# Lipoamide Dehydrogenase from *Malbranchea pulchella*: Isolation and Characterization†

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ABSTRACT: Lipoamide dehydrogenase (EC 1.6.4.3) has been isolated from a total homogenate of frozen mycelium of the thermophilic fungus *Malbranchea pulchella* var. sulfurea by a three-step procedure involving ammonium sulfate fractionation, Procion Brilliant Blue M-R–Sepharose 4B chromatography, and hydroxylapatite chromatography. The second step is the key purification step with the Procion Brilliant Blue M-R dye acting as an affinity ligand for the enzyme. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. The enzyme is a dimer of molecular weight 102000, and each monomer of 51000 molecular weight binds one molecule of flavin adenine dinucleotide. Other properties determined include a pH optimum of 8.2, a strong specificity for the substrates dihydrolipoamide and nicotinamide adenine dinucleotide, the apparent lack of multiple enzymic forms, the presence of diaphorase activity, and resistance to temperature denaturation up to 60 °C. The amino acid composition and absorption spectrum of the enzyme were also determined. The properties of lipoamide dehydrogenase from this source are very similar to those reported for the enzyme from several other sources.

Lipoamide dehydrogenase (LD,[1] EC 1.6.4.3) is a component enzyme of the pyruvate and 2-oxoglutarate dehydrogenase multienzyme complexes, and it may perform other roles in the cell as well [for a review, see Williams (1976)]. Besides the part it plays in the function of these enzyme complexes, LD is an interesting enzyme in its own right. It is a flavoprotein containing FAD, it has an active-site disulfide bond which participates in the enzyme mechanism, and it exhibits transhydrogenase and diaphorase activities in addition to the reduction of $NAD^+$ with dihydrolipoamide.

We have recently begun to investigate the structure and function of the pyruvate dehydrogenase enzyme complex using a new approach involving monofunctional and bifunctional arsenoxides (Stevenson et al., 1978). This approach is now being applied to the well-characterized procaryotic multienzyme complex from *Escherichia coli* and will be extended to the complex from a new eucaryotic source, the thermophilic fungus *Malbranchea pulchella* var. sulfurea. This paper presents a simple three-step procedure for purifying LD from a total homogenate of frozen mycelium of the fungus and the results of the basic characterization of the enzyme. LD from *M. pulchella* is very similar to the enzyme purified from several other sources (Williams, 1976).

## Experimental Procedures

*Enzyme Assays.* LD activity was assayed by measuring the formation of NADH spectrophotometrically at 340 nm from $NAD^+$ (2.5 mM) and DL-dihydrolipoamide (0.30 mM) in Tris-HCl (50 mM, pH 8.0) at 25 °C. DL-Dihydrolipoamide was prepared from DL-lipoamide (Reed et al., 1958). One unit of activity causes the formation of 1 μmol of NADH/min at 25 °C.

The diaphorase activity of LD was assayed essentially as described by Edelhoch et al. (1952). The reduction of 2,6-dichlorophenolindophenol (0.04 mM) was measured spectrophotometrically at 600 nm in the presence of NADH (0.2 mM), EDTA (20 μM), and Tris-HCl (50 mM, pH 7.5). One unit of diaphorase activity causes a decrease in absorbance of 1.0 unit/min at 25 °C.

*Growth Procedure. M. pulchella* var. sulfurea was obtained from Dr. G. M. Gaucher of this department as lyophilized spores and commercially grown up to a 450-L scale in the Biochemistry Department, Imperial College, London, by using a modified version of the procedure of Ong & Gaucher (1976). The lyophilized spores were grown on a yeast–glucose–agar slant and then in a 400-mL primary shake culture as described. Next this vegatative growth was expanded to 5 L and then to 60 L in two successive fermentor cultures on the same medium as the primary shake culture. Finally a 450-L fermentor culture was grown on a 2% casein and salts medium as described by Ong & Gaucher (1976). This culture was harvested when the activity of the extracellular protease, thermomycolin (Gaucher & Stevenson, 1975), had reached a maximum. The mycelium was removed by filtration, thoroughly washed with distilled water, and frozen for transport and storage. The yield was 14 kg of frozen mycelium.

*Enzyme Isolation.* One kilogram of frozen mycelium was suspended in 4 L of 0.25 M sucrose solution containing 20 μM *p*-toluenesulfonyl fluoride and allowed to thaw overnight at 4 °C. The resulting suspension was homogenized in a Waring blender (500-mL aliquots, 100 V, 2 min, room temperature). The homogenate was centrifuged (16000*g* for 30 min), and the resulting supernatant was filtered through glass wool.

