Temperature-dependence of intramolecular coupling of active sites in pyruvate dehydrogenase multienzyme complexes

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Intramolecular coupling of active sites in the pyruvate dehydrogenase multienzyme complexes of *Escherichia coli*, ox heart and *Bacillus stearothermophilus* was measured at various temperatures. As the temperature was raised, the extent of active-site coupling was found to increase, approaching a maximum near the physiological growth temperature of the organism. Under these conditions, a single pyruvate dehydrogenase (lipoamide) dimer appeared able to cause a rapid (20 s) reductive acetylation of probably all 24 polypeptide chains in the dihydrolipoamide acetyltransferase core of the enzyme complex from *E. coli* at 37°C, and of most if not all of the 60 polypeptide chains in the dihydrolipoamide acetyltransferase cores of the enzymes from ox heart and *B. stearothermophilus* at 37°C and 60°C respectively. Experiments designed to measure the inter-core and intra-core migration of enzyme subunits suggested that, in the bacterial enzymes at least, this was not a major contributor to active-site coupling.

The pyruvate dehydrogenase multienzyme complexes from bacterial and mammalian sources all comprise a dihydrolipoamide acetyltransferase (E2) component acting as a structural core to which are bound multiple copies of pyruvate dehydrogenase (lipoamide) (E1) and dihydrolipoamide dehydrogenase (E3) (Reed, 1974). Dihydrolipoamide acetyltransferase from *Escherichia coli* is itself made up of 24 polypeptide chains arranged with octahedral symmetry (Reed, 1974; Danson *et al.*, 1979), whereas the E2 component from most mammalian sources (Reed, 1974; Kresze & Ronft, 1980) and from *Bacillus stearothermophilus* (Henderson *et al.*, 1979; Henderson & Perham, 1980) comprises 60 polypeptide chains arranged with icosahedral symmetry.

Each E2 polypeptide chain in the dihydrolipoamide acetyltransferase from *E. coli* contains two lipoyl-lysine residues that become reductively acetylated (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977; Speckhard *et al.*, 1977; Angelides & Hammes, 1978; White *et al.*, 1980) but the dihydrolipoamide acetyltransferase from mammalian sources (White *et al.*, 1980; Stanley *et al.*, 1981) and from *Bacillus stearothermophilus*

(Perham & Wilkie, 1980; Stanley *et al.*, 1981) have only one such lipoyl group per E2 chain. The lipoyl-lysine residues, which function as swinging arms in the enzyme mechanism (Green & Oda, 1961; Koike *et al.*, 1963; Ambrose & Perham, 1976; Grande *et al.*, 1976), are capable of connexion by means of extensive intramolecular coupling reactions (Bates *et al.*, 1977; Collins & Reed, 1977; Danson *et al.*, 1978a; Cate & Roche, 1979; Stanley *et al.*, 1981). The lipoyl groups are located in parts of the E2 polypeptide chains that protrude from the E2 core between the E1 and E3 subunits (Hale & Perham, 1979a,b; Bleile *et al.*, 1979; Kresze & Ronft, 1980; Perham & Wilkie, 1980; Bleile *et al.*, 1981), and these lipoyl domains are linked by substantial regions of peptide chain that enjoy the conformational mobility of random coils, as judged by ¹H-n.m.r. spectroscopy of the *E. coli* complex (Perham *et al.*, 1981; Packman *et al.*, 1982). Similar regions of polypeptide chain with high conformational mobility appear to exist in 2-oxo acid dehydrogenase complexes from all sources thus far examined (Wawrzynczak *et al.*, 1981; Perham & Roberts, 1981; Duckworth *et al.*, 1982). They presumably underlie the mechanism of active-site coupling and obviate the need for exact stoichiometric arrangement of active sites in the 2-oxo acid dehydrogenase complexes (Perham *et al.*, 1981; Perham & Roberts, 1981; Berman *et al.*, 1981; Stepp *et al.*, 1981; Duckworth *et al.*, 1982).

