Mechanism of action of the pyruvate dehydrogenase multienzyme complex from *Escherichia coli*
(protein modification/subunit interactions/enzyme mechanisms/flavoprotein)

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ABSTRACT The extent of cooperativity among the polypeptide chain components in the overall reaction catalyzed by the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* has been studied. Selective inactivation of the pyruvate dehydrogenase component with thiamin thiazolone pyrophosphate demonstrates that no cooperativity between this component and the overall catalytic reaction occurs: the amount of overall complex activity is directly proportional to the fraction of active pyruvate dehydrogenase component. The transacetylase component has two lipoic acid residues on each of its polypeptide chains that can be modified by Ni^2+ethylmaleimide in the presence of pyruvate and thiamin pyrophosphate. The kinetics of the loss of overall complex activity due to modification of the lipoic residues on the transacetylase component by maleimide reagents shows that not all lipoic acids are coupled into the overall catalytic reaction and that acyl-group and electron pair transfer involving two or more lipoic acids per catalytic cycle must occur. Finally, full complex activity is found when only half the normal flavin content is present. The results indicate that extensive communication among lipoic acids in acyl-group and electron pair transfer must exist in the normal catalytic mechanism. These results are consistent with the average distances between catalytic sites measured by energy transfer experiments.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* that catalyzes the overall reaction

$\text{pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$ [1]

is composed of three enzymes: pyruvate dehydrogenase (E₁) which decarboxylates pyruvate and uses thiamin pyrophosphate (TPP) as a coenzyme; dihydrolipoyl transacetylase (E₂) which contains lipoic acid and transfers the acyl group to CoA; and dihydrolipoyl dehydrogenase (E₃), a flavoprotein that oxidizes the dihydrolipoates formed. The E₃ forms a structural core of the assembled complex to which E₁ and E₂ bind. The E₃ core probably has octahedral symmetry which is consistent with 24 polypeptide chains of E₃ per molecule (cf. ref. 1). The total number of polypeptide chains in the intact complex is still a matter of debate. Reed et al. (2) have proposed 24:24:12 as the E₁:E₂:E₃ polypeptide chain ratio in the native structure. Bates et al. (3) have concluded that the chain ratio varies between 1:1:1 and 2:1:1 for the native complex. Several laboratories have shown that two lipoic acid residues are present per polypeptide chain of E₃ (4–6) and that these lipoic acid residues can be enzymatically acetylated (6–8).

A model for the mechanism of action of this enzyme has been proposed (1) in which a single lipoic acid residue rotates between the catalytic sites of all three enzymes. Previous work in this laboratory, utilizing fluorescence resonance energy transfer measurements, has shown that the average distances between catalytic sites of the three enzymes and between the lipoic acids and the catalytic sites are too long to be consistent with this mechanism (5, 9–11). Other recent results also have suggested a more complex mechanism (6, 8).

The present studies were undertaken to elucidate the degree of interdependence of the individual components in the overall catalytic reaction in the intact pyruvate dehydrogenase complex. Rather than pursuing these relationships by self-assembly of a dissociated enzyme complex, we chose to modify specifically the individual components without dissociating the native complex into individual subunit structures. Three sets of experiments are described. Selective inactivation of E₁ with thiamin thiazolone pyrophosphate (TPP) indicates that no cooperativity between E₁ and the overall reaction occurs and that each catalytic cycle normally requires an E₁ molecule. By means of chemical modification of the lipoic groups with sulfhydryl reagents, the degree of communication among the lipoic acids on the core transacetylase component was examined. The results indicate that not all lipoic acids are coupled into the overall catalytic mechanism, and acylgroup and electron pair transfer involving two or more lipoic acids on the E₃ core must occur. Finally, we find that the oxidation of dihydrolipoate by flavin can occur with one-half the full complement of flavin adenine dinucleotide, further indicating extensive communication among lipoic acids.

EXPERIMENTAL PROCEDURES

Materials. The pyruvate dehydrogenase multienzyme complex from *E. coli*, strain B (Miles Laboratories) was prepared and purified as described (9). The specific activity of the complex, determined by using the NAD⁺ reduction assay at 30°C, was 30–36 μmol of NADH/min per mg of protein.

The N-ethylmaleimide (MalNEt) was from Aldrich, and the titrated compound (140 Ci/mol) was from New England Nuclear. All other biochemicals were from Sigma. Other chemicals were the best reagent grades available, and deionized distilled water was used for all solutions. TPP was prepared as described by Gutowski and Lienhard (12) and was further purified on an Amberlite CG-50 ion-exchange column in the hydrogen-ion form. The product was eluted with deionized distilled water. This preparation of TPP showed a single UV-absorbing component with an Rₑ of 0.48 on thin-layer chromatography [Eastman cellulose plates; ethanol/n-butanol/0.15 M sodium citrate, pH 4, 10:1:6 (vol/vol)].

