Purification and Subunit Characterization of Propanediol Dehydratase, a Membrane-Associated Enzyme†

Dennis E. McGee and John H. Richards*

ABSTRACT: A new isolation procedure for propanediol dehydratase increases by a factor of about 16 the yield of enzyme obtainable from *Klebsiella pneumoniae*; the enzyme thus isolated has a specific activity of 95 ± 4 units/mg. The apoenzyme consists of four subunits with molecular weights of 60000, 51000, 29000, and 15000 (±5%). In this new procedure, care was taken to prevent the partial proteolysis of the propanediol dehydratase which seems to occur in earlier procedures. The other novel aspect recognizes that the enzyme is associated with the cell membrane. Accordingly, after gentle sonication, the membrane fragments are separated from cytosol, and the enzyme is solubilized by extraction with buffers containing detergent. The 60000-dalton subunit has been purified and a partial sequence (34 of the 36 N-terminal residues) determined.

Diol dehydratase [(*RS*)-1,2-propanediol hydrolyase; EC 4.2.1.28] from *Klebsiella pneumoniae* (ATCC 8724) is an example of a large group of enzymes which utilize adenosylcobalamin (coenzyme B_{12}) as a specific cofactor and catalyze a variety of molecular rearrangements. In the case of diol dehydratase, the reaction is the rearrangement of 1,2-propanediol to 1,1-propanediol and its subsequent stereospecific dehydration to propionaldehyde (Zagalak et al., 1966; Retey et al., 1966). The role of the adenosylcobalamin cofactor in the catalytic mechanism is fairly well understood (Abeles & Zagalak, 1966; Frey & Abeles, 1966; Moore et al., 1979); the role of the protein in catalysis (Bachovchin et al., 1977) remains obscure.

An efficient procedure for isolating the enzyme in pure form and in high yield from its bacterial source would greatly assist studies of the role of the protein in catalysis, but existing procedures (Abeles, 1966; Poznanskaya et al., 1979) do not give especially high yields and produce enzyme which seems to have undergone significant proteolysis during isolation. This work focuses on a modified procedure for isolating pure propanediol dehydratase from *Klebsiella pneumoniae* in high yield and the preliminary characterization of the subunit structure of the resulting protein and the N-terminal amino acid sequence of the 60K subunit.

Materials and Methods

Bacteria. Klebsiella pneumoniae (ATCC 8724) were grown in the presence of glycerol and 1,2-propanediol according to the procedure of Lee & Abeles (1962). Autolyzed brewer's yeast used in the growth medium was obtained from Amber Laboratories, Juneau, WI. The bacteria were harvested by using a Beckman J-21C continuous-flow centrifuge equipped with a JCF-Z rotor. The pelleted cells were weighed and then washed with 0.01 M tris(hydroxymethyl)aminomethane (Tris)[1] (5 mL/g of wet cells) and pelleted by centrifugation in a GSA rotor for 1 h at 10000 rpm. The cells were then resuspended in deionized, distilled water and lyophilized.

Isolation of Diol Dehydratase. The lyophilized cells (5–15 g) were suspended in 10 mL/g of 10 mM DTT and 0.1 mM PMSF adjusted to pH 8.2–8.4 with 40% potassium hydroxide. The cells were then sonicated between 5 and 15 °C for a total time of 1 min/g of lyophilized cells by using 1-min bursts (240 W) from a Braunsonic 1510 sonicator, allowing 3 min between each burst.

After centrifugation of the sonicate suspension at 100000*g* for 1 h in a Beckman L2-75B ultracentrifuge, the supernatant was decanted and discarded. The pellet was then suspended in about 100 mL of 1% Triton X-100, 1% potassium cholate, 1 mM EDTA, and 0.1 mM PMSF, pH 8.8. After stirring the mixture in the cold for 10–15 min, it was spun at 100000*g* for 1 h. The detergent extract was decanted and saved, the pellet was again treated with detergents as described above, and the centrifugation was repeated. The second detergent extract was pooled with the first, and they were concentrated to 50 mL with an Amicon Diaflo apparatus on a PM-30 membrane. The concentrated sample was then applied to a 5 × 81 cm Sepharose 6B gel filtration column equilibrated with buffer G.

Fractions comprising the major activity peak (approximately 500000 daltons) of the gel filtration column were pooled (see Figure 1) and applied to a 2.5 × 15 cm Cellex-D (Bio-Rad) ion-exchange column equilibrated with buffer G. After the sample was loaded, the column was washed with two bed volumes of buffer G followed by four bed volumes of 1 M KCl in buffer G. The column was again washed with two bed volumes of buffer G, and then a linear gradient (0–100% DBSGP) was used to elute diol dehydratase from the column. The mixing flask contained 250 mL of buffer G to which was added 250 mL of DBSGP. In this manner, one can elute diol dehydratase in essentially pure form; the pooled fractions have a specific activity of 95 ± 4 units/mg, as shown in Figure 2.

