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COENZYME B_{12}-DEPENDENT PROPANEDIOL DEHYDRATASE SYSTEM

TERNARY COMPLEX BETWEEN APOENZYME, COENZYME, AND SUBSTRATE ANALOG*

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SUMMARY

A dead-end ternary complex was formed between propanediol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) apoenzyme, coenzyme B_{12}, and a substrate analog when a substrate analog such as 1,2-butanediol or styrene glycol was incubated with the apoenzyme and coenzyme B_{12} in the presence of potassium ions. The 1,2-diols used did not show any substrate activity, and behaved as weak competitive inhibitors with respect to the substrate. When the true substrate, 1,2-propanediol, was added in excessive amounts to the ternary complex system, the initially bound substrate analog was readily displaced by the substrate and the propanediol dehydratase reaction normally took place.

The analog ternary complex was relatively stable to oxygen compared with the holoenzyme, and was much more thermostable than the apoenzyme. Like the holoenzyme and reacting holoenzyme, the ternary complex was photostable under the conditions where free coenzyme B_{12} was rapidly photolyzed. The apoenzyme was completely inactivated by incubation with *p*-chloromercuribenzoate, iodoacetamide, or *N*-ethylmaleimide, but not with arsenite, suggesting that sulfhydryl groups, but not vicinal ones, are involved at the active site of the enzyme. Treatment with mercaptoethanol or dithiothreitol reversed the inhibition by *p*-chloromercuribenzoate. In contrast to the mercurial-insensitivity of both the holoenzyme and reacting holoenzyme, the analog ternary complex was considerably sensitive to *p*-chloromercuribenzoate. Upon Sephadex G-25 gel filtration, dissociation of coenzyme B_{12} from the ternary complex occurred more readily than that from the holoenzyme. These results suggest that the apoenzyme–coenzyme–substrate analog ternary complex is a suitable, stable model for the so-called Michaelis complex (intermediate enzyme–substrate complex), and that its structure may be somewhat distorted compared to the holoenzyme and reacting holoenzyme.

Abbreviations: coenzyme B_{12}, α-(5,6-dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide; PCMB, *p*-chloromercuribenzoate; PHMB, *p*-hydroxymercuribenzoate.

* Paper III in this series. The preceding paper in the series is cited as ref. 5.

INTRODUCTION

Propanediol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) from *Aerobacter aerogenes* (ATCC 8724) is an enzyme which catalyzes the conversion of L- or D-1,2-propanediol to propionaldehyde and 1,2-ethanediol to acetaldehyde. This enzyme has been purified and was found to require coenzyme B_{12} and potassium ions[1,2].

During the course of an investigation of the nature of the interaction between the propanediol dehydratase apoprotein and cobalamins, we elucidated the essential role of monovalent cations in the binding of coenzyme B_{12} or its analogs to the apoenzyme, and presented methods for resolving a tight complex of the apoenzyme with cobalamin[3-5]. Using these resolution methods, it has been demonstrated that apoenzyme–coenzyme B_{12} analog complexes are a suitable, stable model for the holoenzyme[5]. A study of the structure and properties of the intermediate complex itself or related model complexes would provide useful information regarding the reaction mechanism. Based on this idea, the formation of a complex between apoenzyme, coenzyme, and substrate analog was studied. A dead-end ternary complex was formed when a substrate analog such as 1,2-butanediol or styrene glycol was incubated with the apoenzyme and coenzyme B_{12} in the presence of potassium ions. This paper deals with the characterization and some properties of the ternary complex which seems to be a stable model for the intermediate complex.

A preliminary report of part of this study has appeared[6].

MATERIALS AND METHODS

Materials

The crystalline vitamin B_{12} coenzyme used in this study was a gift from Yamanouchi Pharmaceutical Co., Tokyo, Japan. Styrene glycol was prepared by reduction of DL-mandelic acid with lithium aluminum hydride[7], and 1,2-butanediol by acid hydrolysis of 1,2-butylene oxide[8]. These two substrate analogs synthesized were identified by infrared and NMR spectra. All other materials were obtained from commercial sources and were used without further purification. Propanediol dehydratase apoenzyme was prepared from *Aerobacter aerogenes* (ATCC 8724) and assayed by the same procedure as described previously[4], which is similar to that of Lee and Abeles[2].

Formation and stability of apoenzyme–coenzyme–substrate analog ternary complex

The ternary complex was formed by the incubation of a substrate analog with the apoenzyme and coenzyme B_{12} in the presence of potassium ions. The stability of the ternary complex to heat, light, and oxygen was estimated from the enzyme activity remaining after the appropriate treatment as described below; that is to say, an excess of the true substrate 1,2-propanediol was added to the ternary complex system, and after incubation at 37 °C for 10 min the amount of propionaldehyde formed was determined colorimetrically by conversion to its 2,4-dinitrophenylhydrazone. Addition of excess substrate led to initiation of the normal enzyme reaction by the replacement of the initially bound analog with added substrate.