Methods. The overall enzyme activity was determined with the NAD⁺ reduction assay (13). The activity of the pyruvate dehydrogenase component was measured with the ferricyanide assay (14), and the lipoamide dehydrogenase activity was de-

Abbreviations: E₁, pyruvate dehydrogenase; E₂, dihydrolipoyl transacetylase; E₃, dihydrolipoyl dehydrogenase; MalNEt, N-ethylmaleimide; TPP, thiamin thiazolone pyrophosphate; TPP, thiamin pyrophosphate.

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The E₁ component of the enzyme complex was specifically inactivated by titration with the active-site-directed inhibitor TTPP. Comparison of the dissociation constants of TTPP and TPP shows that this inhibitor binds at least 20,000 times more tightly than does the coenzyme (12). A specific concentration of TTPP in 0.5 mM MgCl₂, pH 7.0 or 8.0/0.02 M potassium phosphate at 4° was incubated with the enzyme. After 30 and 60 min, aliquots were withdrawn and assayed for overall complex activity and E₁ activity at pH 7.0 and 8.0 (in 0.02 M potassium phosphate). Essentially the same activities were found with both aliquots.

The enzyme complex was pretreated with unlabeled MalNEt for 4 hr at 4° in the absence of substrates and was subsequently labeled with [³H]MalNEt in the presence of TPP and pyruvate to selectively modify the lipoic acids (5). The reaction mixture contained 2.08 mg of enzyme per ml, 0.54 mM TPP, 1.9 mM pyruvate, and 2.3 mM MgCl₂ in 0.02 M potassium phosphate (pH 7.0), and the reaction was initiated by addition of MalNEt to a final concentration of 0.51 mM. At selected time intervals the reaction mixture was quenched with diethiothreitol (100-fold excess over MalNEt) and assayed for overall complex activity. In addition, the activities of E₁ and E₃ were assayed to establish that no inactivation of either component occurred upon incorporation of maleimide. The reaction mixtures were precipitated by injection of cold 10% trichloroacetic acid into the vials. The protein was collected on Whatman glass microfiber filters (GF/C or GF/A) and washed with 25 ml of cold 10% trichloroacetic acid, 10 ml of water, and then 10 ml of petroleum ether. The dry filters were placed in vials of Aquasol and allowed to swell overnight; the radioactivity was determined in a Beckman LS-250 liquid scintillation counter. Analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis indicated that all of the radioactivity was on E₂ (5).

The removal of flavin adenine dinucleotide from the enzyme complex was achieved by treatment of the multi-enzyme complex with 49% saturated (NH₄)₂SO₄ at pH 3.2 for 10 min on ice (16). The precipitated enzyme was resuspended in 0.02 M potassium phosphate (pH 7.0) and dialyzed at 4° to remove the ammonium sulfate. This treatment removed 75–80% of the total flavin adenine dinucleotide content. In an attempt to remove more of the flavin adenine dinucleotide, the modified enzyme complex was sometimes subjected to a second acid and salt treatment. An additional 10% of the flavin adenine dinucleotide was removed by this further treatment. Reincorporation of the flavin adenine dinucleotide into E₃ at 4° was accomplished by either incubating the enzyme with selected amounts of flavin adenine dinucleotide or by adding an excess of flavin adenine dinucleotide to the enzyme and terminating the reincorporation at selected time intervals by passage through a Sephadex G-25 column with 0.02 M potassium phosphate (pH 7.0) as the eluant buffer. The eluant was collected, and the enzyme-containing fractions were identified by monitoring the fluorescence of flavin adenine dinucleotide (360 nm excitation, 520 nm emission). Overall complex activity and E₁ and E₃ activities were then measured. The specific activity of E₃ gives a good measure of the amount of complex destroyed during the procedure. Less than a 5% decrease in E₁ activity was observed. The amount of flavin adenine dinucleotide bound to the reconstituted complex was determined by two methods. One involved measurement of the flavin fluorescence of the reconstituted complex relative to that of the native enzyme. Alternatively, the enzyme was precipitated with 50% trichloroacetic acid and centrifuged at 18,000 × g for 15 min. The precipitate was resuspended in 5% trichloroacetic acid and recentrifuged. The supernatant was collected in the dark, and the flavin adenine dinucleotide in the supernatant was hydrolyzed to flavin mononucleotide either by 1 M HCl at 50° for 1 hr (17) or by enzymatic hydrolysis at pH 7.0 with phosphodiesterase (Naja naja venom; 15 mg/ml in 0.02 M potassium phosphate, pH 7.0). The flavin mononucleotide was measured by comparing its fluorescence with that of standard flavin mononucleotide solutions. Both methods of determining flavin content gave identical results. Flavin fluorescence was measured with a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter with excitation at either 360 nm or 445 nm and the emission scanned from 480 to 580 nm.