Assays. Propanediol dehydratase activity was assayed by using yeast alcohol dehydrogenase (Sigma) and NADH (Sigma) to reduce propionaldehyde to 1-propanol (Bachovchin et al., 1977). The production of propionaldehyde by diol dehydrase was measured by monitoring the decrease of absorbance at 340 nm due to oxidation of NADH to NAD in

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received December 19, 1980.* This is Contribution No. 6366. This work was supported by National Institutes of Health Grant GM16424. D.E.M. also acknowledges support from National Institutes of Health Traineeship GM07616.

[1] Abbreviations used: buffer G, 10% glycerol, 5% 1,2-propanediol, and 0.01 M K_2HPO_4, pH 8.0; Cellex-D, diethylaminoethylcellulose; DBSGP, 1% deoxycholate, 1% Brij 58 [poly(oxyethylene)$_{20}$ cetyl ether], 0.1% $NaDodSO_4$, 0.05 M Tris, 0.02% sodium azide, and 1 mM EDTA in buffer G, pH 8.0; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NAD and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms, respectively; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; RIPA buffer, 1% Triton X-100, 1% sodium deoxycholate, 0.1% $NaDodSO_4$, 0.15 M NaCl, and 0.05 M Tris-HCl, pH 7.2.

0006-2960/81/0420-4293$01.25/0

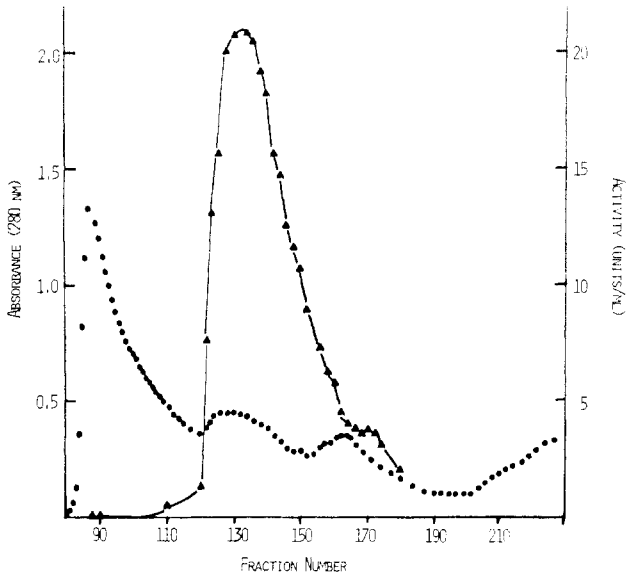

FIGURE 1: Activity–elution profile from a 5 × 81 cm Sepharose 6B column equilibrated with buffer G. Circles denote absorbance, and triangles denote activity. The K_{av} of the major activity peak was 0.30, which corresponds to a molecular weight of 500 000. The minor activity peak has a K_{av} of 0.55, corresponding to a molecular weight of 80 000. Fractions 110–150 were pooled (pool I) and purified further on Cellex-D; a $NaDodSO_4$–polyacrylamide gel of pool I is shown in Figure 4, sample 5. Pool II (fractions 151–180) was not purified further; however, a $NaDodSO_4$ gel is shown in Figure 4, sample 6.

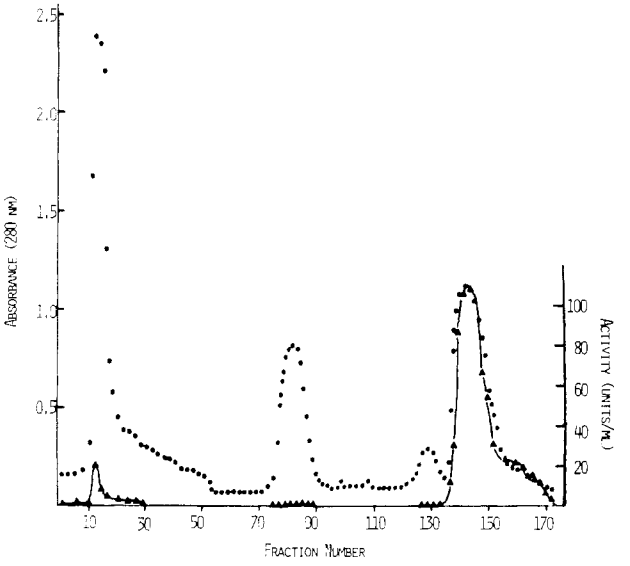

FIGURE 2: Activity–elution profile from a 2.5 × 15 cm Cellex-D anion-exchange column. Circles denote absorbance, and triangles denote activity. Fractions were not collected until the buffer reservoir was switched to 1 M KCl in buffer G. The upper reservoir was switched to buffer G while fraction 41 was being collected, and the gradient was started while fraction 58 was being collected. The gradient flasks were nearly empty while fraction 136 was collected, so DBSGP was added to the upper reseroivr to complete the elution. Fractions 136–170 were pooled together, and the $NaDodSO_4$ gel of the enzyme is shown in Figure 4, sample 7.

the presence of excess alcohol dehydrogenase. Reaction volumes were between 2.0 and 2.5 mL, and the reaction was initiated by the addition of 30 μL of a 2 mg/mL solution of adenosylcobalamin (Sigma) in the dark. All reaction mixtures were equilibrated at 37 °C. Assays were carried out on a Beckman Acta CIII. Mixing was effected by Teflon-coated stirring beads in the bottom of each cuvette. One unit of activity is defined as the amount of enzyme that will decompose 1 μmol of 1,2-propanediol per min at saturating concentrations.