Abbreviations used: TTPP, thiamin thiothiazolone pyrophosphate; E1, pyruvate dehydrogenase (lipoamide) (EC 1.2.4.1); E2, dihydrolipoamide acetyltransferase (EC 2.3.1.12); E3, dihydrolipoamide dehydrogenase (EC 1.8.1.4).

We developed a simple method for analysing the extent of active-site coupling in 2-oxo acid dehydrogenase complexes (Stanley *et al.*, 1981), based on the use of a specific inhibitor, thiamin thiothiazolone pyrophosphate (TTTPP), selectively to inhibit the pyruvate or 2-oxoglutarate dehydrogenase (lipoamide) (E1) components (Gutowski & Lienhard, 1976; Collins & Reed, 1977; Cate & Roche, 1979). In those experiments we noted that partly inhibited pyruvate dehydrogenase complex from ox heart or *B. stearothermophilus* bound exogenous thiamin pyrophosphate in the presence of Mg^{2+} only slowly at 0°C, whereas binding was rapid at higher temperatures (Stanley *et al.*, 1981). This prompted us to study the temperature-dependence of active-site coupling. We show here below that its extent increases with temperature, approaching a maximum as the temperature rises towards the physiological temperature of the organism from which the pyruvate dehydrogenase complex was isolated.

Materials and methods

Enzymes and reagents

The pyruvate dehydrogenase complexes were prepared from a constitutive mutant of *E. coli* (Reed & Mukherjee, 1969; Danson *et al.*, 1979), ox heart (Stanley & Perham, 1980) and *B. stearothermophilus* (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% NaN_3 . Ox heart complex was similarly stored in 20 mM-morpholinepropanesulphonic acid/NaOH buffer, pH 7.0, containing 2.7 mM-EDTA, 0.02% NaN_3 , 1% (v/v) Triton X-100 and 30% (v/v) glycerol.

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

Sodium [2- ^{14}C]pyruvate was obtained from Amersham International, 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 assays

Whole-complex activity was measured spectrophotometrically at 30°C by monitoring the reduction of NAD^+ (Danson & Perham, 1976). Pyruvate dehydrogenase (lipoamide) (E1) activity was measured by following at 600 nm the reduction of 2,6-dichlorophenol-indophenol (Khailova *et al.*, 1977). The reaction was started by addition of

pyruvate dehydrogenase complex to 1 ml of a mixture containing 50 mM-potassium phosphate buffer, pH 7.0, 70 μ M-2,6-dichlorophenol-indophenol, 0.2 mM-thiamin pyrophosphate, 1 mM- $MgCl_2$ and 2 mM-sodium pyruvate.

Inhibition and reductive acetylation of pyruvate dehydrogenase complexes

Each pyruvate dehydrogenase complex (100 μ l; approx. 1 mg/ml in 20 mM-sodium phosphate buffer, pH 7.0, containing 1.5 mM- NAD^+) was inhibited non-covalently in its E1 component by treatment for 30 min at 0°C with TTTPP (0–10 μ M, final concentration) (Stanley *et al.*, 1981). The amounts of inhibitor were chosen so as to give a gradually increasing inhibition of the E1 component. After this time, each sample of pyruvate dehydrogenase complex was reductively acetylated with sodium [2- ^{14}C]pyruvate at 0°C as described previously (Stanley *et al.*, 1981). At temperatures of 20°C and above, acetylation was performed as follows.

To samples (100–115 μ l) of uninhibited or partly inhibited complex was added 5 μ l of a solution of 20 mM- $MgCl_2$ and 4 mM-thiamin pyrophosphate in 20 mM-sodium phosphate buffer, pH 7.0. After incubation for 1.5 min at the appropriate temperature, a small portion was removed for assay of whole-complex activity. After a total incubation time of 2 min, 1 μ l of sodium [2- ^{14}C]pyruvate (26 mM) was added to the remaining enzyme and the incubation was continued for a further 20 s. The reaction was then arrested by adding 2 ml of ice-cold 10% (w/v) trichloroacetic acid, and the precipitated protein was collected by filtration and counted for radioactivity as described previously (Stanley *et al.*, 1981).

