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The role of lipoic acid residues in the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*

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Two lipoic acid residues on each dihydrolipoamide acetyltransferase (E2) chain of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* were found to undergo oxidoreduction reactions with NAD^+ catalysed by the lipoamide dehydrogenase component. It was observed that: (*a*) 2mol of reagent/mol of E2 chain was incorporated when the complex was incubated with *N*-ethylmaleimide in the presence of acetyl-SCoA and NADH; (*b*) 4mol of reagent/mol of E2 chain was incorporated when the complex was incubated with *N*-ethylmaleimide in the presence of NADH; (*c*) between 1 and 2mol of acetyl groups/mol of E2 chain was incorporated when the complex was incubated with acetyl-SCoA plus NADH; (*d*) 2mol of acetyl groups/mol of E2 chain was incorporated when the complex was incubated with pyruvate either before or after many catalytic turnovers through the overall reaction. There was no evidence to support the view that only half of the dihydrolipoic acid residues can be reoxidized by NAD^+. However, chemical modification of lipoic acid residues with *N*-ethylmaleimide was shown to proceed faster than the accompanying loss of enzymic activity under all conditions tested, which indicates that not all the lipoyl groups are essential for activity. The most likely explanation for this result is an enzymic mechanism in which one lipoic acid residue can take over the function of another.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* catalyses the overall reaction:

$$\text{Pyruvate} + NAD^+ + \text{CoASH} \rightarrow \text{acetyl-SCoA} + \text{NADH} + H^+ + CO_2$$

It is composed of multiple copies of three different types of polypeptide chain responsible for the constituent enzymic activities: pyruvate decarboxylase (E1) (EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2) (EC 2.3.1.12) and lipoamide dehydrogenase (E3) (EC 1.6.4.3). An analogous multienzyme complex catalyses the oxidative decarboxylation of 2-oxoglutarate [for reviews see Reed (1974) and Perham (1975)]. In both complexes the dihydrolipoamide acyltransferase (E2) component forms the structural core and appears to comprise 24 polypeptide chains arranged with octahedral symmetry (Reed, 1974; Danson *et al.*, 1979; Fuller *et al.*, 1979). The substrate is carried by lipoic acid residues covalently attached to lysine side chains in the E2 core (Nawa *et al.*, 1960); these cofactors are thought to rotate among the catalytic sites of the three component enzymes of the complex as part of the mechanism (Green & Oda, 1961; Koike *et al.*, 1963; Ambrose & Perham, 1976; Grande *et al.*, 1976).

Each E2 chain of the *E. coli* pyruvate dehydrogenase complex bears two lipoic acid residues that become reductively acetylated in the presence of pyruvate (Danson & Perham, 1976; Collins & Reed, 1977; Speckhard *et al.*, 1977). In contrast, lipoate succinyltransferase from *E. coli* (Collins & Reed, 1977; Angelides & Hammes, 1979; White *et al.*, 1980) and *Azotobacter xylinum* (De Kok *et al.*, 1980) contains only one lipoyl group per E2 chain, as do the dihydrolipoamide acetyltransferases from *Bacillus stearothermophilus* (Perham & Wilkie, 1980; Stanley *et al.*, 1981) and ox heart or ox kidney (Barrera *et al.*, 1972; White *et al.*, 1980; Stanley *et al.*, 1981).

Under certain conditions about 50% of the lipoyl moieties can be excised from the *E. coli* pyruvate

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dehydrogenase complex by treatment with trypsin, leaving the overall catalytic activity hardly impaired (Bleile *et al.*, 1979). Similarly, the kinetics of modification of the lipoic acid residues by *N*-ethylmaleimide in the presence of pyruvate demonstrated the existence of lipoyl groups that are not essential for full catalytic activity (Ambrose-Griffin *et al.*, 1980). We have shown by quenched stopped-flow experiments that both lipoic acid residues per E2 chain can be rapidly acetylated by pyruvate (Danson *et al.*, 1978*a*); furthermore, both acetyl groups can readily be removed by CoASH (Collins & Reed, 1977; Frey *et al.*, 1978; Ambrose-Griffin *et al.*, 1980). However, experiments by Frey *et al.* (1978) suggested that only half of the dihydrolipoyl groups so produced are reoxidized by the E3 component to produce NADH. It was proposed that the other lipoic acid residues might be reoxidized *in vivo* by an additional, unknown, electron acceptor.

