Immobilized Diol Dehydrase and Its Use in Studies of Cobalamin Binding and Subunit Interaction†

Tetsuo Toraya, Kazumoto Ohashi, and Saburo Fukui*

ABSTRACT: Coenzyme B_{12} dependent diol dehydrase from *Aerobacter aerogenes* was immobilized by covalent binding to CNBr-activated Sepharose 4B. The Sepharose-bound enzyme exhibited a markedly high catalytic activity, viz., 75–95% of the specific activity of the original free enzyme. The apoenzyme acquired much greater stability to heat by immobilization. No significant difference between the immobilized and free enzymes was observed in the following properties: the affinity for coenzyme B_{12}; the sensitivity to a sulfhydryl-modifying agent; the absolute requirement for a certain monovalent cation, such as K^+, for catalysis; the susceptibility toward oxygen upon incubation with coenzyme B_{12} in the absence of substrate. These results suggest that the structure and function of the enzyme are not significantly influenced by immobilization on Sepharose. The immobilized enzyme was found to provide a convenient method for a study of ligand interaction with the enzyme. The subunit interaction between two dissimilar subunits, components F and S, was investigated using the component S immobilized on CNBr-activated Sepharose and free component F, and it was demonstrated that the substrate (1,2-propanediol) promotes the hybrid formation between component F and component S, but K^+ alone rather retarded the subunit association to some extent. Na^+ markedly weakens the forces which bind the subunits together. The relationship between cobalamin binding and subunit structure is also discussed.

Coenzyme B_{12}[1] dependent diol dehydrase (D,L-1,2-propanediol hydro-lyase, EC 4.2.1.28) from *Aerobacter aerogenes* (ATCC 8724) catalyzes the conversion of 1,2-propanediol and 1,2-ethanediol to propionaldehyde and acetaldehyde, respectively (Lee and Abeles, 1963). This enzyme is composed of two dissimilar protein components (subunits), designated components F and S, both of which are required for catalysis (Toraya et al., 1973, 1974). Neither component alone binds coenzyme B_{12} or CNB_{12}. In our earlier paper, the holoenzyme of diol dehydrase has been shown to be readily resolved into the apoenzyme and coenzyme B_{12} in the absence of inorganic monovalent cations (Toraya et al., 1971). However, it was not clear whether the coenzyme resolution necessarily resulted from dissociation of the enzyme into components F and S because the factors affecting the dissociation–association of the components have not yet been established.

Recently, methods of immobilizing enzymes on various polymer supports have been extensively developed, and immobilized enzymes have been applied for industrial, analytical, and medical purposes. In order to use an immobilized enzyme in the studies of the ligand interaction with the apoenzyme and of the subunit interaction, we made an attempt to immobilize diol dehydrase or its subunits by covalent binding to CNBr-activated Sepharose 4B particles. Activation of Sepharose by CNBr is known to lead to formation of imidocarbonate groups in this hydrophilic polymer support which are reactive with unprotonated amino groups in the protein (Porath et al., 1967; Cuatrecasas, 1970; Axén and Ernback, 1971).

In this manuscript, we report the preparation and some properties of the immobilized diol dehydrase or immobilized component S and their convenient application for studies of cobalamin binding and subunit interaction. The relationship between cobalamin binding and subunit structure is also discussed in this paper.

Materials and Methods

Materials. Crystalline coenzyme B_{12} and CNB_{12} were obtained from Glaxo Ltd., Greenford, U.K. Sepharose 4B was purchased from Pharmacia Fine Chemical Co., Uppsala, Sweden. All other chemicals used in this study were reagent grade commercial products and were used without further purification. Diol dehydrase apoenzyme was prepared from *Aerobacter aerogenes* (ATCC 8724) by the procedure similar to that of Lee and Abeles (1963), and the partially purified preparations (specific activity, about 0.8–1.5 units/mg of protein) were used for immobilization. Components F and S of diol dehydrase were separated by chromatography of the apoenzyme on DEAE-cellulose, as described before (Toraya et al., 1974). Component S was subjected to immobilization on CNBr-activated Sepharose 4B after being concentrated into a small volume with a Diaflo ultrafiltration apparatus.

