Wasyl, Z., Luchter, E., & Bielanski, W., Jr. (1971) *Biochim. Biophys. Acta 243*, 11.
Wasyl, Z., Luchter-Wasyl, E., & Bielanski, W., Jr. (1972) *Biochim. Biophys. Acta 285*, 279.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Williams, C. H., Jr. (1976) *Enzymes, 3rd Ed. 13*, 89.
Williams, C. H., Jr., Zanetti, G., Arscott, L. D., & McAllister, J. K. (1967) *J. Biol. Chem. 242*, 5226.
Wyckoff, M., Rodbard, D., & Chrambach, A. (1977) *Anal. Biochem. 78*, 459.

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

Danny J. Llewellyn and Geoffrey D. Smith*

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

† From the Department of Biochemistry, Faculty of Science, The Australian National University, Canberra, A.C.T., Australia. *Received May 18, 1979.*

[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.

0006-2960/79/0418-4707$01.00/0

from Sigma Chemical Co. All other chemicals were purchased and were of the highest available purity.

Bacteria. *E. coli* K12 strain JP2312, a constitutive mutant for CMPD, was obtained from J. Pittard via J. F. Morrison, Department of Biochemistry, The John Curtin School of Medical Research, The Australian National University. It was grown on Luria's medium containing bactotryptone (10 g), yeast extract (5 g), and NaCl (10 g) per 1.5 L. Cells were inoculated onto nutrient agar plates and allowed to grow overnight at 30 °C. They were then scraped from the plate and used to inoculate 1.5 L of the liquid medium which was then incubated at 37 °C overnight.

Buffers. Unless otherwise indicated, the buffer used in this work was 0.1 M Tris-HCl, 1 mM Na_2EDTA, and 0.2 mM dithiothreitol, pH 7.5.

Methods

Assays. Chorismate mutase activity was measured by detecting the formation of prephenate after converting it to phenylpyruvate (Koch et al., 1970). Prephenate dehydrogenase activity and the overall coupled activity were determined by measuring spectrophotometrically the formation of NADH from prephenate plus NAD and chorismate plus NAD, respectively, at pH 8.1 (Koch et al., 1972). All substrates were 1 mM. The latter continuous assays were both used in AEC experiments.

Proteolytic activity was measured by the release of acid-soluble peptide fragments from denatured casein as the substrate. The fragments were assayed by their absorbance at 280 nm (Rick, 1965).

Protein concentrations were measured by the method of Hartree (1972) using bovine serum albumin as the standard.

Preparation of Cell Extracts. Cells were harvested from a late log phase culture by centrifugation (1000*g*; 20 min; 0–4 °C). The yield was 3–4 g/1.5 L of culture. Cells were resuspended in buffer (2 mL/g wet weight of cell paste), with or without proteolytic inhibitors, and disrupted in a French pressure cell at 20000 psi. Cell debris was removed by centrifugation (30000*g*; 10 min; 0–4 °C), and the supernatant was treated with either protamine sulfate (1 volume of 2% protamine sulfate per 4 volumes of extract) or streptomycin sulfate (40% w/v to give a final concentration of 6% w/v) and allowed to stand cold for 30 min. Precipitated nucleic acids were removed by centrifugation (30000*g*; 10 min; 0–4 °C), and such extracts are referred to as 30000*g* extracts. However, in some experiments it was necessary to centrifuge this extract further (300000*g*; 1–2 h; 0–4 °C) to pellet microsomal contaminants which interfered with the dehydrogenase assays. This is referred to as the 300000*g* extract. The final extracts were dialyzed overnight against buffer, except where indicated.

Sucrose Gradient Centrifugation. This was performed in a linear sucrose gradient (5–20% w/v) in a volume of 4.6 mL of the appropriate buffer. Extract (0.1 mL; 3 mg of protein), containing 0.2 mg/mL yeast alcohol dehydrogenase, ADH (EC 1.1.1.1), as an internal standard, was applied to the top of the gradient and centrifuged at 120000*g* for 12–16 h in a Beckman SW50.1 rotor. Fractions (6 drops) were collected through the bottom of the tube and assayed for CMPD and ADH activity. The sedimentation coefficient of CMPD was calculated by the method of Martin & Ames (1961) using a sedimentation coefficient of 6.72 S for yeast ADH. The molecular weight of CMPD was calculated by assuming the molecular weights of these globular proteins to be proportional to the 3/2 power of their sedimentation coefficient (Schachman, 1959); the molecular weight of yeast ADH was taken to be 141000.

Density and Viscometry Measurements. These were made pycnometrically and with a Ostwald viscometer, respectively, and used to calculate correction factors for the sedimentation data in the standard way (Schachman, 1957).

Active Enzyme Centrifugation (AEC). All experiments were performed with an MSE Centriscan 75 combination analytical and preparative ultracentrifuge. Overfilling single-sector synthetic boundary cells were used throughout. Experiments were carried out at 30 °C although prior to the experiment the rotor and contents were kept at 37 °C to allow rapid temperature equilibration. The general experimental procedure of Cohen & Mire (1971) was followed. Specifically, cells were filled with 0.4 mL of buffer solution containing the required substrates (1 mM each) for the enzyme's assay, together with 16 or 30% (v/v) glycerol used for density stabilization. Sucrose and D_2O were avoided for this purpose since they have been implicated in causing nonphysiological aggregation of proteins (Kemper & Everse, 1973). The volume of extract layered in the experiments was usually between 2 and 20 μL (up to 0.6 mg of protein). The rotor speed was 55000 rpm in all cases. Sedimentation coefficients were usually measured from the rate of movement of the inflection point of the leading edge of the NADH product distribution curve (measured spectrophotometrically at 340 nm), although difference curves were used when necessary (Llewellyn & Smith, 1978). In calculations of $s_{20,w}$ values, a partial specific volume of 0.739 mL/g was assumed for CMPD, as calculated from the amino acid composition (Koch et al., 1971) by knowing the values for the individual amino acids (Lee & Timasheff, 1974). A partial specific volume of 0.769 was used for yeast ADH (Hayes & Velick, 1954).

