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MICROBIAL LIPOAMIDE DEHYDROGENASE

PURIFICATION AND SOME CHARACTERISTICS OF THE ENZYME DERIVED FROM SELECTED MICROORGANISMS

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SUMMARY

1. Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) was partially purified from selected prokaryotic and eukaryotic organisms. The enzymes from these sources were found to differ in their sensitivity to inhibition by NADH. Lipoamide dehydrogenase from *Serratia marcescens*, like the enzyme from *Escherichia coli*, failed to catalyze a significant initial reduction of lipoamide by NADH in the absence of added NAD^+. This inhibition may be overcome by the prior addition of NAD^+ to the reaction medium. In contrast, the enzymes isolated from *Pseudomonas fluorescens*, *Bacillus subtilis* and *Azotobacter agilis* were unaffected by substrate levels of NADH and the enzymatic activity was unchanged by the addition of NAD^+ to the reaction medium. Eukaryotic lipoamide dehydrogenase was inhibited by NADH, but to a lesser extent than the enzyme from *E. coli* or *S. marcescens*.

2. The enzymes isolated from *Saccharomyces cerevisiae* and *Neurospora crassa* displayed electrophoretic heterogeneity, having at least three diaphorase-reactive bands. In contrast, the enzymes isolated from prokaryotes formed either a single band or a predominant single band upon electrophoresis. Molecular weights of the enzyme isolated from these organisms were estimated by gel filtration and polyacrylamide gel electrophoresis. The molecular weights of the enzyme isolated from both prokaryotic and eukaryotic organisms were found to be 110 000 ± 4400.

INTRODUCTION

Lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) has been isolated from a variety of sources including pig heart[1,2] beef liver[3], human liver[4], pig brain[5], *Escherichia coli*[6,7], *Proteus vulgaris*[8] *Mycobacterium tuberculosis*[9], *Saccharomyces cerevisiae*[10], *Saccharomyces oviformis*[11], and *Candida krusei*[12]. The enzyme from each of these sources is a flavoprotein of approx. 100 000 molecular weight and its

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cellular role involves the catalysis of the oxidation of protein-bound dihydrolipoic acid by NAD^+ in the sequence of reactions concerned with the oxidative decarboxylation of pyruvate and α-ketoglutarate[13,14]. The purified enzyme also catalyzes diaphorase[2,15], transhydrogenase[16], and quinone reductase activities[17]. The enzyme isolated from pig heart and beef liver has been shown to be composed of at least six isoenzymes[18–21]. Recently, evidence has been obtained that three of the six forms of the pig heart isoenzymes originate from the pyruvate dehydrogenation complex while the remainder are derived from the lipoamide dehydrogenase associated with the α-ketoglutarate dehydrogenation complex[20].

Lipoamide dehydrogenases isolated from pig heart and *E. coli* have long been known to be inhibited by excess NADH[7,22–24]. McGARRY[25] demonstrated lipoamide dehydrogenase activity in extracts of twelve species of bacteria and presented evidence that the inhibition of the enzyme by NADH could be related to the fermentative capacity of the organism. The enzyme was not inhibited by NADH in strictly aerobic microorganisms, whereas the enzyme from facultative anaerobes was inhibited to varying degrees. On the basis of these experiments, he attempted to classify the facultative anaerobes into two classes based upon their relative sensitivity to NADH.

This paper describes some of the properties of lipoamide dehydrogenase purified from a number of prokaryotic and eukaryotic organisms, and the main conclusions of McGARRY[25] have been confirmed using partially purified preparations of lipoamide dehydrogenase. In addition, the electrophoretic and molecular size characteristics of the enzymes have been studied. Although lipoamide dehydrogenase appears to have essentially the same molecular size irrespective of source, the enzyme from eukaryotic organisms appears to exist in at least three electrophoretically separable forms, while those isolated from prokaryotes exist either as a single species or as a single major species.

MATERIALS

DL-Lipoamide, dihydrolipoamide, NAD^+, NADH, and sodium pyruvate were obtained from the Sigma Chemical Co. Acetylpyridine nicotinamide dinucleotide and *p*-nitroblue tetrazolium chloride were purchased from Nutritional Biochemicals Corporation. Acrylamide, recrystallized electrophoresis grade, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine were purchased from Eastman Organic Chemicals. Coomassie Brilliant Blue R250 was obtained from Colab Laboratories, Inc. Sephadex G-100 was purchased from Pharmacia. Enzyme grade ammonium sulfate, enzyme grade sucrose, ovalbumin, twice recrystallized, human γ-globulin, crystalline salt-free sperm whale myoglobin, and crystalline bovine serum albumin were from Mann Research Laboratories. All other chemicals used were of reagent grade quality.

METHODS

Growth of microorganisms

Bacillus subtilis, wild type 6009, *Pseudomonas fluorescens*, grown on high peptone medium, and *Serratia marcescens*, grown on minimal medium with glucose, were obtained from General Biochemicals. All cells were harvested at late log phase and the

cell paste was frozen prior to shipment. They were maintained at −20° until used. *E. coli*, Crookes strain, was grown on the medium of HAGER[6] and the cells were harvested after 24 h, washed twice with 0.01 M phosphate buffer (pH 7.2) and stored at −20°. *S. cerevisiae* cells were obtained either as commercial bakers yeast (Red Star Yeast) or were grown in YPE medium[26] at 30° for 24 h. *Azotobacter agilis* cells were provided by Dr. Albert Chung. They were harvested in the late log phase, washed once with 0.01 M phosphate buffer (pH 7.2) and the cell paste was stored at −20°. *Neurospora crassa*, ATCC 9279, was maintained on malt extract–agar slopes to which was added 0.1% peptone I (Nutritional Biochemicals) and 2% glucose. Conidia were grown in a medium consisting of 20 g malt extract, 1 g peptone I, and 20 g succinate per l and harvested after growth at room temperature in shake culture for 72 h. Generally, 200 g wet weight of packed cells were used for each preparation of lipoamide dehydrogenase.