Immobilization Procedures. The apoenzyme or component S of diol dehydrase was immobilized by direct coupling to CNBr-activated Sepharose 4B particles, as described by Axén and Ernback (1971). In order to minimize the number of sites in the enzyme molecule of attachment to the Sepharose matrix, limited amounts of CNBr (30 mg/ml of packed Sepharose 4B) were used; 10 ml of activated Sepharose was added to an enzyme solution (50–80 mg of protein/10 ml) containing 0.05 *M* potassium phosphate buffer (pH 8.0), 0.05 *M* KCl, and 0.10 *M* 1,2-propanediol, in a total volume of 20 ml. The reaction mixture was kept overnight at 2–4° with continuous stirring. To remove unreacted enzyme, a small column was made from the Sepharose reaction mixture and the gel was washed thoroughly with 300

† From the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto, Japan. *Recieved May 1, 1975.* Paper V in this series. The preceding paper in the series is Toraya et al. (1974). Dedicated to the memory of Professor Konrad Bernhauer.

[1] Abbreviations used are: coenzyme B_{12}, adenosylcobalamin or *Coα*-[*α*-(5,6-dimethylbenzimidazolyl)]-*Coβ*-adenosylcobamide; CNB_{12}, cyanocobalamin; PCMB, *p*-chloromercuribenzoate.

Table I: Coupling of Diol Dehydrase to CNBr-Activated Sepharose 4B.[a]

Sample	Diol Dehydrase Activity		Protein		Specific Activity
	units	%	mg	%	(units/mg)
Before coupling reaction (free enzyme)	70	100	82	100	0.85
After coupling reaction					
Sepharose 4B-bound	63	90	44	54	1.43
supernatant	0.7	1	38	46	

[a] Experimental procedure is described in the text.

Table II: Inactivation of Immobilized Apoenzyme by PCMB and Its Reversal with β-Mercaptoethanol.[a]

PCMB Concn (μM)	β-Mercaptoethanol Treatment	Propionaldehyde Formed (μmol per 10 min per 0.3 ml of Sepharose)
0	Without	8.1
0	With	6.4
10	Without	2.6
10	With	6.4
100	Without	2.2
100	With	4.8

[a] The Sepharose-bound apoenzyme (0.3 ml) was treated at 37° for 10 min with the indicated concentration of PCMB, and the mixture was then incubated at 37° for 20 min with and without 20 mM β-mercaptoethanol. The enzyme activity was determined by adding the remaining reactants of the usual assay mixture.

ml of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.10 M substrate, then with 300 ml of 0.30 M potassium phosphate buffer (pH 8.0) containing 0.10 M substrate, and finally with 100 ml of the first buffer. The Sepharose beads were then removed from the column and suspended in the same buffer in a final volume of 20 ml. No appreciable loss in enzyme activity was observed over a period of 2 months when the Sepharose-bound enzyme was stored at 0–4°.

Protein Determination. Protein was routinely determined by the procedure of Lowry et al. (1951). Crystalline bovine serum albumin was used as the standard. Sepharose-bound protein was determined in a similar manner except for the modification that the reaction mixture was stirred for 20 min and then filtered before measuring the absorbance.

Enzyme Assay. The activity of the free apoenzyme of diol dehydrase was assayed as described previously (Toraya et al., 1971). The activity of the immobilized enzyme was assayed in the reaction mixture containing 0.1–0.4 ml of packed Sepharose-bound apoenzyme, 0.2 M 1,2-propanediol, 0.05 M KCl, 15 μM coenzyme B_{12}, and 0.035 M potassium phosphate buffer (pH 8.0), in a total volume of 2 ml. The enzyme reaction was carried out at 37° for 10 min with gentle shaking. The reaction mixture was then cooled in an ice–water bath and filtered on a glass filter. The Sepharose-bound enzyme was washed with small amounts of cold water. The filtrate and the washings were collected and adjusted to 10 ml. The amount of propionaldehyde formed was determined spectrophotometrically after conversion to its 2,4-dinitrophenylhydrazone. One unit is defined as the amount of enzyme activity catalyzing the formation of 1 μmol of propionaldehyde per min under the standard assay conditions (Lee and Abeles, 1963).

