in the binding mode in which hydrogen is removed rather than deuterium. For example, [1(*S*)-^{2}H]dopamine will undergo catalysis at the same rate through the 1*R* mode but at a reduced rate through the 1*S* mode (proportional to an isotope effect of 9.6 or 11.6, depending upon the relationship between C-1 and C-2; Summers et al., 1979). As shown below, the relative flux rate of [1(*S*)-^{2}H]dopamine through the 1*R* vs. 1*S* mode is ca. 90:10. A similar calculation for [1(*R*)-^{2}H]-dopamine leads to relative flux rates of ca. 0.25:0.75.

Calculation of Flux Rates of [1(S)-^{2}H]Dopamine.

	syn cleavage	anti cleavage
1*R* mode	$k_1 = k_1$	$k_1 = k_1$
1*S* mode	$k_{2'} = k_2/9.6$	$k_{2'} = k_2/11.6$
$\frac{1R}{1R + 1S}$	$\frac{k_1}{k_1 + k_{2'}} = 0.925$	$\frac{k_1}{k_1 + k_{2'}} = 0.90$
$\frac{1S}{1R + 1S}$	$\frac{k_{2'}}{k_1 + k_{2'}} = 0.075$	$\frac{k_{2'}}{k_1 + k_{2'}} = 0.10$

Calculation of Flux Rates of [1(R)-^{2}H]Dopamine.

	syn cleavage	anti cleavage
1*R* mode	$k_{1'} = k_1/3.6$	$k_{1'} = k_1/3.0$
1*S* mode	$k_2 = k_2$	$k_2 = k_2$
$\frac{1R}{1R + 1S}$	$\frac{k_{1'}}{k_{1'} + k_2} = 0.26$	$\frac{k_{1'}}{k_{1'} + k_2} = 0.21$
$\frac{1S}{1R + 1S}$	$\frac{k_2}{k_{1'} + k_2} = 0.74$	$\frac{k_2}{k_{1'} + k_2} = 0.79$

As indicated in Table VI, tritium release from [1(*S*)-^{2}H,2-(*R*)-^{3}H]- and [1(*R*)-^{2}H,2(*R*)-^{3}H]dopamine is readily predicted from the flux rates given above.

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Pyruvate Dehydrogenase and 3-Fluoropyruvate: Chemical Competence of 2-Acetylthiamin Pyrophosphate as an Acetyl Group Donor to Dihydrolipoamide†

Douglas S. Flournoy and Perry A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53705

Received December 30, 1985; Revised Manuscript Received June 3, 1986

ABSTRACT: The pyruvate dehydrogenase component (E_1) of the pyruvate dehydrogenase complex catalyzes the decomposition of 3-fluoropyruvate to CO_2, fluoride anion, and acetate. Acetylthiamin pyrophosphate (acetyl-TPP) is an intermediate in this reaction. Incubation of the pyruvate dehydrogenase complex with 3-fluoro[1,2-^{14}C]pyruvate, TPP, coenzyme A (CoASH), and either NADH or pyruvate as reducing systems leads to the formation of [^{14}C]acetyl-CoA. In this reaction the acetyl group of acetyl-TPP is partitioned by transfer to both CoASH (87 ± 2%) and water (13 ± 2%). When the E_1 component is incubated with 3-fluoro[1,2-^{14}C]pyruvate, TPP, and dihydrolipoamide, [^{14}C]acetyldihydrolipoamide is produced. The formation of [^{14}C]acetyldihydrolipoamide was examined as a function of dihydrolipoamide concentration (0.25–16 mM). A plot of the extent of acetyl group partitioning to dihydrolipoamide as a function of 1/[dihydrolipoamide] showed 95 ± 2% acetyl group transfer to dihydrolipoamide when dihydrolipoamide concentration was extrapolated to infinity. It is concluded that acetyl-TPP is chemically competent as an intermediate for the pyruvate dehydrogenase complex catalyzed oxidative decarboxylation of pyruvate.

The pyruvate dehydrogenase complex of *Escherichia coli* catalyzes the decarboxylation and dehydrogenation of pyruvate by the following sequence of reactions:[1]

$$H^+ + CH_3COCO_2^- + E_1\cdot TPP \rightarrow E_1\cdot HETPP + CO_2 \quad (1)$$

$$E_1\cdot HETPP + E_2\text{-lipoyl(S)}_2 \rightarrow E_1\cdot TPP + E_2\text{-lipoyl(SH)SCOCH}_3 \quad (2)$$

† This research was supported by Grant AM 28607 from the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases.

0006-2960/86/0425-6036$01.50/0

$$E_2\text{-lipoyl(SH)SCOCH}_3 + \text{CoASH} \rightleftarrows E_2\text{-lipoyl(SH)}_2 + \text{CH}_3\text{COSCoA} \quad (3)$$

$$E_2\text{-lipoyl(SH)}_2 + E_3\cdot\text{FAD} \rightleftarrows E_2\text{-lipoyl(S)}_2 + \text{dihydro-}E_3\cdot\text{FAD} \quad (4)$$

$$\text{dihydro-}E_3\cdot\text{FAD} + \text{NAD}^+ \rightleftarrows E_3\cdot\text{FAD} + \text{NADH} + \text{H}^+ \quad (5)$$

sum:

$$\text{CH}_3\text{COCO}_2^- + \text{CoASH} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{CH}_3\text{COSCoA} + \text{NADH} \quad (6)$$

The complex is composed of three enzymes: pyruvate dehydrogenase (E_1), the thiamin pyrophosphate (TPP) dependent component; dihydrolipoyl transacetylase (E_2), which contains covalently bound lipoic acid; and dihydrolipoyl dehydrogenase (E_3), a flavoprotein with noncovalently bound FAD.

It is generally accepted that the initial steps of catalysis (eq 1) involve the TPP-dependent decarboxylation of pyruvate catalyzed by E_1 to form 2-(1-hydroxyethylidene)-TPP. It is unclear whether this enamine becomes protonated during catalysis, but 2-(1-hydroxyethyl)-TPP has been isolated from incubations containing pyruvate, TPP, and the intact complex (Scriba & Holzer, 1961).