Dissociation of coenzyme B_{12} from ternary complex upon Sephadex G-25 gel filtration

Dissociation of coenzyme B_{12} from an apoenzyme–coenzyme–substrate analog ternary complex was determined by gel filtration on a Sephadex G-25 column (1.6 cm × 18 cm) using an appropriate buffer[4]. Kinetic estimation of coenzyme dissociation from the complex was performed as described previously[4]. Protein was routinely determined by the procedure of Lowry *et al.*[9]. Crystalline bovine serum albumin was used as the standard.

RESULTS

Formation of ternary complex

It has been reported that aerobic incubation of apopropanediol dehydratase and coenzyme B_{12} in the absence of substrate leads to inactivation of the holoenzyme[2,10], and that this inactivation is dependent on monovalent cations[4]. On the other hand, in the presence of a substrate, the holoenzyme ("reacting holoenzyme*") is stable to oxygen since the enzyme reaction was linear for incubation periods of about 30 min duration. The following experiments were carried out with 1,2-diols, such as 1,2-butanediol and styrene glycol, which have no observable substrate activity. The apoenzyme was incubated for an appropriate period with coenzyme B_{12} and a substrate analog in the presence of potassium ions. After incubation, excess substrate (1,2-propanediol) was added and then incubated again at 37 °C. This initiated the normal propanediol dehydratase reaction. From this fact it was suggested that a dead-end ternary complex, which is somewhat insensitive to oxygen, was formed between the apoenzyme, coenzyme and a substrate analog, and that the initially bound analog was readily displaced by the true substrate, so that the normal reaction proceeded. Hence, protection of the holoenzyme by a substrate analog against oxygen inactivation reflects the complex formation between them. Besides 1,2-butanediol and styrene glycol, the following analogs also protected the holoenzyme, but were not further studied: α- and β-phenylethyl alcohols; 2,3-butanediol. Of the analogs tested, the following compounds which have hydroxyl, amino, or sulfhydryl groups at the 1- and 2-positions did not protect the holoenzyme or protected only slightly: glycerol, methanol, ethanol, *n*- and *i*-propanol, *n*-, *sec*-, and *tert*-butanol, mercaptoethanol, methyl cellosolve, ethanolamine and propylenediamine.

Fig. 1 depicts the effects of varying concentrations of 1,2-butanediol and styrene glycol on protection of the holoenzyme against oxygen inactivation. Inhibitions by these analogs were almost negligible under the conditions employed. The extent of the ternary complex formation can be estimated from the ordinate. Apparent V values obtained from the intersection on the ordinate of the double reciprocal plots are 0.036 μmole/min for 1,2-butanediol and 0.059 μmole/min for styrene glycol. The amount of enzyme used in both experiments catalyzed the formation of propionaldehyde at a rate of 0.060 μmole/min when no oxygen inactivation occurred, *i.e.*, in the presence of substrate, 1,2-propanediol. Hence, styrene glycol is much more effective than 1,2-butanediol for the protection of the holoenzyme. Intersection on the abscissa of the double reciprocal plots gives the apparent dissociation constants of the

* Reacting holoenzyme:holopropanediol dehydratase, in the presence of 1,2-propanediol.