Polyacrylamide Gel Electrophoresis. The disc gel method of Davis (1964) was used, with the omission of the sample and stacking gels. For all gel concentrations the bis(acrylamide) concentration was 5% of the total acrylamide concentration. A Tris–glycine buffer system (pH 8.2) was used in all cases except where noted. Up to 0.9 mg of protein was applied to each gel and run at a constant current of 1 mA/gel until a bromophenol blue marker (in a separate gel) had run off the gel. Protein bands were detected by Coomassie brilliant blue staining (Work & Work, 1969). CMPD or prephenate dehydrogenase activity was detected by coupling the formation of NADH with the reduction of a tetrazolium dye (Koch et al., 1970). Molecular weights were estimated by the method of Hedrick & Smith (1968) using catalase (bovine kidney), ADH (yeast), and hemoglobin (human fraction IV) as standards. Their respective molecular weights were 247000, 141000, and 63000. When samples were electrophoresed in the presence of modifiers, these were either included during the preparation of the gels (tyrosine) or electrophoretically equilibrated before use (chorismate and NAD). During electrophoresis they were present in both the upper and lower electrolytic reservoirs. Before staining the gels were leached for 1 h in Tris–glycine buffer (pH 8.2) with constant agitation to remove residual substrates and effectors.

Ultrafiltration was done with an Amicon cell and a UM10 membrane under 40 psi of N_2.

Column Chromatography. DEAE-cellulose (Sigma Chemical Co.) was prepared (Peterson & Sober, 1962) and equilibrated with buffer. The column size was 15 × 1 cm. Extract containing 30 mg of protein was applied and eluted with a linear KCl gradient (0.05–0.3 M) in a total volume of 500 mL. Fractions (5 mL) were collected and assayed.

Sephadex G-200 (Pharmacia) was prepared, packed into a column (20 × 2.5 cm), and equilibrated with Tris-HCl buffer

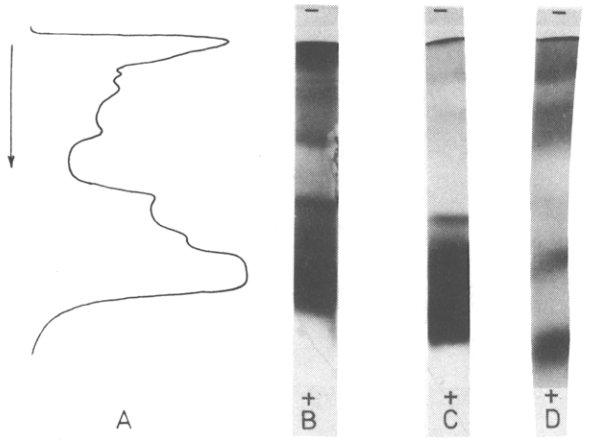

FIGURE 1: Multiple banding of CMPD on 10% polyacrylamide gels. Crude protein (0.9 mg) containing 30% sucrose (w/v) and 0.01 mg/mL bromophenol blue was applied to 5 × 70 mm gels, electrophoresed at 1 mA/gel for 45 min, and stained for CMPD activity. Electrophoresis was carried out in Tris–glycine buffer (pH 8.2) containing no additions (B), 1 mM chorismate and 1 mM NAD (C), or 1 mM tyrosine (D). A densitometer trace of (B) is shown in (A), the arrow indicating the direction of migration. Residual substrates and effectors were removed before staining by agitation in an excess of buffer for 1 h.

(pH 7.5). Extract containing 30 mg of protein was applied and eluted with buffer. Fractions (3 mL) were collected and assayed. The column was calibrated for molecular weight by also running through it the following proteins of known molecular weight: 2 mg of thyroglobulin (660 000–680 000), 2 mg of bovine γ-globulin (150 000–170 000), 2.5 mg of bovine serum albumin (68 000), and 2.5 mg of cytochrome *c* (12 400).

Results

Multiple Oligomeric Forms of CMPD as Determined by Polyacrylamide Gel Electrophoresis. Cell extracts (containing 0.1–1 mg of total protein) were applied to 10% polyacrylamide gels, electrophoresed, and then stained from CMPD activity. In initial experiments gels were stained for 5–10 min at room temperature until a sharp band of dye could be seen. If the staining time was slightly longer, other slower moving bands of activity could occasionally be seen. When the gels were stained over an extended time (3–4 h at 30 °C), several bands were consistently observed, all with mobilities less than the predominant, early staining activity (Figure 1A,B). The heavy stain corresponding to protein that had not entered the gel was observed when NAD alone (but not chorismate or prephenate) was included in the staining solutions and presumably represents direct reduction of the dye system by the respiratory chain components of small microsomes. Those bands that had entered the gel were only observed when chorismate or prephenate was also present in the staining solution. Inclusion of the proteolytic inhibitors phenylmethanesulfonyl fluoride (1 mM), ε-aminocaproic acid (5 mM), and *p*-aminobenzamidine hydrochloride (5 mM) during preparation of the extract did not change the pattern of multiple bands, suggesting that they are not random products of proteolytic digestion. It is of interest that the same multiple bands were seen with a partially purified enzyme prepared by the affinity chromatography procedure of Smith et al. (1977).

Experiments were repeated with gels of varying strength according to the procedure of Hedrick & Smith (1968), and plots of the negative logarithm of mobility (relative to the dye bromophenol blue) against gel concentration were constructed for the fastest moving band of CMPD activity and three standard proteins, catalase (247 000), yeast ADH (141 000), and human hemoglobin (63 000). The slope of such plots is proportional to the molecular weight of the protein and yielded a molecular weight of 90 000 for the predominant form of CMPD. This is presumed to correspond to the 88 000-dalton dimer purified and studied by other workers (Sampathkumar, 1978; Koch et al., 1971). A similar procedure could not be used for the other bands of CMPD activity because to achieve sufficient resolution with all gel concentrations we had to run the dye front off the gel. Mobilities (R_m) of the various bands were therefore expressed relative to the fastest moving band, arbitrarily given a value of unity. The combined data from several experiments plotted as 100 log R_m100 for each band against gel concentration gave a series of straight lines intersecting near the origin. According to the criteria of Hedrick & Smith (1968), the multiple forms of CMPD must therefore be different molecular weight isomers. When the six slower moving bands were assumed to be larger species with molecular weights of integral multiples of 90 000, a plot of molecular weight vs. slope was linear, supporting the initial assumption.