A 60–90% saturated ammonium sulfate fractionation was performed on the homogenate supernatant at 4 °C, and the resulting brown precipitate was dissolved in a minimum volume (usually 100–150 mL) of 10 mM Tris-HCl, pH 7.5. This dark brown solution was dialyzed overnight at 4 °C against several 5-L changes of 10 mM Tris-HCl, pH 7.5.

The dialysate was centrifuged to remove any insoluble material (40000*g* for 5 min), equilibrated to room temperature, and applied to a Procion Brilliant Blue M-R–Sepharose 4B column (6 × 4 cm) which had been equilibrated with dialyzing buffer at room temperature. Procion Brilliant Blue M-R–

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[1] Abbreviations used: LD, lipoamide dehydrogenase; $NaDodSO_4$, sodium dodecyl sulfate.

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Sepharose 4B was prepared from Procion Brilliant Blue M-R dye (Serva) and Sepharose 4B gel (Pharmacia) according to the procedure of Dudman & Bishop (1968) as described by Baird et al. (1976). The column was washed with dialyzing buffer until the absorbance of the eluant at 280 nm dropped to 0.1 unit, and then LD was eluted with 200 mM sodium chloride in dialyzing buffer. The eluted fractions containing LD activity were pooled and dialyzed overnight at 4 °C against several 5-L changes of 10 mM sodium phosphate, pH 6.7.

The resulting dialysate was centrifuged to remove any insoluble material, equilibrated to room temperature, and applied to a hydroxylapatite (Bio-Rad) column (6 × 2 cm) which had been equilibrated with 10 mM phosphate buffer, pH 6.7. The column was washed with 200 mM phosphate buffer until no more material absorbing at 280 nm could be detected, and then LD was eluted with 400 mM phosphate buffer. The eluted fractions containing LD activity were greenish yellow in color. These fractions were pooled and dialyzed exhaustively against 50 mM ammonium bicarbonate at 4 °C to remove variable amounts of contaminating carbohydrate.

The resulting LD solution was either lyophilized and stored in the freezer at −20 °C or concentrated by a 100% saturated ammonium sulfate precipitation, redissolved in a minimum volume of 10 mM Tris-HCl, pH 7.5, and stored at 4 °C.

*Effect of pH on Activity.* A solution of LD (0.2 mg/mL) was dialyzed against deionized and distilled water and assayed for enzyme activity as a function of pH by changing the buffer in the assay procedure. The following buffers were used: 50 mM citrate buffers at pH 3.0, 4.0, and 5.0 (citric acid solutions titrated with sodium phosphate); 50 mM phosphate buffers at pH 6.0, 7.0, and 7.5 (sodium phosphate solutions titrated with citric acid); 50 mM Tris buffers at pH 7.8, 8.0, 8.2, 8.5, 8.7, and 9.0 (Tris solutions titrated with citric acid); 50 mM borate buffers at pH 9.0 and 10.0 (sodium borate solutions titrated with citric acid); and 50 mM carbonate buffer at pH 10.0 (sodium carbonate solution titrated with citric acid). Assays at each pH were done in duplicate.

*Thermal Stability.* An LD solution (0.2 mg/mL) was dialyzed against 50 mM Tris–citrate, pH 8.2, overnight at 4 °C. The resulting solution was brought to room temperature, and 1-mL aliquots were placed in a heating block at the desired temperature. LD activity was measured as a function of time with zero time taken as the instant that the solution reached the desired temperature. The half-life of the enzyme was determined at each temperature with 100% activity taken as the activity at zero time. For testing the effects of substrates, we added $NAD^+$ (1.25 mM) or DL-dihydrolipoamide (45 μM) just prior to placing the aliquot in the heating block and 100% activity was taken as the value of LD at the desired temperature at zero time in the absence of substrates.

*Polyacrylamide Gel Electrophoresis.* For routine gel electrophoresis in the presence of $NaDodSO_4$, the discontinuous pH system of Ornstein (1964) and Davis (1964) as described by Laemmli (1970) was used, except that $NaDodSO_4$ was present only in the protein sample and in the upper electrode buffer as suggested by Wyckoff et al. (1977).