In the present work we have investigated the catalytic interaction of lipoic acid residues with enzymes E1 and E3 of the *E. coli* complex by using a variety of radiolabelling methods. We show that both residues on each E2 chain can be functionally connected to the E3 component (Collins & Reed, 1977) and that lipoic acid residues become modified more rapidly than enzymic activity is lost. In accord with our earlier conclusion (Ambrose-Griffin *et al.*, 1980), it is clear that a full complement of lipoic acid residues is not required for full catalytic activity of the enzyme complex.

Materials and methods

Reagents

N-Ethyl[2,3-^{14}C]maleimide (CFA.293), [1-^{14}C]-acetyl-SCoA (CFA.390) and sodium [2-^{14}C]pyruvate (CFA.79) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Acetyl-SCoA was prepared by the acetylation of CoASH with acetic anhydride as described by Stadtman (1957). All other chemicals were of analytical grade and are listed by Danson *et al.* (1978*b*).

Enzyme and enzyme assays

Pyruvate dehydrogenase multienzyme complex was purified from a pyruvate dehydrogenase-constitutive mutant of *Escherichia coli* K12 as described by Danson *et al.* (1979). The whole-complex and E3 enzymic activities were assayed spectrophotometrically in the direction of NAD^+ reduction at 30°C as described by Danson *et al.* (1978*b*). The stoicheiometry of the polypeptide chains in the purified complex was determined by the radioamidination method of Bates *et al.* (1975) as modified by Hale *et al.* (1979).

Modification of pyruvate dehydrogenase complex with N-ethyl[2,3-^{14}C]maleimide

Pyruvate dehydrogenase complex (1.3 mg/ml) was incubated at 0°C with 0.5 mM-*N*-ethyl[2,3-^{14}C]maleimide in 50 mM-sodium phosphate buffer, pH 7.0, containing 1 mM-pyruvate, 0.5 mM-thiamin pyrophosphate, 5 mM-$MgCl_2$ and 1 mM-NAD^+. At measured time intervals samples were removed and mixed immediately with 2-mercaptoethanol (final concn. 280 mM). The whole-complex and E3 enzymic activities of each sample were assayed. An additional portion of the reaction mixture was added to 1 ml of ice-cold 10% (w/v) trichloroacetic acid to precipitate the complex. The precipitated protein was collected by filtration on nitrocellulose discs and was washed with 10% trichloroacetic acid. The discs were dried before their radioactivity was counted. Treatment with *N*-ethylmaleimide was continued until less than 10% of the original overall complex activity remained. At this point the modification was stopped with 280 mM-2-mercaptoethanol, and the sample was dialysed exhaustively at 4°C against 20 mM-sodium phosphate buffer, pH 7.0, containing 2 mM-EDTA. After dialysis, small volumes were taken for electrophoresis on 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate (Perham & Thomas, 1971). Radioactivity in the three protein bands was measured as described by Brown & Perham (1976). The inactivation is first-order in the concentration of *N*-ethylmaleimide (Brown & Perham, 1976).

In addition to this modification in the presence of the substrate pyruvate, the experiment was repeated in the presence of (*a*) 0.2 mM-NADH and (*b*) 0.2 mM-NADH plus 0.4 mM-acetyl-SCoA. In all these modifications, thiamin pyrophosphate, $MgCl_2$ and NAD^+ were included as before and a control experiment in the presence of these cofactors alone was also performed.

Incorporation of acetyl groups from [2-^{14}C]pyruvate and [1-^{14}C]acetyl-SCoA into pyruvate dehydrogenase complex

[2-^{14}C]Pyruvate (final concn. 0.1 mM) was added to pyruvate dehydrogenase complex (1.6 mg/ml) preincubated at 0°C in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.5 mM-thiamin pyrophosphate, 5 mM-$MgCl_2$ and 1 mM-NAD^+. After 20 s samples were taken and precipitated with trichloroacetic acid for measurement of the radioactivity incorporated into the protein, as described above.

