Intramolecular coupling of active sites in the pyruvate dehydrogenase multienzyme complexes from bacterial and mammalian sources

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A simple method was developed for assessing the intramolecular coupling of active sites in the lipoate acetyltransferase (E2) component of the pyruvate dehydrogenase multienzyme complexes from *Escherichia coli*, *Bacillus stearothermophilus* and ox heart and pig heart mitochondria. Samples of enzyme complex were prepared in which the pyruvate decarboxylase (E1) component was selectively and partly inhibited by treatment with increasing amounts of a transition-state analogue, thiamin thiothiazolone pyrophosphate. The fraction of the E2 component acetylated by incubation with [2-¹⁴C]pyruvate, in the absence of CoA, was determined for each sample of partly inhibited enzyme and was found in all cases to exceed the fraction of overall complex activity remaining. This indicated the potential for transacetylation reactions among the lipoic acid residues within the E2 core. A graphic presentation of the data allowed comparison of the active-site coupling in the various enzymes, which may differ in their lipoic acid content (one or two residues per E2 chain). It is clear that active-site coupling is a general property of pyruvate dehydrogenase complexes of octahedral and icosahedral symmetries, the large numbers of subunits in each E2 core enhancing the effect.

The pyruvate dehydrogenase multienzyme complex catalyses the overall reaction:

In *Escherichia coli*, the multienzyme complex comprises multiple copies of three different types of polypeptide chain responsible for the three constituent enzymic activities: Pyruvate decarboxylase (E1) (EC 1.2.4.1), lipoate acetyltransferase (E2) (EC 2.3.1.12) and lipoamide dehydrogenase (E3) (EC 1.6.4.3). The E2 component forms the structural core of the complex and appears to comprise 24 polypeptide chains arranged with octahedral symmetry (Reed, 1974; Danson *et al.*, 1979). The pyruvate dehydrogenase complexes from other organisms also possess a lipoate acetyltrans-

ferase core, the structure and symmetry of which vary according to the source. The E2 component of the pyruvate dehydrogenase complex from *Bacillus stearothermophilus* (Henderson *et al.*, 1979) and mammalian sources (Reed, 1974) appears to comprise 60 subunits arranged with icosahedral symmetry. One exception to this is found with pig heart lipoate acetyltransferase, which may be of the octahedral, 24-subunit type (Hamada *et al.*, 1975; Randle *et al.*, 1979), although its pentagonal dodecahedral appearance in the electron microscope argues in favour of icosahedral symmetry.

Transfer of electrons and acetyl groups between the catalytic sites of pyruvate dehydrogenase complex is brought about by lipoic acid residues in amide linkage with specific *N*⁶-amino groups of E2 subunits (Green & Oda, 1961; Koike *et al.*, 1963). The number of lipoyl-lysine residues present in each E2 chain of the *E. coli* pyruvate dehydrogenase complex is almost certainly two (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977; White *et al.*, 1980). Similarly, ox kidney complex has been reported to possess two lipoic acid molecules per E2 chain (Cate & Roche, 1979), although other experiments suggest that this complex (White *et al.*,

Abbreviation used: TTTTP, thiamin thiothiazolone pyrophosphate (i.e. thiamin pyrophosphate with its thiazole ring altered to thiothiazolone).

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1980) and the pig heart complex (Hamada *et al.*, 1975) have one. In the *E. coli* pyruvate dehydrogenase complex, the active sites in the E2 core are coupled by means of an extensive system of intramolecular transacetylation reactions involving the lipoyl-lysine 'swinging arms' (Bates *et al.*, 1977; Collins & Reed, 1977). These reactions are not rate-determining in the complex and provide an important clue as to the metabolic advantage of such complicated quaternary structure (Danson *et al.*, 1978*a*).