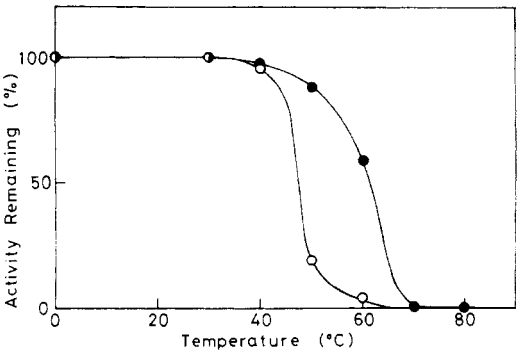

FIGURE 1: Comparison of thermal stability between the immobilized and free apoenzyme. The Sepharose-bound apoenzyme (0.3 ml) (●) was suspended in 0.26 M 1,2-propanediol–0.06 M KCl–0.04 M potassium phosphate buffer (pH 8.0), in a total volume of 1.8 ml. After incubating the suspension for 5 min at the indicated temperature, the enzyme activity was determined by adding 15 μM coenzyme B_{12}. The thermal stability of the apoenzyme (○) was measured as described before (Toraya and Fukui, 1972).

Results

A. Immobilization of Diol Dehydrase on Sepharose 4B

The partially purified apoenzyme of diol dehydrase was immobilized by direct coupling with CNBr-activated Sepharose 4B. As shown in Table I, almost all of the enzyme activity was fixed on Sepharose, while only 54% of the protein was insolubilized. The enzyme activity did not decrease upon extensive washing with 0.30 M potassium phosphate buffer (pH 8.0). This fact indicates that the binding of enzyme to Sepharose is not ionic but covalent since the apoenzyme of diol dehydrase can be eluted from an ion-exchange column (e.g, DEAE-cellulose column) with much lower concentrations of potassium phosphate buffer (pH 8.0). Only a trace of the activity was detected in the supernatant solution after the immobilization reaction. The specific activity of the immobilized diol dehydrase was about 80–95% of the original activity, which seems extraordinarily high as compared with the cases of other enzymes.

B. Properties of Immobilized Enzyme

Thermal Stability of Immobilized Apoenzyme. Figure 1 depicts the comparison of thermal stability between the immobilized and free apoenzymes at different temperatures. As reported before (Toraya and Fukui, 1972), the apoenzyme of diol dehydrase is heat-labile and loses more than 80% of the enzyme activity when heated at 50° for 5 min. In contrast, the Sepharose-bound apoenzyme exhibited much greater stability to heat as compared with the free apoenzyme, and only 10% of the enzyme activity was lost upon heat treatment at 50° for 5 min.

Inactivation of Immobilized Enzyme by Sulfhydryl Inhibitor. Diol dehydrase is a sulfhydryl protein and is readily inactivated by treatment with PCMB (Lee and Abeles, 1963; Toraya and Fukui, 1972). Table II shows that Sepharose-bound apoenzyme is also highly sensitive to PCMB. This inhibition was demonstrated to be reversible, since a large part of the enzyme activity was recovered by incubation at 37° for 20 min with β-mercaptoethanol.

Oxygen Sensitivity of Immobilized Holoenzyme. The apoenzyme of diol dehydrase forms a tight complex (holoenzyme) on incubation with coenzyme B_{12} at 37°. The cobalt–carbon bond in the active holoenzyme is known to be activated by the interaction of coenzyme with the apopro-

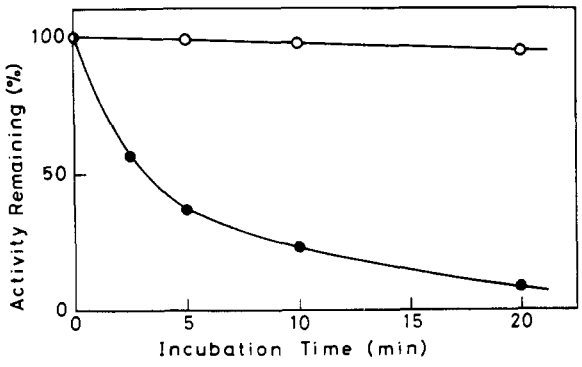

FIGURE 2: Inactivation of the immobilized enzyme upon aerobic incubation with (●) and without (○) coenzyme B_{12} in the absence of substrate. The Sepharose-bound apoenzyme (0.3 ml) was washed with 20 ml of 0.05 *M* potassium phosphate buffer (pH 8.0) to remove the substrate and then suspended in 17 μM coenzyme B_{12}–0.06 *M* KCl–0.04 *M* potassium phosphate buffer (pH 8.0), in a total volume of 1.8 ml. After incubating the suspension aerobically at 37° for the indicated time, the enzyme activity was determined by adding 0.2 *M* 1,2-propanediol.