For molecular weight determinations in the presence of $NaDodSO_4$, the continuous pH system of Weber & Osborn (1969) was used. The buffer was 25 mM sodium phosphate, pH 7.1, in all phases, and $NaDodSO_4$ was present only in the sample and the upper electrode buffer. Coomassie Brilliant Blue G-250 (instead of R-250) was used for protein staining. Ribonuclease (13 700 daltons), chymotrypsinogen (25 000), aldolase (39 500), and bovine serum albumin (67 000) were used as protein standards. Protein standards and LD were run together on 6 and 10% polyacrylamide slab gels. The ratio of acrylamide to "bisacrylamide" was 36:1 in each case. Protein $R_f$ values were determined relative to Pyronine Y.

*Thin-Layer Gel Filtration.* This technique was performed by using a commercially available apparatus (Pharmacia) essentially as described in the accompanying instruction booklet. The paper print taken at the completion of each run was stained with 0.1% Coomassie Blue G-250 in methanol–acetic acid–water (50:10:40 v/v/v) for 10 min at room temperature and destained in several changes of methanol–acetic acid–water (10:10:30 v/v/v) with occasional agitation. The protein spots began to appear immediately after the start of destaining, and more than 90% of background stain was removed in less than 30 min.

To determine the subunit molecular weight of LD in 8 M urea, we used a 1-mm thick layer of Sepharose 6B in 8 M urea buffered with Tris-HCl (50 mM, pH 7.5). The standard proteins were ribonuclease (13 700 daltons), chymotrypsinogen (25 000), aldolase (39 500), and bovine serum albumin (67 000). Thyroglobulin (porcine) was used to measure the void volume, while $N^{\alpha}$-dinitrophenylaspartic acid was used to measure the internal volume of the gel. Prior to use, the proteins were reduced with dithiothreitol and carboxymethylated with iodoacetic acid (Kolb et al., 1974).

To determine the native molecular weight of LD, we used Bio-Gel P-200 (−400 mesh, 0.6-mm thick layer) in 50 mM Tris-HCl, pH 7.5. The standard proteins were ribonuclease (13 700 daltons), chymotrypsinogen (25 000), pepsin (dimer, 69 200), concanavalin A (102 000), bovine serum albumin (dimer, 134 000), and aldolase (158 000). The void volume and internal volume of the gel were measured with thyroglobulin and $N^{\alpha}$-dinitrophenylaspartic acid, respectively.

*Amino Acid Analysis.* The amounts of all amino acids except tryptophan, half-cystine, and methionine in LD were determined by strong acid hydrolysis (6 M HCl and 0.1% phenol, 105 °C, in vacuo) for 24, 48, and 72 h followed by amino acid analysis on a Beckman 121 amino acid analyzer using the single-column system. Tryptophan content was determined by hydrolysis in 4 M methanesulfonic acid containing 0.2% tryptamine (105 °C, 22 h, in vacuo). Methionine and half-cystine were determined as methionine sulfone and cysteic acid by performic acid oxidation (Hirs, 1956) followed by strong acid hydrolysis for 24 h. Half-cystine was also determined as *S*-(carboxymethyl)cysteine by reaction of LD with tributylphosphine and iodoacetamide in 8 M urea (Ruegg & Rudinger, 1977) followed by strong acid hydrolysis for 24 h. A third estimate of half-cystine was obtained by quantitative titration of the cysteine-free thiol groups of LD in 6 M guanidium chloride with aldrithiol-4 (4,4′-dipyridyl disulfide) according to Grassetti & Murray (1967). All determinations were done in triplicate.

*Flavin Analysis.* Lyophilized LD was dissolved in 8 M urea, mixed with an equal volume of 10% trichloroacetic acid, and centrifuged. The supernatant was used for quantitative analysis of FAD by reduction with dithionite (Beinert & Page, 1957). The precipitated protein was redissolved in 8 M urea, dialyzed against distilled water, and determined quantitatively by strong acid hydrolysis for 24 h and amino acid analysis. The flavin content of LD was calculated from these two measurements.

## Results

*Purification of Lipoamide Dehydrogenase.* LD is usually purified by first isolating the pyruvate dehydrogenase multienzyme complex and then resolving the complex into its

Table I: Summary of the Purification of Lipoamide Dehydrogenase

| purification step | vol (mL) | absorbance (280 nm) | total protein[a] (g) | total act. (units) | sp act.[a] (units/mg) | % recovery |
|---|---|---|---|---|---|---|
| homogenate supernatant | 3600 | 11.2 | 41.4 | 813 | 0.0196 | 100 |
| ammonium sulfate | 133 | 50.6 | 6.73 | 744 | 0.115 | 92 |
| Procion Blue–Sepharose | 220 | 1.76 | 0.388 | 605 | 1.56 | 74 |
| hydroxylapatite | 146 | 0.18 | 0.026 | 377 | 14.4 | 46 |