The nature and extent of this active-site coupling has hitherto been most conveniently studied by analysing disassembled and partly reassembled complexes (Bates *et al.*, 1977; Danson *et al.*, 1978*a,b*). In the present paper we describe a method for measuring active-site coupling without the need to dissociate and reassemble the enzyme. This method has the advantage of convenience for all complexes, but is of additional importance for the complex from *B. stearothermophilus*, for which conditions permitting efficient disassembly and reassembly have yet to be found (Henderson *et al.*, 1979; Henderson & Perham, 1980). It is based on the selective inhibition of pyruvate decarboxylase (E1) active sites by treatment with TTPP (Gutowski & Lienhard, 1976), a transition-state analogue of the metastable enamine formed by decarboxylation of pyruvate in the active site of E1. A related analogue, thiamin thiazolone pyrophosphate, has been used to demonstrate high extents of E2 acetylation in pyruvate dehydrogenase complexes from *E. coli* (Collins & Reed, 1977) and ox kidney (Cate & Roche, 1979), in which only a few E1 active sites were functional.

By using this method we have been able to test active-site coupling in the pyruvate dehydrogenase complex from *E. coli*, *B. stearothermophilus*, ox heart and pig heart mitochondria and obtain comparative estimates of this ability in relation to the lipoic acid content of the complexes.

Materials and methods

Enzymes and reagents

The pyruvate dehydrogenase complexes were prepared from: (1) *E. coli* (constitutive mutant) by the method of Reed & Mukherjee (1969), (2) ox heart as described by Stanley & Perham (1980), and (3) *B. stearothermophilus* by the method of Henderson & Perham (1980).

The multienzyme complexes from bacterial sources were typically stored at 4°C at a concentration of 10–25 mg/ml in 20 mM-sodium phosphate buffer, pH 7.0, containing 2.7 mM EDTA and 0.02% NaN₃. Ox heart complex was similarly stored in 50 mM-4-morpholinepropanesulphonic acid/NaOH buffer, pH 7.0, containing 2.7 mM-EDTA,

0.02% NaN₃, 1% (v/v) Triton X-100 and 30% (v/v) glycerol.

TTTPP was prepared as described by Gutowski & Lienhard (1976) and stored at –20°C as a 10 mM solution in 10 mM-sodium phosphate buffer, pH 7.0. The solution was diluted with water to give 100 µM-TTTPP before use, and this was stable for at least 3 days at 0–4°C.

Sodium [2-¹⁴C]pyruvate was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The reagent was prepared as a 26 mM solution in water and had a specific radioactivity of 9.67 Ci/mol. All other reagents were of analytical grade.

Enzyme assay

Whole-complex and lipoamide dehydrogenase activity was measured spectrophotometrically at 30°C (Danson *et al.*, 1978*b*).

Preparation of sample tubes

All reactions were performed in Pyrex test tubes (12 mm × 75 mm). To ensure quantitative recovery of trichloroacetic acid-precipitated protein, the tubes were prepared in the following way. After being washed in detergent (Decon 90), the tubes were extensively rinsed with glass-distilled water, and then rinsed once with acetone. They were dried for at least 1 h at 100°C.

Inhibition of pyruvate dehydrogenase complex by TTPP

Method 1. Stock pyruvate dehydrogenase complex was diluted into 100 µl of 1.5 mM-NAD⁺ in 20 mM-sodium phosphate buffer, pH 7.0, at 0°C to give a final protein concentration of approx. 1 mg/ml. Samples (0–10 µl) of 100 µM-TTTPP were added to the enzyme solution. This was mixed thoroughly and incubated for 30 min at 0°C to ensure full oxidation of lipoyl groups on the protein and allow time for efficient binding of the inhibitor. At the end of this period, 5 µl of a solution of 20 mM-MgCl₂ and 4 mM-thiamin pyrophosphate in 20 mM-sodium phosphate buffer, pH 7.0, were added to give final concentrations of 1 mM-MgCl₂ and 0.2 mM-thiamin pyrophosphate. The enzyme solution was then incubated at 0°C for 1.5 min, at which time a small sample was removed for assay of whole complex activity. (Other experiments showed E3 activity to be unaffected by TTTPP.) After a further 30 s, 1 µl of sodium [2-¹⁴C]pyruvate (26 mM in water) was added and the acetylation was arrested after 20 s by adding 2 ml of ice-cold 10% (w/v) trichloroacetic acid.