The NADH-dependent acetylation of the complex by [1-^{14}C]acetyl-SCoA was performed essentially as described by Collins & Reed (1977). Enzyme complex (1.6 mg/ml) was incubated at 0°C in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.5 mM-thiamin pyrophosphate, 5 mM-$MgCl_2$,

1mM-NAD^+ and 0.2mM-NADH. After 2min [1-^{14}C]acetyl-SCoA was added to a final concentration of 0.4mM, and after a further 2min the protein was precipitated with trichloroacetic acid and collected on nitrocellulose discs as described above. A control experiment in the absence of NADH was performed.

Radioactivity measurements

Radioactivity measurements were made with an LKB Wallac Rackbeta scintillation counter. Aqueous samples were prepared for counting of radioactivity as described by Danson & Perham (1976). The radioactivities of dried nitrocellulose discs were counted as described by Danson *et al.* (1978*a*).

Specific radioactivities of radiochemicals

N-Ethyl[2,3-^{14}C]maleimide. The specific radioactivity of 12.0μCi/μmol quoted by the manufacturers was used.

[2-^{14}C]Pyruvate. The specific radioactivity of the pyruvate was measured after its reductive amination to [2-^{14}C]alanine by the method of Borch *et al.* (1971) as described by Ambrose-Griffin *et al.* (1980). A value of 9.9μCi/μmol was obtained, in close agreement with the value of 9.7μCi/μmol quoted by the manufacturers. The mean, 9.8μCi/μmol, was taken.

[1-^{14}C]Acetyl-SCoA. The concentration of acetyl-SCoA was determined by its quantitative conversion into [5-^{14}C]citrate by pig heart citrate synthase (EC 4.1.3.7); the CoASH thus produced was titrated with 5,5'-dithiobis-(2-nitrobenzoic acid). Other samples were taken for counting of radioactivity. The specific radioactivity was calculated to be 5.2μCi/μmol, in close agreement with the manufacturers' quote of 5.0μCi/μmol. The mean value, 5.1μCi/μmol, was taken.

Results

Modifications with N-ethyl[2,3-^{14}C]maleimide

(*a*) *In the presence of pyruvate.* We have previously described (Ambrose-Griffin *et al.*, 1980) the modification of native pyruvate dehydrogenase complex with *N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate. We wanted to compare this modification with that performed in the presence of other substrates of the complex, and it was thought necessary to repeat the experiment with the present preparation of enzyme and the new stock of *N*-ethyl[2,3-^{14}C]maleimide.

The modification was performed as described in the Materials and methods section. The extent and kinetics of modification and inactivation were very similar to those obtained previously (Ambrose-Griffin *et al.*, 1980). Thus a rapid loss of whole-complex activity was observed, with no effect on the E3 activity. The inactivation fitted a single-exponential curve with pseudo-first-order rate constant 0.096 (S.E. $\pm$ 0.004)min^{-1}. The ^{14}C-incorporation data were fitted to single- and double-exponential curves by a non-linear least-squares procedure (Ambrose-Griffin *et al.*, 1980). As found previously, an appreciably better fit was obtained to a double exponential (variance = 8.4) than to a single exponential (variance = 33.4); the two rate constants were 0.68 (S.E. $\pm$ 0.07)min^{-1} and 0.088 (S.E. $\pm$ 0.004)min^{-1}.

After complete modification, 105 (S.E. $\pm$ 8)nCi of radioactivity was incorporated per mg of complex. Of this, 96–98% was found in the E2 component, consistent with previous evidence that maleimides react specifically with the acetylated dihydrolipoic acid residues on the E2 component under these conditions (Danson & Perham, 1976; Collins & Reed, 1977). The stoicheiometry of the polypeptide chains in the complex was measured to be 1.38 (S.E. $\pm$ 0.04):1:0.79 (S.E. $\pm$ 0.01) (E1:E2:E3). The maximum incorporation of radioactivity corresponds to 2.2 (S.E. $\pm$ 0.2)mol of maleimide/mol of E2 chain.