Enzyme preparations

All buffers used in the preparation and study of lipoamide dehydrogenase were prepared from glass distilled water and contained 3 mM EDTA. Unless otherwise noted, all purification steps were done at 0–4°. Pyruvate and α-ketoglutarate dehydrogenation complexes were isolated from *E. coli* and lipoamide dehydrogenase was resolved and purified from the complexes essentially as described by REED AND WILLMS[27]. Lipoamide dehydrogenase was also purified from *E. coli* by the method of WILLIAMS[24]. A similar isolation procedure was used for preparation of lipoamide dehydrogenase from *A. agilis*. *B. subtilis* and *S. marcescens* lipoamide dehydrogenases were purified by essentially this procedure with the modification that two calcium phosphate gel–cellulose column chromatography steps involving successive washing of a 2.5 cm × 17 cm gel–cellulose column with 0.1 M phosphate (pH 7.2), 0.05 M phosphate buffer (pH 7.2)–1% ammonium sulfate, and 0.05 M phosphate buffer (pH 7.2)–5% ammonium sulfate were substituted for the DEAE-cellulose column step. Lipoamide dehydrogenase from *S. cerevisiae* was isolated by the method of WREN AND MASSEY[10] and a similar procedure was employed for purification of the enzyme from *N. crassa*. Since purification of the enzyme from *Ps. fluorescens* involved a substantially modified procedure, it will be described in more detail.

Ps. fluorescens cell paste (250 g) was suspended in 4 vol. of 0.05 M phosphate buffer (pH 7.5) and sonicated in batches of 350 ml for 5 min each using a Branson W-185 C Sonifier at a setting of 10. The suspension was maintained below 5° during sonication by immersion in an ice–methanol bath. The sonicate was centrifuged at 16 000 × *g* for 30 min to remove unbroken cells and cell debris. Solid ammonium sulfate was added to the supernatant fluid to 0.2 saturation and the resulting precipitate was dissolved in 180 ml 4 M urea–0.05 M phosphate buffer (pH 7.5) and stirred for 1 h. The volume was adjusted to 520 ml with 0.05 M phosphate buffer (pH 7.5) and 0.02 vol. of 2% protamine sulfate adjusted to pH 6.4 was added to the solution. After stirring for 15 min, the resulting precipitate was removed by centrifugation for 15 min at 16 000 × *g*. The supernatant solution was dialyzed for 16 h against 0.05 M phosphate buffer (pH 7.5). A saturated solution of ammonium sulfate, adjusted to pH 8.8, was added to 0.5 saturation. The resulting precipitate was removed by centrifugation and the supernatant solution was made to 0.85 saturation with the alkaline ammonium sulfate saturated solution. This fraction (0.5–0.85 satu-

ration) contained most of the lipoamide dehydrogenase activity and it was dissolved in 200 ml 0.05 M phosphate buffer (pH 7.5) and dialyzed overnight against the same buffer.

The dialyzed enzyme was applied to a 2.5 cm × 17.2 cm calcium phosphate gel–cellulose column[28] which had been equilibrated with 0.05 M phosphate buffer (pH 7.5). The column was washed with 0.1 M phosphate buffer (pH 7.5) and then with 1% ammonium sulfate in the same buffer to remove inert protein. The enzyme was eluted with 5% ammonium sulfate in 0.05 M phosphate buffer (pH 7.5). A broad yellow, highly fluorescent lipoamide dehydrogenase active peak was obtained which was pooled and dialyzed overnight against 0.05 M phosphate (pH 7.5). The dialyzed enzyme was applied to a second calcium phosphate gel–cellulose column, 1 cm × 12 cm. This column was washed with about 200 ml 0.1 M phosphate buffer, and the enzyme was then eluted with a linear gradient composed of 100 ml each of 0.1 M phosphate buffer (pH 7.5)–10% sucrose–1% ammonium sulfate and 0.1 M phosphate buffer (pH 7.5)–6% ammonium sulfate. The eluted yellow, fluorescent enzyme was concentrated by negative dialysis and applied to a Sephadex G-100 column, 2.5 cm × 68 cm, equilibrated with 0.05 M phosphate buffer (pH 7.5). The column was developed with the same buffer and the active enzyme was eluted between 118 and 140 ml. Void volume of the column, as determined by the elution of Blue Dextran 2000, was at 97 ml. The enzyme-containing eluate was concentrated by ultrafiltration[29] using 6 mm diameter Visking cellulose tubing and then stored at −20°.

Enzyme assays

Lipoamide dehydrogenase from *E. coli* and the other microorganisms was assayed by the method of REED AND WILLMS[27]. For rapid assay of the enzyme activity in fractions obtained from column chromatography, this assay was modified as follows. A stock incubation medium of the following composition was prepared: 15 ml 0.3 M phosphate buffer (pH 8.1); 9 ml 3 mM NAD^+ in 0.005 M phosphate buffer (pH 7.5); 3 ml 3 M NADH in 0.05 M phosphate buffer pH 7.5; 0.9 ml DL-lipoamide (22.5 mg/ml) in ethanol; and 1.2 ml glass distilled water. 1 ml of this medium was placed in a 1-ml silica glass cuvette with a 1-cm light path and the reaction was initiated by the addition of 25 μl of the enzyme solution. After rapidly mixing the solution, the initial rate of oxidation of NADH was followed by measuring the initial decrease in absorbance at 340 nm in a Hitachi-Coleman Model 124 dual-beam recording spectrophotometer. Results are expressed in terms of μmoles NADH oxidized per min per fraction. The results of the unmodified lipoamide dehydrogenase assay are expressed in terms of μmoles NADH oxidized per min per mg of protein.