tein and to play an essential role in the enzyme action. In the absence of substrate, the enzyme-bound coenzyme reacts with atmospheric oxygen and the modified coenzyme inactivates the enzyme by forming a tight, undissociable complex with the apoprotein, although free coenzyme B_{12} is quite unreactive toward oxygen. As illustrated in Figure 2, the Sepharose-bound enzyme underwent rapid inactivation when it was incubated aerobically with coenzyme B_{12} at 37° in the absence of substrate. Thus, it is clearly demonstrated that not only the free holoenzyme but also the Sepharose-bound holoenzyme is sensitive to atmospheric oxygen.

K_m Value of Immobilized Enzyme for Coenzyme B_{12}. The affinity of the immobilized enzyme for coenzyme B_{12} was compared with that of the free counterpart. The apparent K_m value of the immobilized enzyme for coenzyme B_{12} estimated by the conventional method was 0.91 μM, being in close agreement with that of the free enzyme (0.99 μM). These data indicate that the affinity of the enzyme for coenzyme B_{12} is not appreciably affected by the direct coupling with the polysaccharide support.

Effect of Monovalent Cations on Activity of Immobilized Diol Dehydrase. Diol dehydrase is reported to require a certain monovalent cation for catalytic activity (Lee and Abeles, 1963; Toraya et al., 1971). Table III indicates that the immobilized enzyme absolutely requires inorganic monovalent cation, such as K^+. Na^+ was also effective as an activator, but to a lesser degree. Hence, the monovalent cation requirement for catalytic activity is not influenced by immobilization of the enzyme.

It can be concluded that properties of the immobilized enzyme are quite similar to those of the free enzyme, except that the former exhibits much greater stability to heat as compared with the latter.

C. Use of Immobilized Enzyme in Study of Cobalamin Binding

Effects of K^+ and Substrate on Cobalamin Binding. From the data described above, it seems likely that the structure and function of the active site of this enzyme is not significantly altered by immobilization on Sepharose. Hence, the immobilized enzyme may be used as a convenient tool for studies of ligand interaction with the apoenzyme. In general, several tedious procedures would be omitted and, thus, much time would be spared by using the immobilized enzyme.

Table III: Monovalent Cation Requirement of Immobilized Enzyme for Catalytic Activity.[a]

Monovalent Cation	Concn (*M*)	Propionaldehyde Formed (μmol per 10 min per 0.2 ml of Sepharose)
None		0.1
Na^+	0.1	5.8
K^+	0.1	7.8

[a] To remove inorganic monovalent cations, the Sepharose-bound apoenzyme (0.2 ml) was washed with 20 ml of 0.05 *M* Tris-HCl buffer (pH 8.0). The enzyme activity was determined in the presence and absence of monovalent cation added.

