Re-Investigation of the Protein Structure of Coenzyme B_{12}-Dependent Diol Dehydrase

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

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We have purified diol dehydrase, an adenosylcobalamin-dependent enzyme, from *Klebsiella pneumoniae* by two different procedures to re-investigate its protein structure; one including its extraction with detergent from the membrane fraction, and the other consisting of only chromatographic separations of the soluble fraction. The enzyme preparations obtained by these two methods were different in the subunit structure, but both are identical in molecular weight, and in enzymological and immunochemical properties. In addition, the enzyme preparation obtained from the membrane fraction dissociated reversibly into two dissimilar protein components (F and S) in the absence of substrate, as did the preparation from the soluble fraction. Although the subunit multiplicity of component S might be partly due to proteolytic cleavage during the enzyme purification as revealed by limited digestion with trypsin, component F is not a product of proteolytic cleavage of component S, but a primordial and essential constituent of the enzyme.

Introduction

Diol dehydrase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) is an adenosylcobalamin (vitamin B_{12} coenzyme)-dependent enzyme which catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to propionaldehyde, acetaldehyde, and β-hydroxypropionaldehyde, respectively (see ref. [1] for a recent review). We have previously shown that the enzyme purified to homogeneity from *Klebsiella pneumoniae* ATCC 8724 has a molecular weight of about 230,000 [2], and is composed of two different components, F (M_r, 26,000) and S (M_r, 200,000), both of which are indispensable for coenzyme binding and catalysis [2–4]. Component S consists of at least four nonidentical subunits [2]. Studies with several chemical modification reagents revealed the presence of essential sulfhydryl group(s) [4–8] probably in component S [4] and a lysyl residue in component F which is essential for interaction between the two components [9]. An essential arginyl residue located at or in close proximity to the active site has also been identified [10].

Diol dehydrase of *K. pneumoniae* ATCC 8724 was originally isolated and purified as a soluble protein occurring in the cytosolic fraction of the cells [2, 11]. However, a new purification procedure has been developed by McGee and Richards [12], based on their observation that nearly 4-fold higher total activities in the sonicate of cells than the previously reported activity could be pelleted by centrifugation at $100{,}000 \times g$, and that the bulk of this cell membrane-associated activity was effectively solubilized with detergents, Triton X-100 and potassium cholate. For prevention of possible proteolysis of the enzyme after extraction from the membrane, they used buffers containing EDTA and PMSF (phenylmethanesulfonyl fluoride) during the purification [12]. They reported that the enzyme purified has a subunit structure different from that of our preparation obtained from the soluble fraction [2], and suggested that the component F is a proteolytic product from one of the subunits [12, 13].

In the present study, we have purified the enzyme from both the membrane and cytosolic fractions to re-examine the subunit structure of the enzyme. Our results show clearly that component F is not an artifact produced by proteolysis during the purification, but an inherent component of the enzyme protein.

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Materials and Methods

Crystalline adenosylcobalamin was purchased from Sigma, St. Louis, Mo., U.S.A. Diol dehydrase was purified from the cytosolic fraction of *K. pneumoniae* ATCC 8724 by the method of Poznanskaja *et al.* [2], and from the membrane fraction by the method of McGee and Richards including detergent extraction [12]. The purification from the membrane fraction was carried out in the presence of 1 mM EDTA and 0.1 mM PMSF in order to protect the enzyme from the proteolytic modification [12]. Rabbit antiserum against diol dehydrase was obtained as described previously with the enzyme purified from the cytosolic fraction [14].

The enzyme was assayed with 3-methyl-2-benzothiazolinone hydrazone [15]. The activities of components F and S were determined after addition of an excess of one component to make the other rate limiting as described previously [4]. Protein concentration was measured by the method of Lowry *et al.* [16] using crystalline bovine serum albumin as the standard and by measurement of absorbance at 278 nm. An absorption coefficient ($A^{1\%}_{1\,cm}$) of 5.27 was used for the apoenzyme [2].