Protein concentrations were determined by the method of Lowry et al. (1951). Bovine serum albumin was used as a standard for the protein assay, and a buffer blank was used for a zero-protein reference because the detergents present interfere with the Lowry assay.

Preparation of Diol Dehydratase Subunits. Purified diol dehydratase was heated for 5 min in gel sample buffer (15 mg/mL DTT, 3% $NaDodSO_4$, and 10% glycerol in 0.125 M Tris, pH 6.8), and approximately 5 mg was electrophoresed according to Laemmli (1970) on a preparative polyacrylamide gel slab (15 × 11 × 0.6 cm), with the separating gel being 12% acrylamide and 0.32% bis(acrylamide) and a stacking gel which was 3% acrylamide and 0.08% bis(acrylamide). Electrophoresis was done at 20 mA for 18 h, after which the gel was stained in 0.25% Coomassie Brilliant Blue, 25% ethanol, and 8% acetic acid for 5–10 min. The bands were visualized over a light box, and the subunits were cut from the gel. The protein was removed from the gel by electroelution into a dialysis bag in 0.19 M glycine, 0.025 M Tris, and 0.1% $NaDodSO_4$, pH 8.5, for 18 h. The protein solutions were dialyzed against several changes of 0.15 M NaCl and 0.02% $NaDodSO_4$ and then against 0.01% $NaDodSO_4$ in distilled, deionized water for 48 h, after which they were lyophilized and aliquots were reelectrophoresed to confirm their purity.

Hydrolysis of the 60K Subunit. The lyophilized protein was taken up in a known volume of glass-distilled deionized water and was frozen at −70 °C until ready for use. Samples containing approximately 0.2 nmol of peptide were hydrolyzed in 6 N HCl or 3 M mercaptoethanesulfonic acid at 106 °C for 12, 24, 48, and 72 h in deoxygenated ignition tubes. At the end of the hydrolysis, samples were frozen at −70 °C until the amino acid analyses could be performed. Amino acid analyses were done on a Durrum D-500 amino acid analyzer. Values for serine and threonine were extrapolated to zero time; values for the hydrophobic residues leucine, isoleucine, and valine were extrapolated to infinite time. No corrections were necessary for amino acids present in buffers (determined by analyzing unhydrolyzed protein).

N-Terminal Sequence Analysis. Automated Edman degradation of the 60K subunit was performed by Dr. M. W. Hunkapiller with a spinning cup sequenator designed and constructed at Caltech (Hunkapiller & Hood, 1980), using a previously described computer program (Hunkapiller & Hood, 1978). Phenylthiohydantoin derivatives of amino acids were identified by high-performance liquid chromatography on a Du Pont Zorbax CN column, using the procedures and standard chromatograms described elsewhere (Johnson et al., 1979; Hunkapiller & Hood, 1980).

Results

A number of factors influence the amount of diol dehydratase which is solubilized by sonication of the bacteria, including the length of bursts, the pH of the suspension, and lyophilization of the cells after harvesting. In contrast, the ionic strength of the sonicate suspension does not seem critical; the same amount of enzyme was solubilized under given conditions of pH and length of bursts at ionic strengths of 0.6 and 0.02 M. Under the conditions described, up to 93% of the diol dehydratase activity initially present in the bacteria could be pelleted by centrifugation of the sonicate suspension at 100000*g* for 1 h. By thus separating the diol dehydratase from proteins in the cytosol, one obtains approximately 6-fold purification.

Three different methods could be used to solubilize diol dehydratase from the sonicate pellet. (i) The pellet can be resuspended and resonicated continuously for 20–30 min (this

Table I: Purification of Diol Dehydratase

Purification Step	Protein (mg)	Activity (u)	Specific Activity (u/mg)	Yield (%)
Method #1				
Crude extract	226	54	0.24	100
Protamine sulfate	154	41	0.27	77
30% ammonium sulfate	1.19	15.4	13	28
52% K_2HPO_4	0.28	13.3	47	24
Cellex-D chromatography	0.19	11.3	60	21
Method #2				
Crude extract	350	266	0.76	100
Protamine sulfate	176	223	1.27	84
40% ammonium sulfate	70	162	2.31	61
First DEAE-cellulose column	18.9	114	6.05	43
Hydroxylapatite chromatography	5.6	82	14.8	31
Sepharose 6B chromatography	2.9	69	23.7	26
Second DEAE-cellulose column	0.52	47.8	91.5	18
Method #3				
Sonicated cells	350	1058[a]	3.02	100
Detergent extract of membrane	57.6	980[b]	17	93
Sepharose 6B chromatography	31.8[c]	889	28[c]	84
Cellex-D chromatography	8.3	802	95	76

[a] This is the maximum amount of activity that could be solubilized by extended periods of sonication with efficient cooling.