To confirm this idea, the immobilized enzyme was used for a study of the binding of cobalamin. The nature of cobalamin binding to the immobilized apoenzyme was studied through both the formation and resolution of the enzyme–cobalamin complexes. Table IV shows the extent of resolution of the immobilized holoenzyme under various conditions. The holoenzyme of the immobilized enzyme was washed with an appropriate buffer which contains K^+ and/or the substrate, and the extent of the resolution was then estimated by assaying the enzyme activity of the Sepharose particles with and without coenzyme B_{12} exogenously added. Although the data were not all-or-none, it is demonstrated that the binding of coenzyme B_{12} to the immobilized apoenzyme is K^+ dependent. The resolution of the immobilized enzyme–CNB_{12} complex was also studied by the method similar to that for the holoenzyme. The Sepharose-bound enzyme–CNB_{12} complex was washed with an appropriate buffer, and then the extent of the resolution was estimated by assaying the activity with coenzyme since the enzyme–CNB_{12} complex is catalytically inactive even in the presence of the coenzyme exogenously added. As shown in Table IV, a fairly large part of the immobilized enzyme–CNB_{12} complex was resolved only in the absence of both K^+ and the substrate. The effects of K^+ and Na^+ ions on the formation of the holoenzyme were investigated based on the sensitivity of the holoenzyme to atmospheric oxygen in the absence of substrate. When the immobilized apoenzyme was aerobically incubated with coenzyme B_{12} at 37° for 20 min in the presence and absence of monovalent cation, a marked inactivation (80%) was observed in the presence of K^+, whereas only a little inactivation of the enzyme occurred in the presence of Na^+ or in the absence of inorganic monovalent cation. This fact indicates that Na^+ is not so efficient as K^+ in promotion of the holoenzyme formation. When the immobilized apoenzyme was incubated with CNB_{12}, the complex formation with CNB_{12} was also found to be dependent on K^+ (data not shown). Thus, it can be concluded that K^+ participates in the binding of coenzyme B_{12} or CNB_{12} to the apoenzyme, resulting in the formation of the active holoenzyme or apoenzyme–CNB_{12} complex, respectively.

The conclusions thus obtained using the immobilized enzyme were essentially the same as those obtained using the free enzyme (Toraya et al., 1971). Although the immobilized enzyme sometimes gave not so clear-cut data as the free enzyme does, the former was demonstrated to have the advantage of permitting much saving of time for a study of ligand interaction with the enzyme.

Table IV: Effects of Monovalent Cation and Substrate on Dissociation of Cobalamin from Immobilized Apoenzyme–Cobalamin Complexes.[a]

Buffer Used for Washing	(I) Sepharose-Apoenzyme	(II) Sepharose-Holoenzyme			(III) Sepharose-Apoenzyme·CNB_{12}	
		Activity		Coenzyme Dissociation (%)		
	Activity[b]	+Coenzyme	−Coenzyme		Activity	CNB_{12} Dissociation (%)
Control	12.7[c]				8.5[d]	(100)
0.05 *M* Tris-HCl–0.1 *M* KCl–0.1 *M* 1,2-propanediol	14.0	10.7	10.7	0	0.0	0
0.05 *M* Tris-HCl–0.1 *M* KCl	12.7	9.2	8.7	6	0.7	9
0.05 *M* Tris-HCl–0.1 *M* 1,2-propanediol	14.0	9.2	5.0	46	0.7	9
0.05 *M* Tris-HCl	12.7	9.8	4.4	55	3.3	39

[a] Sepharose-bound apoenzyme–cobalamin complexes were formed by incubating the immobilized apoenzyme with corresponding cobalamin and the substrate at 37° for 10 min. The Sepharose was washed with 20 ml of an appropriate buffer, and the extent of resolution was then estimated as described in the text. The immobilized apoenzyme itself was also washed with the same buffer to estimate the decrease in activity during this treatment. [b] μmol of propionaldehyde formed per 10 min per 0.3 ml of Sepharose. [c] Activity of the untreated Sepharose-apoenzyme. [d] The Sepharose-apoenzyme was washed with Tris-HCl buffer and its activity was used as control.

Table V: Effect of SH–Modification of Immobilized Apoenzyme on Coenzyme B_{12} Binding.[a]

PCMB Concn (μ*M*)	β-Mercaptoethanol Treatment	Propionaldehyde Formed (μmol per 10 min per 0.3 ml of Sepharose)		Coenzyme B_{12} Binding (%)
		+Coenzyme	−Coenzyme	
0	Without	6.8	5.9	87
0	With	5.5	4.8	92
100	Without	1.3	1.5	
100	With	4.6	0.4	9

[a] The Sepharose-bound apoenzyme (0.3 ml) was treated at 37° for 10 min with 100 μ*M* PCMB, and then washed with 20 ml of 0.05 *M* potassium phosphate buffer (pH 8.0) containing 0.1 *M* 1,2-propanediol. The PCMB-treated Sepharose was incubated at 37° for 10 min with 100 μ*M* coenzyme B_{12} in the presence of 0.3 *M* 1,2-propanediol, 0.1 *M* KCl, and 0.025 *M* potassium phosphate buffer (pH 8.0) and then washed with 20 ml of 0.05 *M* potassium phosphate buffer containing 0.1 *M* 1,2-propanediol. The Sepharose was incubated with and without 20 m*M* β-mercaptoethanol in the presence of 0.2 *M* substrate. After washing the Sepharose with 20 ml of potassium phosphate buffer containing 0.1 *M* substrate, the enzyme activity was determined with and without coenzyme B_{12} exogenously added.