For comparison of the lipoamide dehydrogenase reaction using dihydrolipoamide and NAD^+ as substrates with the lipoamide–NADH system, the assay procedures of MCGARRY[25] were used. The exact conditions used for the assays are given in the legends to the figures.

Protein was assayed by the method of LOWRY *et al.*[30] using bovine serum albumin as the protein standard. Column eluates were monitored for protein by measuring the absorbance at 280 nm and 260 nm (ref. 31).

Physical measurements

Polyacrylamide gel disc electrophoresis was performed routinely by the con-

tinuous method of CLARKE[32] in tubes 0.6 cm by 10 cm using either 4% or 7.5% acrylamide gel with a cross linkage of 1%. The usual polymerization mixture for a 4% gel contained the following components expressed as the final concentrations: acrylamide, 4%; *N,N'*-methylenebisacrylamide, 0.04%; *N,N,N',N'*-tetramethylethylenediamine, 0.035% (v/v); ammonium persulfate, 0.07%; sucrose 10%; glycine, 0.36%; and Tris, 0.075%. Protein (10–50 μg) in volumes between 10 μl and 100 μl of 20% sucrose–0.05 M phosphate (pH 7.5) were layered onto the tops of the gels after transferring them into a Buchler polyanalyst electrophoresis unit containing 0.005 M Tris buffer (pH 8.5) cooled to 3°. Electrophoresis was usually performed at 1.5–2 mA per tube for 1–2 h. After completing the run, diaphorase activity was detected by incubating the gels with 0.3 mg *p*-nitroblue tetrazolium chloride and 0.6 mg NADH per ml 0.1 M phosphate buffer (pH 7.5). The positions of protein bands were visualized by fixing the protein in 12.5% trichloroacetic acid for 16 h, and then staining the gels with 0.2% Coomassie Brilliant Blue R250 in 12.5% trichloracetic acid for about 6 h as described by CHRAMBACH *et al.*[33]. Background stain was removed by washing the gels in 10% trichloroacetic acid which was replaced several times until the background was light and protein bands were sharply defined. The stained gels were photographed using transmitted light with a Polaroid MP3 camera and a red filter.

Molecular sizes of the lipoamide dehydrogenases were estimated using polyacrylamide gel electrophoresis by application of the procedure described by HEDRICK AND SMITH[34]. Suitable aliquots of enzyme were electrophoresed in gels prepared with different percentage concentrations of acrylamide, employing a constant ratio of acrylamide to *N,N'*-methylenebisacrylamide of 30:1. Generally, a range of 3–7% acrylamide concentrations were used. The log of the mobility of the enzyme protein in the various percentage gels divided by the mobility of a marker dye; that is, the relative mobility (R_m), was plotted against the percentage gel concentration. The slope of the resulting curve is a function of the molecular size of the protein, while the intercept at a given percentage gel concentration is a function of the charge on the protein. When the slopes of standard proteins are plotted against molecular weight, this method permits estimation of the molecular weights of impure proteins providing a method is available for specific identification of the protein. In this study, lipoamide dehydrogenases were visualized as diaphorase-reactive protein.

Molecular weights of microbial lipoamide dehydrogenases were also estimated by the method of ANDREW[35] with a 2.5 cm × 68 cm column of Sephadex G-100 in 0.1 M phosphate buffer (pH 7.5). Void volume of the column was estimated using dextran blue, and cytochrome *c*, myoglobin, ovalbumin, bovine serum albumin, and γ-globulin were used as standard proteins. A plot of the log of molecular weight versus V_e/V_o; *i.e.* elution volume divided by void volume, permits estimation of the molecular weight of the protein.

Sedimentation velocity measurements were performed at 2° in a Spinco Model E analytical ultracentrifuge at an operating speed of 59 780 rev./min. Most samples were run in a double sector cell with 0.1 M phosphate buffer (pH 7.5)–3 mM EDTA in one sector.

RESULTS

In order to compare some of the properties of lipoamide dehydrogenase ob-

tained from selected prokaryotic and eukaryotic organisms, the enzyme was purified from *E. coli* and *S. marcescens*, representative of facultative anaerobic prokaryotes; *Ps. fluorescens*, *A. agilis*, and *B. subtilis*, examples of aerobic prokaryotes; and *S. cerevisiae* and *N. crassa*, as representatives of eukaryotes. Table I summarizes the results of purification of the enzyme from *Ps. fluorescens*. A total of 1.26 mg enzymically active protein having a specific activity of 35.7 μmoles NADH oxidized per min

TABLE I

SUMMARY OF THE PURIFICATION OF LIPOAMIDE DEHYDROGENASE FROM *Ps. fluorescens*

Yield from 250 g cells is 1.26 mg. Recovery of enzyme is approx. 2.25%.

Fraction	*Vol. (ml)*	*Protein (mg)*	*Total activity (μmoles NADH oxidized per min)*	*Specific activity (μmoles NADH oxidized per min per mg)*
Crude extract	625	40 000	2000	0.05*
$(NH_4)_2SO_4$ added to 0.2 satn.; 1-h incubation of ppt. in 4 M urea–0.05 M phosphate (pH 7.5)	520	20 800	3100	0.15
Protamine sulfate, followed by $(NH_4)_2SO_4$ fractionation. Collect ppt. between 0.55 and 0.8 satn.	250	900	1000	1.10
Calcium phosphate gel–cellulose eluate from 2.5 cm × 17.2 cm column	27.6	11.7	240	20.5
Sephadex G-100 column, 2.5 cm × 68 cm, developed with 0.05 M phosphate buffer, (pH 7.5). Eluate concentrated	4.5	1.26	45	35.7

* Assay is invalid in crude extract.

per mg protein was obtained from the final step in which the enzyme was eluted from a Sephadex G-100 as described in METHODS. A summary of the results of the preparation of the *N. crassa* enzyme, which generally followed the purification procedure described for the enzyme from *S. cerevisiae* by WREN AND MASSEY[10] is shown in Table II. A yield of 2 mg of enzyme catalyzing the oxidation of 176.8 μmoles NADH per min per mg protein was obtained from 350 g wet weight cells. When assayed using dihydrolipoamide and NAD^+ as substrates at pH 7.6, as described by MCGARRY[25], the enzyme catalyzed the reduction of 54.8 μmoles NAD^+ per min per mg protein.