In order to estimate the amounts of these larger aggregates relative to the predominant CMPD species, we subjected an undiluted extract to DEAE-cellulose and Sephadex G-200 chromatography and also to sucrose gradient centrifugation. In each case a single symmetrical peak was observed. The molecular weights determined from Sephadex G-200 chromatography and density gradient centrifugation were 100 000 and 86 000, respectively. In the latter case the sedimentation coefficient was 4.83 S. Peak broadening on Sephadex was comparable with that observed for ADH and human hemoglobin chromatographed on the same column, and no broadening due to aggregation is indicated.

The aggregates were shown to be in slow equilibrium by the following interrupted electrophoresis experiment. An extract was electrophoresed on a number of gels until the dye front was near the bottom. Half the gels were stained for CMPD activity while the others were incubated at 30 °C for 30 min and electrophoresis was then continued for a further 15 min. The gels were then stained for CMPD activity for 3 h. A complex banding pattern was observed in these latter gels (Figure 2C). Data from a number of such gels were combined (Figure 2B), and it was seen that during the period when electrophoresis had been interrupted the various discrete aggregates (Figure 2A) had reestablished equilibrium with the other components (Figure 2B). Clearly, no covalent linkage is involved in the aggregation.

Active Enzyme Centrifugation of CMPD in Cell Extracts. To understand the role, if any, that these aggregates play in the regulation of CMPD, it was essential to obtain more quantitative data than is possible with polyacrylamide gel electrophoresis, particularly in relation to the relative amounts and activities of the various species and the effect on these of tyrosine. As reported above, column chromatography and sucrose gradient centrifugation failed to resolve any of the aggregates from the predominant CMPD species, possibly because the resolution time (several hours) of these techniques is much greater than the equilibration time of the enzyme species such that a single symmetrical boundary might inevitably be observed (Nichol et al., 1964). AEC, on the other hand, has a resolution time comparable with that of polyacrylamide gel electrophoresis. When a range of extract concentrations was analyzed by this technique, none of the higher molecular weight aggregates was revealed. A typical AEC pattern for this enzyme is shown in Figure 3. An average sedimentation coefficient of 4.67 ± 0.09 S was calculated from many experiments using both dialyzed and

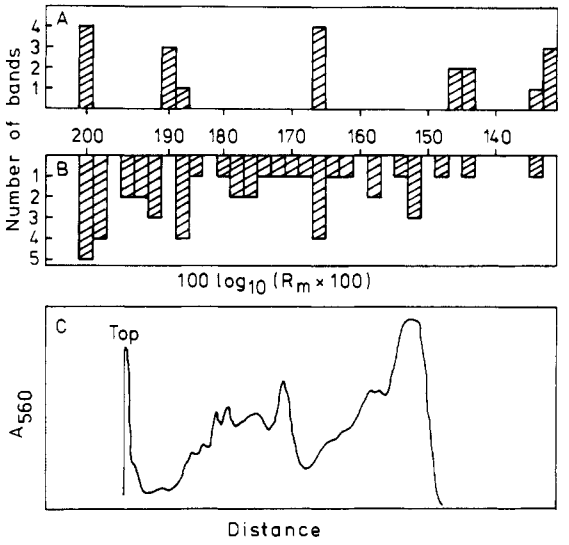

FIGURE 2: Histograms of the CMPD banding patterns constructed from 10% polyacrylamide gels by using four gels electrophoresed and stained in the usual manner for CMPD (A) and five gels electrophoresed as in (A), removed to 30 °C for 1 h, and then further electrophoresed for 15 min before staining (B). Histograms were constructed by calculating 100 log R_m100 for all bands found in all of the gels and then scoring them into a number of small groups with a width of 1.67 (a value chosen to give the best resolution). (C) shows a typical densitometer trace at 640 nm of a gel from (B). In all cases 0.6 mg of crude protein containing 30% sucrose and 0.01 mg/mL bromophenol blue was applied and electrophoresed at 1 mA/gel.

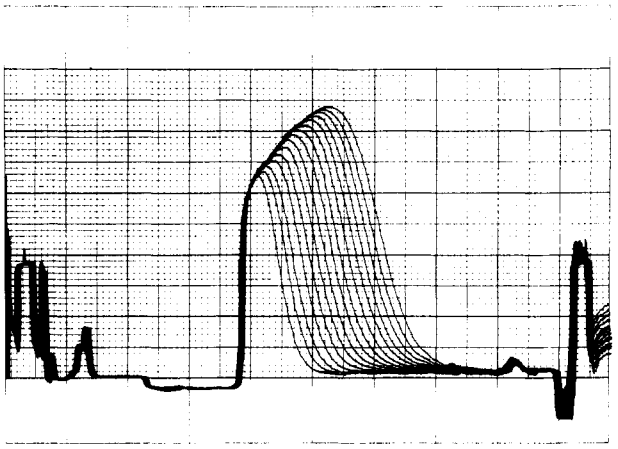

FIGURE 3: A typical AEC experiment on crude extracts of CMPD, showing successive 5-min traces of the absorbance down the cell at 340 nm due to the formation of NADH by prephenate dehydrogenase. The single-sector cell contained 0.4 mL of Tris-HCl buffer (pH 8.1) containing 1 mM prephenate, 1 mM NAD, and 16% glycerol (w/v). Crude protein (0.3 mg/mL) in 25 μL was layered. The experiment was carried out at 30 °C.

undialyzed 30000*g* and 300000*g* extracts. This corresponded to a molecular weight of 86 000 when compared with the measured sedimentation coefficient (6.52 S) for a standard enzyme, yeast ADH, run under similar conditions (141 000) and calculated as already outlined for the sucrose gradient results.