[b] The amount of activity in the detergent extracts was determined by the difference between the amount of activity solubilized by carefully rupturing the cells and the total activity known to be present in the cells.

[c] These values are uncertain due to the interfence of Triton X-100 with the protein assay.

This table summarizes the three main procedures used in purifying diol dehydrase. Method #1 is essentially that of Abeles (1963), method #2 was used by Poznanskaya et al (1979), and method #3 is described in this report. In order to make meaningful comparisons, the values reported have been normalized to one gram, lyophilized weight, of bacteria (this corresponds to approximately five grams of wet cells). Activity is reported in units (u) which were previously defined.

requires efficient cooling if thermal denaturation is to be avoided). (ii) The pellet can be extracted with detergents as described. (iii) The pellet can be washed many times with pH 9.6 buffers containing no detergents. These results suggest that the diol dehydratase is associated with the cell membrane.

Diol dehydratase as purified by this procedure contains no carbohydrate. Thus, an $NaDodSO_4$–polyacrylamide gel of the enzyme shows no staining using the PAS procedure of Fairbanks et al. (1971) to detect carbohydrate. (In this test for carbohydrate, ascites was used as a positive reference and bovine serum albumin as a negative reference.)

Diol dehydratase does not seem to require lipids or phospholipids for catalytic activity. Thus, extraction of the enzyme from the pellet with Triton X-100 and passage through a Sepharose 6B column preequilibrated with buffer G containing 2% Triton X-100 produced an enzyme with undiminished catalytic activity (Jacobs et al., 1966; Ne'eman et al., 1972).

Treatment of the crude extracts with charcoal (Poznanskaya et al., 1979) was unnecessary as no more than 5% of the protein was present as active holoenzyme. Accordingly, further purification of the detergent extracts involved gel filtration, which separates the larger diol dehydratase from smaller proteolytic enzymes. Omission of this step produces diol dehydratase contaminated with a very small amount of protease, which causes noticeable decomposition of the diol dehydratase within 4 weeks (see Figure 3). For the prevention of proteolysis of the enzyme after extraction from the membrane, EDTA and PMSF were included in the detergent solutions; PMSF inhibits serine proteases such as trypsin and chymotrypsin (Fahrney & Gold, 1963), and EDTA inhibits proteases, such as thermolysin, which require divalent metal ions for activity (Matsubara, 1970).

In the absence of DBSGP, diol dehydratase has an exceedingly high affinity for Cellex-D; however, less than 5% of the activity was eluted by washing the column with 1 M KCl in buffer G. This activity probably represents proteolyzed derivatives of the native enzyme.

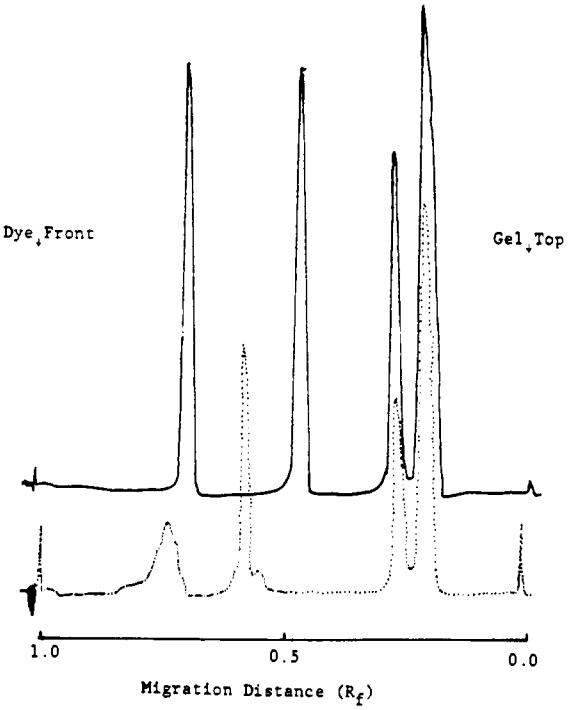

FIGURE 3: Gel scans of diol dehydratase. The scan depicted by the solid line represents freshly purified diol dehydratase in which the Sepharose 6B gel filtration was omitted; the lower scan is of the same sample 4 weeks later. The vertical axis measures the absorbance at 550 nm. Bromophenol Blue was the dye front marker. The approximate molecular weights of the bands in the proteolyzed sample are 60 000, 51 000, 25 000, 23 000, 15 000, and 14 000. For improved resolution, another aliquot of the proteolyzed sample was rerun on a $NaDodSO_4$ exponential gradient (8–18% acrylamide) gel (see Figure 6).