The enzyme from the membrane fraction was resolved into components F and S by DEAE-Sephadex A-50 chromatography as described previously [2] for the enzyme from the soluble fraction: the enzyme was dialyzed at 4 °C against 10,000 volumes of 10 mM potassium phosphate buffer (pH 8.0) with several buffer changes to remove substrates and detergents exhaustively. A high-performance liquid chromatography was also used for resolution of the enzyme into components F and S. The substrate-free enzyme solution (ca. 25 μg/25 μl) was injected to a Toyo Soda TSK-3000SW gel filtration column (7.5 × 600 mm) on a Toyo Soda HLC-803D liquid chromatograph. The protein was eluted at a flow rate of 0.7 ml/min with 10 mM potassium phosphate buffer (pH 8.0) containing 10 mM KCl with or without 2% 1,2-propanediol, and monitored by the absorption at 270 nm with a Toyo Soda UV-8 model II spectrophotometer.

Limited digestion of diol dehydrase purified from the membrane fraction was carried out at 37 °C with bovine pancreas trypsin (TPCK-untreated, Type III-S, Sigma, St. Louis, Mo., U.S.A.) at 2% weight per weight of substrate protein in 10 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol. Time course of the trypsin digestion was followed by the sodium dodecyl sulfate/polyacrylamide gel electrophoresis according to the method of Laemmli [17] with 12% gels.

Results and Discussion

Enzyme purification

We have purified diol dehydrase by both of the different procedures reported previously [2, 12] with closely similar results; the enzyme was purified from the soluble fraction in an about 25% yield with the specific activity of 100 units/mg protein, whereas it was obtained from the membrane fraction in an about 60% yield with the specific activity of 97 units/mg protein. Both the preparations migrated as a single protein band upon polyacrylamide gel electrophoresis in the presence of 2% 1,2-propanediol. It is noteworthy that the homogeneous enzyme preparations obtained by the different methods exhibit essentially the same specific activity. Thus, proteolytic cleavage during purification from the soluble fraction, if any, does not significantly affect the diol dehydrase activity. The enzyme purified from the membrane fraction is designated henceforth as the membrane-bound enzyme, and that from the soluble fraction as the cytosolic enzyme.

Comparison of properties of membrane-bound and cytosolic enzymes

The apparent Michaelis constants of the membrane-bound enzyme for several substrates were identical with the values obtained with the cytosolic enzyme [18]; 0.18 mM for 1,2-propanediol, 1.08 mM for ethanediol, and 1.54 mM for glycerol, although the K_m value for adenosylcobalamin (2.0 μM) of the membrane-bound enzyme was slightly higher than the value (0.8 μM) of the cytosolic one. The mechanism-based inactivation caused by reaction with glycerol [18–20] was also observed for the membrane-bound enzyme (k_{inact} = about 1.3 min^{-1} at 37 °C). The membrane-bound enzyme absolutely required monovalent cations such as potassium and ammonium ions for the catalytic activity (data not shown), as well as the cytosolic enzyme [21].

In addition to their identity in the enzymological properties, the membrane-bound enzyme is indistinguishable from the cytosolic enzyme when examined by Ouchterlony double-diffusion analysis [22] (Fig. 1); the homogeneous and the crude membrane-

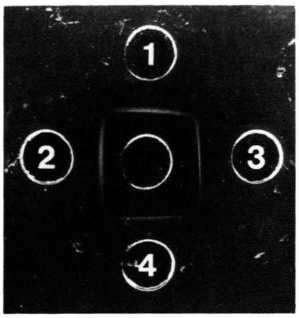

Fig. 1. Ouchterlony double-diffusion analysis of diol dehydrase isolated from the membrane and soluble fractions with antiserum against the homogeneous cytosolic enzyme. The center well was filled with the antiserum (11 μl) and peripheral wells with enzyme solutions containing 50 mM potassium phosphate buffer (pH 8.0), 10% glycerol and 2% 1,2-propanediol: well 1, crude enzyme from the membrane fraction (10 μg protein and 0.06 unit activity); well 2, crude enzyme from the soluble fraction (10 μg protein and 0.06 unit activity); well 3, purified cytosolic enzyme (0.9 unit); and well 4, purified membrane-bound enzyme (0.9 unit). The gel was incubated at room temperature in a humid atmosphere for 24 h.