(*b*) *In the presence of acetyl-SCoA and NADH.* *S*-Acetyldihydrolipoic acid residues may also be generated on the E2 component of the complex from acetyl-SCoA in the presence of NADH through the reverse catalytic reaction via enzymes E3 and E2. We therefore modified the pyruvate dehydrogenase complex with *N*-ethyl[2,3-^{14}C]maleimide in the presence of acetyl-SCoA and NADH, both to investigate the kinetics of modification and inactivation and to determine the extent of incorporation of radiolabel and hence the degree of acetylation of the E2 component.

The modification was performed as described in the Materials and methods section. The results of the experiment are shown in Fig. 1. As the modification proceeded there was a rapid loss of the overall complex activity, whereas the E3 enzymic activity remained unaffected. The inactivation fitted a single-exponential curve with pseudo-first-order rate constant 0.092 ($\pm$0.003)min^{-1}, a value very close to that observed for the modification in the presence of pyruvate (see above).

The ^{14}C-incorporation data for modification in the presence of acetyl-SCoA and NADH gave a better fit to a double exponential (variance = 10.1) than to a single exponential (variance = 21.6). The two rate constants obtained were 0.40 ($\pm$0.04)min^{-1} and 0.084 ($\pm$0.006)min^{-1}.

When the modification by the maleimide was complete, 90–92% of the radioactivity incorporated into the complex [110 ($\pm$9)nCi/mg of complex] was present in the E2 component. From the specific radioactivity of the maleimide and the measured

stoicheiometry of polypeptide chains in the complex (see above), the maximum incorporation of radioactivity corresponds to 2.2 (±0.2) mol of maleimide/mol of E2 chain. This value suggests that all the lipoic acid residues can be reduced by the E3 component of the complex.

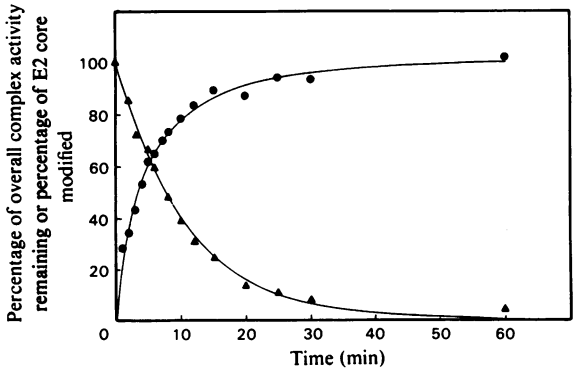

Fig. 1. *Modification of pyruvate dehydrogenase complex with N-ethyl[2,3-^{14}C]maleimide in the presence of acetyl-SCoA and NADH*

Treatment with 0.5 mM-*N*-ethyl-[2,3-^{14}C]maleimide in the presence of 0.4 mM-acetyl-SCoA and 0.2 mM-NADH was performed as described in the text. ▲, Overall complex activity; ●, percentage of the E2 core modified by the maleimide as measured by the incorporation of ^{14}C radioactivity into the protein. The value of 100% modification of the E2 core corresponds to the incorporation of 2.2 (±0.2) mol of maleimide/mol of E2 chain. The lines represent the best fits of the data to exponential(s) as described in the text. The overall complex activity (▲) is best described by a single exponential with rate constant 0.092 min^{-1}, and the modification of the E2 core (●) is best described by two exponentials with rate constants 0.40 min^{-1} and 0.084 min^{-1}.

(*c*) *In the presence of NADH.* The catalytic interaction of the lipoic acid residues with the E3 component was investigated further by studying the modification of the complex in the presence of NADH alone. Under these conditions, dihydrolipoyl residues are generated on the E2 component by the action of enzyme E3, and these can be modified and inactivated by treatment with *N*-ethylmaleimide.

The modification with *N*-ethyl[2,3-^{14}C]maleimide was performed in the presence of NADH as described in the Materials and methods section, and the results are shown in Fig. 2. As in the two previous modifications (see above), the loss of overall complex activity fitted a single-exponential curve with pseudo-first-order rate constant 0.077 (±0.004) min^{-1}. Again, there was no detectable loss of E3 enzymic activity. The maximum incorporation of radioactivity was 191 (±14) nCi/mg, with 90–94% of the radiolabel being in the E2 component. This corresponds to 3.9 (±0.3) mol of maleimide/mol of E2 chain. A molecule of dihydrolipoic acid contains two thiol groups, each of which presumably can react with a molecule of maleimide. Thus the ^{14}C-incorporation data again imply that two lipoic acid residues on each E2 chain can be reduced by NADH through the action of enzyme E3.