Polyacrylamide electrophoresis was repeated with precisely the same solutions used for AEC (i.e., 1 mM chorismate and 1 mM NAD were also present), but the banding pattern of the CMPD aggregates was not altered (Figure 1C). It must therefore be concluded that under the conditions used the concentration of these aggregates is so small as to make an insignificant contribution to the weight-average sedimentation coefficient. Furthermore, the aggregates were insignificant

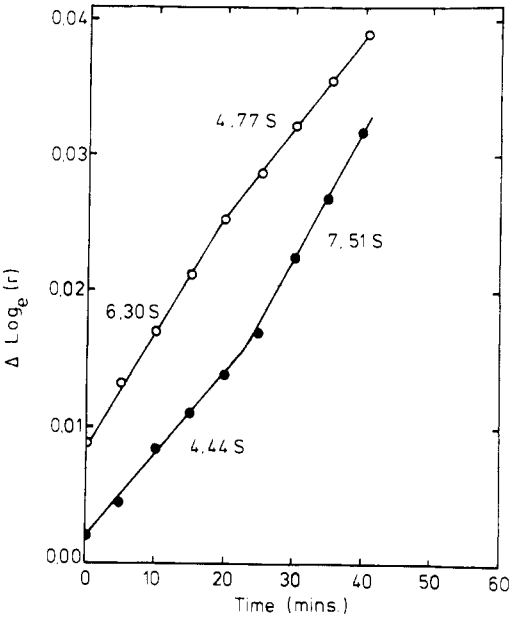

FIGURE 4: Artifacts in the AEC technique. This is a plot of the natural logarithm of the position of the inflection point (r) of successive NADH distributions against scan time. (○) The crude extract contained 1 mM tyrosine and had a protein concentration of 0.75 mg/mL. A total of 25 μL was layered onto 0.4 mL of Tris-HCl buffer (pH 8.1) containing 1 mM chorismate, 1 mM NAD, and 16% glycerol (w/v). (●) An experiment in which 25 μL of crude extract (7.5 mg of protein/mL) was layered onto 0.4 mL of Tris-HCl buffer (pH 8.1) containing 1 mM chorismate, 1 mM NAD, 0.2 mM tyrosine, and 16% glycerol (w/v). Values quoted are apparent sedimentation coefficients.

in their contribution to the overall enzyme activity.

Effect of Tyrosine on the Physical Properties of CMPD. On 10% polyacrylamide gels, tyrosine, the end-product regulator, appeared to have no effect on the number or sizes of the aggregates (Figure 1D).

AEC allows an enzyme in one environment to be sedimented into another environment, and a series of experiments were performed in which an extract prepared in the presence of tyrosine (usually 1 mM) was sedimented onto a reaction mixture containing no, or a lower concentration (0.2 mM) of, tyrosine. Other experiments were performed with these conditions reversed. When extracts were sedimented away from the influence of tyrosine, the ln r vs. t plots indicated an apparent decrease in molecular weight (Figure 4) when chorismate and NAD were the substrates used. However, when prephenate replaced chorismate, no such curvature was observed, and the lower s value only was observed. When corresponding *assays* were performed by diluting CMPD extracts containing tyrosine into assay solutions without it, kinetic lag periods were observed in the coupled assay system (chorismate plus NAD) but not with prephenate plus NAD. In the former case the lag period correlated precisely with the time of curvature in Figure 4, and, hence, the apparent change in molecular weight is almost certainly an artifact brought about by a change in enzyme activity. Such an artifact was previously predicted (Llewellyn & Smith, 1978), using simulated AEC data for an enzyme whose activity was changing during sedimentation. An increasing activity caused an apparent upward deflection of the ln r vs. t plot, as is observed here experimentally.

When an extract without tyrosine was sedimented into 0.2 mM tyrosine, an apparent increase in molecular weight was observed (Figure 4), but this is also interpreted as an artifact, due to the absorbance of the extract at 340 nm. Such an

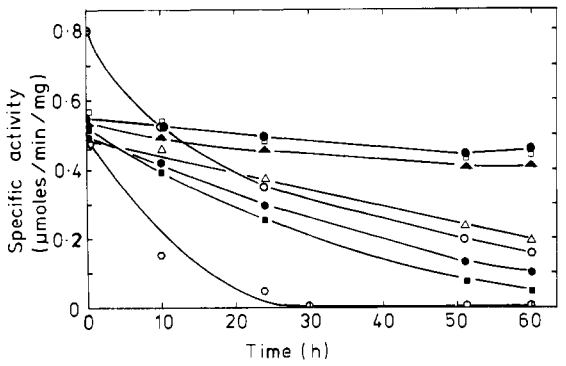

FIGURE 5: Effect of various ligands on the stability of chorismate mutase after dialysis of 0.5 mL of crude extract overnight in 500 mL of Tris-HCl buffer (pH 7.5) containing no additions (○), 10 mM sodium citrate (●), 1 mM tyrosine (△), 10 mM adamantaneacetic acid (◐), 10 mM citrate and 1 mM tyrosine (▲), 10 mM adamantaneacetic acid and 1 mM tyrosine (■), and 5 mM *p*-aminobenzamidine hydrochloride (○). The specific activity of chorismate mutase in a 50-fold dilution was assayed at 30 °C at intervals over 60 h of storage at 0–4 °C. The specific activity of the undialyzed extract (□) was also measured at the same time.

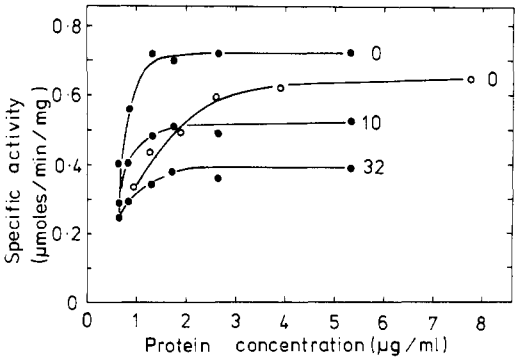

FIGURE 6: Effect of dialysis on the dilution inactivation of chorismate mutase. Crude extract (1.0 mL; 30 mg of protein) was dialyzed overnight against 2 L of Tris-HCl buffer (pH 7.5). The specific activity of chorismate mutase was assayed at various dilutions in this same buffer for both the dialyzed (●) and undialyzed (○) extracts. This was denoted the zero (0) time point. The assays were repeated for the dialyzed extract at 10 and 32 h after storage at 0–4 °C. The protein concentrations denote the final concentration in the assay.

increase was not observed in sucrose gradient centrifugation experiments in the presence of tyrosine. In such experiments a single symmetrical peak was observed corresponding to a molecular weight of 90 000.