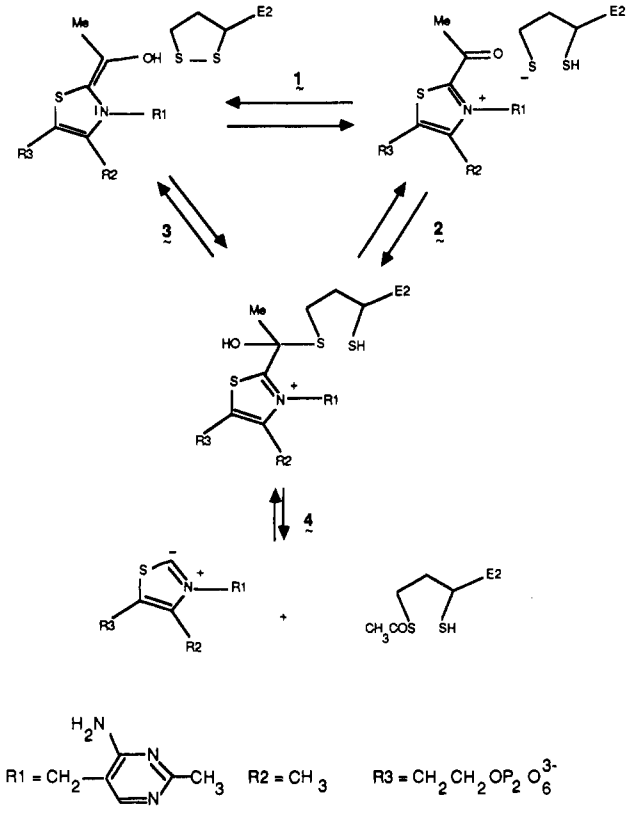
While 2-(1-hydroxyethylidene)-TPP has long been accepted as the immediate decarboxylation intermediate, the mechanistic details of the transfer of the hydroxyethylidene moiety to the covalently bound lipoyl moiety of E_2 are unclear. Two chemically plausible mechanisms for reductive acetylation are under consideration. The "second carbanion" mechanism, originally suggested by White and Ingraham (1962), proposed cleavage of the lipoyl disulfide by the enamine 2-(1-hydroxyethylidene)-TPP, reacting as a carbanion, followed by collapse of the resulting tetrahedral intermediate with the regeneration of E_1·TPP concomitant with reductive acetylation of E_2. A second mechanism, first proposed by Reed et al. (1961), involves the intermediacy of acetylthiamin pyrophosphate (acetyl-TPP). Electron and group transfer are proposed to take place in different steps. Thus acetyl-TPP and dihydrolipoyl-E_2 are generated as discrete intermediates in a redox process, following which transfer of the acetyl group to dihydrolipoyl-E_2 occurs. These two pathways are outlined in Scheme I, the second carbanion mechanism proceeding via steps 3 and 4 and that involving discrete redox and group transfer via steps 1, 2, and 4.

One way to evaluate the chemical competence of acetyl-TPP as an intermediate is to generate this species at the active site of the E_1 component in the presence of dihydrolipoamide. Efficient capture of the acetyl group by dihydrolipoamide would establish its chemical competence, whereas failure to capture would exclude acetyl-TPP from further consideration as an intermediate.

The pyruvate dehydrogenase complex catalyzes the decomposition of 3-fluoropyruvate to CO_2, acetate, and fluoride anion (eq 7). This decomposition has been shown to be TPP-dependent and catalyzed by the pyruvate dehydrogenase component of the complex. The evidence indicates that acetyl-TPP is a reaction intermediate and the precursor of acetate (Leung & Frey, 1978).

$$\text{FCH}_2\text{COCO}_2^- + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{CH}_3\text{CO}_2^- + \text{F}^- + \text{H}^+ \quad (7)$$

Scheme I



We have used 3-fluoropyruvate as a substrate to generate acetyl-TPP at the active site of the pyruvate dehydrogenase component (E_1) of the complex. The efficiency with which *S*-acetyldihydrolipoamide is produced from acetyl-TPP has been determined under various conditions. The results, reported in this paper, establish that acetyl-TPP efficiently reacts with dihydrolipoamide at the active site of pyruvate dehydrogenase to produce *S*-acetyldihydrolipoamide.

Materials and Methods

Chemicals. Sodium metal, sodium 3-fluoropyruvate, ethyl fluoroacetate, diethyl oxalacetate, and triethylamine were all obtained from Aldrich. Oxalic acid was obtained from Mallinkrodt. Benzene and chloroform were obtained from Matheson, Coleman and Bell. Benzene, chloroform, and triethylamine were distilled before use. Oxalic acid was recrystallized from glacial acetic acid. Sodium metal was cleaned according to Fieser and Fieser (1967). [^{14}C]Oxalic acid was purchased from Amersham.

Enzymes. The pyruvate dehydrogenase complex was purified by the method described by Reed and Mukherjee (1969) as modified by Speckhard and Frey (1975). The enzyme was stored at −70 °C after it was frozen in liquid nitrogen at a concentration of 20 mg/mL.

The pyruvate dehydrogenase component (E_1) of the pyruvate dehydrogenase complex was resolved from the E_2·E_3 subcomplex by the method of Reed and Willms (1966), except that the fractions collected from the $CaPO_4$ column were of 5 mL and collected in a tube containing 0.3 mL of 2.0 M KP_i buffer, pH 7.0.

Acetate kinase was purchased from Boehringer Mannheim; lactate dehydrogenase was purchased from Sigma.

Substrates and Cofactors. Coenzyme A, thiamin pyrophosphate, NAD, NADH, sodium pyruvate, and DL-thioctic

[1] Abbreviations: TPP, thiamin pyrophosphate; HETPP, 2-(1-hydroxyethylidene)thiamin pyrophosphate; FAD, flavin adenine dinucleotide; CoASH, coenzyme A; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; acetyl-TPP, 2-acetylthiamin pyrophosphate; dihydrolipoyl-E_2, reduced form of dihydrolipoyl transacetylase; TEAF, triethylammonium formate; bicine, *N*,*N*-bis(hydroxyethyl)glycine; TCA, trichloroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DHLA, dihydrolipoamide.

