

Spin-Label Study of the Mobility of Enzyme-Bound Lipic Acid in the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*

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(Received 2 December 1975)

The lipic acid residues covalently bound to the transacetylase component of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* were selectively modified by reaction with 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl. The electron-spin-resonance spectrum of the spin-labelled enzyme indicates that the bound nitroxide groups have high mobilities relative to the protein molecule. This physicochemical evidence is consistent with the view that the dithiolane ring of a lipoyl residue is capable of rapid migration between the active sites of the component enzymes in the catalytic mechanism.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* is a highly organized multimeric structure that contains three different types of polypeptide chain in multiple copies (Perham, 1975). These chains correspond to the three component enzymic activities of the complex, namely a pyruvate decarboxylase (E1), a lipoyl transacetylase (E2) and lipoamide dehydrogenase (E3). The commonly accepted mechanism proposes that enzyme E1 brings about decarboxylation of the substrate, pyruvate, which leads in turn to reductive acetylation of a lipic acid residue covalently bound to each chain of enzyme E2. The acetyl group is then transferred to CoA from its thio ester linkage with the lipic acid, thereby generating the product, acetyl-CoA, and the resulting dihydrolipoamide is oxidized back to the disulphide form by lipoamide dehydrogenase (E3) at the expense of NAD⁺ [for reviews see Reed & Oliver (1968) and Reed (1974)].

The lipic acid is bound to enzyme E2 by means of an amide bond involving its carboxyl group and the ϵ -amino group of a lysine residue in a unique amino acid sequence in the polypeptide chain (Daigo & Reed, 1961). The dithiolane ring of the lipic acid residue is therefore at the end of a covalent 'arm' that can reach about 1.5 nm from the polypeptide backbone of enzyme E2 and it has long been thought (Green & Oda, 1961; Koike *et al.*, 1963) that this 'swinging arm' can explain how the oxidized lipoyl groups could be reductively acylated at the active sites of the E1 components and the dihydrolipoamide groups subsequently be reoxidized at the active sites of the E3 components.

Such a mechanism implies considerable freedom to move on the part of the lipoyl residue. It has been shown (Brown & Perham, 1976) that in the presence of pyruvate and absence of CoA, maleimides with even quite bulky *N*-substituents can inhibit the complex by specific reaction with the free thiol group of the S⁶-acetyldihydrolipoamide gener-

ated during the part-functioning of the complex. This provides a convenient method for the selective introduction of reporter groups on to the lipic acid. We show here that 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl, a maleimide carrying a nitroxide spin label, is an effective inhibitor under these conditions and, from the e.s.r. (electron-spin-resonance) spectrum of the spin-labelled enzyme, demonstrate that the lipoyl residue has a freedom of movement in keeping with the generally accepted reaction mechanism of the complex.

Materials and Methods

Reagents and enzymes

4-Maleimido-2,2,6,6-tetramethylpiperidino-oxyl was from Synvar Corp., Palo Alto, CA, U.S.A. Acetyl phosphonate (dilithium salt) was kindly given by Dr. H. B. F. Dixon. All other reagents, including *N*-ethyl[2,3-¹⁴C]maleimide, were from the suppliers listed by Brown & Perham (1976).

Pyruvate dehydrogenase multienzyme complex, purified from a mutant of *E. coli* K12 constitutive for pyruvate dehydrogenase (see Brown & Perham, 1976), was kindly provided by Dr. J. P. Brown. The pyruvate dehydrogenase and lipoamide dehydrogenase activities were assayed as described by Brown & Perham (1976).

Reaction of pyruvate dehydrogenase with spin-labelled maleimide

A typical treatment, based on the work of Brown & Perham (1976), was as follows. Pyruvate dehydrogenase complex (2.5 mg/ml) was incubated at 0°C in 50 mM-potassium phosphate buffer, pH 7.0, containing MgCl₂ (5 mM), thiamin pyrophosphate (0.5 mM), sodium pyruvate (1 mM) and 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl (0.3 mM). All buffers were de-gassed before use. After 30-40 min

incubation, inactivation of the pyruvate dehydrogenase activity was effectively complete and the treated complex was dialysed overnight at 2°C against the appropriate buffer. The solution was then concentrated by ultrafiltration to a final protein concentration of approx. 20mg/ml.