The modification of four thiol groups means that there are at least four exponentials (and hence four potentially different rate constants) contributing to the observed ^{14}C-incorporation data given in Fig. 2. We thought it imprudent to try to fit the data to four exponentials, but it is obvious from Fig. 2 that modification initially proceeds appreciably faster

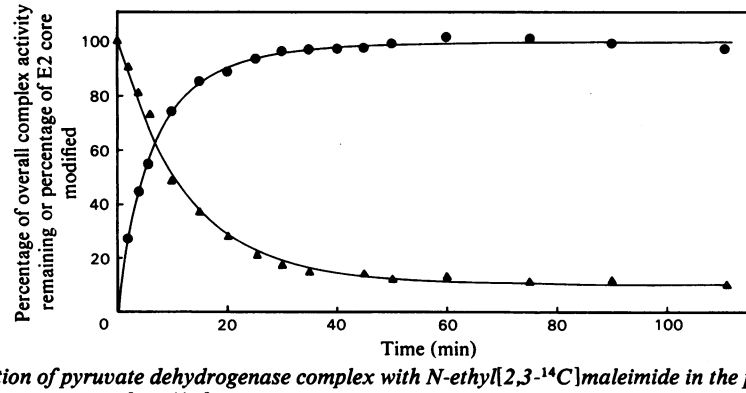

Fig. 2. *Modification of pyruvate dehydrogenase complex with N-ethyl[2,3-^{14}C]maleimide in the presence of NADH*

Treatment with 0.5 mM-*N*-ethyl[2,3-^{14}C]maleimide in the presence of 0.2 mM-NADH was performed as described in the text. ▲, Overall complex activity, where the line represents the best fit of the data to a single exponential with rate constant 0.077 min^{-1} (see the text); ●, percentage of the E2 core modified by the maleimide as measured by the incorporation of ^{14}C radioactivity into the protein. The value of 100% modification of the E2 core corresponds to the incorporation of 3.9 (±0.3) mol of maleimide/mol of E2 chain.

than the accompanying loss of enzymic activity. It is apparent from Fig. 2 that overall complex activity was not completely lost during the modification with *N*-ethylmaleimide in the presence of NADH, some of the activity remaining uninhibited. Subsequent additions of maleimide and of NADH did not lower this value further, whereas addition of pyruvate to the mixture did cause loss of remaining enzymic activity. We are unable fully to account for this finding. It could be that during the exposure of the enzyme to *N*-ethylmaleimide in the presence of NADH, a small proportion of the dihydrolipoic acid residues become oxidized in some way and are revived in the enzyme assay mixture, which contains cysteine. Such thiol-containing compounds cannot of course be present during the treatment of the enzyme with *N*-ethylmaleimide.

Incorporation of acetyl groups from [2-^{14}C]pyruvate and [1-^{14}C]acetyl-SCoA

The modifications of enzyme complex with *N*-ethylmaleimide have provided evidence that all the lipoic acid residues are available as substrates for enzymes E1 and E3. Attempts were made to check this by investigating the incorporation of [^{14}C]acetyl groups into the lipoic acid residues from the radiolabelled substrates pyruvate and acetyl-SCoA. The incorporations were performed and measured as described in the Materials and methods section.

Incubation of native pyruvate dehydrogenase complex with [2-^{14}C]pyruvate resulted in the incorporation of 2.0(±0.1) acetyl groups per E2 chain. Pretreatment of the complex with 0.5mM-*N*-ethylmaleimide for 30min at 0°C in 50mM-sodium phosphate buffer containing 0.5mM-thiamin pyrophosphate, 5mM-$MgCl_2$ and 1mM-NAD^+ did not significantly decrease the extent of acetylation, 1.9(±0.1) acetyl groups per E2 chain being incorporated on subsequent incubation with [2-^{14}C]-pyruvate.