The precipitated protein was kept at 0°C for at least 2 min and then collected on Whatman GF/C glass microfibre filters, the incubation tube being rinsed out with a total of 10 ml of trichloroacetic acid. The filter was washed with a further 15 ml of

ice-cold 10% trichloroacetic acid, rinsed with 10 ml of acetone and finally dried under vacuum for 5 min. The filters were added to 3 ml of toluene containing diphenyloxazole (5 g/l) and counted for radioactivity in an LKB Rackbeta liquid-scintillation counter.

Method 2. Inhibition of pyruvate dehydrogenase complex with TTTTPP was carried out as described in Method 1. Thiamin pyrophosphate and MgCl_2 were added to the enzyme solution as for Method 1, but the sample was incubated at 37°C (ox heart enzyme) or 60°C (*B. stearothermophilus* enzyme) for 2 min. At the end of this period, the tube was kept at 0°C for 5 min. Assays of enzyme activity and acetylation of the complex were then performed as described in Method 1.

On all multienzyme complexes tested, more than 98% removal of protein-bound acetyl groups could be achieved by adding CoA and cysteine (final concns. 0.8 mM and 16 mM respectively) after the 20 s incubation of the protein with 0.26 mM-sodium [$2\text{-}^{14}\text{C}$]pyruvate. The reaction was quenched by adding 2 ml of ice-cold 10% trichloroacetic acid. Enzyme complex inhibited with TTTTPP behaved similarly.

Results

Binding of TTTTPP to pyruvate dehydrogenase complex

Titration of *E. coli* pyruvate dehydrogenase complex with TTTTPP produced a linear decrease in whole-complex activity with increasing inhibitor concentration, up to 85–90% inhibition. Larger additions of inhibitor thereafter resulted in a correspondingly smaller degree of inhibition (Fig. 1). Lipoamide dehydrogenase activity was unaffected.

The rate of inhibitor binding was studied by comparing the decrease in whole-complex activity with time of incubation in the presence of various amounts of TTTTPP. At concentrations of TTTTPP up to 4 μM , inhibitor binding was rapid and complete within 10 min. The titration curve (Fig. 1) is linear over this range of inhibitor concentration. At high concentrations of TTTTPP, two phases of binding were observed; a rapid phase was followed within 10 min by a much slower phase (Fig. 2). Enzyme activity was found eventually to approach a value expected by interpolation of the linear portion of the titration curve (Fig. 1) to the concentration of TTTTPP in the sample under investigation. The slow phase of binding took place in the presence and absence of 0.2 mM-thiamin pyrophosphate and 1 mM- MgCl_2 ; it was essentially complete in 2 h.

Uninhibited enzyme was assayed in the absence of exogenous thiamin pyrophosphate. A value equivalent to 10–15% of the control activity was observed. It is therefore likely that 10–15% of the thiamin

Fig. 1. Inhibition of *E. coli* pyruvate dehydrogenase complex by thiamin thiothiazolone pyrophosphate. *E. coli* pyruvate dehydrogenase complex (1 mg/ml) was incubated with 0–6 μM -TTTTPP in 20 mM-sodium phosphate buffer containing 1.5 mM- NAD^+ , final pH 7.0, at 0°C. After 30 min, MgCl_2 and thiamin pyrophosphate were added to final concentrations of 0.2 mM and 1 mM respectively. After a further 1.5 min, samples (2 μl) of the mixture were assayed for whole complex activity as described in the Materials and methods section.

pyrophosphate-binding sites are occupied by the cofactor in the isolated complex. An explanation for the slow phase of TTTTPP binding may be that the inhibitor is slowly displacing tightly bound endogenous thiamin pyrophosphate. This idea is supported by the observation that, among the several preparations of *E. coli* pyruvate dehydrogenase complex studied, there was an approximate correlation between the percentage activity at which the titration curve departed from linearity and the catalytic activity measured in the absence of exogenous thiamin pyrophosphate.

The *B. stearothermophilus* and ox heart complexes demonstrated similar properties in their inhibition by TTTTPP.

