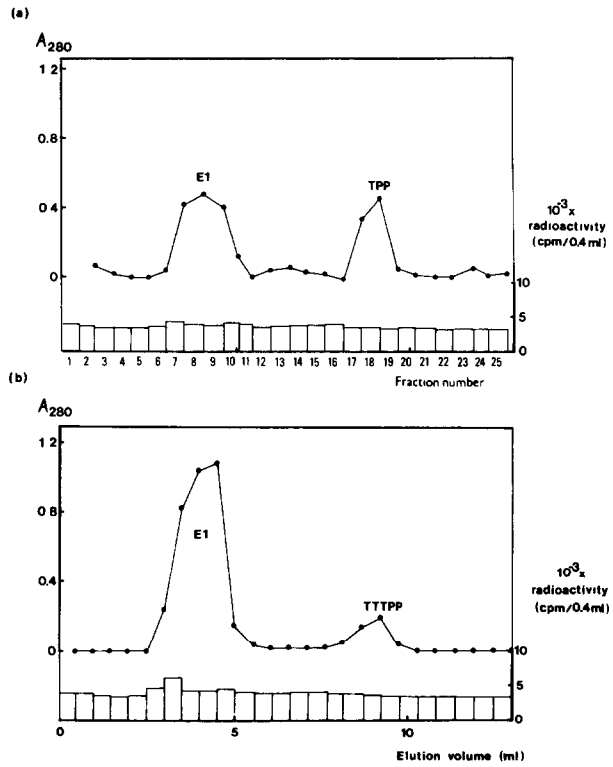

Fig.1. Equilibrium binding of radiolabelled lipoyl domains to E1p. Column conditions were as described in section 2. The figures display the radioactivity present in column fractions, and the absorbance at 280 nm of a 1:10 dilution of each fraction (●). (a) The column buffer was supplemented with TPP (1 mM) and magnesium chloride (5 mM). The sample of E1p (0.13 μmol of subunit) was first dissolved with TPP (0.78 μmol) and magnesium chloride (4 μmol) in a small volume of column buffer and incubated at 20°C for 15 min. A precipitate which formed during this incubation was removed by centrifugation. The concentration of E1p in the supernatant was determined by amino acid analysis and this sample (volume 0.14 ml, 0.066 μmol of E1p subunit) was applied to the column. K_s was estimated to be greater than 0.48 mM (see text). (b) The column buffer was supplemented with TTTPP (1 μM) and magnesium chloride (50 μM). The sample of E1p (0.13 μmol of subunit) was dissolved with TTTPP (0.3 μmol) and magnesium chloride (1.5 μmol) in 0.2 ml of column buffer and incubated at 20°C for 15 min. No precipitate formed, and the whole sample was applied to the column. K_s was estimated to be greater than 0.34 mM (see text).

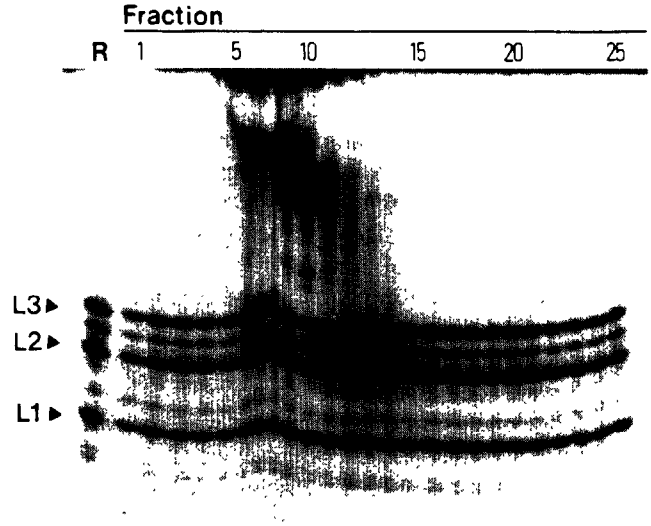

Fig.2. Analysis by polyacrylamide gel electrophoresis of effluent from an equilibrium binding experiment. Samples (75 μl) of fractions from the equilibrium experiment of fig. 1b were analysed by non-denaturing polyacrylamide gel electrophoresis (27%T, 0.3%C, Tris/glycine buffer) and stained with Coomassie blue. Track R is a reference sample of lipoyl domains. The different types of lipoyl domain are identified as L1(LpV1), L2(LpV2) and L3(LpV3) [24].

ml/g [15], the maximum error in the calculated K_s values which could arise from displacement of the domains by E1p was below 2%, and no attempt was made to take such displacement into account. In any case, it is necessary to point out that the shape and location of the radioactive peaks, and the absence of corresponding troughs, suggest that not even the extent of binding appropriate to K_s values of around 0.4 mM was, in fact, occurring.