In the presence of 0.2mM-NADH and 1.0mM-NAD^+, 1.5(±0.1) acetyl groups were incorporated per E2 chain on incubation of complex with 0.4mM-[1-^{14}C]acetyl-SCoA. This value is less than 2 acetyl groups per E2 chain predicted from the modification experiments with *N*-ethylmaleimide, but is significantly greater than the value of 1.0 expected if only half of the lipoyl groups are coupled to enzyme E3. However, the presence of NAD^+ (included in the incubation mixture to prevent the formation of the catalytically inactive 4-electron form of enzyme E3) in addition to the NADH could lend to an equilibrium being established between oxidized and reduced lipoic acid residues. Therefore less than complete acetylation might be expected under these conditions. To test this hypothesis, 0.5mM-*N*-ethylmaleimide was added to the incubation mixture after the addition of [1-^{14}C]acetyl-SCoA, in the hope that modification of the *S*-acetyldihydrolipoyl groups would displace the equilibrium towards the fully acetylated form. When whole-complex activity had fallen to less than 5% of the original value (approx. 40min), the extent of acetylation was measured and found to be 2.1(±0.1) acetyl groups incorporated per E2 chain. Incubation for this length of time in the absence of the maleimide did not increase the incorporation of acetyl groups from the initial value of 1.5.

As expected, the acetylation by [1-^{14}C]acetyl-SCoA was NADH-dependent. In the absence of NADH only 0.1 acetyl group was incorporated per E2 chain, irrespective of the presence of *N*-ethylmaleimide.

Overlap of acetylation from pyruvate and acetyl-SCoA

Having demonstrated that two acetyl groups can be incorporated per E2 chain from either pyruvate or acetyl-SCoA, we set up experiments to test whether the same two lipoic acid residues are involved in each case.

Pyruvate dehydrogenase complex was modified for 30min at 0°C with 0.5mM-*N*-ethylmaleimide in the presence of unlabelled 2.5mM-pyruvate, as described above. At 30min, when whole-complex activity was less than 5% of its original value, the modification was stopped by the addition of 2-mercaptoethanol. The NADH-dependent acetylation by [1-^{14}C]acetyl-SCoA was then measured exactly as described above for the native complex. Acetylation of the modified enzyme was less than 3% of that observed with the native complex.

The converse experiment was also performed. Enzyme complex was modified for 30min at 0°C with 0.5mM-*N*-ethylmaleimide in the presence of 0.2mM-NADH and 0.14mM-acetyl-SCoA as described above. After quenching of the reaction with 2-mercaptoethanol, the modified enzyme was incubated in 0.1mM-[2-^{14}C]pyruvate for 30s. The acetylation was less than 4% of that found with unmodified complex.

Enzyme complex was also modified with *N*-ethylmaleimide in the presence of 0.2mM-NADH (in the absence of acetyl-SCoA), as described in the Materials and methods section. After 30min the overall complex activity was 10% of its original value. The incorporation of [2-^{14}C]pyruvate into this modified enzyme was 7% of that found with native enzyme.

From these experiments it appears that the lipoic acid residues that connect with the E3 component are the same as those that connect with enzyme E1.

Direct demonstration that all lipoic acid residues can interact with enzyme E3

The evidence presented so far that all the lipoic

acid residues can interact catalytically with the E3 component of the enzyme complex has come from the use of part reactions of the complex. We therefore investigated the interaction in the enzyme engaged in the forward overall catalytic reaction.

Enzyme complex was incubated at 0°C in 50mM-sodium phosphate buffer, pH 7.0, containing 0.5mM-thiamin pyrophosphate, 5mM-$MgCl_2$, 1mM-NAD^+ and 0.03mM-[2-^{14}C]pyruvate. After 30s the extent of acetylation was measured to be 2.0 (±0.1) acetyl groups incorporated per E2 chain. After 1min, CoASH (final concn. 0.15mM, a 5-fold molar excess over pyruvate) was added to the reaction mixture. All the substrates and cofactors were then present, and the overall complex reaction should proceed until the pyruvate is used up. As expected, therefore, after 2min in the presence of CoASH, measurements of radioactivity in the enzyme complex showed that 95% of the radioactivity initially incorporated into the complex has been removed, leaving 0.1 acetyl group per E2 chain. A second addition of [2-^{14}C]pyruvate was then made to give a final concentration of 0.6mM-pyruvate (a 5-fold molar excess over remaining CoASH). After 30s it was found that 1.9 (±0.1) acetyl groups had been incorporated per E2 chain.