Effect of Sulfhydryl Groups of Apoenzyme on Cobalamin Binding. Table V summarizes the effect on cobalamin binding of modification of sulfhydryl groups of the immobilized enzyme by PCMB. A large portion of the activity was restored by β-mercaptoethanol treatment and this activity was dependent on exogenously added coenzyme B_{12}. In a similar experiment carried out using the Sepharose particles which were not treated with PCMB (control), almost all of the enzyme existed in the form of the holoenzyme. Hence, it is obvious that the enzyme loses the capacity of the cobalamin binding upon modification of sulfhydryl groups with PCMB. This conclusion obtained without tedious and time-consuming prodecures is the same as that with the free enzyme (Toraya et al., 1972).

D. Study of Subunit Interaction Between Components F and S Using Immobilized Component S

Diol dehydrase is composed of two dissimilar protein components (subunits), designated components F and S (Toraya et al., 1973, 1974). We have reported that neither component F nor S alone binds coenzyme B_{12} or CNB_{12} (Toraya et al., 1974), and that coenzyme B_{12} is readily resolved from the holoenzyme in the absence of K^+, irrespective of the presence of substrate (Toraya et al., 1971). There exists a possibility that the enzyme may dissociate into components F and S in the absence of K^+, resulting in resolution of the holoenzyme into the apoenzyme and coenzyme B_{12}. Thus, it seems interesting to know the factors affecting dissociation or reassociation of the components of diol dehydrase. The immobilized enzyme itself seems not to be an adequate material for a study of the subunit interaction because some parts of the enzyme molecules may be attached to Sepharose via both components F and S. Hence, we attempted to approach these problems using the immobilized component S and free component F. Component S was separated from component F by DEAE-cellulose chromatography (Toraya et al., 1974), and immobilized on CNBr-activated Sepharose 4B according to the method similar to that for immobilizing the apoenzyme. A trace of activity of the immobilized component S alone must be due to contamination with component F. Table VI summarizes the extent of association upon incubation of the immobilized component S with free component F in various buffers. The highest degree of association occurred in the presence of both K^+ and substrate. Neither K^+ nor substrate seems to be absolutely required for association since the immobilized component S was associated with component F even in the absence of both K^+ and substrate. However, it is obvious that the substrate strongly facilitates association of the components in the presence of K^+. K^+ alone rather retarded the association to some extent. In the absence of substrate, Na^+ strongly inhibited the hybrid formation between components F and S, but in the presence of substrate Na^+ had very little effect. Also, in the absence of monovalent cations, the substrate had very little effect. The effects of monovalent cation and/or substrate on dissociation of the enzyme into the components were also studied using the immobilized apoenzyme which was reconstituted from the immobilized component S and free component F. The reconstituted apoenzyme was incubated at 37° in various buffers, and then washed with the same buffer. The extent of dissociation was estimated from the decrease in the enzyme activity. As shown in Table VII, the substrate depressed the dissociation, and this effect of the substrate was enhanced by the presence of K^+. However, the subunit dissociation seems to be slightly stimulated by the presence of K^+ alone, as compared with that in the absence of both substrate and

Table VI: Effects of Monovalent Cation and Substrate on Association of Immobilized Component S and Free Component F.[a]