A lipoyl domain from the *E. coli* pyruvate dehydrogenase complex contains at most 10 reactive amino groups, and therefore only one domain in every hundred is labelled in a sample which has been amidinated to a level of 0.1%. If modification causes the domains to become impaired in their ability to bind to E1p, the radioactive elution profile observed in the equilibrium binding experiments could be misleading, and extensive binding of unlabelled domains could occur without being detected. However, it is clear from fig. 2, which reflects the elution profile in terms of total domain concentration, that such binding did not take place to any appreciable extent. Fig. 2 also confirmed that no degradation of radiolabelled lipoyl domains was occurring during the binding experiments.

4. DISCUSSION

It is evident that lipoyl domains bind with only low affinity to E1p subunits under the conditions tested. It is possible that lipoyl domains do bind tightly to the E1p-hydroxyethylidene-TPP complex, but that the E1p-TTTPP complex tested in the binding experiments does not mimic this species closely enough to elicit binding. However, other observations do not support this idea. Comparison of the molar activity of E1p bound in the wild-type complex, in several mutant complexes [19], and as an unbound component, suggests that acetylated or unlipoylated lipoyl domains in assembled complexes do not engage in dead-end competitive inhibition with exogenous substrates (such as DCPIP or purified lipoyl domains) for complex-bound E1p [20].

Experiments where E1p activity with exogenous substrates is measured before and after reconstitution with E2E3 subcomplex also suggest this [20]. Similarly, proteolytic removal of intrinsic lipoyl domains from wild-type and mutant complexes results only in small and unsystematic increases in the activity of complex-bound E1p with exogenous substrates, and these increases seem to arise from direct action of the proteinase on the E1p subunits [20]. Thus, it is unlikely that acetylated or unlipoylated lipoyl domains do bind tightly to E1p-hydroxyethylidene-TPP, even at the effective domain concentrations achieved within assembled pyruvate dehydrogenase complexes. This rather surprising conclusion is in agreement with the results of biophysical studies [21,22], which have shown that the lipoyl groups in assembled particles of *E.coli* pyruvate dehydrogenase complex spend little time in the vicinity of the E1p active sites. Thus, the lipoyl domain does not appear to promote the reductive acetylation of the lipoic acid simply by enhancing its binding to the E1p subunit.

For a lipoyl domain preparation exhibiting a K_m value of 33 μM, generous estimates for binding lead to K_s values around 0.4 mM. There are, however, important differences between the methods used to calculate these two constants. In particular, the determination of K_m is independent of the concentration or amount of active enzyme present during catalysis, whereas the amount of functional macromolecule present in a ligand-binding experiment features as a term in the Scatchard equation from which K_s is calculated. Assay of E1p suggests that some inactivation occurs during the purification of the E1p component: the molar activity of purified E1p with purified lipoyl domains as substrates is about 68% of that obtained with the unpurified subunit [20]. Correction of the minimum K_s values obtained in the equilibrium binding experiments by this factor gives revised estimates of about 0.3 mM. Only 60–80% of the lipoyl domains in any preparation appear active as substrates for E1p, as judged by their ability to incorporate ^{14}C-acetyl groups from [2-^{14}C]pyruvate [8]; if any inactive domains are still ligands for E1p, our value for K_m (but not K_s) will be an overestimate, and the difference between the true values of K_s and K_m will actually be greater than that calculated here. It seems safe to conclude that the values of K_s for the lipoyl domains exceed those of K_m by at least an order of magnitude. Such a difference between K_s and K_m indicates that the rate constant, k_{off}, describing the formation of reductively acetylated lipoyl domains from E1p-hydroxyethylidene-TPP and their subsequent release, cannot be a negligible term in the equation which defines K_m for lipoyl domains [23].

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