These artifacts were avoided by using prephenate plus NAD as the substrates and an extract of high specific activity. Under these conditions extracts prepared under a variety of conditions, both in the presence and in the absence of tyrosine, gave an average sedimentation coefficient of 4.65 ± 0.07 S.

Inclusion of proteolytic inhibitors during preparation of extracts and during subsequent experiments altered none of these findings.

Stability of CMPD in Extracts. Work described in the following sections was based entirely on assays of chorismate mutase activity alone, due to problems in the dehydrogenase assay caused by contaminating respiratory components with NADH oxidase activity. This problem was not satisfactorily overcome by using 300000*g* supernatants or by using specific inhibitors (Nester et al., 1974; Davis et al., 1955).

Pure CMPD has been shown to be very unstable although it can be stabilized to some extent by prephenate, tyrosine (Koch et al., 1971) and particularly citrate (Sampathkumar, 1978). The enzyme is also very labile in crude cell extracts, despite the endogenous presence of each of these metabolites (Figure 5). When the extract was stored at room temperature, its loss of activity over 24 h was only slightly greater than that at 0–4 °C. The rate of loss of activity was unaffected by phenylmethanesulfonyl fluoride (1 mM) in the cold but was significantly enhanced at room temperature. When all endogenous small molecules were removed from the extract by dialysis against buffer, there was an initial activation, followed by a rapid loss of CM activity on subsequent storage at 0–4 °C; the half-life was 24 h. This enhanced instability after dialysis was not relieved by 1 mM citrate in the dialysis buffer but was significantly reduced by 10 mM citrate, which stabilized the mutase activity almost to the same level as undialyzed extract (Figure 5). Tyrosine (1 mM) did not give any stabilization. When both citrate (10 mM) and tyrosine (1 mM) were present during dialysis, tyrosine was not an antagonist of citrate in stabilizing the enzyme, suggesting that citrate does not bind at the tyrosine regulatory site on the enzyme. It has also been shown not to bind at the active site since it is not inhibitory of CMPD activity (Sampathkumar, 1978). The putative transition-state analogue of the mutase reaction, adamantaneacetic acid (Andrews et al., 1977), also had no significant stabilizing effect and in combination with tyrosine resulted in slightly enhanced instability.

Several proteolytic inhibitors were included in the dialyzed extract, and their various effectiveness in controlling general proteolysis in *E. coli* extracts was determined with denatured bovine casein as the substrate. *p*-Aminobenzamidine hydrochloride (5 mM) gave the best inhibition (99%), followed in order by 1 mM EDTA (49%), 1 mM phenylmethanesulfonyl fluoride (37%), and 5 mM ε-aminocaproic acid (no inhibition). A mixture of all inhibitors gave 96% inhibition. However, when all of the inhibitors were included in the dialysis buffer, there was a rapid loss of CMPD activity, caused specifically by benzamidine hydrochloride, as determined by testing the compounds individually (Figure 5). Phenylmethanesulfonyl fluoride had a marginal stabilizing effect.

Dilution Inactivation of CMPD. The specific activity of CMPD (measured by either activity) was found to decrease sharply when diluted to a certain concentration (undialyzed control in Figure 6).

When the glassware which the enzyme came into contact with in the assays was treated with trimethylchlorosilane, the dilution inactivation was still observed, suggesting that it is not caused by adsorption of the enzyme to glass. In many of these experiments the specific activity appeared to drop by no more than half of its value at higher concentrations. When the extract was dialyzed against such a volume of buffer that its dialyzable components were of the same concentration as for the highest dilution of the undialyzed control of Figure 6, dilution inactivation was observed over the same protein concentration range (Figure 6) and continued to be observable during the course of the time-dependent inactivation described under Stability of CMPD in Extracts. The initial activation after dialysis is also evident in Figure 6.

That the inactivation was not caused by dilution of small molecules in the extract was confirmed by adding back to a diluted extract sample a filtrate of the initial extract prepared by ultrafiltration through a membrane chosen to retain molecules of molecular weight greater than 10 000. This filtrate did not prevent dilution inactivation when used as the diluent. Furthermore, citrate (10 mM), which had a pronounced effect on the time-dependent loss of activity, and tyrosine had no effect on dilution inactivation, indicating that the two processes are independent. These results suggest that dilution inactivation is an innate property of the CMPD protein itself and its interaction with the solvent. Addition of glycerol

(16%) or poly(ethylene glycol) (2%) did not prevent the inactivation.

Kinetic analysis at both high (20 μg/mL protein) and low (1.3 μg/mL) extract concentrations (i.e., on each side of the inactivation transition) gave linear Lineweaver–Burk plots. Kinetic constants showed considerable variability between preparations, as noted for *Aerobacter aerogenes* by Heyde & Morrison (1978), but were consistent for a given preparation. Dilution inactivation lowered the V_{max} but did not appear to affect the K_m (0.3 ± 0.1 mM).

Using AEC, with increasing dilutions of extract, we reached a point (over a narrow concentration range) where no activity was observed, presumably due to the inactivation transition of Figure 6. In some experiments the inactivation could be observed directly during sedimentation as diffusional dilution took the extract below this critical protein concentration. When experiments were performed with initial concentrations of extract just above those at which dilution inactivation was observed, a consistent sedimentation coefficient of 4.4 S was observed. This evidence, considered in relation to simulated data (Llewellyn & Smith, 1978), suggests that dilution inactivation is not due to dissociation, since this would be apparent as a decrease in the weight-average sedimentation coefficient.