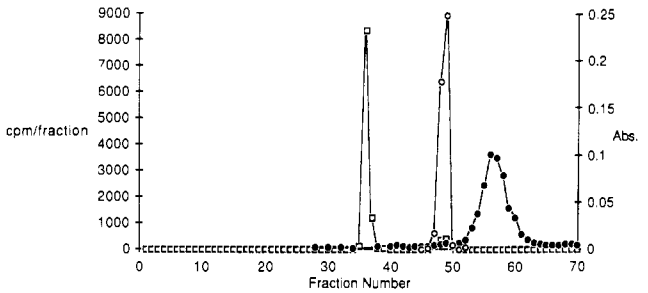


FIGURE 1: Chromatographic separation of [^{14}C]acetate, 3-fluoropyruvate, and acetyl-CoA. One micromole of each component, in a volume of 2 mL, was applied to a DEAE A-25 Sephadex column (0.7 × 17 cm, formate) and developed as described under Materials and Methods except that 0.5-mL fractions were collected. Analysis of the fractions was as follows: 0.001 mL was removed for radiochemical analysis (□), 0.01 mL was removed for analysis of lactate dehydrogenase reducible material at 340 nm (○), or 0.05 mL was removed to determine the absorbance at 260 nm (●).

amide (lipoamide) were all purchased from Sigma. Dihydrolipoamide was synthesized as described by Gunsalus and Razzell (1957) and generously provided by Dr. Claire CaJacob. The concentrations of dihydrolipoamide solutions were determined by reaction with DTNB, and measurement of the absorbance was done at 412 nm, assuming a molar extinction coefficient of 13 600 (Ellman, 1959). The concentration of DL-lipoamide (thioctic amide) was determined from the molar extinction coefficient reported by Reed (1966), for thioctic acid, of 150 at 330 nm.

Assays. Pyruvate dehydrogenase complex was assayed according to Maldonado et al. (1972). The E_1 component was assayed under the conditions described by Maldonado et al. (1972), except that initial rates were used; 1 unit is defined as the amount of enzyme required to oxidize 1 μmol of pyruvate/min to acetate under the stated conditions. Protein concentrations of E_1 were estimated by the method of Lowry as described by Cooper (1977). Protein concentrations of the purified complex were established by using the dry weight absorbance at 280 nm as determined by Speckhard (1974).

Concentrations of pyruvate and 3-fluoropyruvate were established by using lactate dehydrogenase and NADH. Acetate was determined by the method described by Rose et al. (1954). Concentrations of various cofactors were established by using the following molar extinction coefficients: TPP, 7700 at 269 nm (Goedde et al., 1962); CoASH, 16 000 at 260 nm (Decker, 1959); NAD, 18 000 at 260 nm (Siegel et al., 1959); NADH, 6220 at 340 nm (Horecker & Kornberg, 1958).

Chromatography. DEAE A-25 Sephadex was permitted to swell at least 24 h in water, prior to use. It was converted to the formate form by passing a minimum of 20 column volumes of 1 M sodium formate through the packed column and then washing with water.

Chromatographic separations of acetate, 3-fluoropyruvate, and acetyl-CoA were performed by application of the sample to be analyzed, at pH 5, to a column of DEAE A-25 Sephadex (11.0 × 0.7 cm, formate), followed by stepwise elution with distilled water (11 mL), 0.1 M TEAF at pH 3.6 (17 mL), and 0.6 M TEAF at pH 3.6 (20 mL). In all cases base line separations were obtained, acetate emerging first, followed by 3-fluoropyruvate and acetyl-CoA, respectively. A typical elution profile is shown in Figure 1.

Chromatographic separations of *S*-acetyldihydrolipoamide, acetate, and 3-fluoropyruvate were performed by the same procedure, acetyldihydrolipoamide emerging in the first six fractions of the water wash, followed by acetate and 3-fluoropyruvate as shown in Figure 1.

Radiochemical Assays. Samples to be counted were placed in a scintillation vial, and the volume was brought to 1 mL with water. Aquasol, 15 mL, was added and the sample allowed to stand in the dark a minimum of 15 min prior to counting. Samples were counted in a Beckman LS-1000 liquid scintillation spectrometer.

Spectroscopy. All UV–vis spectroscopy was performed on a Cary 118C or a Hitachi 100-80 A spectrophotometer. NMR spectroscopy was performed on a Brucker WH-270 MHz NMR spectrometer, field frequency locked on the deuterium resonance of 99.8 atom % D_2O or Me_2SO-d_6. Chemical shifts were referenced to a 0.1% tetramethylsilane (Me_4Si) external standard.

Anaerobic Conditions. Anaerobic conditions were established by evacuating the reaction vessel with a lab pump and subsequently flushing with argon a minimum of 3 times. Partitioning experiments were carried out in an argon atmosphere; the argon was freed of O_2 by passage through a Deoxo catalytic hydrogen purifier.

Partitioning Experiments. Pyruvate dehydrogenase complex was incubated under the stated conditions and the reaction initiated by the addition of 3-fluoro[1,2-^{14}C]pyruvate. At the indicated times 1 mL of the reaction mixture was removed and delivered into a conically shaped tube containing 75 μL of 1.17 M formic acid, which adjusted the pH to 3.5. The samples were then spun in a tabletop centrifuge to pellet the protein, and the supernatant fluid was removed. After addition of carrier acetate, CO_2 was bubbled through the sample for 1 min. The pH was adjusted to 5 by using a 5% aqueous solution of triethylamine. These samples were then applied to individual DEAE columns, prepared as described above, and eluted as described under Chromatography.

In cases where anaerobic conditions were employed, NADH, pyruvate, and 3-fluoro[1,2-^{14}C]pyruvate were separately deaerated in individual chambers of the reaction vessel, apart from the protein and other reaction components. In time studies using pyruvate as the reducing system, individual reaction vessels were used for each time point and anaerobic conditions broken only at the time of quenching.

Partitioning experiments carried out with the pyruvate dehydrogenase component (E_1) of the complex were initiated by the addition of 3-fluoro[1,2-^{14}C]pyruvate after preincubation with all other components for 5 min at room temperature (ca. 22 °C). After 15 min the reactions were quenched with formic acid. After CO_2 was bubbled through the sample for 1 min, the pH was adjusted to 5 and the sample applied to a DEAE-Sephadex column (formate) and eluted as described above.

Synthesis of 3-Fluoro[3-^{3}H]pyruvate. The sodium salt of 3-fluoro[3-^{3}H]pyruvate was synthesized according to Goldstein et al. (1978).