Table III presents a summary of the specific activities of the most highly purified fractions obtained from all of the microorganisms studied and the sedimentation coefficient, $s_{20,w}$, of the enzymically active material wherever it was practical to perform analytical ultracentrifugation. Amounts of enzyme available ranged from 2.1 to 3.5 mg per ml 0.1 M potassium phosphate buffer (pH 7.5)–3 mM EDTA. The enzymes were centrifuged in a double sector cell in the Spinco Model E analytical ultracentrifuge operated at a rotor speed of 59 780 rev./min at 2°. In no case was sufficient protein available to determine the dependence of the sedimentation coefficient on the protein concentration. However, it has been established that the sedimentation coefficient of the pig heart enzyme is not altered markedly by the protein concentration[36]. With this limitation, the calculated $s_{20,w}$ values range from 5.8 to 7.6. Much

TABLE II

SUMMARY OF THE PURIFICATION OF LIPOAMIDE DEHYDROGENASE FROM *N. crassa*

Yield from 350 g cells is 2 mg. Recovery of enzyme is approx. 23.4%.

Fraction	*Vol. (ml)*	*Protein (mg)*	*Total activity (μmoles NADH oxidized per min)*	*Specific activity (μmoles NADH oxidized per min per mg)*
Crude extract	4000	3000	1506	0.5*
Heated 10 min at 60° in 10% $(NH_4)_2SO_4$	3960	2800	1428	0.55
Protamine sulfate, followed by $(NH_4)_2SO_4$ fractionation. Collect ppt. between 0.55 and 0.8 satn.	200	1120	1350	1.21
Calcium phosphate gel–cellulose eluate from 2.5 cm × 30 cm column	40	56	400	7.14
Concentrate from first column applied to calcium phosphate gel–cellulose column. Eluate collected and concentrated	3.5	2	353.6	176.8

* Assay is unreliable in crude extract.

greater variation is observed in the specific activities of the preparations, ranging from a value of 11 for the *B. subtilis* enzyme to a maximal specific activity of 176.8 μmoles NADH oxidized per min per mg *N. crassa* enzyme. The specific activities of the enzymes isolated from *E. coli* and *S. cerevisiae* are generally comparable to those reported by REED AND WILLMS[27] and WREN AND MASSEY[10], respectively. In the latter case, the enzyme was also assayed using acetylpyridine NAD^+ and dihydrolipoamide as substrates[10]. A specific activity of 232 μmoles acetylpyridine NAD^+ reduced per

TABLE III

SUMMARY OF SPECIFIC ACTIVITY AND SEDIMENTATION COEFFICIENTS OF LIPOAMIDE DEHYDROGENASE FROM MICROBIAL SPECIES

Organism	*Specific activity (μmoles NADH oxidized per min per mg)*	$s_{20,w}$ *(calc.)*
Escherichia coli	102*	—
	104**	6.2
Serratia marcescens	34	—
Pseudomonas fluorescens	35.7	7.6
Azotobacter agilis	40	7.0
Bacillus subtilis	11	5.8
Saccharomyces cerevisiae	56.2	6.6
Neurospora crassa	176.8	6.9

* Purified by the method of WILLIAMS[24].
** Purified by the method of REED AND WILLMS[27].

min per mg *S. cerevisiae* protein was obtained. The relative degree of purification of the other enzymes may be estimated in part from the enzyme specific activities and in part from the sedimentation velocity ultracentrifugal patterns. The latter was used to estimate the approximate degree of purification by measuring the area under the peak corresponding to enzymically active material as determined by direct sampling and enzyme assay. By these criteria, the *B. subtilis* and *A. agilis* may be approx. 35–41% pure, while the enzyme from *Ps. fluorescens* may be approx. 70% pure. Ultracentrifugal sedimentation velocity analysis of the *E. coli*, *S. cerevisiae*, and *N. crassa* enzymes failed to detect significant amounts of contaminating inert protein.

NADH inhibition of lipoamide dehydrogenase

E. coli lipoamide dehydrogenase is known to be very sensitive to inhibition by NADH when assayed using lipoamide or lipoic acid as substrate[7,24,25]. Addition of NAD^+ to the reaction mixture relieves this inhibition[24]. As shown in Fig. 1, using the

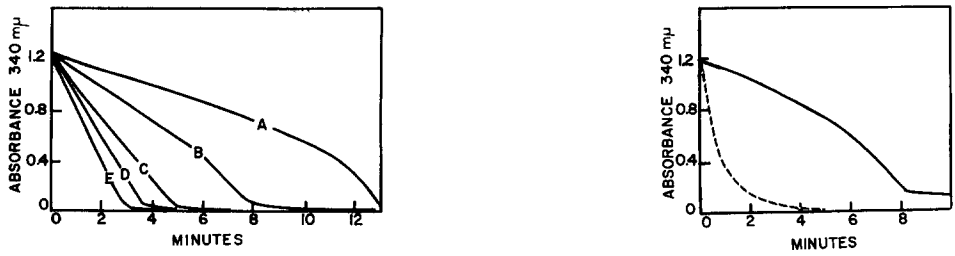
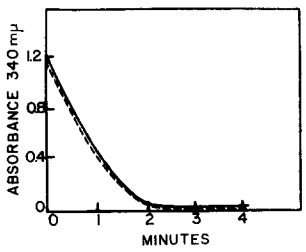


Fig. 1. Inhibition of *E. coli* lipoamide dehydrogenase by NADH and its reversal by NAD^+. *E. coli* lipoamide dehydrogenase (20 μg) were assayed by measuring the oxidation of NADH using lipoamide as hydrogen acceptor in the presence of varying amounts of NAD^+. Incubation medium[25] consisted of 0.2 mM NADH, 1.33 mM DL-lipoamide in 0.05 M phosphate buffer (pH 7.2) with addition of NAD^+ as indicated. A, 0.2 mM NADH alone; B, 0.2 mM NADH *plus* 0.013 mM NAD^+; C, 0.2 mM NADH *plus* 0.026 mM NAD^+; D, 0.2 mM NADH *plus* 0.039 mM NAD^+; E, 0.2 mM NADH *plus* 0.052 mM NAD^+.