Inhibition of the E2 component of B. stearothermophilus pyruvate dehydrogenase complex

The pyruvate dehydrogenase complex of *B. stearothermophilus* (5 mg/ml) was inhibited covalently in its E2 component by treating the native enzyme with 0.5 mM-*N*-ethylmaleimide, in the presence of the substrate pyruvate, at 0°C under N_2 . This procedure irreversibly modifies the free thiol group of the *S*-acetyldihydrolipoic acid residue generated in the acyl-enzyme intermediate (Brown & Perham, 1976; Perham & Wilkie, 1980).

Results

Inhibition of pyruvate dehydrogenase complexes by TTTPP

The selective inhibition of each pyruvate dehydrogenase complex by limiting amounts of TTTPP gave a linear decrease in whole-complex activity as the inhibitor concentration was increased. At high degrees of inhibition (80–100%), the inactivation

Fig. 1. Inhibition of *B. stearothermophilus* pyruvate dehydrogenase complex by TTPP

B. stearothermophilus pyruvate dehydrogenase complex (1 mg/ml) was incubated with 0–10 μM-TTPP in 20 mM-sodium phosphate buffer containing 1.5 mM-NAD⁺, final pH 7.0, at 0°C. After 30 min, MgCl₂ and thiamin pyrophosphate were added to final concentrations of 1 mM and 0.2 mM respectively, and the temperature of the incubation was raised to 60°C. After a further 1.5 min, samples (2 μl) of enzyme were removed for assay of whole-complex activity at 30°C.

Fig. 2. Reductive acetylation of *E. coli* pyruvate dehydrogenase complex after inhibition with TTPP

Samples of *E. coli* pyruvate dehydrogenase complex were inhibited with increasing amounts of TTPP and then reductively acetylated for 20 s with sodium [¹⁴C]pyruvate at 0°C (○) and 37°C (●) as described in the Materials and methods section. The reaction was arrested by adding ice-cold 10% (w/v) trichloroacetic acid. The precipitated protein was collected on glass-fibre filters, washed and counted for radioactivity. The broken line denotes the relationship expected between the extents of acetylation and inhibition in the absence of active-site coupling within the E2 core of the enzyme.

curve departed from linearity. This has been noted before, and may be due to the slow displacement of a small amount of thiamin pyrophosphate that remains bound to the enzyme during the purification procedure (Stanley *et al.*, 1981). In the present study, titration of the *B. stearothermophilus* complex with TTPP showed a linear loss of activity up to about 96% inhibition (Fig. 1). With the *E. coli* and ox heart enzymes departure from linearity, which became less pronounced at higher temperatures owing to increased inhibitor binding (Stanley *et al.*, 1981), occurred at about 90% and 80% inhibition respectively (results not shown).

Active-site coupling in pyruvate dehydrogenase complexes at different temperatures

Samples of pyruvate dehydrogenase complex from *E. coli*, *B. stearothermophilus* and ox heart were inhibited to gradually increasing extents with TTPP and then reductively acetylated by treatment with sodium [¹⁴C]pyruvate for 20 s. All complexes were reductively acetylated at 0°C and also at the physiological growth temperature of the relevant organism, i.e. 60°C for enzyme from *B. stearothermophilus* and 37°C for enzyme from *E. coli* and ox heart. The protein was precipitated with ice-cold

10% (w/v) trichloroacetic acid, collected and assayed for radioactivity. By plotting the amount of radioactive acetylation against the extent of inhibition of enzyme activity, both measured relative to an uninhibited control sample of enzyme, an acetylation curve is produced (e.g. Fig. 2). The deviation of this curve from linearity is a measure of the extent of active-site coupling within the complex (Bates *et al.*, 1977; Stanley *et al.*, 1981).

