Biochem. J. (1980) **187**, 393–401
Printed in Great Britain

Kinetic Analysis of the Role of Lipoic Acid Residues in the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*

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(*Received 15 November 1979*)

The catalytic roles of the two reductively acetylatable lipoic acid residues on each lipoate acetyltransferase chain of the pyruvate dehydrogenase complex of *Escherichia coli* were investigated. Both lipoyl groups are reductively acetylated from pyruvate at the same apparent rate and both can transfer their acetyl groups to CoASH, part-reactions of the overall complex reaction. The complex was treated with *N*-ethylmaleimide in the presence of pyruvate and the absence of CoASH, conditions that lead to the modification and inactivation of the *S*-acetyldihydrolipoic acid residues. Modification was found to proceed appreciably faster than the accompanying loss of enzymic activity. The kinetics of the modification were fitted best by supposing that the two lipoyl groups react with the maleimide at different rates, one being modified at approximately 3.5 times the rate of the other. The loss of complex activity took place at a rate approximately equal to that calculated for the modification of the more slowly reacting lipoic acid residue. The simplest interpretation of this result is that only this residue is essential in the overall catalytic mechanism, but an alternative explanation in which one lipoic acid residue can take over the function of another was not ruled out. The kinetics of inactivation could not be reconciled with an obligatory serial interaction between the two lipoic acid residues. Similar experiments with the fluorescent *N*-[*p*-(benzimidazol-2-yl)phenyl]maleimide supported these conclusions, although the modification was found to be less specific than with *N*-ethylmaleimide. The more rapidly modified lipoic acid residue may be involved in the system of intramolecular transacetylation reactions that couple active sites in the lipoate acetyltransferase component.

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

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoASH} \rightarrow \text{acetyl-SCoA} + \text{NADH} + \text{H}^+ + \text{CO}_2$$

The complex is composed of 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) [for reviews see Reed (1974) and Perham (1975)]. 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 substrate is carried in thioester linkage by lipoic acid residues covalently attached to lysine side chains on the E2 polypeptide chains (Nawa *et al.*, 1960). These cofactors are thought to rotate among the catalytic sites of the three component enzymes of the complex (Green & Oda, 1961; Koike *et al.*, 1963), and measurements of the mobility of spin-labelled lipoic acid residues are consistent with this 'swinging-arm' mechanism (Ambrose & Perham, 1976; Grande *et al.*, 1976).

Each E2 chain bears two lipoic acid residues that become reductively acetylated in the presence of pyruvate (Danson & Perham, 1976; Collins & Reed,

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1977; Speckhard *et al.*, 1977). It is also known that these acetylatable lipoyl-lysine 'swinging arms' form a novel interacting network on the E2 core of the complex. Thus, by examining the fate of pyruvate decarboxylated within the complex in the absence of CoASH, we found that a single E1 dimer can catalyse the reductive acetylation of multiple copies of the E2 chains in the core (Bates *et al.*, 1977; Danson *et al.*, 1978*a*). We proposed that acetyl groups can be passed between lipoic acid residues by intramolecular transacetylation reactions. Further evidence for such a network of interacting lipoic acid residues was presented by Collins & Reed (1977) and by Hale *et al.* (1979*a*). Pulsed-quenched-flow measurements demonstrated that these intramolecular transacetylations are not rate-limiting in the normal reaction mechanism of the complex (Danson *et al.*, 1978*b*). There is therefore the potential for rapid multiple coupling of active sites in the E2 core by means of the lipoic acid swinging arms.

The precise interaction of the two acetylatable lipoic acid residues with each other in the normal enzyme reaction and in the intramolecular coupling remains to be established. One method of investigating their functions would be to modify them specifically with a chemical reagent. Then, following the kinetic analysis described by Ray & Koshland (1961), a comparison of their rates of modification with the rates of loss of enzymic activity might permit an assessment of their respective roles; in particular, it might become clear whether they operate in a series or a parallel mechanism. In the present paper we describe this type of approach for the modification of the acetylatable lipoic acid residues with *N*-ethylmaleimide. In the presence of pyruvate and the absence of CoASH, the lipoic acid residues on each E2 chain become reductively acetylated and the enzymic reaction then ceases. The thiol group of the *S*-acetyldihydrolipoamide so generated can be made to react rather specifically with *N*-ethylmaleimide, which inactivates the enzyme (Brown & Perham, 1976).

Our results are explained most simply if the two acetylatable lipoic acid residues of each E2 chain have different catalytic roles.