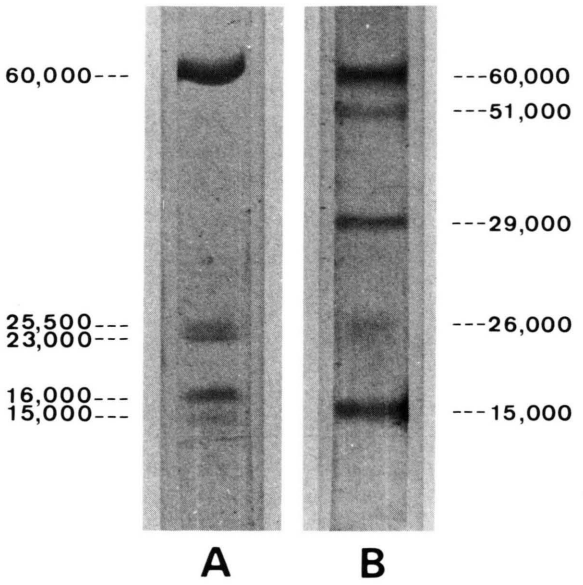

Fig. 2. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the cytosolic (A) and membrane-bound (B) diol dehydrase. The purified membrane-bound enzyme (15 μg) (B) and the purified cytosolic enzyme (20 μg) (A) were treated with 1% sodium dodecyl sulfate in 20 mM 2-mercaptoethanol at 100 °C for 5 min and electrophoresed as described by Laemmli [17] with 12% gels. Numbers beside each gel indicate molecular weight of the protein bands determined by comparison with standard proteins; phosphorylase *b* (M_r, 97,400), bovine serum albumin (68,000), egg albumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and α-lactoalbumin (14,400).

bound enzymes reacted with the polyclonal antiserum against the cytosolic enzyme to form a single precipitin band, which fused with the band formed between the cytosolic enzyme and the antiserum. This finding indicates that both the purification procedures do not affect the antigenic structures of enzyme molecule.

Subunit structure

As suggested by electrophoresis under non-denaturating conditions, diol dehydrase is composed of several dissimilar subunits, and their association is markedly promoted by the presence of 1,2-propanediol [2]. However, subunit composition of the membrane-bound enzyme, which was analyzed by electrophoresis under denaturating conditions, was reported by McGee and Richards [12] to be considerably different from that of the enzyme purified from the soluble fraction [2]. The two preparations obtained in this study also showed different patterns of subunit composition upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2). Notable differences are that the subunits with molecular weights of 51,000 and 29,000 are found only in the membrane-bound enzyme, and those with lower molecular weights (23,000 and 16,000−15,500) are exclusively in the cytosolic enzyme. The largest (M_r, 60,000) and the smallest (M_r, 15,000−14,000) subunits occur in both the enzymes. The faint protein band with a molecular weight of 25,000−26,000 detectable in both probably corresponds to component F, which is only poorly stained with Coomassie Brilliant Blue [23]. We used the buffers containing no protease inhibitors in the previous purification procedure, and two smaller subunits (M_r, 23,000 and 16,000) were found instead of two larger subunits (M_r, 51,000 and 29,000). Thus, it is likely that the enzyme (particularly subunits of M_r, 51,000 and 29,000) underwent a limited proteolysis during purification from the soluble fraction [2], as suggested by McGee and Richards [12]. The subunit stoichiometry of the membrane-bound enzyme was determined by them to be

$$(60{,}000)_2(51{,}000)_1(29{,}000)_2(15{,}000)_2$$

by quantitative analysis of amino terminal sequences of the whole enzyme [13].