E.s.r. spectra

E.s.r. spectra were obtained with a Varian E3 spectrometer, kindly made available by Dr. J. C. Metcalfe (Department of Biochemistry, University of Cambridge). Samples were injected into fine capillary tubes and maintained at 20°C in a steady stream of N₂.

Other techniques

All other techniques have been described in detail by Brown & Perham (1976).

Results and Discussion

In the presence of pyruvate and the absence of CoA, *N*-ethylmaleimide inhibits the pyruvate dehydrogenase complex by selective reaction with the transacetylase component (E2) and there is good evidence that the reaction is in fact with the lipoic acid residues covalently bound to that component (Brown & Perham, 1976). 4-Maleimido-2,2,6,6-tetramethylpiperidino-oxyl was found to be an effective replacement for *N*-ethylmaleimide in this reaction. The kinetics of the inhibition of the pyruvate dehydrogenase activity were simple pseudo-first-order and the lipoamide dehydrogenase activity was left unaffected. In view of this similarity to the reaction of the multienzyme complex with *N*-ethylmaleimide, no more elaborate study of the kinetics was undertaken at this stage.

The spin-labelled maleimide was not available in radiolabelled form. However, we showed that the reactions of *N*-ethylmaleimide and 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl with the pyruvate dehydrogenase complex were mutually exclusive by the following experiment. The treatment of the complex with pyruvate and spin-labelled maleimide was set up as usual and samples were removed at various time-intervals. To each sample was then added *N*-ethyl[2,3-¹⁴C]maleimide (7.1mCi/mmol) to a final concentration of 1mM. With these relative concentrations of spin-labelled maleimide and *N*-ethylmaleimide, reaction with the latter dominates. After incubation for 20min at 0°C, 2-mercaptoethanol (final concentration 0.1M) was added to react with any free maleimides and the protein was analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Scintillation counting of the radioactivity of the stained protein bands showed that all the radioactivity was associated with the transacetylase component (Brown & Perham, 1976) and, further,

that the extent of reaction with ¹⁴C-labelled maleimide was inversely proportional to the percentage loss of enzyme activity at the time of sampling. We conclude therefore that the two maleimides react at the same site on the transacetylase component.

An e.s.r. spectrum of spin-labelled pyruvate dehydrogenase complex is shown in Fig. 1. Superimposed are the spectra of the addition product of cysteine and 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl and of spin-labelled pyruvate dehydrogenase complex that had been digested for 30min at 37°C with trypsin (1%, w/w) and subtilisin (1%, w/w). All samples were in the same buffer, 50mM-potassium phosphate, pH7.0, containing MgCl₂ (5mM) and thiamin pyrophosphate (0.5mM). No difference in the spectrum of the spin-labelled enzyme was detected if a buffer of 20mM-potassium phosphate, pH7.0, containing EDTA (1mg/ml) was substituted.

These e.s.r. spectra indicate that the nitroxide group has a high degree of mobility on the spin-labelled enzyme. Little additional mobility was gained from the proteolytic digestion of the complex. From the widths of the hyperfine lines (Carrington & McLachan, 1967), we calculate the correlation times of the nitroxide group on the cysteine adduct and on the enzyme-bound lipoyl residue as approx. 10⁻¹¹s and 2×10⁻¹⁰s respectively. The rotational correlation time, *t_c*, of the pyruvate dehydrogenase complex was estimated from the Debye expression: $t_c = 4\pi\eta r^3/3kT$ (Carrington & McLachan, 1967). For this purpose, the enzyme complex is assumed to be a sphere of effective radius *r*, undergoing isotropic Brownian rotational diffusion. Taking values of the diameter as 30nm (Reed & Oliver, 1968), 40nm (Dennert & Höglund, 1970) and 43nm (Durchschlag, 1975), we obtained values for *t_c* of 0.35×10⁻⁵, 0.83×10⁻⁵ and 1.05×10⁻⁵s respectively. The nitroxide group attached to the enzyme has a freedom of movement far in excess of this and seems effectively unrestricted in its motion, which may have implications for the number and arrangement of active sites of component enzymes serviced by a given lipoic acid residue.