RESULTS

Selective Inactivation of E₁. The inhibitor TTPP was found to inactivate the overall complex activity and the activity of E₁ stoichiometrically. The activity of E₃ was unaffected by TTPP. The results obtained at pH 7.0 and 8.0 are summarized in Fig. 1A. Fig. 1B shows the variation of the overall enzyme activity

![Figure 1](https://example.com/image1.png)
Displacement of the T TPP did not occur within the time course (<2 min) of the assay because no evidence of a lag period was observed upon addition of T PP. However, when 4.4 mM T PP was incubated for 10–20 min with the T TPP-inactivated complex, about 10% of both overall and E1 activities were restored, indicating that some T PPP can be displaced by T PP. A sample to which no T PPP was added served as a control, and all activities are expressed relative to the activity of this control.

**T PPP-Pyruvate-Dependent Incorporation of MalNEt**. Inactivation of the complex was achieved by reductive acetylation of the lipoic acid moieties on the transacetylase followed by maleimide incorporation onto the free sulfhydryl group of the lipoic acid. A total of 10 nmol of MalNEt was incorporated per mg of protein, which corresponds to about 46 mol of label per mol of complex if a molecular weight of $4.6 \times 10^6$ is assumed. The kinetics of inactivation are complex: a typical plot of the loss of overall complex activity vs. time is shown in Fig. 2. Under identical conditions, inactivation of the complex was more rapid with aromatic maleimides [pyrene- and N-(4-dimethylamino-3,5-dinitrophenylmaleimide)] than with MalNEt. Both E1 and E2 activities were unaffected by reaction with MalNEt but the overall complex activity was decreased. A plot of the overall activity vs. the fraction of unmodified lipoic acids is presented in Fig. 3. The sigmoidal character of this plot indicates that the loss of overall complex activity was not proportional to the loss of free lipoic acid. Furthermore, only after approximately 10% of the lipoic acids were modified by the maleimides did a loss occur in the overall catalytic activity of the complex. The remainder of the time course of the inactivation required at least two exponential decay terms for a mathematical description of the kinetic curve. The overall activity did not drop to zero even after 4 hr, although it did so either at sufficiently long times or after addition of fresh T PP and pyruvate. A Hill-type plot of the logarithm of the relative

**FIG. 2.** Time course of the inactivation of the pyruvate dehydrogenase complex by MalNEt. The specific activity (in μmol of NADH/min per mg of protein) vs. time is shown for a solution containing 0.31 mM [3H]MalNEt, 2.08 mg of prelabeled enzyme per ml, 1.9 mM pyruvate, 0.54 mM TPP, 2.3 mM MgCl2, and 0.02 M potassium phosphate (pH 7.0) at 4°C.

with the concentration of T PPP at both pH 7.0 and 8.0. On the basis of a molecular weight of $4.6 \times 10^6$ for the enzyme complex, complete loss of overall activity corresponds to 24 mol of T PPP per mol of complex.

**FIG. 3.** Percentage overall complex activity vs. fraction of unmodified lipoic acid. Labeling and stoichiometry measurements were as described in Experimental Procedures, and the reaction conditions were similar to those in Fig. 2.

**FIG. 4.** Percentage of pyruvate dehydrogenase complex activity vs. fraction of flavin adenine dinucleotide reconstituted. The line drawn is a theoretical curve calculated as described in the text.
activity vs. the logarithm of the fraction of unmodified lipoic acids had a maximum slope of 2.2, indicating that ≥2.2 lipoic acids are required for each catalytic cycle.

Dependence of Overall Complex Activity on Flavin Adenine Dinucleotide. When the overall complex activity was measured with varying amounts of flavin adenine dinucleotide present on the enzyme, full activity was attained under conditions such that not all of the flavin adenine dinucleotide of the native structure was restored. The results obtained are shown in Fig. 4 as a plot of the percentage of overall activity vs. the fraction of reconstituted flavin. The turnover rate of the fully reconstituted enzyme was identical, within experimental error, to that of an untreated "native" complex, provided that the native structure was restored. The results obtained are consistent with previous measurements using TTPP (12) and reconstitution experiments (8). The suggestion has been made that a single E1 can service multiple lipoic acids (6, 8). Collins and Reed (6) have found that the complex can be fully acetylated even though E1 was 90% inhibited by TTPP. However, the acetylation was quite slow (reaction time, ~30 min) and might be due to dissociation or migration of TTPP on the complex. Such a slow rate cannot be of significance in the normal activity of the enzyme. Bates et al. (8) have found that a complex of E2 and E3 partially reconstituted with E1 can be fully acetylated, although this might be due to migration of E1 on the complex. The fact that the loss in activity due to dissociation or migration of TTPP on the complex. Such a mechanism is consistent with the average distances between catalytic sites measured by energy transfer experiments.

In summary, we have demonstrated that cooperativity (presumably transacetylation) among a minimum of two lipoic acids is essential for normal catalytic activity. Furthermore, a significant number of lipoic acids is not essential for overall catalytic activity although they can be reductively labeled. Oxidation-reduction among lipoic acids also can occur, providing further evidence for an extensive communication network between lipoic acids on the E3 core. Finally a single E1 normally services a single catalytic cycle. Such a mechanism is consistent with the average distances between catalytic sites measured by energy transfer experiments.

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