[a] Assumed 1.0 $A$ at 280 nm ≡ 1 mg/mL protein.

component enzymes. In the present case, frozen mycelium did not yield any active complex and so this general approach could not be used. Instead a procedure has been developed for isolating LD alone. This procedure produces pure LD from the homogenate supernatant in three main steps (see Experimental Procedures). The key step in the procedure uses Procion Brilliant Blue M-R–Sepharose 4B, which binds only LD and four or five other proteins. These contaminating proteins are then easily separated from LD by using hydroxylapatite chromatography. The results of a typical purification from 1 kg of frozen mycelium are summarized in Table I. Approximately 25 mg of lyophilized LD was routinely obtained from 1 kg of frozen mycelium with greater than 700-fold purification and 40–50% yield.

*Purity.* Purified LD produced a single sharp protein band on 5, 7.5, and 10% polyacrylamide gels in the presence of $NaDodSO_4$. In the absence of $NaDodSO_4$ under the same electrophoresis conditions a single diffuse protein band was obtained. On the basis of these results, the purified LD was considered to contain a single protein. The LD fractions obtained from hydroxylapatite chromatography contained variable amounts of nonprotein contaminating material, probably carbohydrate, which interfered with the tryptophan determinations and grossly inflated the yields of lyophilized material. This contaminating material was removed by exhaustive dialysis (2 or 3 days) at 4 °C prior to lyophilization.

*Storage and Stability.* Dilute solutions of purified LD (less than 1 mg/mL) lost most of their activity in a few days at 4 °C. Concentrated solutions (greater than 2 mg/mL) were more stable, requiring longer than a month for complete loss of activity.

Lyophilized LD was poorly soluble in aqueous solutions, but the small amount that did redissolve retained much of its initial activity.

*Effect of pH on Activity.* Under the experimental conditions used, maximum LD activity occurred at pH 8.2. This pH optimum was sharp with 90% of maximum activity occurring at pH 8.0 and 8.5. Activity declined more rapidly when the pH increased above the optimum value (10% of maximum at pH 10.0 for carbonate buffer but at pH 9.0 for borate buffer) than when the pH decreased below the optimum value (10% of maximum at pH 5.0). In the physiological pH range of pH 7.0–7.5, the LD activity was 60–70% of the maximum value at the pH optimum.

*Temperature Stability.* The temperature stability of LD was determined at 65, 70, 75, and 80 °C, and the results are summarized in Table II. Purified LD is very stable to increases in temperature. Substrates $NAD^+$ and DL-dihydrolipoamide were tested for their effects on LD stability at 75 °C. $NAD^+$ slightly increased the stability of LD, while DL-dihydrolipoamide greatly destabilized the enzyme at 75 °C.

*Determination of Subunit Molecular Weight.* The subunit molecular weight of LD was estimated by using polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ on 10 and 6% polyacrylamide slab gels. The data for the standard proteins were used to construct a linear plot of log $M_r$ vs. $R_f$ by linear regression methods. From this plot and the data for LD, an estimate of the subunit molecular weight of LD and the 95% confidence limits of the estimate were determined for each polyacrylamide gel concentration. The results are summarized in Table III. The estimated values decreased as the polyacrylamide gel concentration decreased, but the difference between the two estimates is not statistically significant at the 95% confidence level.

Table II: Temperature Stability of Lipoamide Dehydrogenase

| temp (°C) | half-life[a] (min) |
|---|---|
| 80 | 6 |
| 75 (LD) | 15 |
| 75 (LD + $NAD^+$) | 20 |
| 75 (LD + dihydrolipoamide) | <1 |
| 70 | 50 |
| 65 | 170 |

[a] Half-life = time for 50% loss in activity.

Table III: Estimates of Subunit and Native Molecular Weights

| method | $M_r$ estimate | 95% confidence limits |
|---|---|---|
| $NaDodSO_4$-PAGE[a] | | |
| 10% gels | 59 200 | ±3 100 |
| 6% gels | 53 600 | ±5 600 |
| TLG[b] on Sepharose 6B in 8 M urea | 49 800[c] | ±7 500 |
| | 51 000[d] | ±6 100 |
| TLG[b] on Bio-Gel P-200 in Tris-HCl | 114 000[c] | ±19 000 |
| | 116 000[e] | ±15 000 |