Materials and Methods

Reagents

N-Ethyl[2,3-^{14}C]maleimide (CFA.293) and [2-^{14}C]pyruvate (sodium salt) (CFA.79) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. DL-Dihydrolipoamide was prepared by the reduction of lipoamide with $NaBH_4$ (Reed *et al.*, 1958). [^{3}H]DNA, isolated from mouse L cells cultured in the presence of [*Me*-^{3}H]thymidine, was a gift from Dr. A. Newton of this Department. *N*-[*p*-(Benzimidazol-2-yl)phenyl]maleimide was obtained from Teikaseiyaku Co., 250 Arakawa, Toyama, Japan. All other chemicals were of analytical grade and are listed by Danson *et al.* (1978*a*).

Enzyme and enzyme assays

Pyruvate dehydrogenase multienzyme complex was purified from a pyruvate dehydrogenase-constitutive mutant of *Escherichia coli* K12 by the method of Reed & Mukherjee (1969) 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*a*). 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*b*).

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

Pyruvate dehydrogenase complex (1.6mg/ml) was incubated at 0°C with 0.3mM-*N*-ethyl[2,3-^{14}C]maleimide in 50mM-sodium phosphate buffer, pH 7.0, containing 1mM-sodium pyruvate, 0.5mM-thiamin pyrophosphate, 5mM-$MgCl_2$, 1mM-NAD^+ and 30nCi of [^{3}H]DNA (the [^{3}H]DNA was included as an internal standard with which to compare the incorporation of [^{14}C]maleimide into the protein). At measured time intervals 0.1ml samples were removed and mixed immediately with 2-mercaptoethanol and EDTA (final concentrations 140mM and 10mM respectively). The whole-complex and E3 enzymic activities of each sample were assayed, and the modification was continued until less than 10% of the original overall complex activity remained. To each sample was added 0.25ml of 20mM-sodium phosphate buffer, pH 7.0, containing 2mM-EDTA, and all samples were then dialysed exhaustively against this same buffer containing 0.1% sodium dodecyl sulphate. After dialysis, small volumes were taken from each sample for measurements of radioactivity and for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

A control experiment was carried out exactly as described in the above procedure except that the pyruvate dehydrogenase complex was added to the reaction mixture containing *N*-ethyl[2,3-^{14}C]maleimide and [^{3}H]DNA after the addition of 2-mercaptoethanol. This control permitted the measurement of any [^{14}C]maleimide that was incorporated into the sample of DNA or was not removed by the dialysis. The ^{14}C radioactivity in these controls was approx. 5% of that in the test samples.

Modification of pyruvate dehydrogenase complex with N-[p-(benzimidazol-2-yl)phenyl]maleimide

The complex (13mg/ml) was first treated for 2h with 1mM-*N*-ethylmaleimide at 0°C in the absence of pyruvate under the conditions described above. The enzyme activity remained constant throughout this time, after which 2-mercaptoethanol was added and the reagents were removed by dialysis against 50mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA. The treated complex (0.09mg/ml) was then incubated at 24°C with 10μM-*N*-[*p*-(benzimidazol-2-yl)phenyl]maleimide in 50mM-potassium phosphate buffer, pH7.0, containing 1mM-EDTA, 6mM-$MgCl_2$, 0.33mM-thiamin pyrophosphate, 1.0mM-NAD^+ and 1.0mM-sodium pyruvate. In control experiments the pyruvate was omitted. Overall complex activity was monitored during the course of the incubation as described above.

Fluorescence measurements were made with an Aminco–Bowman fluorimeter with the cuvette maintained at 24°C. The excitation wavelength was 315nm and emission was observed at 360nm.

Modification of pyruvate dehydrogenase complex with N-ethylmaleimide in the presence of [2-^{14}C]-pyruvate

The complex was modified with 0.5mM-*N*-ethylmaleimide in the presence of 0.25mM-[2-^{14}C]-pyruvate at 0°C as described above except that no [^{3}H]DNA was included. Samples (0.12ml) taken at measured time intervals were added to 2-mercaptoethanol (final concentration 45mM) and, after 1min at 0°C, 30μl of 50mM-sodium phosphate buffer, pH7.0, containing 16mM-sodium arsenite, 20mM-sodium pyruvate, 1.4mM-CoASH and 30mM-cysteine hydrochloride was added to each. After 5min at 0°C, the reaction was stopped with 1ml of ice-cold 10% (w/v) trichloroacetic acid. The precipitated proteins were collected by filtration on nitrocellulose discs and were washed with 25ml of 10% trichloroacetic acid. The discs were dried before their radioactivity was counted.

In a control experiment, the time course of removal of [1-^{14}C]acetyl groups by CoASH in the absence of maleimide was measured. In addition, the maximum incorporation of acetyl groups into the lipoic acid residues from [2-^{14}C]pyruvate was measured in the absence of modification by *N*-ethylmaleimide and with no CoASH treatment.