Synthesis of Diethyl [1,2-^{14}C]Oxalate. Generation of diazoethane was by the following modification of the procedure of Arndt (1943a). Aqueous sodium hydroxide (18 mL, 40% w/v) and ether (45 mL) were placed in a diazoethane-generating apparatus consisting of a round bottom flask with no ground glass joints equipped with an open side arm that had been drawn to a fine tip. The flask was placed in an ice–water bath and allowed to cool to 5–7 °C. *N*-Ethyl-*N*-nitrosourea (10 g) (Arndt, 1943b) was added and the mixture allowed to stir for 15-min. At this time the ethereal layer, containing the diazoethane, was distilled through the side arm, which was connected to a section of Tygon tubing and whose other end passed through a rubber septum into a 50-mL round bottom

flask suspended in an ice–water bath. The collected diazoethane was kept on ice and used within 15 min.

Esterification was carried out by placing [1,2-^{14}C]oxalic acid (2 mmol, 250 μCi) into ether (100 mL) and cooling in an ice bath for 15 min. Diazoethane was added as an ethereal solution slowly over a period of several minutes until a light yellow color remained. This mixture was stirred for 15 min and the ether removed by rotary flash evaporation. The residue was taken up in ether (5 mL) and filtered into a small flask, and the ether was evaporated, leaving a clear colorless liquid, diethyl [1,2-^{14}C]oxalate, in quantitative yield. The nuclear magnetic resonance spectrum (Me_2SO-d_6) of six different nonradioactive samples prepared exactly as described above showed signals only at 1.5 (3 H, t, J = 7 Hz) and 4.4 ppm (2 H, q, J = 7 Hz).

Synthesis of Sodium 3-Fluoro[1,2-^{14}C]pyruvate. This compound was prepared by the following modification of the procedure of Nair and Bush (1958). To sodium ethoxide (3.5 mmol) is added benzene (0.9 mL) followed by diethyl [1,2-^{14}C]oxalate (2 mmol) and ethyl fluoroacetate (2 mmol). The reaction mixture is heated to 35–37 °C with stirring for 42–48 h and the solvent removed by rotary flash evaporation. The precipitated sodium enolate of diethyl 3-fluoro[1,2-^{14}C]oxalacetate is washed with anhydrous ether until the washings are colorless and then suspended in ether (7 mL) and cooled to −20 °C. Hydrochloric acid (0.24 mL, 5 N), cooled to −20 °C, is added to the etheral suspension and the mixture shaken well. Water (10 mL) and ether (10 mL) are added, and the diethyl 3-fluoro[1,2-^{14}C]oxalacetate is extracted into the ethereal layer. The aqueous layer is separated and washed with ether (10 mL). The organic layers are combined, and the ether is removed by rotary flash evaporation. The ester if refluxed with hydrochloric acid (3 mL, 3 N) for 3 h. After removal of H_2O and HCl by rotary flash evaporation, the residue, taken up in ether, is placed in a sublimation apparatus and the ether evaporated by using a stream of nitrogen. 3-Fluoro[1,2-^{14}C]pyruvic acid sublimes at 65–80 °C, 0.25–0.5 torr (lit. 70–80 °C, 0.5–0.7 torr; Nair & Bush, 1958). The acid is washed from the cold finger with water and the resulting solution titrated with 1% sodium hydroxide (2.1 mL) to pH 7.0. Evaporation of the solvent yields sodium 3-fluoro[1,2-^{14}C]pyruvate (0.5 mmol) in an overall yield of 25%. The nuclear magnetic resonance spectrum of nonradioactive samples (six) showed two pairs of doublets at 4.41 and 5.51 ppm (J = 47.8 Hz.), relative intensities 0.90 and 0.10, respectively. The downfield shift is assigned to the keto form and the upfield shift to the hydrate. Our calculation of the equilibrium constant for formation of the hydrate is then 9, in agreement with the value obtained by using authentic samples of 3-fluoropyruvate.

Results

The work of Leung and Frey (1978) provides a means by which the chemical competence of acetyl-TPP as an intermediate may be assessed. Acetyl-TPP can be generated at the active site of E_1 when 3-fluoropyruvate is used as a substrate. When acetyl-TPP is generated in the presence of reduced dihydrolipoyl transacetylase (E_2) and CoASH, the fate of this intermediate can be examined. If the acetyl group is transferred to the lipoyl moieties, it should subsequently be transferred to CoASH and appear as acetyl-CoA, whereas if the acetyl moiety is transferred to water, it will appear as acetate.

In a preliminary experiment the pyruvate dehydrogenase complex was incubated with NADH (0.5 mM), NAD (1.5 mM), TPP (0.1 mM), $MgSO_4$ (1 mM), CoASH (10 mM), and 3-fluoro[3-^{3}H]pyruvate (0.43 mM) in sodium bicinate buffer. After 30 min the reaction was quenched by the addition of formic acid to pH 3.5. After the precipitated protein was removed, the supernatant fluid was analyzed for [^{3}H]-acetate and [^{3}H]acetyl-CoA by chromatography through a column of DEAE-Sephadex, as described under Materials and Methods. Under these conditions it was expected that the lipoyl moieties on the enzyme would be reduced by NADH. A large excess of CoASH was used to protect the small amount of [^{3}H]acetyl-CoA formed from being hydrolyzed to CoASH and [^{3}H]acetate, a reaction the enzyme is known to catalyze (CaJacob et al., 1985). In this experiment, of the counts that were partitioned between acetate and its CoA ester, 83% appeared as [^{3}H]acetyl-CoA and 17% as [^{3}H]acetate. This experiment was performed under aerobic conditions, a state in which a significant portion of the lipoyl moieties may be oxidized. Also, the supernatant was applied to the column at pH 3.5, a condition in which not all the acetate may bind; in subsequent experiments the supernatant was always applied to the column at pH 5. In subsequent control experiments the partitioning of the acetyl moiety was shown to depend on the presence of TPP, CoASH, NADH, and enzyme (see Table I).

Table I: Partitioning of Acetyl-TPP on the Pyruvate Dehydrogenase Complex: Substrate Dependence[a]

control	% cpm recovered as acetate	3-fluoropyruvate	acetyl-CoA
complete	15.0	36.6	44.5
−TPP	0.13	92.8	4.2
−CoASH	6.6	89.8	1.3
−NAD/NADH	13.1	56.0	25.7
−enzyme	0.11	89.1	4.7

[a] Pyruvate dehydrogenase complex (2 mg/mL) was incubated with $MgSO_4$ (1 mM), TPP (0.1 mM), CoASH (5 mM), NADH (0.5 mM), NAD (1.5 mM), and sodium 3-fluoro[1,2-^{14}C]pyruvate (0.5 mM) in 50 mM sodium bicinate buffer (pH 8.0) in the complete reaction mixture under aerobic conditions. In the control experiments various components were omitted. The partitioning was initiated with sodium 3-fluoro[1,2-^{14}C]pyruvate. After 25 min the reactions were quenched with formic acid and subjected to chromatographic analysis for [^{14}C]-acetate, 3-fluoro[1,2-^{14}C]pyruvate, and [^{14}C]acetyl-CoA as described in the text.