Preliminary experiments were also carried out to investigate the effect of substrates and cofactors on the e.s.r. spectrum. Spectra were taken of spin-labelled complex in the presence of (i) 1mM-sodium pyruvate, (ii) 1mM-acetyl phosphonate and (iii) 50μM-dihydrolipoamide, all dissolved in 50mM-potassium phosphate, pH7.0, containing MgCl₂ (5mM) and thiamin pyrophosphate (0.5mM). Acetyl phosphonate is a powerful inhibitor of the enzyme, competitive with pyruvate (*K_i* approx. 4×10⁻⁶M) (R. A. Harrison, R. N. Perham & P. M. Slater, unpublished work). These spectra were all identical in shape with that of the spin-labelled enzyme in the absence of substrates or cofactors, indicating

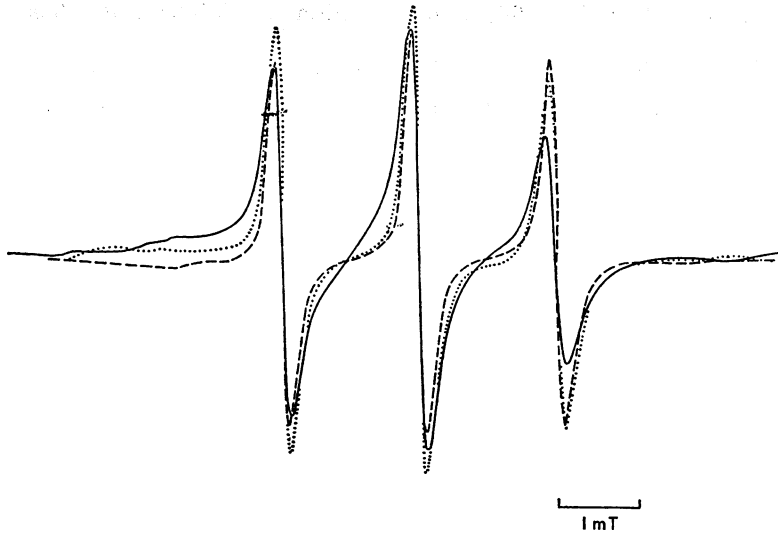


Fig. 1. E.s.r. spectra of pyruvate dehydrogenase multienzyme complex in which the lipoyl residues have been spin-labelled by reaction with 4-maleimido-2,2,6,6-tetramethylpiperidino-oxyl

—, Spin-labelled enzyme; ····, spin-labelled enzyme after digestion with trypsin and subtilisin; ----, adduct of free cysteine with spin-labelled maleimide. For other details, see the text.

that the particular compounds tested cause no decrease in the mobility of the nitroxide group bound to the lipioic acid.

The experiments described in the present paper have demonstrated that the lipoyl residues bound to the transacetylase component of the pyruvate dehydrogenase multienzyme complex of *E. coli* have virtually unrestricted freedom of motion, which provides convincing physicochemical evidence in support of the 'swinging arm' hypothesis of substrate channelling between the subunits. The mobility of the lipoyl residues can, of course, only be examined when they have been chemically modified, but our present results are consistent with the view that thermal energy is sufficient to drive the lipoyl residues on their visits to the active sites of the three component enzymes. However, the geometrical arrangement of the sites is still unknown and it remains to be determined whether these visits are random in the normal course of the catalytic reaction. On the basis of fluorescence-energy-transfer experiments, Moe *et al.* (1974) have raised doubts about the ability of a single lipioic acid residue to span the distance they measured (about 4.5 nm) between the active sites of the E1 and E3 subunits. Further experiments to study the mobility of the spin-labelled lipoyl residues in the presence of various substrates should provide more detailed information about the subunit interactions in the multienzyme complex.

Note Added in Proof (Received 12 March 1976)

Since this paper was submitted for publication, conclusions similar to ours have been reported for the mobility of the lipioic acid in the pyruvate dehydrogenase complex of *Azotobacter vinelandii* (Grande *et al.*, 1975).

We thank Dr. T. R. Hesketh for advice on the use of the e.s.r. spectrometer and Mr. W. G. Griffin, Dr. A. Stone and Dr. R. M. Lynden-Bell for helpful discussion. We also thank the Science Research Council for a Studentship (M. C. A.) and financial support (Grant B/SR/9480).

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