Specific radioactivities of N-ethyl[2,3-^{14}C]maleimide and [2-^{14}C]pyruvate

N-Ethyl[2,3-^{14}C]maleimide. The concentration of maleimide was determined by titration with cysteine [measured by titration with 5,5'-dithiobis-(2-nitrobenzoic acid)] and a sample was taken for counting of radioactivity. The specific radioactivity was thus determined to be 4.8 ($\pm$0.2) μCi/μmol, in excellent agreement with the value of 4.7μCi/μmol quoted by the manufacturers.

[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).

A sample of [2-^{14}C]pyruvate (3μmol) was freeze-dried and dissolved in 0.5ml of methanol containing NH_4Cl (130μmol) and $NaBH_3CN$ (51μmol). The solution was shaken at room temperature for 48h. Then 0.13ml of 12M-HCl (AristaR) was added and, after being stirred for a further 1h, the solution was evaporated *in vacuo*. The residue was dissolved in 0.5ml of distilled water and added to a Zerolit 225 (H^+ form) column. After the column had been washed with 5ml of distilled water, the alanine was eluted with 1M-NH_3. The fractions containing radioactivity were pooled, dried down and washed several times with distilled water to remove all the NH_3. A sample was then applied to a Rank Hilger Chromaspek amino acid analyser and the effluent from the column was collected. Alanine was the only ninhydrin-positive compound observed, and from the measured radioactivity eluted in this peak the specific radioactivity of the [2-^{14}C]pyruvate was calculated to be 9.9 ($\pm$0.2)μCi/μmol. This agrees closely with the value of 9.7μCi/μmol quoted by the manufacturers.

Radioactivity measurements

Radioactivity measurements were carried out in a Nuclear–Chicago Unilux II-A scintillation counter. Radioactivities of aqueous samples were counted as described by Danson & Perham (1976). For the simultaneous counting of ^{3}H and ^{14}C, the settings on the counter were adjusted so that no ^{3}H radioactivity appeared in the ^{14}C channel and only 15% of the ^{14}C radioactivity was found in the ^{3}H channel.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis in 7.5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate was carried out as described by Perham & Thomas (1971). The stained gels were scanned for protein on a Joyce–Loebl densitometer. Radioactivity in the three protein bands was measured as described by Brown & Perham (1976).

Results

Modification with N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate

Native pyruvate dehydrogenase complex was modified with 0.3mM-*N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate as described in the Materials

and Methods section. Consistent with our previous observations (Brown & Perham, 1976; Danson & Perham, 1976), the modification produced a rapid loss of whole-complex activity (Fig. 1), whereas the E3 activity remained completely unaffected. All the evidence suggests that the maleimide reacts with the acetylated dihydrolipoic acid residues on the E2 component under these conditions (Danson & Perham, 1976; Collins & Reed, 1977). In the present experiment the rate of incorporation of *N*-ethyl-[2,3-^{14}C]maleimide into these lipoic acid residues was measured in two ways. At known time intervals during the course of the modification samples were taken and the reaction was quenched with 2-mercaptoethanol. After removal of excess of maleimide by dialysis, the ^{14}C radioactivity incorporated into the complex was counted relative to the ^{3}H radioactivity in the internal standard of DNA. The presence of this [^{3}H]DNA throughout the whole experiment allowed for any variation in sampling volume and for possible differential dilutions during dialysis. To obtain the degree of modification of the lipoic acid residues from these incorporations, a correction is required for the small percentage of non-specific modification of the E1 and E3 components (Brown & Perham, 1976; Ambrose & Perham, 1976; Danson & Perham, 1976). Therefore each sample was subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the percentage of the radioactivity incorporated into the E2 component (85–95%) was determined.

An independent estimate of the extent of modification of the lipoic acid residues was obtained solely from the results of the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. To correct for any variations in the amounts of protein loaded on to the gels, the measured radioactivity in the excised E2 band was expressed relative to the amount of protein in that band as recorded by prior densitometric scanning of the Coomassie Blue-stained gel. Since the loadings of the gels were closely similar, linearity of Coomassie Blue-staining could safely be assumed. Both measurements of the incorporation of [^{14}C]maleimide into the lipoic acid residues are shown in Fig. 1. Excellent agreement between the two sets of data for the incorporation was observed.