Other workers have noted that the kinetics of CMPD are markedly affected by bovine serum albumin (BSA) and other globular proteins, both with enzyme from *A. aerogenes* (Heyde & Morrison, 1978) and *E. coli* (Sampathkumar, 1978). BSA and bovine casein (both 1 mg/mL) abolished dilution inactivation. Casein which had been denatured by boiling (Rick, 1965) also abolished dilution inactivation, whereas addition of the free amino acids from an acid hydrolysate of casein (casamino acids; Difco) had no such effect.

AEC experiments, in the presence of BSA, with diluted extracts, to which BSA (1 mg/mL) was also added, gave an average sedimentation coefficient of 4.67 S over a wide range of concentrations above and below that of the inactivation transition. Kinetic experiments at an extract concentration of 20 μg/mL protein, both in the presence and in the absence of BSA (1 mg/mL), showed the V_{max} of the enzyme to be unchanged but its K_m to be considerably reduced (from 0.29 to 0.11 mM) when BSA was present.

Dilution inactivation could be reversed, in the short term at least, since BSA restored a considerable part of the activity of enzyme which had been diluted and stored on ice for 1 h. Restoration was only partially successful at very low extract concentrations. Inactivation was also observed when the Tris buffer was replaced by 0.1 M *N*-ethylmorpholine hydrochloride buffer (also pH 7.5) in the dilution and assay of the extract.

Discussion

It is seen from Figures 1 and 2 that CMPD can exist as a series of discrete aggregates of integral multiples of a basic structure of molecular weight 90 000 reported for purified CMPD (Koch et al., 1971; Sampathkumar, 1978). The multiple molecular forms are in equilibrium with each other (Figure 2), and so the system appears to conform to an indefinite self-association (Holloway & Cox, 1974). The equilibrium must be slowly established in relation to the time of electrophoresis; otherwise, species would not have been observed. Also, the association equilibrium constant(s) for the system must be very low because the aggregates were not observed in AEC or sucrose gradient sedimentation experiments or in chromatography. The alternative explanation, that the aggregates are present in significant quantities but are inactive, is unlikely because the specific activity does not increase with dilution and also because on sucrose gradient centrifugation, which has a slow resolution time, the peak of activity was symmetrical about a position corresponding to the molecular weight of the dimer. Furthermore, for a protein which exists as an equilibrium mixture of different oligomeric species, even inactive species can influence the weight-average sedimentation coefficient as determined by AEC (Llewellyn & Smith, 1978); in the present study a constant sedimentation coefficient was observed, even over a range of concentrations.

The very existence of such an equilibrium mixture, even where under nonperturbed conditions one species is predominant, offers a potential regulatory system for the pathway in which it is involved, if the equilibrium can be shifted by the binding of a ligand [e.g., Dunne & Wood (1975)]. Such a system has been found with acetyl-CoA carboxylase (Gregolin et al., 1966) where the modifier citrate causes the inactive enzyme to associate into very large active oligomers. On the other hand, a number of enzymes are capable of existing as very large aggregates of a basic subunit but for which no regulatory mechanism has been demonstrated (Mansour, 1972; Klotz et al., 1970). In the present study, it was seen that tyrosine, the major physiological effector of CMPD, has no effect on its sedimentation coefficient and therefore presumably on the equilibrium between the aggregates. It could also be noted that the substrates chorismate, prephenate, and NAD have no effect on the equilibrium since the sedimentation coefficient, determined by AEC in their presence, is almost identical with that obtained for the pure enzyme in their absence (Koch et al., 1971; Sampathkumar, 1978). Neither tyrosine, NAD, nor chorismate affects the number or size of the aggregates (Figure 1). It therefore appears necessary to conclude that the indefinite self-association of CMPD is not of physiological importance but merely reflects a physical property of this protein. It has not been possible to determine whether the aggregates differ in activity from the dimer since their low concentrations prevented their detection except by activity staining on electrophoresis. Even in these experiments the aggregates reequilibrated with the active dimer so that it cannot be concluded that the aggregates themselves are active.

CMPD exhibits three classes of instability in crude extracts: a slow denaturation (Figure 5), a loss of activity caused by dialysis (Figure 5), and dilution inactivation (Figure 6). Slow denaturation occurred in concentrated extracts that had not been dialyzed and which therefore presumably contained all endogenous small molecules. It was not affected by the proteinase inhibitor phenylmethanesulfonyl fluoride nor greatly enhanced by raising the storage temperature from 4 °C to room temperature, each of which indicates that the process does not involve proteolysis. It probably involves a complex interplay of factors related to the removal of the enzyme from its strictly regulated cellular millieu.

Loss of activity of CMPD after dialysis can be noted during purification procedures of this enzyme (Koch et al., 1971; Sampathkumar, 1978; Smith et al., 1977), but its time course has not previously been examined. As seen in Figure 5, following dialysis there is an initial activation, followed by a decay of activity with a half-life of some 24 h. The initial activation is presumably due to the removal of endogenous inhibitors such as prephenate. Of the ligands tested, citrate (10 mM) was the only one which stabilized the enzyme after dialysis. The concentration of citrate required for stabilization was 1 order of magnitude greater than that reported by Sampathkumar (1978) for the pure enzyme, but the number of proteins which in a crude extract binds citrate is probably large, thereby reducing the effective concentration available

for stabilization of CMPD. The mode of action of citrate is unknown, although that it binds to a site other than the active site (Sampathkumar, 1978) is confirmed by the observations that the enzyme is not stabilized by compounds (adamantaneacetic acid and tyrosine) which do bind to the active site and that tyrosine did not act as an antagonist of citrate in stabilizing the enzyme (Figure 5). It is interesting that enzyme inhibited by tyrosine or adamantaneacetic acid is the least stable after dialysis (Figure 5).