Separation of membrane-bound enzyme into two components

Although the reason 1,2-propanediol is added to the buffer ("buffer G" in ref. [12]) used for the purification of the membrane-bound enzyme was not clearly described, we believe that the presence of 1,2-propanediol is essential to maintain the active structure of diol dehydrase. The substrate prevents unfavorable formation of component F, a small protein which is dissociable from the cytosolic apoenzyme complex on ion-exchange chromatography in the absence of substrate and is very labile [2–4]. Although McGee *et al.* [12, 13] suggested that component F is a proteolytic product of the membrane-bound enzyme, we have shown that the membrane-bound enzyme is also dissociated into two protein components by DEAE-Sephadex chromatography (Fig. 3). We used a fresh enzyme preparation with a specific activity of about 22 units/mg which was purified rapidly from the membrane fraction in the presence of protease inhibitors. As is the case with the cytosolic enzyme [2], no fractions alone possessed any significant activity, except a low activity (at most 10% of the total activity applied) in fractions 38–42. The enzyme activity was restored when both the proteins of fractions of the first small and the second large peaks were recombined. This also unambiguously shows that F is a primordial component of the membrane-bound diol dehydrase. The failure of McGee *et al.* [13] in separation of the membrane-bound enzyme into two components may be due to incomplete removal of substrate from the enzyme solution before the ion-exchange column chromatography. The presence of substrate even at a low concentration (1–2 mM) markedly retards the dissociation of enzyme into components (T. Toraya, unpublished results).

The membrane-bound enzyme was dissociated into two protein components as well by the high-performance size-exclusion chromatography (Fig. 4). When 1,2-propanediol (2%) was contained in the elution buffer, a symmetrical single protein peak was observed at retention time of 22.3 min. In contrast, two protein peaks, a large peak at retention time of 23.0 min and a small one at retention time of 31.0 min appeared when the substrate was not added to the elution buffer. By comparison with the retention time of standard proteins (Fig. 4, inset), the molecular weights of proteins of these three peaks

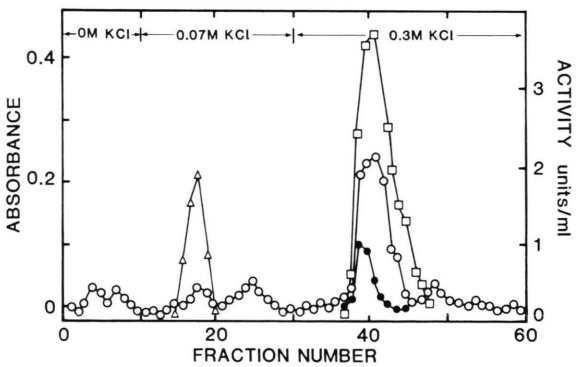

Fig. 3. Separation of membrane-bound diol dehydrase into components F and S by DEAE-Sephadex A-50 chromatography. About 4.5 mg (100 units) of the enzyme partially purified from the membrane fraction was applied to a column (1.0 × 10 cm) of DEAE-Sephadex A-50 pre-equilibrated with 10 mM potassium phosphate buffer (pH 8.0). After washing with 30 ml of the same buffer, the column was successively developed with the buffer containing 0.07 and 0.3 M KCl. The eluate was collected in 3 ml fractions at a flow rate of about 0.5 ml per min. ●, diol dehydrase activity; △, component F activity; □, component S activity; ○, absorbance at 280 nm.