Table I compares this isolation procedure with previous methods. This procedure yields about 16 times the enzyme obtained by Poznanskaya et al. (1979) and 44 times that reported by Abeles (1963). Figure 4 shows polyacrylamide

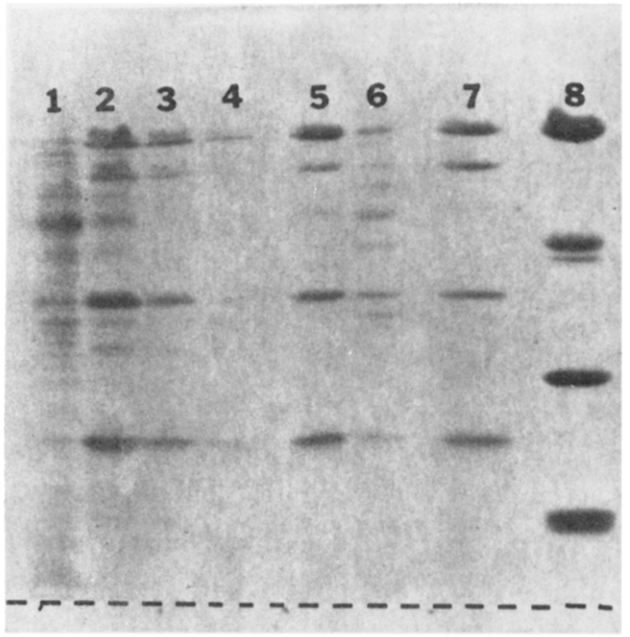

FIGURE 4: $NaDodSO_4$–polyacrylamide slab gel (12% acrylamide). Sample 1, crude sonicate (supernatant of 25000*g* spin); samples 2–4, detergent extracts of the membrane; sample 5, pool I (Figure 1); sample 6, pool II (Figure 1); sample 7, purified diol dehydratase; sample 8, diol dehydratase purified by the method of Abeles (1966). The dashed line depicts the migration of Bromophenol Blue.

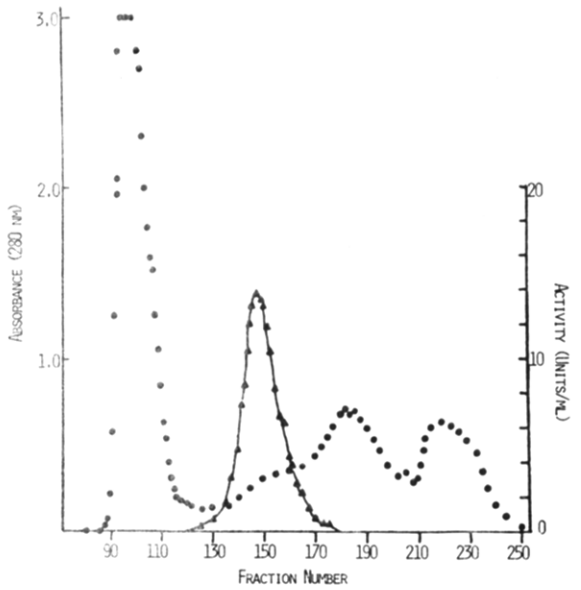

FIGURE 5: Activity–elution profile from a 5 × 81 cm Sepharose 6B column equilibrated with DBSGP. Circles denote absorbance, and triangles denote activity. The K_{av} of the activity peak was 0.39, which corresponds to a molecular weight of about 250 000. This sample came from a sonicate pellet that was extracted with DBSGP.

gels of the enzyme during isolation. Enzyme isolated by the earlier procedures invariably shows evidence of proteolysis.

Molecular Weight and Subunits. Previously reported molecular weights for diol dehydratase vary from 230 000 (Poznanskaya et al., 1977, 1979) to 250 000 (Essenberg et al., 1971). In the presence of DBSGP [which is similar to the RIPA buffer used by Collett & Erikson (1978) to prevent protein aggregation], gel filtration on Sepharose 6B gives a relatively symmetrical elution peak corresponding to a molecular weight of 250 000 (Figure 5). In the absence of DBSGP, the enzymatic activity elutes in a less symmetrical peak corresponding to an approximate molecular weight of 500 000 (Figure 1). (Figure 1 also shows a small shoulder of

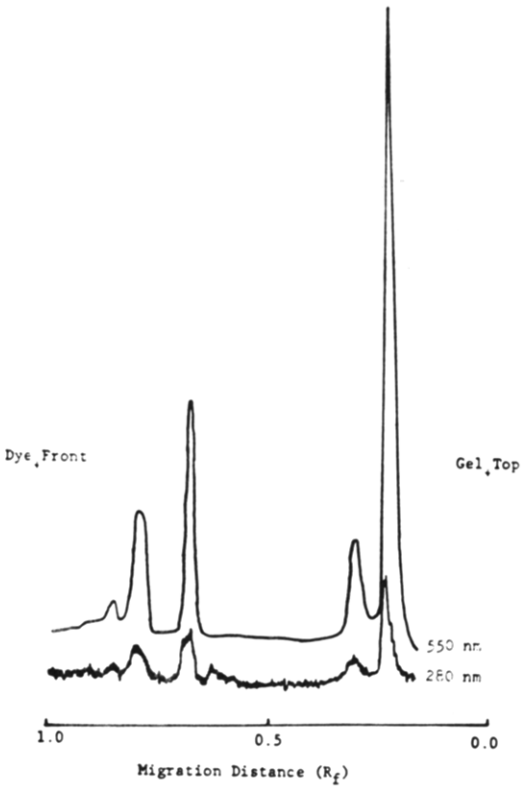

FIGURE 6: Gel scan of a $NaDodSO_4$–8–18% polyacrylamide exponential gradient slab gel. The sample is the same proteolyzed enzyme that is shown in Figure 3. The scans at 280 and 550 nm demonstrate that the 25 000-dalton peptide does not stain very well with Coomassie Brilliant Blue. The gradient also helps to resolve the 15 000-dalton peptide from the 14 000-dalton peptide.