The specific radioactivity of the *N*-ethyl[2,3-^{14}C]maleimide was determined to be 4.8 (±0.2)μCi/μmol. The stoicheiometry of polypeptide chains in the complex was measured to be 1.49 (±0.03):1.0:0.90 (±0.04) (E1:E2:E3). Therefore, from the maximum incorporation of radioactivity [44 (±0.4)nCi/mg of complex] we cal-

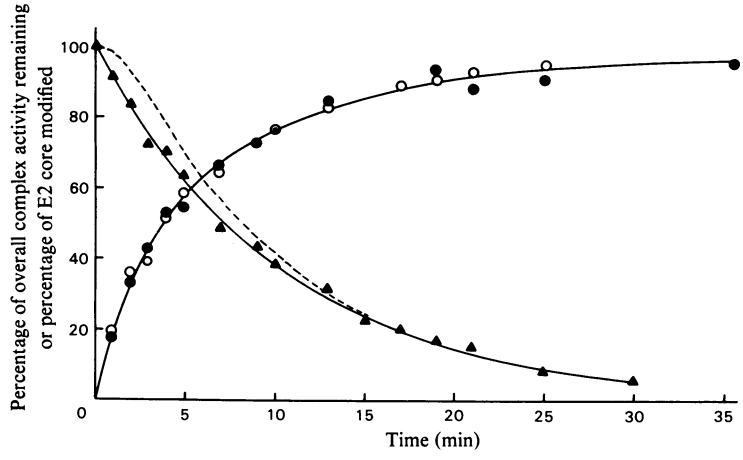

Fig. 1. *Modification of pyruvate dehydrogenase complex with N-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate*

Treatment with 0.3mM-*N*-ethyl[2,3-^{14}C]maleimide in the presence of pyruvate was carried out as described in the text. ▲, Overall complex activity; ●, percentage of the E2 core modified by the maleimide where the ^{14}C radioactivity incorporated into the protein was counted relative to the [^{3}H]DNA internal standard; ○, percentage of the E2 core modified by the maleimide in the same experiment but where the ^{14}C radioactivity incorporated was determined after electrophoresis of the modified complex on sodium dodecyl sulphate/polyacrylamide gels and was expressed relative to the amount of E2 protein on each gel. The value of 100% modification of the E2 core corresponds to the incorporation of 2.2 molecules of maleimide per E2 chain. The lines represent the best fits of the data to exponential(s) as described in the text. The whole-complex activity (▲) is best described by a single exponential with rate constant 0.096min^{-1}, and the modification of the E2 core (● and ○) is best described by two exponentials with rate constants 0.340min^{-1} and 0.090min^{-1}. The broken line shows the loss of activity expected for a mechanism in which an unmodified lipoic acid residue could fully take over the function of a modified one.

culate that 2.2 (±0.2) mol of maleimide was incorporated per mol of E2 chain. This value is in good agreement with our previous measurement of two acetylatable lipoic acid residues on each E2 chain (Danson & Perham, 1976).

The loss of enzyme activity fitted a single exponential curve with pseudo-first-order rate constant 0.096 (±0.003) min^{-1}. However, a single exponential fit to the incorporation of ^{14}C-labelled *N*-ethylmaleimide gave a rate constant of 0.195 (±0.011) min^{-1}, i.e. faster than the loss of enzymic activity. According to the classical analysis by Ray & Koshland (1961), the modification of a group essential for enzyme activity cannot proceed faster than the loss of that activity. This suggests that the observed incorporation of radioactivity is in fact the sum of at least two exponential curves, the rate constant of one being equal to that for the loss of enzyme activity. Therefore we compared the fit of the data to single- and double-exponential curves by using a non-linear least-squares procedure. The ^{14}C-incorporation data were found to give an appreciably better fit to a double exponential (variance = 14.0) than to the single exponential (variance = 19.3); the two rate constants obtained were 0.340 (±0.030) min^{-1} and 0.090 (±0.006) min^{-1}. As expected, the smaller rate constant agrees, within experimental error, with that measured for the loss of enzyme activity. The ratio of the two rate constants is approximately 3.5.

Modification with N-ethylmaleimide in the presence of [2-^{14}C]pyruvate

The previous experiment relied on measuring the substrate-induced modification of lipoic acid residues with *N*-ethylmaleimide. It produced the unexpected result that modification of these residues appears to proceed faster than loss of enzymic activity. We therefore did the experiment the other way round, with the use of radioactive substrate, [2-^{14}C]pyruvate, to label the dihydrolipoic acid residues and then treatment with unlabelled *N*-ethylmaleimide. The [^{14}C]acetyl group cannot be removed by CoASH and arsenite from an *S*-acetyldihydrolipoic acid residue that has reacted with *N*-ethylmaleimide (Collins & Reed, 1977), and this property was used to monitor the reaction of these residues with the maleimide, as described in the Materials and Methods section.