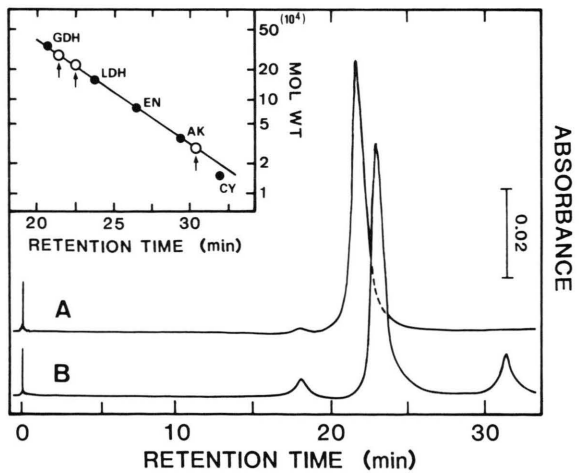

Fig. 4. Separation of membrane-bound enzyme into components by high-performance size-exclusion chromatography. The enzyme solution (25 μl) was injected (at 0 min) to a column of TSK-3000SW. The elution was carried out in the presence (A) or absence (B) of 2% 1,2-propanediol. Other experimental conditions are given in "Materials and Methods". The inset shows molecular weight determination with standard proteins; GDH (glutamate dehydrogenase, M_r, 290,000), LDH (lactate dehydrogenase, 142,000), EN (enolase, 70,000), AK (adenylate kinase, 32,000), and CY (cytochrome *c*, 12,400). The arrows indicate the positions of three protein peaks.

were estimated to be about 250,000 (the single peak in Fig. 4A), 210,000 (the large peak in Fig. 4B), and 26,000 (the small peak in Fig. 4B). These molecular weights are closely similar to the reported values [2, 12] for the enzyme complex, and components F and S, respectively. The association of the latter two components depends solely on the presence of substrate in the elution buffer; the dissociation of the membrane-bound enzyme into two components is reversible. Thus, it can be concluded that the diol dehydrase purified from the membrane fraction also is composed of two dissimilar protein components, which are separable from each other on the basis of their difference in not only ionic properties but also molecular size.

Fluorometric detection of component F

Since component F is characterized by its low stainability [23] with dyes commonly used for detection of proteins in polyacrylamide gel electrophoresis, attempts were made to find out more sensitive methods to detect it in the gels. A reactive amino group of a lysine residue which plays an essential role for the interaction between two components has been identified in component F by a chemical modification study with pyridoxal 5′-phosphate [9]. The phosphopyridoxyllysine residue produced by reduction with $NaBH_4$ of the Schiff base between pyridoxal 5′-phosphate and the amino group is highly fluorescent. Thus, component F isolated from the membrane-bound enzyme by the ion-exchange chromatography as described above was treated with pyridoxal 5′-phosphate at pH 8.0 for 30 min followed by reduction with $NaBH_4$. Electrophoresis of this labeled component F gave a sharp fluorescent peak corresponding to a molecular weight of about 26,000 (Fig. 5B). Component F in the complex was also detected by the similar procedure without isolation (Fig. 5C), since the isolated component S gave only a few minor fluorescent peaks (Fig. 5A).

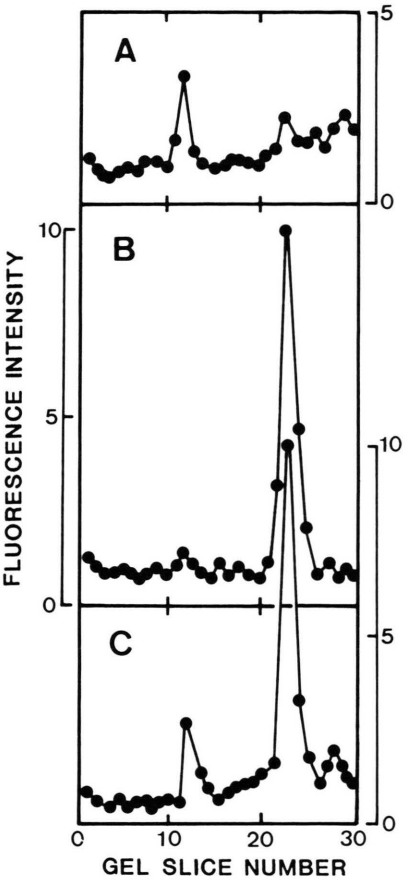

Fig. 5. Fluorometric detection of component F by amino-group modification with pyridoxal 5′-phosphate. Components S (0.9 mg) (A), F (20 μg) (B), or the homogeneous membrane-bound enzyme (1 mg) (C) was incubated with 0.14 mM pyridoxal 5′-phosphate in 5 mM potassium phosphate buffer (pH 8.0) at 25 °C for 30 min (the enzyme activity decreased to about 50% of the initial activity), followed by reduction with 1 mg/ml $NaBH_4$ at 25 °C for 20 min. After dialysis of the solution against 1,000 volumes of 5 mM potassium phosphate buffer (pH 8.0), aliquots of the dialyzed sample were subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The gels were sliced into 2-mm sections and directly measured with a Hitachi MPF-4 spectrofluorophotometer at 390 nm (excitation at 330 nm). Gel slices were numbered from the top of gel.