AMINO ACID SEQUENCE

```
MET-ARG-SER-LYS-ARG-PHE-GLU-ALA-LEU-ALA-LYS-ARG-PRO-VAL-ASN
                   5                  10                  15
GLN-ASP-GLY-PHE-VAL-LYS-GLU-(?)-ILE-GLU-GLU-GLY-PHE-ILE-ALA
                  20                  25                  30
MET-GLU-SER-(PRO)-ASN-ASP-
```

FIGURE 7: N-Terminal sequence for the 60K subunit of diol dehydratase. Residues in parentheses reflect uncertainties in the identity of a residue.

activity with a molecular weight of approximately 80 000; due to its small amount, further studies of this activity have not been undertaken.)

$NaDodSO_4$–polyacrylamide gel electrophoresis of diol dehydratase shows four types of subunits of 60 000, 51 000, 29 000, and 15 000 daltons. This composition differs somewhat from that reported previously (Poznanskaya et al., 1979) of four subunits of 60 000, 23 000, 15 500, and 14 000 daltons in "fraction S" and a fifth subunit of 25 500 daltons in "fraction F", which is reported to stain poorly with Coomassie Brilliant Blue (Poznanskaya et al., 1977). Figures 3 and 6 suggest that the polypeptides of 25 500, 23 000, and 14 000 daltons may be products of proteolysis of the native subunits.

Every preparation of diol dehydratase has the 60 000-dalton subunit [Figure 4 and Poznanskaya et al. (1979)] which has been further characterized by determination of its amino acid composition (Table II) and N-terminal sequence (Figure 7).

Discussion

Previous procedures for the purification of diol dehydratase (Abeles, 1966; Poznanskaya et al., 1979) were developed on the supposition that the enzyme existed in the cytosol, which

Table II: Amino Acid Composition of the 60K Subunit of Diol Dehydratase

amino acid	% composition	no. of residues
Asx	11.4	63
Thr	4.8	26
Ser	6.1	34
Glx	11.5	64
Pro	5.5	30
Gly	9.1	50
Ala	10.5	58
Cys	0.5	3
Val	7.8	43
Met	3.7	20
Ile	5.1	28
Leu	7.0	39
Tyr	2.7	15
Phe	3.2	18
His	1.4	8
Trp	0.4	2
Lys	4.4	24
Arg	4.9	27
		552 total

seemed confirmed by finding that soluble, active enzyme could in fact be obtained by mechanically rupturing the bacteria. However, Table I shows that no more than 25% of the total diol dehydratase activity was solubilized by their procedures. Detergents can solubilize the remaining activity, which suggested that the enzyme may, in fact, be associated with the cell membrane. Accordingly, we developed conditions of sonication which allowed separation of the cytosol and membrane components of the cell. By these procedures, only 7% of the total enzyme was solubilized when the bacteria were ruptured. The cytosol could be separated from the membrane fragments and the bulk of the activity liberated on detergent treatment of the membrane fragments. This procedure not only allows recovery of more activity (by more than an order of magnitude) than previously possible but also produces an initial preparation of higher purity so that fewer subsequent steps are necessary to obtain pure enzyme. Homogeneity of the final diol dehydratase was further significantly improved by prevention of proteolysis during isolation by inhibition of serine proteases with PMSF and of enzymes requiring metal ions by EDTA.

Two general classes of proteins associated with membranes have been termed "extrinsic" and "intrinsic" (Singer, 1971). In many cases, an unambiguous assignment to one of these two limiting categories is not possible (Vanderkooi, 1974). Diol dehydratase lacks at least two properties often characteristic of intrinsic membrane proteins; it apparently lacks carbohydrate and does not require phospholipids for catalytic activity. On the other hand, the enzyme, as isolated by the procedure described here, has a relatively low solubility (<10 mg/mL) and aggregates in the absence of mixed micellular detergents; these two behaviors characterize intrinsic membrane proteins (Vanderkooi, 1974). The amount of diol dehydratase solubilized during sonication seems insensitive to the ionic strength of the aqueous phase; cytochrome *c*, an extrinsic protein which is associated with the membrane surface primarily through electrostatic interactions, is solubilized to an appreciably greater extent at higher ionic strength (Jacobs & Sandai, 1960). Increasing the pH of the sonicate suspension increases the amount of diol dehydratase which is solubilized, and, also, the enzyme can be removed by nondetergent buffers. All these observations taken together suggest that the enzyme is similar to other bacterial membrane proteins which, though essentially extrinsic, nevertheless have some interaction with the hydrophobic region of the lipid bilayer (Panefsky & Tzagoloff, 1971). A specific analogue might be the protein which causes the ATPase complex to be sensitive to oligomycin (MacLennon & Tzagoloff, 1968).