For acetyl-TPP to be seriously considered as a possible intermediate in the pyruvate dehydrogenase catalyze reaction, essentially quantitative trapping of the acetyl moiety by reduced dihydrolipoyl transacetylase should be observed. However, the conditions in these trapping experiments differ in a number of ways from the usual enzymatic reaction, including the amount of enzyme used and the incubation time. For example, acetyl-CoA is known to be hydrolyzed to acetate and CoA in the presence of the enzyme, a reducing system (e.g., NADH), Mg^{2+}, and TPP. To determine if this could have affected the result, a time study was performed and the partitioning measured as a function of time of incubation.

When the initial CoASH concentration was reduced to 5 mM and 3-fluoro[1,2-^{14}C]pyruvate was used as the substrate, extrapolation of the partitioning ratio to zero time showed that 78 ± 1% of the counts appeared in acetyl-CoA, under aerobic conditions (data not shown). The data also indicated that very little if any hydrolysis of acetyl-CoA took place over the time course of the experiment.

Trapping of counts as acetyl-CoA is dependent on complete reduction of the lipoyl moieties that service the 24 pyruvate dehydrogenase subunits of the multienzyme complex. Should a significant fraction of the lipoyl moieties be oxidized rather than reduced they would be chemically incompetent to accept

acetyl groups from acetyl-TPP. In such cases the acetyl moiety will be transferred to water and appear as acetate. If lowering the concentration of reductant in the system leads to a higher concentration of oxidized lipoyl groups, this should be reflected in a decrease in the percentage of counts appearing in acetyl-CoA. When the experiment was repeated at a NADH concentration of 0.25 mM, the percentage of counts appearing as acetyl-CoA was unchanged, 78 ± 1% (data not shown). Therefore, either all of the lipoyl moieties on the enzyme must have been reduced and complete trapping is not possible or the fraction of reduced lipoyl moieties is independent of NADH in this concentration range.

For the results of partitioning experiments to have meaning, all the lipoyl moieties on the complex must be in the reduced state prior to initiation of partitioning. The pyruvate dehydrogenase complex is known to possess NADH oxidase activity, which would interfere with reduction of the lipoyl moieties. In an effort to maximize reduction of the transacylase the enzyme was incubated with NADH at various higher concentrations than previously used and under anaerobic conditions. NADH and 3-fluoro[1,2-^{14}C]pyruvate were incubated separately in individual side arms apart from the enzyme and the remaining reaction components until anaerobic conditions were established. NADH was then added to the reaction mixture, followed 5 min later by the addition of 3-fluoro[1,2-^{14}C]pyruvate, and the reaction quenched 5 min later. The reaction mixture consisted of pyruvate dehydrogenase complex (80 units/mL), 0.1 mM TPP, 1 mM $MgSO_4$, 5 mM CoASH, 0.5 mM 3-fluoro[1,2-^{14}C]pyruvate, and 0.5–1.5 mM NADH. In these experiments 85–88% of the ^{14}C was found to be associated with acetyl-CoA.

A partitioning of 88% to acetyl-CoA is substantially higher than that observed above but does not suggest chemical competence for acetyl-TPP as an intermediate in the overall reaction. Although the reaction times were 5 min at each NADH concentration, other data indicate that substantial hydrolysis of acetyl-CoA does not take place under these conditions (data not shown). Therefore, 88% trapping may be the highest that can be achieved by using this reducing system.

To determine if this is the maximal trapping attainable on the complex, an alternative reducing system was sought. The pyruvate dehydrogenase complex has been shown to be reductively acetylated, to the extent of 48 equiv of acetate per equivalent of complex, in the presence of pyruvate, Mg^{2+}, and TPP (Speckhard et al., 1977). The complex can be subsequently deacetylated by the addition of CoASH. In the absence of NAD and under anaerobic conditions the lipoyl moieties will remain in the reduced state. This provides a second means by which to reduce the dihydrolipoyl transacetylase component: generating dihydrolipoyl groups under anaerobic conditions where they should not be subject to oxidation by oxygen. Subsequent addition of 3-fluoro[1,2-^{14}C]pyruvate would generate [1-^{14}C]acetyl-TPP on E_1.

The results of such an experiment are depicted in Figure 2. The percent counts appearing as acetyl-CoA have been plotted as a function of time of incubation with 3-fluoro-[1,2-^{14}C]pyruvate. Extrapolation of the line to zero time gives a result of 87 ± 2% of ^{14}C appearing as acetyl-CoA. Therefore, using the intact complex to examine the partitioning of 2-[1-^{14}C]acetyl-TPP between water and CoASH gives a maximum of 88% of the ^{14}C appearing as acetyl-CoA when the experiment is performed under anaerobic conditions and two different systems are used for reducing the dihydrolipoyl groups on dihydrolipoyl transacetylase.

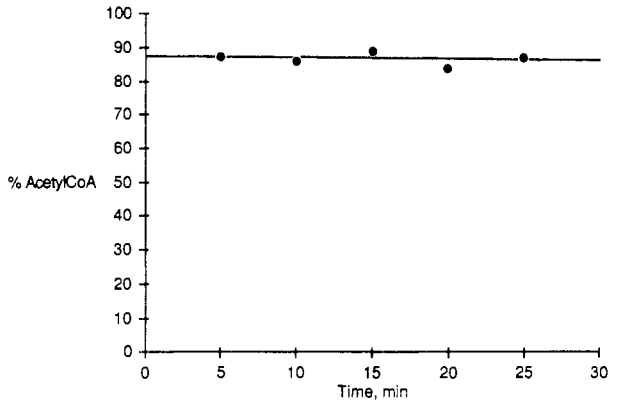
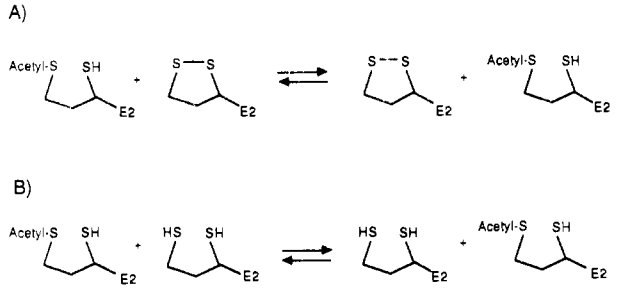