The collected results of two identical experiments are shown in Fig. 2. As before, there was no loss of E3 enzymic activity, whereas the overall catalytic activity of the complex declined rapidly. This was accompanied by an increasing inability of the treatment with CoASH and arsenite to remove the [^{14}C]acetyl groups from the enzyme. As expected,

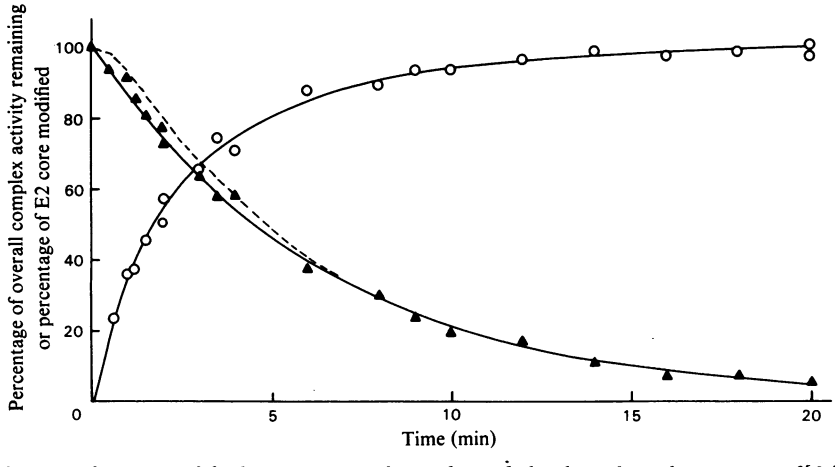

Fig. 2. *Modification of pyruvate dehydrogenase complex with N-ethylmaleimide in the presence of* [2-^{14}C]*pyruvate*
Treatment with 0.5 mM-*N*-ethylmaleimide in the presence of [2-^{14}C]pyruvate was carried out as described in the text. Data from two independent but identical experiments are included. ▲, Overall complex activity; O, percentage of the E2 core modified by the maleimide as measured by the radioactivity not removed by CoASH and arsenite from the modified [1-^{14}C]acetyl-lipoic acid residues (see the text). The maximum incorporation of [1-^{14}C]acetyl groups corresponds to 2.0 lipoic acid residues per E2 chain. The lines represent the best fits of the data to exponential(s) as described in the text. The whole-complex activity (▲) is best described by a single exponential with rate constant 0.154 min^{-1}, and the modification of the E2 core (O) is best described by two exponentials with rate constants of 0.692 min^{-1} and 0.206 min^{-1}. The broken line shows the loss of activity expected for a mechanism in which an unmodified lipoic acid residue could fully take over the function of a modified one.

when the modification by the maleimide was complete, the radioactivity left in the acetylated complex was identical (98 ± 2%) with the radioactivity [67 (±1)nCi/mg of complex] found in a control sample of the complex that was not treated with *N*-ethylmaleimide or with CoASH and arsenite. From the measured specific radioactivity of the [2-^{14}C]pyruvate [9.9 (±0.2)μCi/μmol] and the measured polypeptide-chain stoicheiometry of the complex (see above), this maximum incorporation of [1-^{14}C]acetyl groups corresponds to 2.0 (±0.1) lipoic acid residues per E2 chain.

The kinetic data from this experiment were analysed as before and similar results were obtained. The loss of enzyme activity fitted a single exponential curve with a rate constant of 0.154 (±0.003)min^{-1}. Again, the modification with *N*-ethylmaleimide (measured by [1-^{14}C]acetyl groups not removable by CoASH and arsenite) gave a better fit to a double exponential (variance 6.9) than to a single exponential (variance 9.5). The two rate constants were 0.692 (±0.049)min^{-1} and 0.206 (±0.015)min^{-1}. The agreement between the smaller rate constant (0.206min^{-1}) and that for the loss of enzymic activity (0.154min^{-1}) is fair.

There is a slight complication in the interpretation of these results. If both lipoic acid residues can pass their [1-^{14}C]acetyl groups direct to CoASH, then the measured radioactivity should exactly match the labelling of these residues by *N*-ethylmaleimide. However, if only one lipoyl group (the slower-reacting, essential, one) can interact direct with CoASH and the other must be deacetylated via this one, then the observed kinetics should be slightly different. At any time there will be a proportion of those lipoic acid residues that react more quickly with the maleimide, but are not yet modified themselves, which have [1-^{14}C]acetyl groups that cannot be removed by CoASH because the other, essential, lipoic acid residue has been modified. The expected faster rate constant would then be the sum of the two rate constants observed in the first experiment, and therefore the ratio of rate constants in this second experiment would be about 4.5 rather than 3.5. We cannot decide between the two possibilities from our data. The ratio of the rate constants obtained from the double-exponential fit to the modification data is 3.4, but the ratio obtained from the rate constant for the loss of enzyme activity as a measure of the lower rate (which should be more accurate) is 4.5.