Until removal of Triton X-100, the specific activity of the enzyme could not be accurately determined; the aromatic ring of this detergent interferes with the protein assayed by the method of Lowry et al. (1951). Chromatography on Cellex-D produced a symmetrical absorbance band which eluted at low ionic strength and at the beginning of the detergent gradient (Figure 2, fractions 75–90). This band contained no protein (by $NaDodSO_4$ gel electrophoresis) and had a UV spectrum similar to that of Triton X-100 in the same buffer. These facts suggest that this band is due to a nonionic detergent (Triton X-100) which has been displaced from the diol dehydratase by another detergent (Tanford & Reynolds, 1976).

In the absence of detergent, diol dehydratase has a molecular weight of about 500000. This resembles the molecular weight of ethanolamine deaminase (Kaplan & Stadtman, 1968) which has two active sites per molecule (Babior & Li, 1969; Babior, 1969). Interestingly, the enzyme isolated by Poznanskaya et al. (1979) does not aggregate in the absence of detergents and has a significantly higher solubility than the enzyme isolated by the procedure described here. Proteolytic removal of hydrophobic regions could account for these properties of the diol dehydratase obtained earlier.

The 60000-dalton peptide seems always to be associated with the diol dehydratase complex; in our work, it appears in every preparation of active enzyme and, moreover, has an amino acid composition and sequence which can account for the results of Poznanskaya et al. (1979). For example, the amino acid composition indicates that this subunit contains three cysteines. Since the subunits are not held together by disulfide bonds (Poznanskaya et al., 1979; K. W. Moore and D. E. McGee, unpublished experiments), one cysteine at least of the 60000-dalton peptide should be available for reaction with alkylating agents, and the S fraction is known to be inactivated by alkylation of a free sulfhydryl group (Toraya et al., 1974). Furthermore, Figure 7 shows that methionine is the N-terminal residue of the 60000 subunit; methionine has previously been found to be one of the N-terminal residues upon dansylation of the S fraction (Poznanskaya et al., 1977).

The apparent association of diol dehydratase with the bacterial membrane in vivo may be relevant to the irreversible inactivation in vitro of diol dehydratase by glycerol (Bachovchin et al., 1977) and to the recent report (Honda et al., 1980) that the in vivo system continually regenerates active enzyme which, under in vitro conditions, is irreversibly inactivated.

Acknowledgments

We thank Vince Farnsworth for performing the amino acid analyses.

References

Abeles, R. H. (1966) *Methods Enzymol. 9*, 686.
Abeles, R. H., & Zagalak, B. (1966) *J. Biol. Chem. 241*, 1245.
Babior, B. M. (1969) *J. Biol. Chem. 244*, 2917.
Babior, B. M., & Li, T. K. (1969) *Biochemistry 8*, 154.
Bachovchin, W. W., Eagar, R. G., Jr., Moore, K. W., & Richards, J. H. (1977) *Biochemistry 16*, 1082.
Collett, M. S., & Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2021.
Essenberg, M. K., Frey, P. A., & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242.
Fahrney, D. E., & Gold, A. M. (1963) *J. Am. Chem. Soc. 85*, 997.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.
Frey, P. A., & Abeles, R. H. (1966) *J. Biol. Chem. 241*, 2732.
Honda, S., Toraya, T., & Fukui, S. (1980) *J. Bacteriol. 143*, 1458.
Hunkapiller, M. W., & Hood, L. E. (1978) *Biochemistry 17*, 2124.
Hunkapiller, M. W., & Hood, L. E. (1980) *Science (Washington, D.C.) 207*, 523.
Jacobs, E. E., & Sandai, O. R. (1960) *J. Biol. Chem. 235*, 231.
Jacobs, E. E., Andrews, E. C., Cunningham, W., & Crane, F. L. (1966) *Biochem. Biophys. Res. Commun. 25*, 87.
Johnson, N. D., Hunkapiller, M. W., & Hood, L. E. (1979) *Anal. Biochem. 100*, 335.
Kaplan, B. H., & Stadtman, E. R. (1968) *J. Biol. Chem. 243*, 1794.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lee, H. A., & Abeles, R. H. (1962) *J. Biol. Chem. 236*, 2347.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
MacLennon, D. H., & Tzagoloff, A. (1968) *Biochemistry 7*, 1603.
Matsubara, H. (1970) *Methods Enzymol. 19*, 642.
Moore, K. W., Bachovchin, W. W., Gunter, J. B., & Richards, J. H. (1979) *Biochemistry 18*, 2776.
Ne'eman, Z., Kahane, I., Karvartovsky, J., & Razin, S. (1972) *Biochim. Biophys. Acta 266*, 255.
Panefsky, H. S., & Tzagoloff, A. (1971) *Methods Enzymol. 22*, 204.
Poznanskaya, A. A., Tanizawa, K., Soda, K., Toraya, T., & Fukui, S. (1977) *Bioorg. Khim. 3*, 111.
Poznanskaya, A. A., Tanizawa, K., Soda, K., Toraya, T., & Fukui, S. (1979) *Arch. Biochem. Biophys. 194*, 379.
Retey, J., Umani-Ronchi, A., Seibl, J., & Arigoni, D. (1966) *Experientia 22*, 502.
Singer, S. J. (1971) *Structure and Function of Biological Membranes* (Rothfield, L. I., Ed.) pp 145–222, Academic Press, New York.
Tanford, C., & Reynolds, J. A. (1976) *Biochim. Biophys. Acta 457*, 133.
Toraya, T., Uesaka, M., & Fukui, S. (1974) *Biochemistry 13*, 3895.
Toraya, T., Shirakashi, T., Kosoga, T., & Fukui, S. (1976) *Biochem. Biophys. Res. Commun. 69*, 745.
Vanderkooi, G. (1974) *Biochim. Biophys. Acta 344*, 307.
Zagalak, B., Frey, P. A., Karabatsos, G. L., & Abeles, R. H. (1966) *J. Biol. Chem. 241*, 3028.