E. coli pyruvate dehydrogenase complex

By using the radioamidination method (Bates *et al.*, 1975; Hale *et al.*, 1979) the proportions of the

Fig. 2. Time course of the inhibition of *E. coli* pyruvate dehydrogenase complex by thiamin thiothiazolone pyrophosphate. *E. coli* pyruvate dehydrogenase complex (1 mg/ml) was incubated at 0°C in 20 mM-sodium phosphate buffer containing 1.5 mM-NAD⁺ and 0–5 μM-TTTPP (final pH 7.0). Samples (2 μl) were removed at intervals and assayed for whole complex activity as described in the Materials and methods section. O, Enzyme activity in the absence of TTTPP (A); ●, enzyme activity in the presence of TTTPP at final concns. of 2 μM (B), 3 μM (C), 4.5 μM (D) and 5 μM (E).

polypeptide chains of the *E. coli* pyruvate dehydrogenase complex were found to be 1.45:1:0.77 (E1:E2:E3). Acetylation of uninhibited complex by [2-¹⁴C]pyruvate in the absence of CoA produced an incorporation of 2.0 ± 0.2 mol of [¹⁴C]acetyl groups/mol of E2 chain. After each addition of inhibitor the whole-complex enzyme activity and the acetylation by [2-¹⁴C]pyruvate were measured, both values being expressed relative to the values observed for uninhibited enzyme (Fig. 3). Since binding of TTTPP to the complex is seen to be linear (Fig. 1) and is stoichiometric with thiamin pyrophosphate-binding sites (Angelides & Hammes, 1978), one may expect the fraction of whole-complex activity to equal the fraction of active E1 molecules in the complex. In the absence of transacetylation reactions in the E2 core, the decrease in [¹⁴C]acetylation of the complex should match the inhibition of E1 activity. Conversely, deviation from linearity of the plot of [¹⁴C]acetylation against complex activity reveals the presence of transacetylation reactions, and the departure from linearity is a measure of their extent (Bates *et al.*, 1977). It is obvious from Fig. 3 that there is a substantial network of intramolecular transacetylation reactions in the E2 core of the *E. coli* pyruvate dehydrogenase complex, as reported previously (Bates *et al.*, 1977; Collins & Reed, 1977).

The experimental conditions used in these experiments were those of Method 1, described in the

Materials and methods section above. At the higher extents of inhibition (above 90%), increasing the concentrations of TTTPP did not cause a further linear decrease in the enzymic activity (Fig. 1). However, the experimental points relating the extent of acetylation to the enzymic activity remaining, in samples inhibited by more than 90%, continued to lie on the same smooth curve (see Fig. 3). Enzyme complex inhibited to approx. 95% could be prepared in two ways: (1) by incubation with 5 μM-TTTPP for 30 min, or (2) by incubation with 4.5 μM-TTTPP for about 2 h. Acetylation of enzyme samples inhibited by means of the second method produced the same results as those obtained by using the first method. This strongly suggests that the pyruvate decarboxylase (E1) active sites from which thiamin pyrophosphate is evidently difficult to remove by incubation with TTTPP do not differ from the other E1 active sites in their interaction with lipoic acid residues.

Ox heart pyruvate dehydrogenase complex

The pyruvate decarboxylase component from ox heart pyruvate dehydrogenase complex differs from that of *E. coli* in comprising two polypeptide chains, E1α and E1β (Reed, 1974). The polypeptide-chain proportions of this complex were found to be 1.5:1.7:1:0.5 (E1α:E1β:E2:E3) by the radioamidination method (Bates *et al.*, 1975; Hale *et al.*, 1979). Incorporation of [¹⁴C]acetyl groups into uninhibited

Fig. 3. Acetylation of samples of inhibited pyruvate dehydrogenase complex from *E. coli*

Samples of *E. coli* pyruvate dehydrogenase complex (approx. 100 μ g) were inhibited with increasing amounts of TTPP as described in the Materials and methods section. Each sample was then incubated with 0.2 mM-thiamin pyrophosphate and 1 mM-MgCl₂ for 2 min at 0°C and reductively acetylated for 20 s with 0.26 mM-sodium [2-¹⁴C]-pyruvate. Acetylation was arrested by precipitation of the protein with 10% trichloroacetic acid at 0°C. Protein was collected on glass-fibre filters, washed and counted for radioactivity as described in the text. In the absence of active-site coupling reactions within the E2 core of the enzyme, one would expect the data points to lie on the broken line, as described in the text.