[a] $NaDodSO_4$-PAGE = $NaDodSO_4$-polyacrylamide gel electrophoresis. [b] TLG = thin-layer gel filtration. [c] From a plot of log $M_r$ vs. $K_d$, where $M_r$ = molecular weight, $K_d = (1/x_1 - 1/x_2)/(1/x_i - 1/x_2)$, $x_2$ = migration distance of thyroglobulin (void volume), $x_1$ = migration distance of protein, and $x_i$ = migration distance of dinitrophenylaspartic acid (internal volume). [d] From a plot of $[(x_2 - x_1)/x_1]^{1/3}$ vs. $M_r^{1/2}$. [e] From a plot of $[(x_2 - x_1)/x_1]^{1/3}$ vs. $M_r^{1/3}$.

The subunit molecular weight of LD was also determined by using thin-layer gel filtration on Sepharose 6B in 8 M urea. The combined data from five separate runs were analyzed by constructing linear plots of log $M_r$ vs. $K_d$ (Klaus et al., 1972; Ackers, 1970) and $[(x_2 - x_1)/x_1]^{1/3}$ vs. $M_r^{1/2}$ (Wasyl et al., 1972), using linear regression methods. The results are summarized in Table III. The two estimates agree well with each other and put the subunit molecular weight in the 50 000–51 000 range.

On the basis of the combined results from thin-layer gel filtration and $NaDodSO_4$–polyacrylamide gel electrophoresis, the best estimate of the subunit molecular weight of LD is 51 000. Although this value may be in error by as much as 10–20% as suggested by the 95% confidence limits of the various estimates, nevertheless as a best estimate 51 000 is used in all subsequent work.

*Native Molecular Weight of LD.* The native molecular weight of LD was determined by using thin-layer gel filtration on Bio-Gel P-200 (–400 mesh). The combined data of six runs

Table IV: Amino Acid Composition of Lipoamide Dehydrogenase[a]

| amino acid | residues/subunit | nearest integer |
|---|---|---|
| Asp | 40.7 ± 2.2 | 41 |
| Thr[b] | 22.7 ± 1.4 | 23 |
| Ser[b] | 25.4 ± 1.6 | 25 |
| Glu | 55.8 ± 1.8 | 56 |
| Pro | 19.1 ± 1.9 | 19 |
| Gly | 55.8 ± 4.7 | 56 |
| Ala | 44.6 ± 2.9 | 45 |
| $^1/_2$-Cys[c] | 6.6 ± 1.3 | 7 |
| Val[d] | 54.4 ± 4.8 | 54 |
| Met[e] | 4.0 ± 1.7 | 4 |
| Ile[d] | 29.7 ± 1.5 | 30 |
| Leu | 36.0 | 36 |
| Tyr | 10.8 ± 3.5 | 11 |
| Phe | 9.4 ± 0.7 | 9 |
| His | 12.1 ± 1.9 | 12 |
| Lys | 38.5 ± 2.8 | 39 |
| Arg | 15.7 ± 1.2 | 16 |
| Trp | 1.6 ± 1.3 | 2 |
| total | | 485 |

[a] Based on 36.0 residues of leucine per subunit of 51 000 molecular weight. The values are means ± standard deviations, except for threonine, serine, valine, and isoleucine where values are linear regression estimates ± estimated standard errors. [b] Corrected to zero time for destruction during hydrolysis. [c] Determined as cysteic acid. [d] Corrected to 72 h for incomplete hydrolysis. [e] Determined as methionine sulfone.

were used to construct linear plots of log $M_r$ vs. $K_d$ (Klaus et al., 1972; Ackers, 1970) and $[(x_2 - x_1)/x_1]^{1/3}$ vs. $M_r^{1/3}$ (Wasyl et al., 1971) by using linear regression methods. The results are summarized in Table III. The two estimates agree well with each other and when compared to the subunit molecular weight estimates clearly indicate that LD is a dimer. Since only one protein band was obtained with $NaDodSO_4$–polyacrylamide electrophoresis, then the two subunits are very similar if not identical. When the best estimate of the monomer molecular weight is taken as 51 000, then the best estimate of the dimer molecular weight is 102 000. This value is within the 95% confidence limits of both of the estimates of the native molecular weight.