Synthesis and Characterization of a New Fluorogenic Active-Site Titrant of Serine Proteases†

D. Campbell Livingston, John R. Brocklehurst, John F. Cannon,‡ Steven P. Leytus, John A. Wehrly,§ Stuart W. Peltz, Gary A. Peltz,⊥ and Walter F. Mangel*

ABSTRACT: The molecule 3′,6′-bis(4-guanidinobenzoyloxy)-5-[*N*′-(4-carboxyphenyl)thioureido]spiro[isobenzofuran-1-(3*H*),9′-[9*H*]xanthen]-3-one, abbreviated FDE, was designed and synthesized as a fluorogenic active-site titrant for serine proteases. It is an analogue of *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) in which a fluorescein derivative is substituted for *p*-nitrophenol. FDE and NPGB exhibit similar kinetic characteristics in an active-site titration of trypsin in phosphate-buffered saline, pH 7.2. The rate of acylation with FDE is extremely fast (k_2 = 1.05 s^{-1}) and the rate of deacylation extremely slow (k_3 = 1.66 × 10^{-5} s^{-1}). The K_s is 3.06 × 10^{-6} M, and the $K_{m(app)}$ is 4.85 × 10^{-11} M. With two of the serine proteases involved in fibrinolysis, the rate of acylation with FDE is also fast, k_2 = 0.112 s^{-1} for urokinase and 0.799 s^{-1} for plasmin, and the rate of deacylation is slow, k_3 = 3.64 × 10^{-4} s^{-1} for urokinase and 6.27 × 10^{-6} s^{-1} for plasmin. The solubility limit of FDE in phosphate-buffered saline is 1.3 × 10^{-5} M, and the first-order rate constant for spontaneous hydrolysis is 5.1 × 10^{-6} s^{-1}. The major difference between FDE and NPGB is the detectability of the product in an active-site titration. *p*-Nitrophenol can be detected at concentrations no lower than 10^{-6} M whereas fluorescein can be detected at concentrations as low as 10^{-12} M. Thus, FDE should be useful in quantitatively assaying serine proteases at very low concentrations.

The role of proteolytic enzymes in a wide variety of biological processes is being increasingly recognized (Reich et al., 1975). Proteolysis is involved in the temporal and spatial control of enzyme function, metabolic and secretory pathways, morphogenesis, and cell surface interactions. Proteases are intimately involved not only in such processes as blood coagulation, fibrinolysis, and digestion but also in ovulation (Beers et al., 1975), cell migration (Ossowski et al., 1973a,b, 1975), tumorigenicity (Unkeless et al., 1973; Ossowski et al., 1973a,b; Pollack et al., 1974; Christman et al., 1975; Jones et al., 1975), embryogenesis (Strickland et al., 1976), recombination and derepression (Miskin & Reich, 1980), etc. For many proteins, proteolysis occurs at an early step in their synthesis, is required for activation of enzymatic activity, and is the final step leading to their destruction. The discovery of additional roles for proteases in biological processes is, in part, dependent upon the availability of more sensitive and quantitative assays.

† From the Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England (D.C.L.), Standard Telecommunication Laboratories, Ltd., Harlow, Essex, England (J.R.B.), and the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801 (J.F.C., S.P.L., J.A.W., S.W.P., G.A.P., and W.F.M.). *Received November 24, 1980.* This investigation was supported by Grant CA 25633 from the National Institutes of Health and by a Biomedical Research Support Grant from the University of Illinois.

‡ Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.
§ Present address: E. I. du Pont de Nemours & Co., Inc., Experimental Station, Wilmington, DE 19898.
⊥ Present address: Stanford University School of Medicine, Stanford, CA 94305.

0006-2960/81/0420-4298$01.25/0