Limited digestion with trypsin

The only significant difference of enzyme preparations obtained by the two purification procedures was found in the subunit composition (particularly the composition of component S [2]) as described above. To examine the possibility that the cytosolic enzyme has suffered from proteolysis during purification, the homogeneous membrane-bound enzyme was digested with trypsin at 37 °C in 10 mM potassium phosphate buffer (pH 8.0) containing 2% 1,2-propanediol. Time course of the limited digestion was followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 6). By prolonged incubation, two polypeptide bands with molecular weight of 51,000 and 29,000 notice-

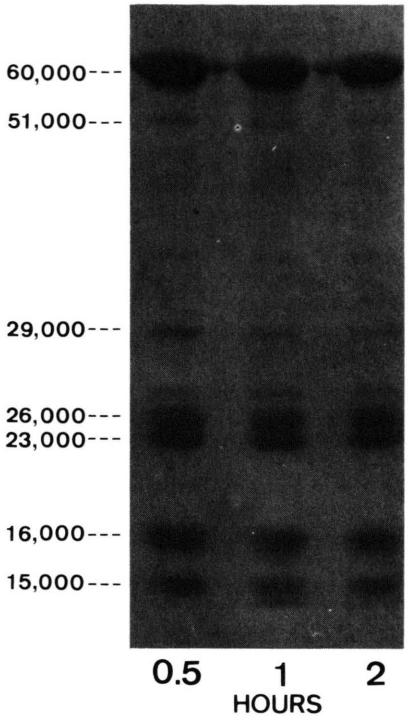

Fig. 6. Time course of limited proteolysis of the membrane-bound diol dehydrase. The homogeneous enzyme (50 μg/30 μl) isolated from the membrane fraction was digested for the indicated hours with trypsin (see "Materials and Methods" for conditions of proteolysis), and aliquots of the digested enzyme (15 μg each) were electrophoresed on 12% polyacrylamide gels in the presence of sodium dodecyl sulfate.

ably disappeared with a concomitant appearance of two bands with lower molecular weight, 23,000 and 16,000. Although the dye-stained intensity of these latter bands did not increase in proportion to the incubation time, the electrophoretic pattern of the trypsin-digested enzyme became similar to that of the cytosolic enzyme. In contrast to such a change in the electrophoretic pattern, the specific activity of the membrane-bound enzyme remained constant at least for five hours during limited digestion with trypsin, and in the presence of 2% 1,2-propanediol the digested enzyme migrated as a single protein band when electrophoresed under non-denaturating conditions.

The results obtained in the present work confirm that diol dehydrase occurs inherently in the membrane fraction, and is nicked by an intracellular proteolytic enzyme(s) if solubilized in the absence of protease inhibitors. McGee *et al.* [13] predicted that the enzyme is a peripheral membrane protein on the basis of the hydrophobicity of each subunit calculated from their amino acid compositions. The limited proteolysis occurring probably on two subunits with M_r, 51,000 and 29,000 causes no change in the enzyme activity and the structure of the apoenzyme complex. Several proteins are known which consist of two independently folding domains interconnected by a hinge peptide that is particularly susceptible to proteolysis [24–26]. One of them is the tryptophan synthase α subunit [27, 28], and its activity is not influenced by proteolytic cleavage. The two subunits of diol dehydrase (M_r, 51,000 and 29,000) may have hinge regions which are sensitive to proteolysis and not essential for enzyme activity as well.

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