Fig. 2. Effect of added NAD^+ on the activity of *S. marcescens* lipoamide dehydrogenase. *S. marcescens* lipoamide dehydrogenase (50 μg) was assayed using conditions described in Fig. 1, except that 0.26 mM NADH was used. ———, 0.26 mM NADH alone; — — —, 0.26 mM NADH *plus* 0.26 mM NAD^+.

assay conditions of McGARRY[25], omission of NAD^+ results in a low reaction rate with a long lag period (Curve A). However, addition of small increments of NAD^+ prior to initiating the reaction increases the initial rate of the reaction with a significant initial rate observed at a NAD^+ to NADH ratio of 0.065–1 (Curve B). The initial rate is increased 2.5 fold when the NAD^+ to NADH ratio is increased to 0.13–1 (Curve C). The rate of reaction at the highest NAD^+ to NADH ratio shown here (Curve E) is approx. 10% of the optimal rate observed when a 3 to 1 ratio of NAD^+ to NADH is included in the reaction medium[27]. McGARRY[25] reported that NAD^+ did not relieve the NADH inhibition of crude cell extracts when assayed at pH 7.0. The discrepancy between his results and ours may be due to loss of NAD^+ from the reaction medium due to the activity of other enzymes utilizing NAD^+ in the crude extract.

A similar rate curve is obtained using the enzyme from *S. marcescens* when NAD^+ is omitted from the reaction medium as shown in Fig. 2. Addition of an amount



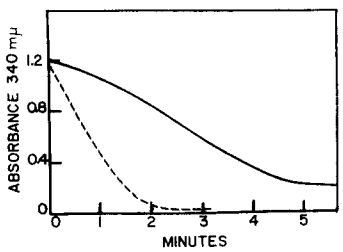


Fig. 3. Effect of added NAD^+ on the activity of *Ps. fluorescens* lipoamide dehydrogenase. *Ps. fluorescens* lipoamide dehydrogenase (50 μg) was assayed using conditions described in Fig. 1, except that 0.26 mM NADH was used. ———, 0.26 mM NADH alone; — — —, 0.26 mM NADH *plus* 0.26 mM NAD^+.

Fig. 4. Effect of added NAD^+ on the activity of *S. cerevisiae* lipoamide dehydrogenase. *S. cerevisiae* lipoamide dehydrogenase (40 μg) was assayed as described in Fig. 1 except that reaction was run at pH 7.0. ———, 0.2 mM NADH alone; — — —, 0.2 mM NADH *plus* 0.26 mM NAD^+.

of NAD^+ equivalent to NADH in the reaction mixture overcomes the inhibition.

E. coli and *S. marcescens*, two similar facultatively anaerobic species[36], thus possess lipoamide dehydrogenases which are very sensitive to NADH. In contrast, as shown in Fig. 3, lipoamide dehydrogenase isolated from *Ps. fluorescens* is not inhibited by NADH when assayed under the same conditions used for assay of *S. marcescens*. Addition of 0.26 mM NAD^+ is without effect on the reaction rate. Similar results were obtained with the enzymes isolated from *B. subtilis* and *A. agilis*.

NADH inhibition is observed with lipoamide dehydrogenases isolated from *S. cerevisiae*[38] and from pig heart[23], although it is observed readily only at pH values below 6.8. Fig. 4 shows the reduction of lipoamide by NADH using the enzyme from *S. cerevisiae*. The lag in the initial reaction rate in the absence of NAD^+ is more readily overcome than with the *E. coli* enzyme, but the reduction of lipoamide is still markedly inhibited. Again, addition of 0.26 mM NAD^+ to the reaction effectively overcomes the inhibition. A detailed analysis of the reduction of lipoamide by NADH and the effect of added NAD^+ has been presented by WREN AND MASSEY[38]. Identical results were obtained with the *N. crassa* enzyme.

Electrophoretic characteristics of microbial lipoamide dehydrogenases

In contrast to the electrophoretic heterogeneity of pig heart lipoamide dehydrogenase manifested by its resolution into at least six electrophoretically distinguishable forms[18–20], total lipoamide dehydrogenase[24] and the enzyme derived from either of the two α-keto acid dehydrogenation complexes of *E. coli*[27] exhibited a single species in polyacrylamide gel disc electrophoresis. These results and the conditions of electrophoresis are shown in Fig. 5, Tubes A–C. When the enzymes derived from the two complexes were mixed and electrophoresed at pH 8.5 a single enzymatically active band was obtained. No additional protein bands were detected when the gel was stained for protein with Coomassie Brilliant Blue (Tube D). The results shown here confirm the findings of PETTIT AND REED[39]. Gel electrophoresis was also performed at pH's 6.5, 7.5, 8.5, and 9.5. A single band was observed at each of these pH values when the gel was reacted with *p*-nitro blue tetrazolium–NADH. Electrophoretic mobility was greatest at pH 8.5 and movement was much retarded at pH 6.5.