The acetylation curves for the *E. coli* pyruvate dehydrogenase complex at 0°C and 37°C are shown in Fig. 2. The higher temperature evidently promoted more extensive active-site coupling in the complex. The maximum value for the incorporation of acetyl groups corresponded to approx. 2.0 mol of acetyl group/mol of E2 chain (Bates *et al.*, 1977; Stanley *et al.*, 1981). The effect of temperature on active-site coupling in the ox heart pyruvate dehydrogenase complex was similar though less pronounced (Fig. 3). For this complex, the maximum value for the incorporation of acetyl groups corresponded to approx. 0.9 mol of acetyl group/mol of E2 chain (Stanley *et al.*, 1981).

The most striking effect was observed with the

Fig. 3. Reductive acetylation of ox heart pyruvate dehydrogenase complex after inhibition with TTTTPP. Samples of ox heart pyruvate dehydrogenase complex were inhibited with increasing amounts of TTTTPP. Each sample of enzyme was reductively acetylated for 20 s at 0°C (○) and 37°C (●). The protein was then precipitated and counted for radioactivity as described in the legend to Fig. 2.

pyruvate dehydrogenase complex from *B. stearothermophilus*. At 60°C, the E2 core of this complex could be fully acetylated when only 5% of its complement of E1 molecules remained active, whereas at 0°C an identical sample achieved only 15% acetylation of the E2 core. The maximum value for the incorporation of acetyl groups corresponded to approx. 1.0 mol of acetyl group/mol of E2 chain (Stanley *et al.*, 1981). An experiment performed at an intermediate temperature (30°C) showed an intermediate extent of active-site coupling (Fig. 4). In a further experiment, a sample of *B. stearothermophilus* pyruvate dehydrogenase complex was titrated with TTTTPP until only 7% of the original whole-complex activity remained. Identical portions of this partly inhibited enzyme were then reductively acetylated with sodium [2-¹⁴C]pyruvate for 20 s at temperatures ranging from 0°C to 60°C, as before. The incorporation of acetyl groups, relative to that for a sample of uninhibited enzyme at the matching temperature, was found to rise with increasing temperature, reaching a maximum value of approx. 1 mol of acetyl group/mol of E2 chain at about 50°C (Fig. 5). There is no sudden break in this curve to indicate an abrupt transition from one form of the enzyme complex with limited ability for active-site coupling to another form in which it is fully expressed. Similar results were obtained with a sample of ox heart pyruvate dehydrogenase complex (80% inhibited with TTTTPP) when it was tested at

Fig. 4. Reductive acetylation of *B. stearothermophilus* pyruvate dehydrogenase complex after inhibition with TTTTPP.

Samples of *B. stearothermophilus* pyruvate dehydrogenase complex were inhibited with increasing amounts of TTTTPP. Each sample of enzyme was reductively acetylated for 20 s at 0°C (Δ), 30°C (○) and 60°C (●). The enzyme was then precipitated and counted for radioactivity as described in the legend to Fig. 2.

various temperatures between 0°C and 45°C. For this enzyme, incorporation of acetyl groups approached a maximum at about 40°C.

If E1 molecules were able to dissociate rapidly from partly inhibited pyruvate dehydrogenase complexes, migration of active E1 subunits between E2 cores during the 20 s incubation with pyruvate could bring about acetylation of E2 chains not otherwise participating in the active-site network, thereby giving a falsely high indication of the extent of intramolecular active-site coupling. The low rate of E1 subunit exchange in the *E. coli* pyruvate dehydrogenase complex has ruled this out as an explanation of active-site coupling in that enzyme (Collins & Reed, 1977; Hale *et al.*, 1979). However, it has been suggested (Cate & Roche, 1979; Cate *et al.*, 1980) that migration of E1 molecules ($\alpha_2\beta_2$ tetramers) can occur between E2 cores of the ox kidney pyruvate dehydrogenase complex, the dissociation step being rate-limiting with a first-order rate constant of about 1.3–2.2/min at 30°C (Cate *et al.*, 1980). It has not proved possible thus far to disassemble *B. stearothermophilus* pyruvate dehydrogenase complex to yield catalytically active subunits, and so the straightforward experiments to measure E1 exchange rates (Collins & Reed, 1977; Hale *et al.*, 1979; Cate & Roche, 1979; Cate *et al.*, 1980) cannot be performed. A different type of