Fig. 4. Acetylation of samples of inhibited pyruvate dehydrogenase complex from ox heart

Samples of pyruvate dehydrogenase complex from ox heart were inhibited with increasing amounts of TTPP as described in the Materials and methods section. Each sample was then incubated with 0.2 mM-thiamin pyrophosphate and 1 mM-MgCl₂ for (a; ○) 2 min at 0°C or (b; ●) 2 min at 37°C followed by 5 min at 0°C. The enzyme was reductively acetylated, precipitated and counted for radioactivity as described in the legend to Fig. 3.

complex by means of Method 1 was equivalent to 0.86 ± 0.10 acetyl group/mol of E2 chain.

The acetylation curve of this enzyme (Fig. 4a) was at first sight rather puzzling. Although trans-acetylation was evident up to 70% inhibition of enzyme activity, the incorporation of acetyl groups thereafter fell more sharply than the activity. A similar result was observed with pyruvate dehydrogenase complex from pig heart (kindly provided by Professor P. J. Randle). This was found to be due to the inability of TTPP-inhibited complex fully to bind thiamin pyrophosphate and Mg²⁺ at 0°C during the 2 min incubation allowed in the standard procedure. The rate of cofactor binding decreased with increasing inhibition. No such slow binding was evident during the assay of inhibited complex carried out at 30°C.

The practical solution to this problem was to raise the temperature of the incubation with thiamin pyrophosphate to the physiological temperature, 37°C. At all degrees of inhibition of complex, 2 min

incubation at this temperature produced full binding of cofactor. The complex was then assayed and its acetylation measured after keeping it in an ice bath for 5 min (see Method 2 in the Materials and methods section). The effect of this modified procedure was further to increase the deviation of the acetylation curve from linearity, with all determinations of [¹⁴C]acetylation exceeding the fraction of enzymic activity remaining (Fig. 4b). It is of ancillary interest to note that the linear portion of the TTPP-titration curve (not shown) for this enzyme was unaffected by binding thiamin pyrophosphate at 37°C, which confirms that the cofactor was unable to displace bound inhibitor under these conditions. As expected, the non-linear portion of the titration curve was less pronounced, owing to an increased rate of TTPP binding at the higher temperature.

Bacillus stearothermophilus pyruvate dehydrogenase complex

This four-component enzyme (Henderson & Perham, 1980) was found to have polypeptide proportions of 1.3:1.0:1.0:0.5 (E1 α :E1 β :E2:E3), the designations of E1 α and E1 β being presumptive (Henderson *et al.*, 1979). Uninhibited complex incorporated 0.95 ± 0.10 mol of [¹⁴C]acetyl groups/

Fig. 5. Acetylation of samples of inhibited pyruvate dehydrogenase complex from *B. stearothermophilus*. Samples of *B. stearothermophilus* pyruvate dehydrogenase complex were inhibited with increasing amounts of TTTPP as described in the Materials and methods section. Each sample was then incubated with 0.2 mM-thiamin pyrophosphate and 1 mM- MgCl_2 for 2 min at 60°C, followed by 5 min at 0°C. The enzyme was reductively acetylated, precipitated and counted for radioactivity as described in the legend to Fig. 3.

mol of E2 chain when treated with $[2\text{-}^{14}\text{C}]$ pyruvate.

As with the mammalian enzyme, inhibited pyruvate dehydrogenase complex from *B. stearothermophilus* failed to bind thiamin pyrophosphate and Mg^{2+} completely in 2 min at 0°C. Incubation for 2 min at 60°C (the optimum growth temperature of the organism) followed by equilibration at 0°C for 5 min achieved complete binding of the cofactor. The acetylation curve thus obtained by Method 2 is shown in Fig. 5. Extensive transacetylation reactions clearly occur in this enzyme.

Influence of various parameters on transacetylation

The technique described here for demonstrating transacetylation reactions in several pyruvate dehydrogenase complexes may in principle be applied to the enzyme from any source. Since the storage conditions of the enzyme may not always be identical, we varied chosen parameters to determine what effect they have on the active-site coupling in a complex.

Transacetylation was independent of protein concentration over the range tested, 0.2–2 mg/ml. The shape of the acetylation curve was also unaffected by the presence of 2-oxoglutarate

dehydrogenase complex (up to 1.4-fold). From this we conclude that purity of the pyruvate dehydrogenase complex preparation is not an important factor in demonstrating active-site coupling. This should permit analysis of a complex that is difficult to separate from 2-oxoglutarate dehydrogenase complex during purification.