*Amino Acid Analysis.* The amino acid composition of LD based on 36 residues of leucine per subunit of molecular weight 51 000 is shown in Table IV. There are ~485 amino acid residues/subunit. Half-cystine was determined by three different methods: as cysteic acid (6.6 ± 1.3 residues, see Table IV), as *S*-(carboxymethyl)cysteine (3.1 ± 0.3 residues from three estimates), and as free thiol (6.6 ± 0.4 residues from two estimates). The free thiol value must be increased by 2 (to 8.6 ± 0.4 residues) to correct for the two half-cystines involved in the active-site disulfide bond (Williams, 1976). The agreement among the three estimates is poor and puts the half-cystine content between 3 and 9 residues/subunit. The estimates for tryptophan, tyrosine, and methionine also contain considerable error.

*Flavin Analysis.* Pure LD produced greenish yellow solutions and yellow lyophilized protein, suggesting that it was a flavoprotein. When lyophilized protein was dissolved in 8 M urea and the resulting solution was made 5% in trichloroacetic acid and centrifuged, the flavin remained in solution while the protein precipitated. The flavin in the supernatant was indistinguishable from FAD by its absorption spectrum and by high-voltage paper electrophoresis at pH 2.0 and 3.5. From these results it was concluded that the flavin component of LD was noncovalently bound FAD. The FAD in the supernatant and the precipitated protein were each determined quantitatively, yielding 0.85 ± 0.02 mol of FAD per subunit molecular weight of 51 000. Thus, LD contains one noncovalently bound molecule of FAD per subunit of LD or two molecules of FAD per molecule of LD.

*Absorption Spectrum.* The absorption spectrum of purified LD had maxima at 453, 355, and 270 nm, shoulders at 480 and 430 nm, and minima at 390, 310, and 250 nm. Absorbance ratios of the maxima were $A_{270}/A_{453} = 7.4$ and $A_{355}/A_{453} = 0.87$.

*Substrate Specificity.* LD is quite specific for the substrates DL-dihydrolipoamide and $NAD^+$. Three other dithiols tested, 1,4-dithiothreitol, 1,4-dithioerythritol, and 2,3-dithiopropanol (British AntiLewisite), were inactive as substrates. Similarly, $NADP^+$ could not replace $NAD^+$.

*Multiple Forms.* No evidence for the existence of more than one form of LD was obtained during purification or on polyacrylamide gel electrophoresis at pH 9.5. This suggests that LD from this eucaryotic source exists in only one enzymic form.

*Diaphorase Activity.* Diaphorase activity accompanied LD activity throughout the purification procedure, and purified LD possessed diaphorase activity. The relative amounts of LD and diaphorase activities varied from preparation to preparation and also depended on the age of the purified LD.

## Discussion

The purification procedure developed for LD from *M. pulchella* is not directly comparable to procedures that have been reported for LD from other sources for several reasons. Firstly, LD is usually obtained by first isolating the pyruvate dehydrogenase multienzyme complex and then resolving the complex into its component enzymes. This approach could not be used for *M. pulchella* because the frozen starting material yielded no active multienzyme complex. Secondly, for eucaryotic sources the mitochondria are usually isolated first and used as the starting material. For *M. pulchella*, attempts to isolate intact mitochondria were unsuccessful and LD activity was found in the total mycelium homogenate supernatant. Thirdly, when procedures for purifying LD only were developed, many of the high-resolution techniques now in use for purifying proteins were not available [cf. Ide et al. (1967) and Lusty (1963)].

Several aspects of the purification procedure for LD from *M. pulchella* are noteworthy. The procedure yields homogeneous LD from a total mycelium homogenate by three main purification steps in 6 days. The key step in the procedure employs the newly introduced technique of Procion Brilliant Blue M-R–Sepharose 4B chromatography (Baird et al., 1976). This Procion dye acts like a specific affinity ligand for LD from *M. pulchella*, and it may be of general use for the purification of LD from other sources. In addition, this Procion dye may be useful for purifying some of the other enzymes of the EC 1.6.4 group such as glutathione reductase and thioredoxin reductase. It was observed that at least one of the few other proteins that bound to the Procion dye but were separated from LD by hydroxylapatite chromatography was yellow, indicating the presence of flavin. Since the enzymes of the EC 1.6.4 group have several structural features in common (William, 1976), perhaps the Procion Brilliant Blue M-R dye is specific for one of these. We have not pursued this possibility.

The properties of LD from several diverse sources are very similar (Williams, 1976). The enzyme is a dimer of molecular weight 100 000. The subunits are very similar if not identical and have molecular weights of 50 000. Each subunit contains one molecule of noncovalently bound FAD which gives the enzyme a very characteristic absorption spectrum. LD is very specific for its substrates $NAD^+$ and dihydrolipoamide and

has a pH optimum in the pH range 8.0–8.5. The corresponding properties determined for LD from *M. pulchella* fit this general pattern also.