Fig. 5. Reductive acetylation of partly inhibited pyruvate dehydrogenase complex from *B. stearothermophilus* at various temperatures

A sample of *B. stearothermophilus* pyruvate dehydrogenase complex was titrated with TTPP until only 7% of the original whole-complex activity remained. Identical portions of this enzyme were reductively acetylated with sodium [¹⁴C]-pyruvate for 20 s at various temperatures. The experiment was repeated with uninhibited enzyme. The incorporation of [¹⁴C]acetyl groups by partly inhibited enzyme at each temperature was expressed relative to the uninhibited control.

Fig. 6. Intermolecular diffusion of E1 subunits in the pyruvate dehydrogenase complex from *B. stearothermophilus*

Samples of E1-inhibited complex from *B. stearothermophilus* (●), E2-inhibited complex from *B. stearothermophilus* (△) and uninhibited complex from *E. coli* (■) were incubated at 30°C in the presence of 0.2 mM-thiamin pyrophosphate, 2.7 mM-EDTA and 3 mM-MgCl₂, as described in the text. At the time shown by the vertical arrow, samples of the E1-inhibited complex were mixed with E2-inhibited complex (○) and with uninhibited complex (▲). The latter incubation demonstrates that no inhibitor is diffusing from the *B. stearothermophilus* enzyme to the *E. coli* enzyme. The increase in activity of the mixture of inhibited *B. stearothermophilus* enzymes must be due to intermolecular diffusion of E1 subunits. The theoretical final activities of this mixture of *B. stearothermophilus* complexes are shown for complete random diffusion of E1 subunits between E2 cores (←A) and for no diffusion of E1 subunits between E2 cores (←B).

experiment was therefore performed with this enzyme complex, as follows.

B. stearothermophilus pyruvate dehydrogenase complex was inhibited in its E1 component by treatment with TTPP (E1-inhibited enzyme) or in its E2 component by treatment with *N*-ethylmaleimide in the presence of pyruvate (E2-inhibited enzyme), as described in the Materials and methods section. Samples of E1-inhibited complex (200 µl; 4.1 mg/ml) and E2-inhibited complex (100 µl; 4.3 mg/ml) were incubated separately at 30°C in the presence of 0.2 mM-thiamin pyrophosphate, 3 mM-MgCl₂ and 2.7 mM-EDTA in 20 mM-sodium phosphate buffer, pH 7.0. The inactivated enzymes showed a slow increase in whole-complex and E1 activities, for which we have no explanation, but these stabilized after about 5 h (Fig. 6). (A similar activation is also observed with uninhibited samples of this complex.) At this point, a sample (50 µl) of the E1-inhibited enzyme was added to a sample (50 µl) of the E2-inhibited enzyme. Incubation at 30°C for a further 3 h caused a further slow increase in whole-complex activity without any change in the E1 activity of the mixture. The whole-complex activity approached a maximum value equal to that expected for random distribution of active E1 molecules among the complexes (Fig. 6). No

proteolysis was observed during the incubation, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

These results could not be attributed to diffusion of TTPP between the E1-inhibited and E2-inhibited complexes, because no change in activity of the pyruvate dehydrogenase complex was observed when a sample (50 µl; 4.1 mg/ml) of E1-inhibited *B. stearothermophilus* pyruvate dehydrogenase complex was added to a sample (50 µl; 1.8 mg/ml) of uninhibited *E. coli* pyruvate dehydrogenase complex and the incubation at 30°C was repeated (Fig. 6). Since the specific activity of the *E. coli* enzyme is about 20 times that of the *B. stearothermophilus* complex under these conditions (see, e.g., Henderson & Perham, 1980; Stanley *et al.*, 1981), any transfer of TTPP from the latter enzyme to the former enzyme would appear as a significant decrease in the

total catalytic activity of the mixture. We conclude that the slow increase in catalytic activity of the mixture of differently inhibited *B. stearothermophilus* complexes (Fig. 6) must have been due to migration of E1 molecules between the enzyme particles. A rate constant for E1 diffusion between E2 cores of the *B. stearothermophilus* complex molecules was calculated from these experimental data to be $1.8 \times 10^{-2}/\text{min}$, corresponding to a half-time of 38 min. This rate is clearly much higher than that observed for the *E. coli* enzyme (Collins & Reed, 1977; Hale *et al.*, 1979) but substantially lower than that reported for the ox kidney enzyme at the same temperature (Cate *et al.*, 1980).