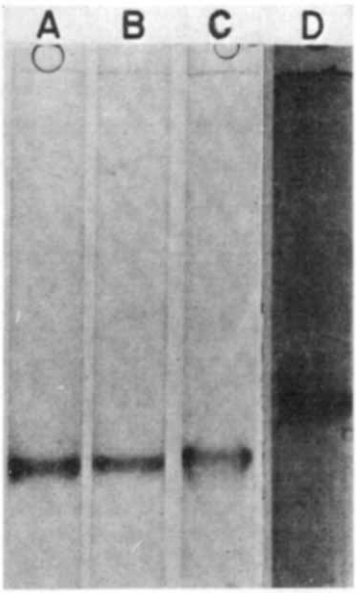


Fig. 5. Polyacrylamide gel disc electrophoresis of *E. coli* lipoamide dehydrogenase. Electrophoresis was performed in 4% polyacrylamide gels for 1 h 15 min at 2.0 mA/tube in 0.005 M Tris–glycine buffer (pH 8.5). Movement of protein is toward the anode. Tube A–C were run with 10 μg protein, and reacted with *p*-nitroblue tetrazolium chloride and NADH. Tube A is enzyme derived from pyruvate dehydrogenation complex. Tube B is enzyme derived from α-ketoglutarate dehydrogenation complex. Tube C is total lipoamide dehydrogenase prepared by WILLIAMS[24]' method. Tube D is enzyme derived from α-ketoglutarate dehydrogenation complex and stained for protein with Coomassie Blue R 250. This tube was run separately from Tubes A–C.

Because lipoamide dehydrogenase from *E. coli* did not exhibit microheterogeneity, whereas pig heart[18–20] as well as beef liver[21] lipoamide dehydrogenases exist as electrophoretically separable multiple forms, examination of the electrophoretic behavior of other microbial lipoamide dehydrogenases was of interest. Such examination would give some insight into (1) whether or not all microbial lipoamide dehydrogenases lack microheterogeneity and so resemble the *E. coli* enzyme, and (2) whether or not there is a fundamental difference between prokaryotes and eukaryotes with respect to electrophoretic heterogeneity.

Fig. 6 shows the electrophoretic patterns obtained when aliquots of the partially purified enzymes from the prokaryotic organisms were subjected to disc gel electrophoresis and the enzymes were visualized as diaphorase-reactive bands. All enzymes were electrophoresed for 1–2 h at pH 8.5 in 4% polyacrylamide at 2 mA per tube. Partially purified *S. marcescens* lipoamide dehydrogenase gave one major diaphorase-reactive band (Tube A) which moved somewhat behind the *E. coli* enzyme (Tube D) under the same conditions at pH 8.5. *Ps. fluorescens* (Tube B) showed a sharp upper band and a weak, more diffuse lower band which moved 1–2 mm ahead of the upper band. *B. subtilis* lipoamide dehydrogenase (Tube E) gave only one diaphorase-reactive band as did the *A. agilis* enzyme (Tube C). The latter enzyme had a much lower mobility than the enzyme from the other microorganisms.

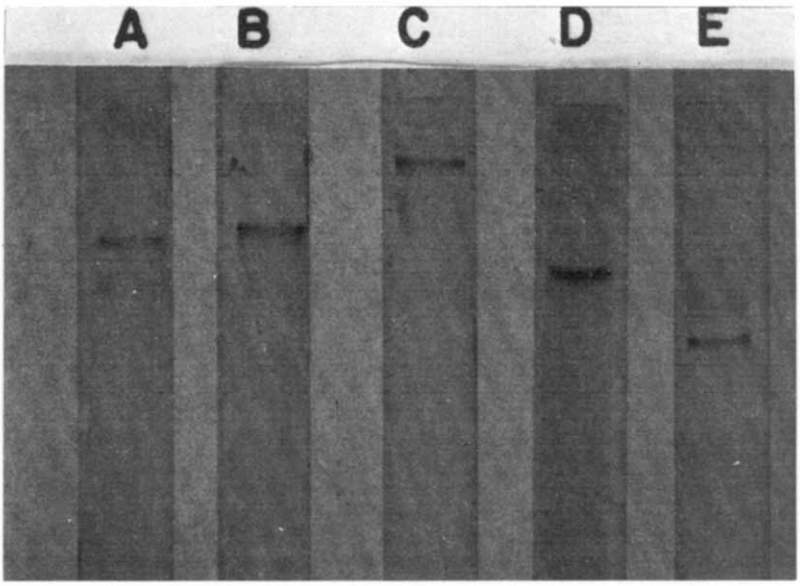


Fig. 6. Polyacrylamide gel disc electrophoresis of some microbial lipoamide dehydrogenases. Approx. 10 μg of protein were applied to 4% polyacrylamide gel and gels were electrophoresed for 1 h at 2 mA/tube in 0.005 M Tris–glycine buffer (pH 8.5). Movement is toward anode. Enzyme was visualized with *p*-nitroblue tetrazolium chloride and NADH. (A) *S. marcescens*; (B) *Ps. fluorescens*; (C) *A. agilis*; (D) *E. coli*; and (E) *B. subtilis*.

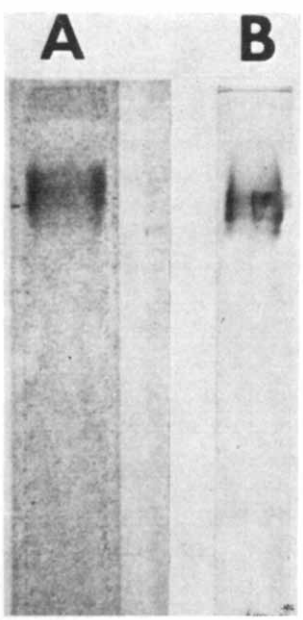


Fig. 7. Polyacrylamide gel disc electrophoresis of lipoamide dehydrogenase from *S. cerevisiae* and *N. crassa*. Approx. 15 μg of each preparation was electrophoresed in 4% polyacrylamide gel 1 h 45 min at 2 mA/tube in 0.005 M Tris–glycine (pH 8.5). Diaphorase activity of enzyme was visualized with *p*-nitroblue tetrazolium chloride and NADH. (A) *S. cerevisiae*; (B) *N. crassa*.