These results provide a direct demonstration that all the lipoic acid residues acetylatable by pyruvate via enzyme E1 can be deacylated by CoASH and reoxidized by enzyme E3, thus permitting continued reacetylation via enzyme E1.

Discussion

The results of our experiments indicate that two lipoyl groups per E2 chain in the *E. coli* pyruvate dehydrogenase complex can be reductively acetylated by pyruvate, that both acetyl groups can then be transferred to CoASH, and that both dihydrolipoic acid residues so generated can be reoxidized by NAD^+ through the action of the E3 component. These conclusions confirm and extend previous observations on the number of lipoic acid residues per E2 chain and their coupling with the E1 and E3 components (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977; Speckhard *et al.*, 1977; Ambrose-Griffin *et al.*, 1980). We can find no evidence for the proposal that there are two classes of lipoyl groups, both of which are coupled to the E1 component but only one of which is coupled to the E3 component (Frey *et al.*, 1978). Nor is there any hint of a functional role for a third lipoic acid residue (Hale & Perham, 1979). In contrast with these indications of two lipoyl groups per E2 chain that participate in the enzyme reaction, only one amino acid sequence including a lipoyl-lysine residue has yet been found, despite the use of several different techniques in two laboratories (Daigo & Reed, 1962; Hale & Perham, 1980). Further structural work will be needed to resolve this problem.

A previous study of the *E. coli* pyruvate dehydrogenase complex demonstrated that lipoic acid residues could be inactivated by chemical modification more rapidly than the enzyme complex lost overall catalytic activity (Ambrose-Griffin *et al.*, 1980). Similarly, it was noted that about half of the lipoyl moieties could be excised from the complex by treatment with trypsin, yet almost all the catalytic activity remained (Bleile *et al.*, 1979). The further chemical modifications that we have studied in the present experiments (Figs. 1 and 2) again reveal that lipoic acid residues can be modified more rapidly than catalytic activity is lost. It is abundantly clear that a full complement of lipoic acid residues is not required by the enzyme complex.

Two explanations for this surprising result can be envisaged: that only one of the two lipoic acid residues per E2 chain is essential for the catalytic reaction, or that one lipoic acid residue can take over the role of another. The kinetics of modification do not allow a clear choice to be made (Ambrose-Griffin *et al.*, 1980). However, two lines of evidence now cause us to opt for the latter. First, during the removal of lipoic acid residues from the complex by treatment with the enzyme lipoamidase from *Streptococcus faecalis* (Reed *et al.*, 1958), the correlation of enzymic activity with lipoic acid content favours the take-over model (Berman *et al.*, 1981). Secondly, the use of proton n.m.r. spectroscopy has revealed that the lipoic acid residues are themselves attached to large regions of the E2 polypeptide chains with unusually high internal mobility akin to random coils (Perham *et al.*, 1981). This high mobility of the polypeptide chain could permit a given active site of component E1 to be served by more than one lipoic acid residue, perhaps even one bound to a different E2 chain, allowing loss of catalytic activity to lag behind modification or excision of lipoic acid residues (Ambrose-Griffin *et al.*, 1980; Cate *et al.*, 1980).

The lipoic acid residues in the E2 core have been shown to provide a system in active-site coupling in the complex by means of intramolecular transacetylation reactions (Bates *et al.*, 1977; Collins & Reed, 1977) that are not rate-limiting in the overall enzymic reaction (Danson *et al.*, 1978*a*). Far from being simply the structural core for bringing together three successive enzymes in a fixed geometrical arrangement, the dihydrolipoamide acetyltransferase component appears to have the property of ensuring that the three activities are coupled as effectively as possible. This could be of selective advantage for an enzyme particle that has been found to have variability in its subunit proportions (Bates *et al.*, 1975) and to demonstrate physical heterogeneity in hydrodynamic studies (Schmitt &

Cohen, 1980; Gilbert & Gilbert, 1980). It would also accommodate problems caused by low concentrations of substrates for the sequential reactions of the enzyme complex (Danson *et al.*, 1978*a*).

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