We conclude that E1 diffusion does not contribute significantly to the acetylation curve of the *B. stearothermophilus* complex (Fig. 4) at 30°C, which was measured after 20 s incubations with pyruvate. At 60°C, however, a contribution by subunit diffusion cannot be excluded, since we do not have information on subunit exchange at that temperature. Unfortunately, the above method of analysing inter-core migration of E1 molecules in *B. stearothermophilus* complex cannot be performed at 60°C because *E. coli* pyruvate dehydrogenase complex, which is used to monitor TTPP diffusion between complexes, is unstable at this temperature.

We also measured the incorporation of [^{14}C]-acetyl groups into uninhibited and partly E1-inhibited pyruvate dehydrogenase complexes at several temperatures, with the incubation time with [2- ^{14}C]pyruvate varied between 10 s and 45 s. The incorporation of [^{14}C]acetyl groups into uninhibited ox heart and *E. coli* complexes was constant over the period 10–45 s at 0°C, 30°C and 37°C. After partial inhibition of E1 with TTPP (about 7% activity remaining), the ox heart complex exhibited less than 4% increase in [^{14}C]acetyl incorporation between 10 s and 45 s incubation with [2- ^{14}C]pyruvate at 30°C. Similar results were obtained when the same experiment was performed with *E. coli* complex that was partly inhibited with TTPP (about 8% activity remaining). It is therefore unlikely that intra-core migration of E1 molecules is playing much part in active-site coupling, as measured by our 20 s experiments, since any such migration should have led to a significant increase in the incorporation of [^{14}C]acetyl groups into these partly inhibited complexes when the incubation time was lengthened to 45 s. When this experiment was repeated with the *B. stearothermophilus* complex at 60°C, the incorporation of [^{14}C]acetyl groups into uninhibited enzyme was unstable at the longer incubation times. A possible explanation for this is hydrolysis of the thioester bond in *S*-acetyl-dihydrolipoic acid, since some protection was afforded by treating the acetylated complex with *N*-ethylmaleimide. Similar protection against loss of acetyl

groups was reported for the *E. coli* and ox heart complexes (Collins & Reed, 1977; Bleile *et al.*, 1981).

Discussion

Intramolecular coupling of active sites in the E2 component has been observed in every 2-oxo acid dehydrogenase complex tested (Bates *et al.*, 1977; Collins & Reed, 1977; Cate & Roche, 1979; Stanley *et al.*, 1981). Initial attempts to quantify the extent of this coupling in the pyruvate dehydrogenase complex of *E. coli* suggested that a single E1 dimer could bring about the rapid reductive acetylation of about 12 E2 chains in the 24-chain E2 core at 0°C, though the difficulties in estimating this number were emphasized (Bates *et al.*, 1977). A much slower coupling process was noted between the rapidly acetylated lipoic acid residues and those that did not react in the initial, faster, phase (Danson *et al.*, 1978a). Other experiments (Collins & Reed, 1977) also suggested that about half the lipoic acid residues in the E2 core of the *E. coli* complex could be rapidly acetylated by means of a small number of active E1 dimers bound to it, with acetylation of the remaining lipoic acid residues following a much slower time course.

Our present results demonstrate that temperature has an important bearing on the extent of active-site coupling. With the theoretical acetylation curves derived by Bates *et al.* (1977) as a model, the acetylation curves for the *E. coli* pyruvate dehydrogenase complex (Fig. 2) indicate that, as the temperature is raised towards the physiological value of 37°C, the extent of active-site coupling increases. The curves approach the limit for a single E1 dimer being able to cause a rapid (20 s) reductive acetylation of all 24 E2 chains in the enzyme core.