No attempt was made to stain these gels for protein since the enzymes were only partially purified.

As shown in Fig. 7, both *S. cerevisiae* and *N. crassa* lipoamide dehydrogenases produced somewhat diffuse patterns following electrophoresis at pH 8.5 for 2 h. However, both enzymes showed at least three diaphorase reactive species, reminiscent of the patterns observed with the enzyme derived from pig heart pyruvate or α-ketoglutarate dehydrogenation complexes[20].

Molecular size determinations

As an additional criterion for comparing some of the characteristics of lipoamide dehydrogenase in prokaryotic and eukaryotic organisms, the molecular sizes of the partially purified microbial lipoamide dehydrogenases were studied by molecular sieve chromatography and electrophoretic molecular sieve analysis. As was suggested by the $s_{20,w}$ values referred to earlier in this study (Table III), the results obtained by these two techniques indicate that the lipoamide dehydrogenases all have approximately the same molecular sizes. In an extensive study of the enzyme from *E. coli*, KOIKE *et al.*[7,40] reported a molecular weight of approx. 112 000 based on ultracentrifugal analysis. A molecular weight of 98 000 to 106 000 was obtained by WREN AND MASSEY[10] for the enzyme from *S. cerevisiae*. Evidence for molecular weights approximately that determined for the *E. coli* enzyme for the enzymes from *S. marcescens* and *Ps. fluorescens* was obtained by molecular sieve chromatography on a

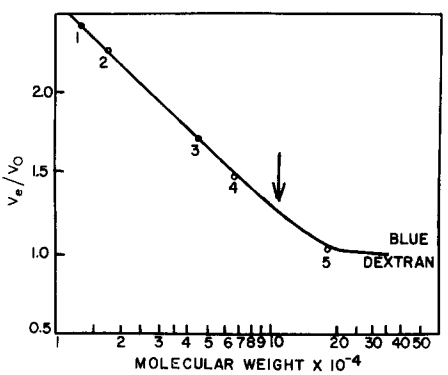


Fig. 8. Chromatography of *Ps. fluorescens* and *S. marcescens* lipoamide dehydrogenases and standard proteins on 2.5 cm × 6.8 cm. Sephadex G-100 column. 1, cytochrome *c*; 2, myoglobin; 3, ovalbumin; 4, bovine serum albumin; 5, γ-globulin. Lipoamide dehydrogenase from the two microorganisms eluted at position shown by the arrow.

Sephadex G-100 column[35]. Fig. 8 illustrates the chromatographic behavior of lipoamide dehydrogenase from these two organisms as compared to the behavior of a series of standard proteins of known molecular weight. 1 mg of each of the proteins was dissolved in 0.1 M phosphate buffer (pH 7.5)–3 mM EDTA and applied to a 2.5 cm × 68 cm Sephadex G-100 column which was equilibrated and developed with 0.1 M phosphate buffer (pH 7.5)–3 mM EDTA. The V_e/V_o was plotted against the log of the molecular weights of the standard proteins. A V_e/V_o value of 1.35 was obtained

for the two microbial lipoamide dehydrogenases which corresponds to a molecular weight of about 110 000.

Lipoamide dehydrogenases prepared from the microorganisms were subjected to disc electrophoresis in 4–7% polyacrylamide gels for 1 h at 2 mA per tube. Their relative mobilities were plotted against the percent gel concentration as described by HEDRICK AND SMITH[34] and the slopes were compared with that obtained for the *E. coli* enzyme. If parallel lines are obtained, then the proteins are of the same molecular size. If they differ in size, the change in slopes of the lines will be directly related to their molecular size. HEDRICK AND SMITH[34] have reported an average precision of 4% using this method.

Fig. 9 shows the results of such an analysis of the enzymes isolated from *S. marcescens*, *Ps. fluorescens*, *B. subtilis*, *S. cerevisiae*, and *N. crassa*. In all cases, the slopes are parallel to that of the *E. coli* enzyme, and the molecular sizes are therefore

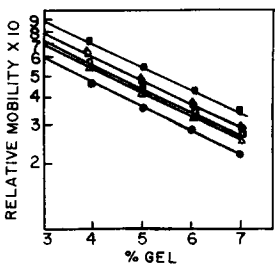


Fig. 9. HEDRICK–SMITH[34] plot of microbial lipoamide dehydrogenases. Protein (10 μg) was electrophoresed for 1 h at 2 mA/tube in 0.005 M Tris–glycine buffer (pH 8.5) in 3%, 4%, 5%, 6%, and 7% polyacrylamide gels. Enzyme was visualized with *p*-nitroblue tetrazolium chloride–NADH. ●—●, *E. coli*; ▲—▲, *S. marcescens*; ○—○, *Ps. fluorescens*; ■—■, *B. subtilis*; □—□, *S. cerevisiae*; △—△, *N. crassa*.

the same within the stated limits of the method. When the mobilities of the additional bands of the enzyme from *S. cerevisiae* and *N. crassa* which have more than one resolvable species were plotted in this manner, the resulting lines were parallel, demonstrating that these are charge isomers rather than dimeric or trimeric species of the enzyme.