The stability of the enzyme at higher temperatures is remarkable. This property could be an adaptation of *M. pulchella* LD to the fact that the fungus is grown at 45 °C or it could be a common characteristic of LD from most sources. The latter possibility seems to be the case. Pig heart LD lost only 10% of its initial activity when heated at 70 °C for 5 min (Straub, 1939), and the procedure for purifying bovine liver LD has a heat-treatment step of 80 °C for 2 min (Lusty, 1963). Similarly, the procedure for spinach LD has a heat-treatment step of 60 °C for 1 min (Basu & Burma, 1960). In addition to its temperature stability, the pig heart enzyme is also quite stable to denaturation in urea (Massey et al., 1962). Oxidized LD (i.e., with its active-site disulfide intact) is very stable in 6.5 M urea. The substrate $NAD^+$ which stabilizes the active-site disulfide helped to stabilize the enzyme, while the substrate dihydrolipoamide which reduces the active-site disulfide greatly destabilized the enzyme in urea. These results with pig heart LD in urea are similar to the results that these substrates produced with the enzyme from *M. pulchella* at 75 °C. Thus, oxidized LD is a very stable protein while the reduced enzyme is considerably less stable.

Amino acid compositions have been determined for LD from *E. coli* (Williams et al., 1967; Vogel & Henning, 1973), from pig heart (Massey et al., 1962; Matthews et al., 1974), and now from *M. pulchella*. Comparison of the three compositions is not very informative except to reveal that they are not radically different. The half-cystine content is of particular interest for several reasons. An active-site disulfide peptide has been isolated and sequenced from the pig heart (Brown & Perham, 1974; Matthews et al., 1974) and the *E. coli* (Burleigh & Williams, 1972; Brown & Perham, 1972) enzymes. Peptides containing 9 of a possible 10 of the half-cystines in pig heart LD have been sequenced (Brown & Perham, 1974; Matthews et al., 1974), and 2 of these half-cystines have been implicated in the ability of cupric ion to inhibit LD activity while simultaneously stimulating diaphorase activity (Matthews & Williams, 1974). The half-cystine content of LD from *E. coli* is 2–4 residues/subunit (determined by performic acid oxidation), from pig heart is 8–10 (determined by performic acid oxidation, carboxymethylation, thiol titration, and peptide sequencing), and from *M. pulchella* is 3–9 (determined by performic acid oxidation, carboxymethylation, and thiol titration). Peptide mapping and sequencing of the half-cystine containing thiol peptides may improve the accuracy of the half-cystine estimates for *M. pulchella* LD. It will be interesting to determine whether any of the half-cystines other than those involved in the active-site disulfide are conserved.

LD exists as a single molecular species in procaryotes, while multiple forms of the enzyme are usually obtained from eucaryotes (Williams, 1976). In *E. coli* it has been established that all LD molecules are the product of a single gene, and it has been suggested that the multiple forms observed in many eucaryotes were derived from a single LD gene product. In support of this view, the enzyme obtained from a total homogenate of *M. pulchella* mycelium exists as a single species. A similar result was obtained for the enzyme from human liver (Ide et al., 1967). In both cases no evidence for the existence of multiple forms was obtained during purification or on anionic polyacrylamide gel electrophoresis. While it is very difficult to demonstrate conclusively that multiple forms of a protein do not exist, in those cases where multiple forms of LD have been found they were readily observable during purification and on gel electrophoresis [e.g., Lusty (1963)]. Thus, the *M. pulchella* and human liver results probably indicate the existence of a single gene product of LD in these organisms and suggest that reported cases of multiple forms of LD from other sources should be more carefully investigated to determine their origin.

The results presented here show that the basic properties of LD from the thermophilic fungus *M. pulchella* are quite similar to those reported for the enzyme from many diverse sources. Accordingly, *M. pulchella* LD is a typical representative of this class of enzymes. Now that the enzyme has been characterized, its structural–functional relations alone and as part of the pyruvate dehydrogenase multienzyme complex are being investigated by using various monofunctional and bifunctional arsenoxides (Stevenson et al., 1978).