The third form of instability is that caused by dilution. This has been observed previously with the enzyme in *A. aerogenes* (Cotton & Gibson, 1967) and ascribed to subunit dissociation. Our studies with the *E. coli* enzyme show that it is not caused by dissociation since a constant sedimentation coefficient was observed in AEC experiments at concentrations above and below the inactivation region. Theoretical considerations of the AEC technique (Llewellyn & Smith, 1978) indicate that such dissociations would be registered by it even if the dissociated monomer were inactive, provided a rapid equilibrium was maintained. That no slow dissociation occurs is shown by the linearity in the chorismate mutase assay product–time plots and by the linear Lineweaver–Burk plots observed at all extract concentrations; the reduced V_{max} observed at high dilutions does not alter this argument. The inactivation is also not caused by glass adsorption, since silane treatment did not affect it, or by dissociation of a bound activating ligand, since extensive dialysis did not shift the transition concentration (Figure 6), nor is it influenced by adding back the small molecule component of a cell extract to a diluted extract. The inactivation must therefore be an innate property of the secondary or tertiary structure of the protein itself, caused by a change in its environment on dilution. It is not a specific inactivation by Tris buffer since it occurred when other buffers were used.

Interestingly, the inactivation was reversed by the presence of BSA and by both native and denatured casein, whereas a free amino acid mixture did not suffice. This stabilization does not involve direct binding to CMPD because the sedimentation coefficient of the enzyme measured in the presence of even a 1000-fold excess of BSA was the same (4.67 S) as that measured in its absence. It must be concluded that the inactivation is due to a solvent effect in which the solvent structure and/or properties necessary for the maintenance of an active enzyme are influenced by the concentration of protein contained within it. The inactivation may be due to a solvation effect *alone* or in combination with some contaminant in the diluent buffer, possibly a trace heavy metal.

The artifacts of the AEC technique are of interest in the light of their prediction from the analysis of simulated data (Llewellyn & Smith, 1978). The apparent decrease in sedimentation coefficient observed when the enzyme was sedimented away from the influence of the inhibitor tyrosine (Figure 4) is due to the kinetic lag in the overall reaction from chorismate, as observed in independent kinetic experiments. It was not observed when prephenate replaced chorismate. The apparent increase in sedimentation coefficient observed in the presence of tyrosine (Figure 4) is a result of the very low activity of the enzyme in the presence of this inhibitor and the strong absorbance of *E. coli* extracts at 340 nm which distorts the product distributions. It can be obviated by using lower concentrations of higher activity extracts.

Finally, this work has shown that extracts both prepared and submitted to AEC under a variety of conditions, with added stabilizers and proteolytic inhibitors, never exhibited sedimentation coefficients corresponding to species with molecular weights greater than 86 000. Although it is always difficult to be sure that specific proteolysis has been prevented, the three inhibitors used in this study in combination certainly substantially inhibited proteolytic activity by *E. coli* extracts. The evidence from this work is that CMPD in vivo is not associated with other proteins or enzymes as a multienzyme complex or as a higher order multifunctional protein such as the *arom* pentafunctional enzyme in *Neurospora* (Gaertner & Cole, 1976; Lumsden & Coggins, 1977). Many of the AEC experiments performed for this communication were carried out within 1 h of cellular disruption. In the light of our work on this and other enzymes of the aromatic amino acid pathway in *E. coli* and other bacteria, it is becoming apparent that views (Stark, 1977; Welch, 1977) concerning the ubiquity of large multifunctional proteins might be more correctly confined to eucaryotes than to procaryotes. In the latter the degree of complexity may be limited to the bifunctional or possibly trifunctional level. The structural complexity of eucaryotic cells may require the high degree of regulatory control conferred by large multifunctional proteins, which becomes quite plausible when one considers the variety of posttranscriptional control in splicing and ordering of mRNA as currently proposed for higher organisms (Doolittle, 1978).

Acknowledgments

We thank Arlene Daday for competent technical assistance and Dr. J. F. Morrison for helpful discussions.

References

Andrews, P. R., Cain, E. N., Rizzardo, E., & Smith, G. D. (1977) *Biochemistry 16*, 4848.
Cohen, R., & Mire, M. (1971) *Eur. J. Biochem. 23*, 267.
Cohen, R., Giraud, B., & Messiah, A. (1962) *Biopolymers 5*, 203.
Cotton, R. G. H., & Gibson, F. (1965) *Biochim. Biophys. Acta 100*, 76.
Cotton, R. G. H., & Gibson, F. (1967) *Biochim. Biophys. Acta 147*, 222.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.
Davis, B., Gilvarg, C., & Mitsuhashi, S. (1955) *Methods Enzymol. 2*, 307.
Doolittle, W. F. (1978) *Nature (London) 272*, 581.
Dunne, C. P., & Wood, W. A. (1975) *Curr. Top. Cell. Regul. 9*, 65.
Gaertner, F. H., & Cole, K. W. (1976) *Arch. Biochem. Biophys. 177*, 566.
Gibson, F. (1970) *Methods Enzymol. 17A*, 362.
Gibson, F., & Pittard, J. (1968) *Bacteriol. Rev. 32*, 465.
Ginsburg, A., & Stadtman, E. R. (1970) *Annu. Rev. Biochem. 39*, 429.
Gregolin, C., Ryder, F., Warner, C., Kleinschmidt, A., & Lane, M. (1966) *Proc. Natl. Acad.* Sci. *U.S.A. 65*, 1751.
Hartree, E. F. (1972) *Anal. Biochem. 48*, 422.
Hayes, J. E., & Velick, S. F. (1954) *J. Biol. Chem. 207*, 225.
Hedrick, J. L., & Smith, A. J. (1968) *Arch. Biochem. Biophys. 126*, 155.
Heyde, E., & Morrison, J. F. (1978) *Biochemistry 17*, 1573.
Holloway, R. R., & Cox, D. J. (1974) *Arch. Biochem. Biophys. 160*, 595.
Kemper, D., & Everse, J. (1973) *Methods Enzymol. 27*, 67.
Kirschner, K., & Bisswanger, H. (1976) *Annu. Rev. Biochem. 45*, 143.
Klotz, I. M., Langerman, N. R., & Darnall, D. W. (1970) *Annu. Rev. Biochem. 39*, 25.
Koch, G. L. E., Shaw, D. C., & Gibson, F. (1970) *Biochim. Biophys. Acta 212*, 375.