FIGURE 2: The pyruvate dehydrogenase complex (48 units/mg, 80 units/mL) was incubated with the following components in a final volume of 1 mL: TPP (0.1 mM), $MgSO_4$ (1 mM), CoASH (5 mM), sodium pyruvate (0.16 mM), and 3-fluoro[1,2-^{14}C]pyruvate (0.5 mM) in sodium bicinate buffer (50 mM, pH 8). Individual reaction mixtures were prepared for each time point indicated. Anaerobic conditions were established, and the pyruvate was added to the reaction mixture first from one side arm of the cell. After 5 min the 3-fluoro[1,2-^{14}C]pyruvate was added from a second side arm to initiate the partitioning. Following the addition of 3-fluoro[1,2-^{14}C]pyruvate the reactions were quenched at the indicated times and analyzed for [^{14}C]acetate, 3-fluoro[1,2-^{14}C]pyruvate, and [^{14}C]acetyl-CoA as described under Materials and Methods. The line shown is the least-squares line through the points; the intercept and associated standard error are 87 ± 2%.

Scheme II



The trapping experiments described above utilize the intact complex and are complicated by two unknown factors; (i) the site occupancy (distribution) of the reduced lipoyl moieties is unknown, and (ii) the transacetylation mechanism among lipoyl groups will be different in the presence of a reducing system than that involved in the normal catalytic process (see Scheme II and Discussion). These uncertainties can be avoided by employing the pyruvate dehydrogenase component (E_1) of the complex and exogenously added dihydrolipoamide in place of E_2·E_3 and an external reducing system. The use of chemically reduced lipoamide should eliminate uncertainties with respect to the state of oxidation of the lipoyl groups, site occupancy, and the distribution of lipoyl moieties associated with the complex. And, by measuring the formation of *S*-[1-^{14}C]acetyldihydrolipoamide rather than that of [1-^{14}C]-acetyl-CoA, we eliminate uncertainties associated with the transacetylation mechanism which are inherent in studies conducted with the intact complex.

When E_1 is incubated with Mg^{2+}, TPP, dihydrolipoamide, and 3-fluoro[1,2-^{14}C]pyruvate, the products [1-^{14}C]acetate and *S*-[1-^{14}C]acetyldihydrolipoamide can be isolated. The formation of *S*-[1-^{14}C]acetyldihydrolipoamide is strictly dependent on the presence of TPP, dihydrolipoamide, and E_1 (see Table II). In the absence of dihydrolipoamide and the presence of TPP the major product is [1-^{14}C]acetate, as expected from the data of Leung and Frey (1978). The ap-

Table II: Partitioning of Acetyl-TPP on E_1: Substrate Dependence[a]

	% cpm recovered as		
control	acetyldihydrolipoamide	acetate	3-fluoropyruvate
complete	12.6	3.06	78.0
-DHLA	0.57	3.1	94.2
-TPP	0.68	0.47	96.4
-enzyme	0.49	0.52	95.5

[a] The pyruvate dehydrogenase component (0.1 unit/mL) of the pyruvate dehydrogenase complex was incubated with $MgCl_2$ (1.2 mM), TPP (0.1 mM), dihydrolipoamide (6.2 mM), lipoamide (oxidized form, 0.08 mM), and sodium 3-fluoro[1,2-^{14}C]pyruvate (0.13 mM) in 30 mM sodium bicinate buffer (pH 8.0) in the complete reaction mixture. The reaction mixtures contained 10% ethanol (v/v). In control experiments various reaction components were omitted. Enzyme was preincubated with all components except 3-fluoro[1,2-^{14}C]pyruvate for 5 min, and partitioning was initiated by the addition of 3-fluoro[1,2-^{14}C]pyruvate. After 15 min the reactions were quenched with formic acid and submitted to analysis for [^{14}C]acetyldihydrolipoamide, [^{14}C]-acetate, and 3-fluoro[1,2-^{14}C]pyruvate as described in the text.

pearance of both [1-^{14}C]acetate and *S*-[1-^{14}C]acetyldihydrolipoamide is dependent on the presence of TPP and E_1, consistent with an E_1-directed transacetylation from acetyl-TPP to dihydrolipoamide.

The appearance of *S*-[1-^{14}C]acetyldihydrolipoamide was examined as a function of the dihydrolipoamide concentration. The appearance of *S*-[1-^{14}C]acetyldihydrolipoamide exhibits saturation behavior, as expected of an enzymatic reaction. This is shown in Figure 3. When the extent of trapping is plotted as a function of 1/[dihydrolipoamide], the plot is biphasic. Extrapolation of the linear portion gives a value of 95 ± 2% trapping of the [1-^{14}C]acetyl moiety at infinite dihydrolipoamide concentration (see inset, Figure 3).

That the enzyme shows selectivity towards DL-dihydrolipoamide was demonstrated by several experiments in which dithioerythritol was the accepting species. Dithioerythritol at 5 mM trapped 48% of the [1-^{14}C]acetyl groups, and it trapped 20% at 0.5 mM, compared with 78% at 4.86 mM and 46% at 0.25 mM trapped by DL-dihydrolipoamide.

The result of the experiment with E_1 and dihydrolipoamide indicate that transfer of the acetyl group from acetyl-TPP to dihydrolipoamide at the active site of E_1 is highly favored relative to hydrolysis. This is also true of the intact complex. The maximal trapping observed with the intact complex and NADH or pyruvate to reduce the transacylase is 88%, whereas with the simplified system involving the E_1 component and reduced lipoamide trapping is increased to 95 ± 2%. We attribute this difference to the uncertainties associated with the use of the complex, i.e., the extent to which the lipoyl groups on the E_2 component are reduced, their distribution among the active sites of the complex, and the difference in the chemistry we expect the transacetylase to conduct in the presence of a reducing system compared with that expected in normal catalytic turnover.

Discussion

The mechanism by which the lipoyl moieties of the pyruvate dehydrogenase complex are reductively acetylated is not well understood. Little experimental evidence in support of any of the steps in Scheme I can be found in the literature.