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# Study of Chorismate Mutase–Prephenate Dehydrogenase in Crude Cell Extracts of *Escherichia coli*†

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ABSTRACT: Polyacrylamide gel electrophoresis of cell extracts of *Escherichia coli* K12 (JP2312) revealed that chorismate mutase–prephenate dehydrogenase can exist in several discrete aggregation states of the basic dimeric enzyme of molecular weight 90 000. The pattern of aggregation is characteristic of an indefinite self-association, all forms being in slow equilibrium with one another. A study of the same extracts by active enzyme centrifugation was undertaken to determine whether these states of aggregation, or changes thereof, might be influenced by the end-product inhibitor tyrosine and, hence, be physiologically significant. Over a range of extract concentrations this technique failed to reveal the forms of the enzyme with molecular weights greater than 86 000 (4.67 S). Sucrose gradient centrifugation also failed to detect these higher molecular weight aggregates. The concentrations of the larger aggregates must be considered to be so small as to be insignificant relative to the active dimer in their contribution to the weight-average sedimentation coefficient. At high protein concentration tyrosine did not appear to alter the equilibrium between the aggregates, as determined by its lack of effect on the sedimentation coefficient. Addition of proteolytic inhibitors altered none of the observed phenomena. The enzyme exhibits three classes of instability in solution. The first is a slow denaturation, the second is a rapid loss of activity upon dialysis, and the third is a dilution inactivation. The latter is not due to dissociation of the enzyme into subunits. Finally, two limitations in the application and interpretation of active enzyme centrifugation are reported.

Chorismate mutase–prephenate dehydrogenase, CMPD[1] (EC 1.3.1.12), is a regulatory enzyme in the segment of the aromatic amino acid biosynthetic pathway leading to tyrosine (Gibson & Pittard, 1968; Pittard & Gibson, 1970). Its prephenate dehydrogenase activity is strongly inhibited by tyrosine, possibly at an allosteric site (Koch et al., 1971). An investigation of the structure and properties of this enzyme in crude cell extracts and the effect on them of tyrosine was undertaken for the following reasons. The regulation of many well characterized oligomeric regulatory enzymes by end products has been found to be associated with changes in their quaternary structure, as reflected by changes in their weight-average molecular weight (Klotz et al., 1970; Dunne & Wood, 1975). Also, the similar enzyme chorismate mutase–prephenate dehydratase (EC 4.2.1.51) from *Salmonella typhimurium* undergoes a dimerization to the tetramer in the presence of very low concentrations of its end-product inhibitor, phenylalanine (Schmit & Zalkin, 1971). Finally, an increasing number of enzymes in general, and in the aromatic amino acid pathway in particular, are being shown to exist as multienzyme complexes and/or multifunctional proteins (Kirschner & Bisswanger, 1976; Welch, 1977; Reed & Cox, 1970; Ginsburg & Stadtman, 1970; Umbarger, 1978). Chorismate mutase is known to exist as a bifunctional enzyme with both prephenate dehydrogenase and prephenate dehydratase in *Escherichia coli* and *S. typhimurium* (Cotton & Gibson, 1965; Schmit & Zalkin, 1969) and with deoxy-D-*arabino*-heptulosonate phosphate synthetase, DAHPS, in *Bacillus subtilis* strain 168 (Nester et al., 1967); the latter enzyme is further complexed with shikimate kinase (EC 2.7.1.71), also of the aromatic pathway (Nakatsukasa & Nester, 1972). It is increasingly clear, at least in eucaryotes, that many in vivo associations of enzymes are not observed in vitro because of dissociation or proteolysis during purification (Kirshner & Bisswanger, 1976; Gaertner & Cole, 1976; Lumsden & Coggins, 1977). Crude extracts contain all of the macromolecular components of the cell, and so a study of such extracts may uncover associations of enzymes normally considered independent and inert. Such crude-extract studies are more difficult to interpret but may disclose regulatory mechanisms which may be missed in studies of the pure components alone.

The approach used in the present work has been to employ techniques which enable determination of the molecular weight or sedimentation coefficient of CMPD under a variety of conditions in crude extracts. The major quantitative technique was active enzyme centrifugation, AEC, in which the sedimentation coefficient of the active form(s) of an enzyme is determined from the unique expression of its activity *during* centrifugation (Cohen et al., 1962; Cohen & Mire, 1971; Llewellyn & Smith, 1978).

## Experimental Section

### *Materials*

*Chemicals.* Chorismic acid was prepared by the method of Gibson (1970). Prephenic acid and NAD were purchased

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[1] Abbreviations used: CMPD, chorismate mutase–prephenate dehydrogenase (EC 1.3.1.12); DAHPS, deoxy-D-*arabino*-heptulosonate phosphate synthetase; AEC, active enzyme centrifugation; ADH, yeast alcohol dehydrogenase (EC 1.1.1.1); BSA, bovine serum albumin.

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