Conditions[b]	Activity (μmol of Propionaldehyde Formed per 40 min per 1.0 ml of Sepharose)	Association (%)
Control (immobilized component S alone)	2.1	(0)
Control (free component F alone)	0.0	
0.05 *M* Tris-HCl–0.1 *M* KCl–0.1 *M* 1,2-propanediol	8.6	(100)
0.05 *M* Tris-HCl–0.1 *M* NaCl–0.1 *M* 1,2-propanediol	5.0	44
0.05 *M* Tris-HCl–0.1 *M* KCl	3.1	16
0.05 *M* Tris-HCl–0.1 *M* NaCl	1.0	0
0.05 *M* Tris-HCl–0.1 *M* 1,2-propanediol	4.8	41
0.05 *M* Tris-HCl	4.5	37

[a] The component S (28 mg) was immobilized on 14 ml of CNBr-activated Sepharose 4B. The Sepharose-bound component S (1.0 ml) was freed from inorganic monovalent cations by extensive washing with 0.05 *M* Tris-HCl buffer (pH 8.0), and then incubated at 37° for 1 hr with 1.5 mg of free component F under the indicated conditions, in a total volume of 3.7 ml. After the Sepharose was washed with 50 ml of an appropriate buffer, the enzyme activity of the Sepharose was determined by the usual procedure. [b] pH 8.0.

Table VII: Effects of Monovalent Cation and Substrate on Dissociation of Immobilized Component S-Free Component F Complex into Components.[a]

Conditions[b]	Activity (μmol of Propionaldehyde Formed per 40 min per 0.9 ml of Sepharose)	Dissociation (%)
0.05 *M* Tris-HCl–0.1 *M* KCl–0.1 *M* 1,2-propanediol	3.8	(0)
0.05 *M* Tris-HCl–0.1 *M* NaCl–0.1 *M* 1,2-propanediol	1.9	49
0.05 *M* Tris-HCl–0.1 *M* KCl	1.6	57
0.05 *M* Tris-HCl–0.1 *M* NaCl	0.7	82
0.05 *M* Tris-HCl–0.1 *M* 1,2-propanediol	3.1	17
0.05 *M* Tris-HCl	2.0	48

[a] The Sepharose-bound component S (6 ml) was incubated at 37° for 1 hr with 11.8 mg of free component F in the presence of 0.1 *M* 1,2-propanediol and 0.1 *M* KCl. The reconstituted Sepharose-apoenzyme thus obtained was divided into six aliquots containing 0.9 ml of the Sepharose-apoenzyme. The Sepharose was washed with 20 ml of an appropriate buffer and then resuspended in 6 ml of the same buffer. After incubating the suspension at 37° for 1.4 hr, the Sepharose was washed with 25 ml of the same buffer. The enzyme activity of the Sepharose was determined by the usual procedure. [b] pH 8.0.

K^+. Na^+ promoted the subunit dissociation strongly. These data are consistent with those obtained in the association experiment. The effect of K^+ on the subunit interaction will be discussed in detail in the Discussion section.

These results clearly indicate that the conditions necessary for dissociation of the apoenzyme into components F and S are different from those for resolution of the holoenzyme into the apoenzyme and coenzyme B_{12}. Therefore, it can be concluded that the resolution of the holoenzyme into the apoenzyme and coenzyme B_{12} in the absence of K^+ does not necessarily result from the dissociation of the enzyme into components F and S. It is likely that some changes in the conformation of the cobalamin binding site are induced by the absence of K^+, irrespective of the subunit structure of the enzyme.

Discussion

Coenzyme B_{12} dependent diol dehydrase was immobilized on CNBr-activated Sepharose 4B. The water-insoluble diol dehydrase would be useful for purely investigative purposes, e.g., studies of ligand binding and subunit interaction. The highly active immobilized diol dehydrase was obtained in extraordinarily high yields by this procedure, suggesting that the direct coupling with the polymer support causes only a minor change around the vicinity of the active site of this enzyme. The specific activity of the partially purified enzyme was increased slightly by the immobilization. This may imply that the apoprotein of this enzyme has a relatively high reactivity toward the CNBr-activated Sepharose. Except that the immobilized enzyme was thermally more stable than the free counterpart, no significant difference between the immobilized and free counterparts was found in the following properties: the affinity (K_m value) for coenzyme B_{12}; the sensitivity to a sulfhydryl inhibitor (PCMB); the oxygen sensitivity of the holoenzyme in the absence of substrate; the absolute requirement for a certain monovalent cation, such as K^+, for catalysis. These lines of evidence strongly suggest that the structure and function of the active site of this enzyme are not appreciably altered by the immobilization.