Similar results were obtained with the pyruvate dehydrogenase complex of ox heart, which has a 60-subunit icosahedral E2 core (Reed, 1974). Again, as the temperature was raised, the acetylation curve departed further from linearity (Fig. 3), although at 37°C the curve appeared not to have quite reached the theoretical limit (Bates *et al.*, 1977) for a single E1 dimer causing a rapid (20 s) reductive acetylation of all 60 E2 chains in the enzyme core. It is difficult to analyse these curves rigorously when the extent of active-site coupling is high (Bates *et al.*, 1977), but the coupling reactions in the ox heart complex are clearly very extensive.

The pyruvate dehydrogenase complex from *B. stearothermophilus* also has a 60-subunit icosahedral E2 core (Henderson *et al.*, 1979; Henderson & Perham, 1980), and temperature has a dramatic effect on the active-site coupling observed with this enzyme (Figs. 4 and 5). At temperatures above

50°C, indeed, it appears that active-site coupling is effectively complete, the acetylation curve being indistinguishable from that expected for a full coupling of all 60 subunits. Thus, in all three enzymes studied, the extent of intramolecular active-site coupling increases with temperature, approaching a maximum as the temperature rises towards the normal growth temperature of the organism from which the pyruvate dehydrogenase complex was isolated. This may be of physiological importance.

Intercore migration of E1 subunits has been reported to be rapid ($t_{1/2} \approx 20$ –30 s at 30°C) for the pyruvate dehydrogenase complex of ox kidney (Cate *et al.*, 1980) compared with the very slow exchange of E1 subunits ($t_{1/2} \approx 27$ h at 15°C) described for the *E. coli* complex (Collins & Reed, 1977; Hale *et al.*, 1979). The experiments that we have performed with the *B. stearothermophilus* pyruvate dehydrogenase complex (Fig. 6) indicate an intermediate rate of inter-core migration of E1 subunits for that enzyme ($t_{1/2} \approx 38$ min at 30°C). Thus, in the bacterial enzymes at least, inter-core migration of E1 subunits cannot be the sole or even major explanation of active-site coupling measured by incubation of the enzymes with [2-¹⁴C]pyruvate for 20 s (Figs. 2–5). Similarly, since the incorporation of [¹⁴C]acetyl groups into partly E1-inhibited pyruvate dehydrogenase complexes from *E. coli* and ox heart did not increase significantly when the incubation time was lengthened from 10 s to 45 s, it is unlikely that intra-core migration of E1 subunits is contributing much to our measurements of active-site coupling. The most likely explanations remain those of some form of intramolecular transacetylation reaction (Bates *et al.*, 1977; Collins & Reed, 1977) and the effects of the remarkable polypeptide-chain mobility discovered in the E2 cores (Perham *et al.*, 1981; Wawrzynczak *et al.*, 1981; Perham & Roberts, 1981; Berman *et al.*, 1981; Stepp *et al.*, 1981; Duckworth *et al.*, 1982). Raising the temperature has already been demonstrated to increase the mobility of the lipoic acid residues of the *E. coli* pyruvate dehydrogenase complex, as measured by e.s.r. experiments (Ambrose-Griffin *et al.*, 1978).

It should be emphasized that our measurements of active-site coupling as a function of temperature rest on acetylation curves generated by 20 s incubation of the enzyme complexes with [2-¹⁴C]pyruvate. This is, of course, time enough for many turnovers of the enzyme. Rapid-reaction studies by pulsed-quenched-flow experiments (Danson *et al.*, 1978b; Cate *et al.*, 1980; Akiyama & Hammes, 1980) have demonstrated that active-site coupling is not rate-limiting in the *E. coli* and ox kidney complexes. It is likely that further dissection of the mechanism and extent of active-site coupling, including an accurate assessment of the part if any played by movement of E1 subunits in the first few catalytic turnovers, will have

to come from renewed application of such rapid-reaction techniques.

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