Modification with N-[p-(benzimidazol-2-yl)phenyl]-maleimide in the presence and absence of pyruvate

N-[*p*-(Benzimidazol-2-yl)phenyl]maleimide is a reagent that becomes fluorescent after reaction with thiol groups (Kanaoka, 1977). Thus incorporation of this maleimide into pyruvate dehydrogenase complex can be monitored directly by the increase in fluorescence, by using an excitation wavelength of 315nm and an emission wavelength of 360nm. Parallel modifications of the enzyme with *N*-[*p*-(benzimidazol-2-yl)phenyl]maleimide were carried out, one set being used to measure the loss of total complex activity with time and the other set to measure the increase in fluorescence with time. The extent of incorporation of the maleimide was assumed to be directly proportional to the intensity

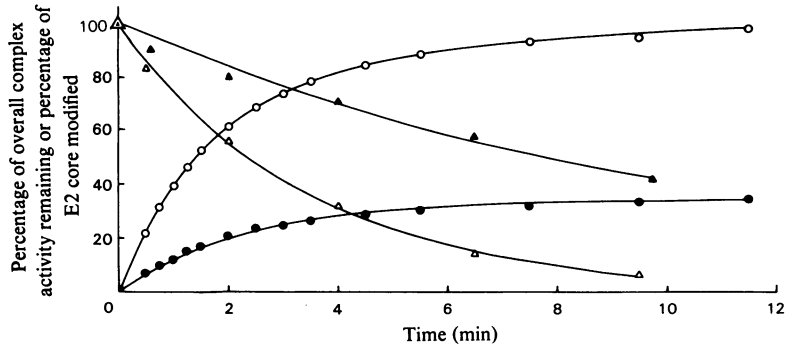

Fig. 3. *Modification of pyruvate dehydrogenase complex with N-[p-(benzimidazol-2-yl)phenyl]maleimide*
The complex (pretreated with *N*-ethylmaleimide in the absence of pyruvate) was treated with 0.01mM-*N*-[*p*-(benzimidazol-2-yl)phenyl]maleimide at 24°C in the presence and absence of 1mM-pyruvate as described in the text. ▲, Overall complex activity in the absence of pyruvate; △, overall complex activity in the presence of pyruvate; ●, relative fluorescence emission at 360nm (excitation at 315nm) in the absence of pyruvate; ○, relative fluorescence emission in the presence of pyruvate. The value of 100% modification of the E2 core corresponds to the end point of the increase in fluorescence measured in arbitrary units. The lines represent best fits of the data to exponential(s), as described in the text.

of fluorescence, after it had been checked that in this concentration range there was no self-quenching of fluorescence of the benzimidazolylphenyl chromophore.

The results of this experiment are shown in Fig. 3. The stoicheiometry of the incorporation of the maleimide could not be determined because the fluorescence measurements were not calibrated. However, as before, the rate of incorporation of the maleimide in the presence of pyruvate was greater than the rate of loss of overall enzyme activity. The latter fitted a single exponential with rate constant 0.29 ($\pm$0.01)min^{-1}. The incorporation data give a better fit to a double exponential (variance = 0.53) than a single exponential (variance = 1.84); the two rate constants were 0.80 ($\pm$0.02)min^{-1} and 0.28 ($\pm$0.01)min^{-1}.

In this experiment, unlike the modification with *N*-ethylmaleimide (Brown & Perham, 1976; Danson & Perham, 1976), there was a considerable degree of modification of the complex in the absence of pyruvate, this resulting in loss of enzyme activity and an increase in fluorescence (Fig. 3). The results are therefore difficult to interpret quantitatively. When an attempt was made to allow for the labelling in the absence of pyruvate, it became impossible to decide between single- and double-exponential fits. However, it was still clear that the complex was modified more rapidly than overall enzyme activity was lost.