In chemical model studies, acetylthiazolium salts have been found to undergo reversible addition of nucleophiles such as water, phosphate dianion, and thiols to the carbonyl group in aqueous solutions; however, very little transfer to acceptors other than water occurs (Lienhard, 1966). In earlier work Daigo and Reed (1962) observed efficient transfer of the acetyl group of an acetylthiazolium salt to hydroxylamine and dihydrolipoamide in dimethoxyethane–water mixtures. These

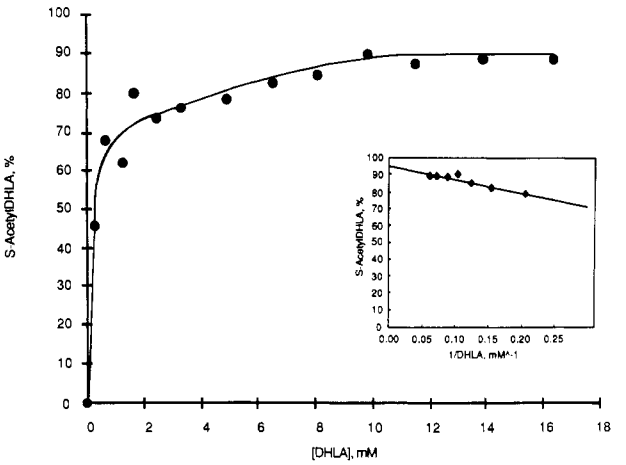


FIGURE 3: The pyruvate dehydrogenase component (0.1 unit/mL) of the pyruvate dehydrogenase complex was incubated with $MgCl_2$ (1.2 mM), TPP (0.1 mM), dihydrolipoamide, and sodium 3-fluoro-[1,2-^{14}C]pyruvate (0.13 mM) in 30 mM sodium bicinate buffer (pH 8.0). The concentration of lipoamide (oxidized form) varied between 1% and 5% of the concentration of the dihydrolipoamide, depending on the preparation. The reaction mixtures contained 10% ethanol (v/v). Enzyme was preincubated with all components sans fluoropyruvate for 5 min. The partitioning was initiated by the addition of sodium 3-fluoro[1,2-^{14}C]pyruvate. After 15 min the reactions were quenched with formic acid and submitted to analysis for [^{14}C]-acetyldihydrolipoamide, [^{14}C]acetate, and 3-fluoro[1,2-^{14}C]pyruvate as described in the text. The inset is a replot of the data at high [dihydrolipoamide], showing the maximum trapping of 95 ± 2%. The 2% uncertainty is the standard error in the intercept of the least-squares line shown in the inset.

findings established the chemical plausibility of acetyl-TPP serving as an acetyl group transfer intermediate in enzymatic reactions. In other chemical studies Rastetter et al. (1979) generated a enamine analogous in structure to 2-(1-hydroxyethlidene)-TPP in aqueous solution and studied its reactivity toward a variety of disulfides. They found that the enamine reacted as a carbanion in cleaving a variety of linear disulfides; however, they observed no cleavage of methyllipoate. Despite the failure of methyllipoate to react in the above experiments, the cleavage of linear disulfides by the enamine provided chemical precedent for the second carbanion mechanism (step 3, Scheme I). Taken together, the chemical models support the plausibility of many of the steps in Scheme I, with the exception of step 1, the oxidation of 2-(1-hydroxyethylidene)-TPP by lipoamide to acetyl-TPP and dihydrolipoamide.

In enzyme studies on the pyruvate and 2-ketoglutarate dehydrogenase complexes, acetyl- and succinyl-TPP, respectively, appear to be produced under conditions of reversal of the reactions (CaJacob et al., 1985; Steginsky & Frey, 1984). Under these conditions, the presence of acetyl- or succinyl-CoA and NADH, the acyl-CoA undergoes TPP-dependent hydrolysis. The process requires the catalytic actions of all three components of the complexes as well as TPP, which evidently is converted to the relevant acyl-TPP as a consequence of reaction with acyldihydrolipoyl groups. Abortive hydrolysis of the acyl-TPP's accounts for the TPP-dependent hydrolysis.

In the present work we have shown that acetyl-TPP is chemically competent as an acetyl group donor to dihydrolipoamide in the active site of the pyruvate dehydrogenase component (E_1). Therefore, steps 2 and 4 of Scheme I are verified as competent processes at the active site.

The difference between the efficiencies with which dihydrolipoyl groups on E_2 and added dihydrolipoamide capture acetyl groups from acetyl-TPP can be rationalized as follows: In the pyruvate dehydrogenase complex the matter of site

occupancy by dihydrolipoyl groups must be considered. There are at least three classes of lipoyl binding sites within the pyruvate dehydrogenase complex, one on each component. The dihydrolipoyl moieties will be partitioned among these sites in unknown ratios. Should acetyl-TPP form at a pyruvate dehydrogenase subunit-active site that is not occupied by or available to a dihydrolipoyl group, the acetyl group will be transferred to water. Another problem that arises is the chemical difference that exists between the lipoyl moieties undergoing normal turnover and those that are present in the partitioning experiments. Acetyl group transfer to coenzyme A during normal catalytic turnover proceeds with acetyl relays among lipoyl moieties according to reaction A in Scheme II, wherein a reduced acetylated lipoyl moiety reacts with a second oxidized lipoyl moiety in a reductive transacetylation (Collins & Reed, 1977; Danson et al., 1978a,b). At least two lipoyl moieties are required for each turnover when measured by the formation of NADH (Angelides & Hammes, 1978). Under the conditions of the above described partitioning experiments, a translipoyl relay of acetyl groups to coenzyme A would proceed according to reaction B in Scheme II, a nonreductive acetyl group transfer between dihydrolipoyl moieties. It is not known whether this process would be as efficient as the normal reaction. Reduced efficiency in this reaction would increase the fraction of E_1 sites occupied by acetyl-TPP and serviced only by *S*-acetyllipoyl moieties on E_2. Acetyl-TPP in such sites would react with water to form acetate. To circumvent these problems, we used the pyruvate dehydrogenase component and dihydrolipoamide as acetyl acceptor and observed much higher trapping efficiencies.