Koch, G. L. E., Shaw, D. C., & Gibson, F. (1971) *Biochim. Biophys. Acta 229*, 795.
Koch, G. L. E., Shaw, D. C., & Gibson, F. (1972) *Biochim. Biophys. Acta 258*, 719.
Lee, J. C., & Timasheff, S. N. (1974) *Arch. Biochem. Biophys. 165*, 268.
Llewellyn, D. J., & Smith, G. D. (1978) *Arch. Biochem. Biophys. 190*, 483.
Lumsden, J., & Coggins, J. R. (1977) *Biochem. J. 161*, 599.
Mansour, T. E. (1972) *Curr. Top. Cell. Regul. 5*, 1.
Martin, R. G., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372.
Nakatsukasa, W. M., & Nester, E. W. (1972) *J. Biol. Chem. 247*, 5972.
Nester, E. W., Lorence, J. H., & Nasser, D. S. (1967) *Biochemistry 6*, 1553.
Nester, E. W., Dale, B., Montoya, A., & Vold, B. (1974) *Biochim. Biophys. Acta 361*, 59.
Nichol, L. W., Bethune, J. L., Kegeles, G., & Hess, E. L. (1964) *Proteins, 2nd Ed. 2*, 305.
Peterson, E. A., & Sober, H. A. (1962) *Methods Enzymol. 5*, 3.
Pittard, J., & Gibson, F. (1970) *Curr. Top. Cell. Regul. 2*, 29.
Reed, L. J., & Cox, D. J. (1970) *Enzymes, 3rd Ed. 1*, 213.
Rick, W. (1965) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., Ed.) p 800, Academic Press, New York and London.
Sampathkumar, P. (1978) Ph.D. Thesis, Australian National University.
Schachman, H. K. (1957) *Methods Enzymol. 4*, 32.
Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry*, Academic Press, New York and London.
Schmit, J. C., & Zalkin, H. (1969) *Biochemistry 8*, 174.
Schmit, J. C., & Zalkin, H. (1971) *J. Biol. Chem. 246*, 6002.
Smith, G. D., Roberts, D. V., & Daday, A. (1977) *Biochem. J. 165*, 121.
Stark, G. R. (1977) *Trends Biochem. Sci. 2*, 64.
Umbarger, H. E. (1978) *Annu. Rev. Biochem. 47*, 533.
Welch, G. R. (1977) *Prog. Biophys. Mol. Biol. 32*, 103.
Work, T. S., & Work, E. (1969) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 1, p 61, North-Holland Publishing Co., Amsterdam.

Purification and Properties of the Flavoenzyme D-Lactate Dehydrogenase from *Megasphaera elsdenii*†

Steven T. Olson and Vincent Massey*

ABSTRACT: A pyridine nucleotide independent D-lactate dehydrogenase has been purified to apparent homogeneity from the anaerobic bacterium *Megasphaera elsdenii*. The enzyme has a molecular weight of 105 000 by sedimentation equilibrium analysis with a subunit molecular weight of 55 000 by sodium dodecyl sulfate gel electrophoresis and is thus probably a dimer of identical subunits. It contains approximately 1 mol of FAD and 1 g-atom of Zn^{2+} per mol of protein subunit, and the flavin exhibits a fluorescence 1.7 times that of free FAD. An earlier purification [Brockman, H. L., & Wood, W. A. (1975) *J. Bacteriol. 124*, 1454–1461] results in substantial loss of the enzyme's zinc, which is required for catalytic activity. The new purification yields greater than 5 times the amount of enzyme previously isolated. The enzyme is specific for D-lactate, and no inhibition is observed with L-lactate. Surprisingly, the enzyme has a significant oxidase activity, which depends on the ionic strength. V_{max} values of 190 and 530 min^{-1} were obtained at a $\Gamma/2$ of 0.224 and 0.442, respectively. Except for this atypically high oxygen reactivity, D-lactate dehydrogenase resembles other flavoenzyme dehydrogenases in that the flavin does not react with sulfite, the tryptophan content is low, and a neutral blue semiquinone is formed upon photochemical reduction. The enzyme flavin is reduced either by dithionite, by oxalate plus catalytic 5-deazaflavin in the presence of light, or by D-lactate. Two electrons per flavin were consumed in a dithionite titration, implying no other oxidation–reduction active groups. From equilibrations of the enzyme with varying ratios of D-lactate and pyruvate, an E_{m7} of -0.219 ± 0.007 V at 20 °C was calculated for the flavin. The enzyme requires dithiothreitol for stability. Rapid inactivtion results when the enzyme is incubated with a substoichiometric level of Cu^{2+}. This inactivation can be reversed by dithiothreitol. It is proposed that the enzyme possesses a pair of cysteine residues capable of facile disulfide formation.

Baldwin & Milligan (1964) first reported the purification of a pyridine nucleotide independent lactate dehydrogenase from the anaerobic rumen bacterium *Megasphaera elsdenii* (formerly known as *Peptostreptococcus elsdenii*). Brockman later purified the enzyme to near homogeneity and found it to be a flavoprotein, specific for the D isomer of lactate. Its metabolic role is to couple the oxidation of D-lactate to the reduction of short-chain α,β-unsaturated acyl-CoA's, two other flavoproteins serving as intermediary electron carriers (Brockman, 1971; Brockman & Wood, 1975). These flavoproteins are an electron-transferring flavoprotein (ETF)[1]

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109. *Received March 1, 1979; revised manuscript received June 20, 1979.* This work was supported by a grant from the U.S. Public Health Service.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; DTT, dithiothreitol; DCIP, 2,6-dichloroindophenol; TLC, thin-layer chromatography; BSA, bovine plasma albumin; EFl_{ox}, $EFlH\cdot$, and EFl_{red}, oxidized, one-electron reduced, and two-electron reduced forms of D-lactate dehydrogenase, respectively; ETF_{ox} and ETF_{red}, the oxidized and reduced forms of electron transferring flavoprotein; BDH_{ox} and BDH_{red}, the oxidized and reduced forms of butyryl-CoA dehydrogenase.

0006-2960/79/0418-4714$01.00/0