Lack of effect of various experimental conditions on the rate of inactivation by N-ethylmaleimide

NAD^+ was included in both modification experiments with *N*-ethylmaleimide to ensure that the lipoic acid residues were all in the fully oxidized form on the addition of pyruvate. However, Angelides & Hammes (1978) have described the comparable modification of *E. coli* pyruvate dehydrogenase complex with *N*-[^{3}H]ethylmaleimide in the absence of NAD^+. The rate of inactivation that they observed was roughly 10-fold slower than we have found in the present work and in earlier studies (Ambrose & Perham, 1976; Brown & Perham, 1976; Danson & Perham, 1976). Therefore we repeated the pyruvate-induced inactivation of complex by 0.3mM-*N*-ethylmaleimide in the presence and absence of NAD^+. [This concentration of maleimide is the same as that used to obtain Fig. 1 of the present paper and by Angelides & Hammes (1978).] Under both sets of conditions the inactivation was a pseudo-first-order process, with rate constants of 0.096 ($\pm$0.003)min^{-1} in the presence of NAD^+ and 0.098 ($\pm$0.007)min^{-1} in the absence of the cofactor. We conclude that the presence of NAD^+ does not affect the rate of inactivation.

A further difference in experimental conditions is that Angelides & Hammes (1978) prepared their stock solution of *N*-ethylmaleimide in dimethyl sulphoxide, giving a final concentration of 5% (v/v) of this solvent in the modification reaction (see Angelides & Hammes, 1979). In the experiments reported in the present paper, *N*-ethylmaleimide was dissolved in dry acetone and the concentration of this solvent in the reaction mixture was never greater than 0.5% (v/v). However, we find that the rate of pyruvate-induced inactivation of the complex by *N*-ethylmaleimide is unaffected by the presence of 5% (v/v) dimethyl sulphoxide, in either the absence or the presence of NAD^+.

Similarly, we repeated our experiments with the use of enzyme complex that had been pretreated with unlabelled *N*-ethylmaleimide in the absence of pyruvate, as recommended by Angelides & Hammes (1978). The results we obtained were very similar to those we have described for the untreated complex.

Discussion

The existence of two reductively acetylatable lipoic acid residues on each E2 polypeptide chain of the pyruvate dehydrogenase complex of *E. coli* (Danson & Perham, 1976; Collins & Reed, 1977; Speckhard *et al.*, 1977) invites speculation about the parts they play in the reaction mechanism. Both residues are reductively acetylated by pyruvate in a rapid pseudo-first-order process (Danson *et al.*, 1978*b*) in the absence of CoASH, and both acetyl groups can be removed by subsequent addition of CoASH (Speckhard *et al.*, 1977; Collins & Reed, 1977; the present work).

The simplest proposal is that they function either in 'parallel', i.e. two independent active sites in each E2 chain, or in 'series', i.e. acetyl groups pass from a first to a second lipoic acid molecule en route to CoASH. If the mechanism were a simple series one, the rate constant for inactivation would be equal to the sum of the rate constants for modification of the two *S*-acetyldihydrolipoic acid residues with *N*-ethylmaleimide [see Case I of Ray & Koshland (1961)]. If the mechanism were a simple parallel one in which both lipoic acid residues contributed equally to the enzymic activity, the rate of inactivation should equal the rate of modification [see Case IV of Ray & Koshland (1961), with $F_1 = F_2 = 0.5$ and $F_3 = 0$]. In fact, the rate of inactivation is roughly half that of modification (Figs. 1–3).

We can explain this highly unusual result by noting that the modification with maleimide under our conditions is in fact better described as two exponential processes, the ratio of the rate constants being about 3.5, and that the rate of inactivation of the enzyme happens to match the slower process. Only one lipoic acid residue per E2 chain (the more slowly reacting) would then be described as 'essential' for the normal catalytic action as measured by NAD^+ reduction.

However, the pyruvate dehydrogenase complex has the hitherto unconsidered property of active-site coupling, whereby lipoic acid residues on adjacent E2 subunits can be linked by internal transacetylation reactions (Bates *et al.*, 1977; Danson *et al.*, 1978*a*,*b*; Collins & Reed, 1977). If we allow a mechanism in which an unmodified lipoic acid residue can take over part or all of the function of a modified one (whether on the same E2 subunit or an adjacent one), the loss of activity can lag behind the incorporation of modifying reagent. The plot of activity versus time would not then be a simple exponential [Case II of Ray & Koshland (1961)]. If we adopt our measured rate constants for the modification of the two species of lipoic acid residues (Figs. 1 and 2) and assume that the unmodified lipoic acid residue can fully take over the function of the modified one, we are able to calculate the curve that would describe the loss of activity with time [Equation 4 of Ray & Koshland (1961)]. This is shown as the broken line in Figs. 1 and 2. The experimental data appear to fit the single exponential curve better than this composite exponential, although a fit to the latter curve is not excluded. We therefore prefer the earlier explanation but do not rule out the latter.