The maximum trapping efficiency in our experiments (95 ± 2%) is not within experimental uncertainty of 100%. Moreover, in control experiments we have not observed the production of [^{14}C]acetate when [^{14}C]pyruvate was the substrate, whereas we always observe a small amount of [^{14}C]-acetate in experiments with 3-fluoro[1,2-^{14}C]pyruvate. Slightly less than 100% capture can be attibuted to several factors that can be expected to reduce efficiencies: (a) We have supplied dihydrolipoamide as the racemic mixture rather than as the pure enantiomer. (b) Dihydrolipoamide always contains a small percentage of oxidized lipoamide. (c) And reductive acetylation via steps 1, 2, and 4 in Scheme I involves the monothiolate form of dihydrolipoamide, whereas we have supplied the neutral form. While the enzyme may or may not preferentially bind the monthiolate, production and reaction of this species under steady-state conditions in the normal reaction is expected to be under kinetic control, whereas in our experiments it would have been under thermodynamic control. The monthiolate would certainly capture acetyl groups with 100% efficiency. Acetyl-TPP has been implicated as an intermediate in other reactions catalyzed by the pyruvate dehydrogenase complex. The oxidation of hydroxyethylidene-TPP by ferricyanide (Das et al., 1961; Holzer & Giedde, 1957), 2,6-dichloroindophenol (Holzer & Goedde, 1957; Alkonyi et al., 1976), methylene blue, and oxygen (Jagannathan & Schweet, 1952) has been reported. Each reaction yields acetate as one of the products and is thought to be derived from acetyl-TPP. As discussed above, acetyl-TPP has also been implicated in the TPP- and NADH-dependent hydrolysis of acetyl-CoA (CaJacob et al., 1985) and in the decomposition of fluoropyruvate (Leung & Frey, 1978).

Direct evidence of the formation of acetyl-TPP from pyruvate has recently been obtained in this laboratory. The pyruvate dehydrogenase complex was incubated with CoASH, NAD, $MgSO_4$, TPP, and 2-[^{14}C]pyruvate in sodium bicinate buffer (50 mM, pH 8.0) at 0 °C for 2 s and quenched with TCA. A [^{14}C]thiamin pyrophosphate derivative possessing the hydrolytic properties expected of [1-^{14}C]acetyl-TPP (production of [^{14}C]acetate at pH 7) was isolated in yields corresponding to about 1% of the E_1 sites.[2] In a hybrid complex constituted from E_1 of the pyruvate dehydrogenase complex and the E_2·E_3 subcomplex from the 2-ketoglutarate dehydrogenase complex, [1-^{14}C]acetyl-TPP accumulated to 2–3% of the E_1 active sites in similar experiments.[3]

3-Fluoropyruvate, in addition to being an alternative substrate for the E_1 component of the pyruvate dehydrogenase complex (Leung & Frey, 1978; this work) is also a suicide substrate. The suicide inactivation mechanism is the subject of a separate study to be reported in the near future. Suicide inactivation by 3-fluoropyruvate in no way affects the findings or interpretations in the present work.

Acknowledgments

We thank Dr. Claire CaJacob for providing the dihydrolipoamide used in this work.

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Crystalline Quinoprotein Glucose Dehydrogenase from *Acinetobacter calcoaceticus*†

Otto Geiger and Helmut Görisch*
Institut für Mikrobiologie der Universität Hohenheim, 7000 Stuttgart 70, West Germany
Received March 4, 1986; Revised Manuscript Received May 19, 1986

ABSTRACT: The soluble form of quinoprotein glucose dehydrogenase (EC 1.1.99.17) from *Acinetobacter calcoaceticus* has been purified 2430-fold to electrophoretic homogeneity. The purified enzyme shows a specific activity of 2600 units/mg of protein, and 45% of the starting activity is recovered. In the presence of polyethylene glycol 6000 the purified glucose dehydrogenase crystallizes readily. Glucose dehydrogenase possesses a molecular weight of 110 000 as determined by sedimentation–equilibrium centrifugation. The enzyme is a dimer of identical subunits. The subunit molecular weight was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis to be 54 000. Each subunit contains one molecule of pyrroloquinoline quinone. Steady-state kinetic measurements with glucose and the one-electron acceptor Wurster's blue indicate that glucose dehydrogenase operates according to a hexa uni ping-pong mechanism. The K_m values were found to be 3.3 mM for glucose and 0.12 mM for Wurster's blue. At higher concentrations both substrates, glucose and Wurster's blue, cause substrate inhibition.

Glucose dehydrogenase from *Acinetobacter calcoaceticus* is an NAD(P)-independent enzyme belonging to the quinoproteins, a novel class of dehydrogenases that possesses a pyrroloquinoline quinone (methoxatin) as the prosthetic group. The enzyme oxidizes glucose and several other aldoses to their corresponding lactones (Hauge, 1960a,b), is associated with the cytoplasma membrane, and transfers the electrons to a cytochrome *b* (Hauge, 1966b). Glucose dehydrogenase from *A. calcoaceticus* can be isolated in a particulate (Hauge, 1966a) and in a soluble form (Hauge, 1966b). The soluble enzyme accepts artificial electron acceptors like 2,6-dichlorophenolindophenol (Hauge, 1960a) or Wurster's blue (Duine et al., 1979) to reoxidize the reduced pyrroloquinoline quinone. Procedures for the purification of quinoprotein glucose dehydrogenase are reported for *Gluconobacter suboxidans* (Ameyama et al., 1981), *Pseudomonas fluorescens* (Matsushita et al., 1980), and *A. calcoaceticus* (Hauge, 1964; Hauge 1966b; Duine et al., 1979).

We present an improved purification procedure for the soluble glucose dehydrogenase from *A. calcoaceticus* resulting for the first time in an homogeneous, crystalline enzyme with an overall yield of 45%.

Materials and Methods

Chemicals. The inorganic chemicals used were of analytical reagent grade. Sodium succinate, glycine, Triton X-100, and DCIP[1] were obtained from Merck (Darmstadt, Germany), and DEAE-Sephacel, CM-Sepharose CL-6B, phenyl-Sepharose CL-4B, and the LMW calibration kit were from Pharmacia (Uppsala, Sweden). Tris and tricine were purchased from

† This work was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. Dedicated to Prof. Dr. G. Pfleiderer on the occasion of his 65th birthday.

[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; LMW, low molecular weight; Tris, tris(hydroxymethyl)aminomethane; tricine, *N*-[tris(hydroxymethyl)methyl]glycine; PEG, polyethylene glycol; DMSI, dimethyl suberimidate; DNase, deoxyribonuclease; WB, Wurster's blue; PQQ, pyrroloquinoline quinone; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-6043$01.50/0