DISCUSSION

Inhibition of pig heart lipoamide dehydrogenase by NADH has been studied extensively by MASSEY[41], who showed that this inhibition was reversed by the addition of NAD^+. KOIKE *et al.*[7] and WILLIAMS[24] showed that *E. coli* lipoamide dehydrogenase was also sensitive to NADH inhibition and that the inhibition was reversed by NAD^+. Within bacterial species MCGARRY[25] reported an interesting apparent grouping of lipoamide dehydrogenase with respect to the degree of inhibition by NADH. He reported that when crude cellular extracts were used as a source of lipoamide dehydrogenase activity, the enzyme activity from extracts of fermentative bacteria was subject to NADH inhibition, whereas the activity in extracts of nonfermentative bacteria was not inhibited. This correlation has been substantiated in this study with the use of partially purified enzymes. The inhibition by NADH is

partially reversed by addition of small amounts of NAD^+ although optimal activity is obtained when the ratio of NAD^+ to NADH approaches 3:1. It is possible that McGARRY[25] failed to observe this effect due to the effective removal of NAD^+ from his reaction medium by enzymes in his crude extract that catalyzed other reactions involving NAD^+.

Several possible mechanisms have been proposed[24,41,42] to explain the role of NAD^+ in overcoming the NADH inhibition. The following features are pertinent to this study. The catalytically important enzymic intermediate on reduction with excess substrate is a 2 electron reduction intermediate with NAD^+ bound to the enzyme which then prevents further reduction of the enzyme to its fully reduced form. When the enzyme is fully reduced, either by artificial reducing agents or by NADH in the presence of NADase, the enzyme will not catalyze the reduction of lipoamide by NADH. NAD^+ thus acts to stabilize the half-reduced catalytically active form of the enzyme.

Our present observations indicate a need for further study of the mechanism of NADH inhibition and the role of NAD^+ with respect to the differences in the degree of inhibition among lipoamide dehydrogenases derived from different species. It is possible that these differences are due to differences in the ability of the half-reduced form to be stabilized by binding NAD^+ or to differences in the rate of reaction of the semireduced form with NADH to form the fully reduced, enzymatically inactive form.

Studies on the gel electrophoretic properties of lipoamide dehydrogenase have shown, confirming similar findings by PETTIT AND REED[39], that *E. coli* lipoamide dehydrogenases derived from the pyruvate and α-ketoglutarate dehydrogenase complexes are electrophoretically a single species. This was true when electrophoresis was performed over the pH range of 6.5–9.5 and it was shown to be independent of the method of isolation of the enzyme.

Since it had already been shown that lipoamide dehydrogenases from several mammalian sources exist as multiple forms upon electrophoresis[5,18–21] it was postulated that all eukaryotic lipoamide dehydrogenases might be isoenzymic, while prokaryotic lipoamide dehydrogenases exist as a single electrophoretic form.

Lipoamide dehydrogenases isolated from five prokaryotes have been shown to exist as a single species, although *Ps. fluorescens* lipoamide dehydrogenase may have two electrophoretically distinguishable species. However, only a single form is detected upon electrophoresis of lipoamide dehydrogenase from *E. coli*, *A. agilis*, *S. marcescens*, and *B. subtilis*. Although lipoamide dehydrogenases derived from all mammalian species thus far studied show an ioenzymic pattern consisting of multiples of three; *i.e.* 3 or 6 forms[18,20,21], no such pattern is apparent among the prokaryotic lipoamide dehydrogenases.

Eukaryotic lipoamide dehydrogenase does show microheterogeneity and at least 3 isoenzymic forms are resolvable by polyacrylamide gel electrophoresis. Electrophoresis of lipoamide dehydrogenase from *S. cerevisiae* results in its separation into three diaphorase-reactive bands. This finding is of particular interest in view of the report that DEAE- cellulose chromatography of lipoamide dehydrogenase isolated from another yeast, *C. krusei*, resolves the enzyme into three enzymatically active peaks[12]. *S. cerevisiae* has been shown by VITOLS AND LINNANE[43] to have low levels of α-ketoglutarate dehydrogenase unless especially adapted to growth on tricarboxylic acid cycle intermediates, while it does possess pyruvate dehydrogenase in significant

amounts. Thus, it is possible that *S. cerevisiae* lipoamide dehydrogenase may arise primarily from pyruvate dehydrogenase[20].

Present studies show that the different lipoamide dehydrogenases differ by charge and not by molecular size. In addition, the multiple forms of lipoamide dehydrogenase isolated from eukaryotes appear to have identical molecular weights as indicated by the identity of the slopes which result when the electrophoretic mobilities in several percentage gels are plotted as described by HEDRICK AND SMITH[34]. BAPTIST *et al.*[44] have shown that the differences in electrophoretic mobility of eight enzymes are directly related to their taxonomic variation. While it was not the purpose of this study to relate electrophoretic mobility to taxonomy, it is apparent that some of the microbial species examined here have lipoamide dehydrogenases with different electrophoretic mobilities, while not differing in molecular size. In contrast, additional studies have shown that the enzymes isolated from *E. coli* K 12 and *E. coli* AB 1157 have electrophoretic mobilities identical to that of the enzyme isolated from *E. coli*, Crookes strain. This provides some additional evidence for the view that electrophoretic analysis of selected enzymes may aid in distinguishing bacterial genera.

The lipoamide dehydrogenases all appear to be associated *in situ* with multienzyme complexes, although this has not been rigorously proven. It was shown by MASSEY[2], ISHIKAWA *et al.*[45] and by KOIKE *et al.*[46] that the enzymes from pig heart, beef kidney and *E. coli* are derived from multienzyme pyruvate and α-ketoglutarate dehydrogenation complexes. In these studies it was found that the enzyme from *B. subtilis* was associated with a fraction which sediments at 144 000 × *g* in 2 h and possesses pyruvate dehydrogenase activity. In addition, the isolation procedure used for *Ps. fluorescens* exploits its association with the α-keto acid dehydrogenation complexes. These observations add substance to the probability, already evident from the work on the mammalian and *E. coli* α-keto acid dehydrogenation complexes, that these complexes are principal sources of lipoamide dehydrogenase.

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