The results of our experiments differ from those of Angelides & Hammes (1978), who have also made a study of the kinetics of the pyruvate-induced inactivation of the complex with *N*-ethylmaleimide. They concluded that at least three classes of lipoic acid residues exist: one class (approx. 10% of the lipoic acids) is modified before catalytic activity begins to fall, after which activity is lost more rapidly than lipoic acid residues are modified. The complexity of the kinetic curve required at least two exponential decay terms to describe it (Angelides & Hammes, 1978). We have not been able to account for these differences from our results. The explanation may yet be found in the fact that the rate of inactivation observed by Angelides & Hammes (1978) is some 10-fold slower than that found by us (Brown & Perham, 1976; Danson & Perham, 1976; Ambrose & Perham, 1976; the present work) and by others (Collins & Reed, 1977), which may reflect some difference in the enzyme preparation or unrecognized difference of experimental conditions. Similar experiments with the *Azotobacter vinelandii* (Grande *et al.*, 1975) and *Bacillus stearothermophilus* (Henderson *et al.*, 1979) complexes have given rates of inactivation comparable with those that we describe in the present paper.

From their results, Angelides & Hammes (1978) proposed that there is an obligatory serial interaction between the two acetylatable lipoic acid residues in the normal catalytic mechanism. Ray & Koshland (1961) point out that any group which is modified more rapidly than enzymic activity is lost can be excluded from participation in the mechanism. We have been unable to reconcile our experimental results on modification of the lipoic acid residues with an obligatory series mechanism, even allowing for extensive active-site coupling.

Several other lines of investigation point to the existence of at least two species of lipoic acid residue. Grande *et al.* (1976) and Ambrose-Griffin *et al.* (1978) infer from a study of the e.s.r. spectra of pyruvate dehydrogenase complex in which the lipoyl groups have been spin-labelled that the swinging arms can inhabit two environments. In one, the lipoic acid residues are highly mobile (correlation time approx. 0.3 ns), whereas in the other a correlation time greater than 50 ns indicates a large degree of immobilization (Ambrose-Griffin *et al.*, 1978). Other experiments (Frey *et al.*, 1978) have led to the surprising conclusion that only half the lipoic acid residues in the E2 core are coupled with the E3 (lipoamide dehydrogenase) component of the complex, despite the fact that all the lipoic acid residues are reductively acetylated by means of the E1 component and can transfer their acetyl groups to CoASH. However, it should be noted that there is a substantial discrepancy between the results obtained by Frey *et al.* (1978) and Collins & Reed (1977) for an important experiment on which this conclusion is based, namely the extent of acetylation of lipoic acid residues in the complex brought about by acetyl-SCoA in the presence of NADH.

Another puzzling feature of the complex is the apparent distance apart of the catalytic sites of the component enzymes. Fluorescence energy-transfer experiments suggest that the catalytic sites of the E1 and E3 components are separated by approx. 4.5 nm (Moe *et al.*, 1974) and that the CoASH-binding site on the E2 component and FAD in the active site of the E3 component are not less than 5 nm apart (Shepherd *et al.*, 1976). Both these distances are greater than 2.8 nm, twice the length of a single lipoyl-lysine swinging arm. A series interaction between at least two lipoic acid residues to connect up the active sites is therefore attractive. However, we cannot reconcile the experiments described in the present paper with an obligatory acyl transfer between the reductively acetylated lipoic acid residues. It is conceivable that other functional groups on the enzyme, perhaps a third lipoic acid residue (Hale & Perham, 1979*a*), could act to pass reducing equivalents to the active site of the E3 component, thereby spanning the physical gap suggested by the fluorescence energy-transfer measurements. In other experiments (Hale & Perham, 1979*b*,*c*) it has been shown that the reductively acetylated lipoic acid residues are readily excised from the complex by limited proteolysis, suggesting that they are attached to exposed loops of polypeptide chain. If these loops were very flexible, they

too could add to the span of the swinging arms (Hale & Perham, 1979*c*).

If only one of the two reductively acetylated lipoic acid residues per E2 chain is essential in the overall complex reaction, the conclusion that our experiments above appear to favour, it is likely that the second reductively acetylated lipoic acid residue is a component of the active-site-coupling mechanism. As we have described it (Bates *et al.*, 1977; Danson *et al.*, 1978*a*), this mechanism could be called into play at low substrate concentrations or for an enzyme complex that is only partly assembled. It would not necessarily be operating in the normal enzymic assay.

We are grateful to the Science Research Council for research grants (to R. N. P. and M. J. D.) and the award of an Advanced Fellowship (to M. J. D.). We thank Dr. K. Sargeant and Mr. A. R. Whitaker of the Microbiological Research Establishment, Porton Down, Wilts., U.K., for growing the bacteria.

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