In such a case, the immobilized enzyme is expected to be applicable for a study of the ligand interaction with the enzyme. To confirm this idea, cobalamin binding properties of the immobilized enzyme were investigated, and essentially the same conclusion as that obtained using the free enzyme was reached. Thus, it was demonstrated that the immobilized enzyme can be successfully used as a convenient tool for a study of the ligand interaction with the enzyme, and that it has advantage of permitting much saving in time and tedium. However, the immobilized enzyme sometimes gave relatively ambiguous data as compared with the free enzyme. This might be due to partial restriction of the conformational change of the enzyme to be induced by the absence of K^+ by immobilization on the insoluble matrix.

The immobilized enzyme was applied for a study of the subunit interaction. The component S, the more stable subunit, was immobilized on CNBr-activated Sepharose, and the factors influencing the dissociation or association of the immobilized component S and the free component F were investigated. The method for investigating the subunit interaction using an immobilized subunit, which has been developed in this paper for diol dehydrase, would provide useful, convenient means for other enzymes composed of different subunits because a partially purified enzyme can also be used for this purpose. Association of the components occurred even in the absence of both monovalent cation and substrate. Of the factors tested, the substrate facilitated association and retarded dissociation. Such an effect of the substrate was markedly enhanced by K^+, but K^+ alone seems to slightly weaken the forces which bind components F and S together. This seems not inconsistent with the fact that the apoenzyme is separated into the components by chromatography on DEAE-cellulose through developing the column with a linear gradient of KCl, while the separation is retarded by the substrate. Na^+ enhanced the subunit dis-

sociation and inhibited the subunit association markedly. In the cases of other enzymes, e.g., glycerol dehydrase (Stroinski et al., 1974), tryptophanase (Morino and Snell, 1967), and arginine decarboxylase (Boeker and Snell, 1968), it has also been reported that K^+ and Na^+ facilitate the subunit dissociation. Since a monovalent cation activator is absolutely required by diol dehydrase action, this effect may be one of the most essential roles played by monovalent cations in the enzyme catalysis.

The holoenzyme of diol dehydrase is easily resolved into the apoenzyme and coenzyme B_{12} by Sephadex G-25 filtration in the absence of K^+ (Toraya et al., 1971). The apoenzyme is dissociated by chromatography on DEAE-cellulose into two dissimilar protein components (subunits), neither of which alone binds cobalamins (Toraya et al., 1974). Thus, there has been a possibility that the absence of K^+ causes dissociation of the enzyme into the components, resulting in the coenzyme dissociation. However, since the conditions necessary for dissociation of the apoenzyme into components were found to be different from those for resolution of the holoenzyme into the apoenzyme and coenzyme B_{12}, the above-mentioned possibility can be excluded. A change in the conformation of the cobalamin binding site of the enzyme may be induced by the absence of K^+, resulting in dissociation of the coenzyme from the enzyme.

It may be possible that K^+ occupies appropriate position(s) of the enzyme and induces the conformational change from a disordered random state to a well-ordered state, resulting in alteration of the forces which connect the subunits. These effects of K^+ on both the tertiary and quaternary structures may slightly weaken the forces which bind the subunits and generate the coenzyme binding site, resulting in activation of the cobalt–carbon bond of bound coenzyme B_{12} for catalysis. A new electrostatic repulsion might be introduced by monovalent metal ion binding to the apoenzyme, which might be a reasonable cause of the dissociation. Although the binding site for monovalent cation in enzymes remains at present to be elucidated, the carbonyl oxygen atom of peptide linkage might be one of the possible candidates for the ligand on the alkali metal ions (Rao et al., 1974).

Stabilization of the apoenzyme by 1,2-propanediol may be due to its facilitating effect on association of the components F and S since the component F alone is extremely unstable and not stabilized by the substrate (Toraya et al., 1974). Thus, the ligand (e.g., substrate and K^+ or Na^+)-mediated interaction of the subunits may be proposed to be a possible control mechanism of enzyme activity and to be important for the regulation of enzyme quantity through stabilization and labilization of diol dehydrase in *A. aerogenes*.

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