Pyruvate dehydrogenase complex is sometimes stored in the presence of reducing agent. Inclusion of 2 mM-dithiothreitol in stock enzyme solutions (10–25 mg/ml) of complex from *E. coli* did not affect subsequent measurements of acetylation curves relative to an untreated control. With the ox heart enzyme, the acetylation curve was independent of the presence of dithiothreitol (2 mM) and/or Triton X-100 (up to 8%, v/v) in the stock solution.

We examined a few changes in acetylation conditions and found that all complexes were unaffected by up to 2 h incubation with 1.5 mM- NAD^+ at 0°C before addition of exogenous cofactor. Prolonged incubation (overnight) with NAD^+ , however, decreased both enzymic activity and the extent of acetylation. Inclusion of EDTA (2.7 mM) in the acetylation mixture caused a marked decrease in active-site coupling, owing to a large fall in the free Mg^{2+} concentration (1 mM down to about 2 μM), which then becomes limiting compared with the concentration of thiamin pyrophosphate-binding sites (up to 7 μM for uninhibited complex).

Discussion

The ability of the lipoate acetyltransferase (E2) component to bring about intramolecular acyl-transfer reactions between reductively acetylated and oxidized lipoic acid residues was described first for the *E. coli* pyruvate dehydrogenase complex (Bates *et al.*, 1977; Collins & Reed, 1977; Danson *et al.*, 1978b). A similar network of interacting lipoic acid residues is also present in the lipoate succinyl-transferase component of the 2-oxoglutarate dehydrogenase complex from *E. coli* (Collins & Reed, 1977). Both these enzymes are based on octahedral symmetry, 24 polypeptide chains comprising the E2 core (Reed, 1974; Danson *et al.*, 1979). However, whereas the E2 chain of the pyruvate dehydrogenase complex carries two acetyltable lipoic acid residues (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977), that of the 2-oxoglutarate dehydrogenase complex probably bears only one (Collins & Reed, 1977).

It has been reported that the pyruvate dehydrogenase complex of ox kidney also possesses a comparable system of interacting lipoic acid residues, with two lipoyl groups per E2 chain in the lipoate acetyltransferase component (Cate & Roche, 1979). By contrast with the *E. coli* enzyme, this lipoate acetyltransferase component is probably

icosahedral in symmetry (Reed, 1974), as is that of the pyruvate dehydrogenase complex from *B. stearothermophilus* (Henderson *et al.*, 1979; Henderson & Perham, 1980). Our present experiments clearly demonstrate the existence of active-site coupling in the pyruvate dehydrogenase complexes of ox heart (Fig. 4) and *B. stearothermophilus* (Fig. 5). In each complex the extent of reductive acetylation with [2-¹⁴C]pyruvate was equivalent to approx. 1 acetyltable lipoic acid residue per E2 chain. It is difficult to compare our results with those of Cate & Roche (1979) on the ox kidney complex, since other results (Reed, 1974; White *et al.*, 1980) indicate the existence of only one lipoic acid residue per E2 chain in that complex too. However, it is safe to conclude that 2-oxo acid dehydrogenase complexes of differing symmetry (octahedral and icosahedral) and differing lipoic acid content (1 or 2 mol/mol of E2 chain) share the property of intramolecular acyl-group transfer. The generality of this property lends force to the argument that it has some physiological purpose, ensuring the efficient coupling of a multistep reaction for which some substrate may be in low concentration or the enzyme complex incompletely assembled (Danson *et al.*, 1978a).

The method that we have described permits the system of active-site coupling to be investigated for 2-oxo acid dehydrogenase complexes available only in small quantities. It avoids the need to prepare disassembled and partly reassembled complexes. A curious feature that we have noted is that the property of active-site coupling may diminish on long-term storage of the enzyme complex without an accompanying significant change in the specific catalytic activity of the enzyme assayed conventionally. The acetylation-activity curve may therefore be regarded as a sensitive test of the structural and functional integrity of the enzyme complex whose use can be exploited in studies of the enzyme mechanism.

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