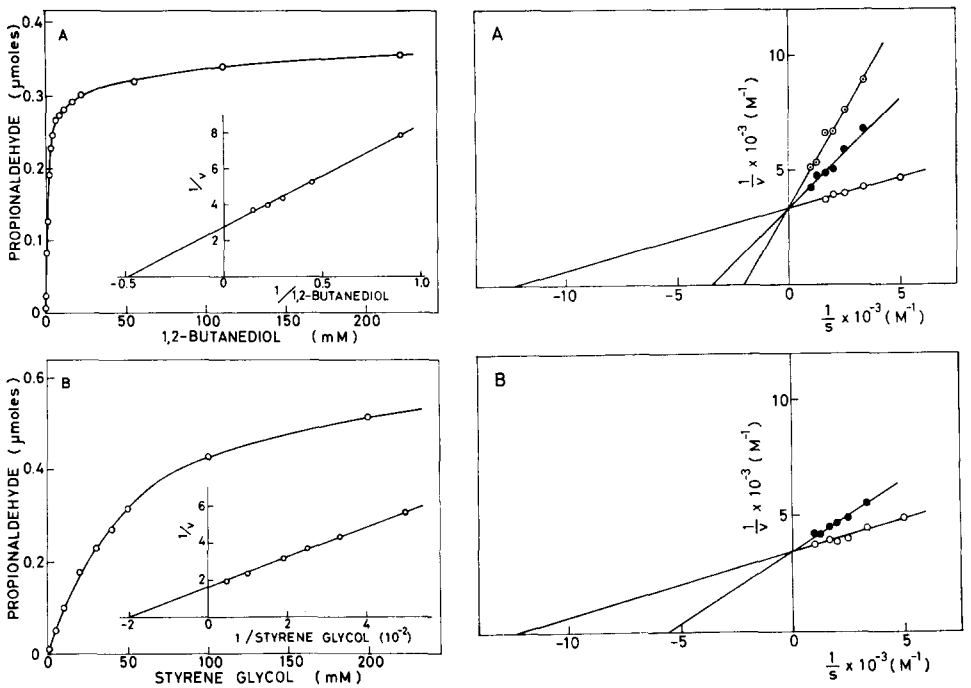


Fig. 1. Effect of varying concentrations of 1,2-butanediol (A) and styrene glycol (B) on the protection of holoenzyme against inactivation. The reaction mixture (0.9 ml), containing 0.060 unit of apoenzyme, 15 nmoles of coenzyme B_{12}, 50 μmoles of KCl, 25 μmoles of potassium phosphate buffer (pH 8.0), and the indicated amount of 1,2-butanediol (A) or styrene glycol (B), was incubated at 37 °C for 5 min. Activity was then assayed by adding 200 μmoles of 1,2-propanediol.

Fig. 2. The Lineweaver–Burk plots for 1,2-propanediol in the presence and absence of 1,2-butanediol (A) or styrene glycol (B). The mixture (1.0 ml) contained 0.92 unit of apoenzyme, 15 nmoles of coenzyme B_{12}, 50 μmoles of KCl, 20 μmoles of potassium phosphate buffer (pH 8.0), varying amounts of 1,2-propanediol, and the indicated amount of 1,2-butanediol (A) or styrene glycol (B). Enzyme reaction was carried out at 37 °C for 3 min. A. Additions: none (○); 5 mM 1,2-butanediol (●); 10 mM 1,2-butanediol (⊙). B. Additions: none (○); 45 mM styrene glycol (●).

holoenzyme–substrate analog complex. The apparent K_i values, which seem equal to the dissociation constants, are shown in the third column of Table I. The type of inhibition by these substrate analogs was studied in Fig. 2. Both 1,2-butanediol and styrene glycol acted as typical competitive inhibitors with respect to substrate, and their K_i values calculated from the double reciprocal plots are presented in the second column of Table I. Their larger K_i values as compared to K_m value for the substrate 1,2-propanediol indicate that their inhibitory actions are very weak. It is noteworthy that apparent K_i values for these analogs obtained from their protecting effects on the holoenzyme agreed reasonably well with those obtained from the kinetic study of inhibition. These results may suggest that the apoenzyme–coenzyme–substrate analog ternary complex is structually similar to the so-called Michaelis complex, *i.e.*, the intermediate enzyme–substrate complex. It can be well interpreted by the data given in Table I, that excess added substrate readily displaced the substrate analog in the complex, leading to the normal propanediol dehydratase reaction.

TABLE I

KINETIC PARAMETERS FOR THE SUBSTRATE AND SUBSTRATE ANALOGS

Substrate or its analog	K_m *(mM)*	K_i* *(mM)*	K_i** *(mM)*	$-\Delta G$ *(cal/mole)*
1,2-Propanediol	0.0814			5800
1,2-Butanediol		1.97	2.08	3840
Styrene glycol		38.4	50.2	2010

* Values obtained from the kinetic study of inhibition.
** Values obtained from the protecting effect on holoenzyme against inactivation.

Stability of ternary complex

To investigate the sensitivity of the complex to oxygen, the rate of inactivation of apoenzyme–coenzyme–substrate analog ternary complexes at 37 °C under aerobic conditions was compared with those of the apoenzyme and holoenzyme in Fig. 3. The ternary complexes, especially the styrene glycol complex, were relatively stable to oxygen, while the holoenzyme underwent rapid inactivation. The apoenzyme itself was insensitive to air. Fig. 4 shows the stabilities of various forms of enzyme at 0 °C. Complexes were formed at 37 °C by incubating the apoenzyme and corresponding ligands, and were used for this experiment after being cooled. The apoenzyme appears

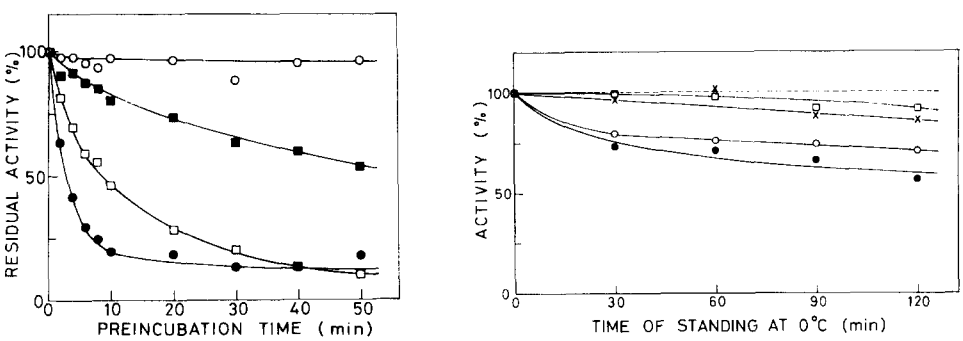


Fig. 3. Inactivation rates at 37 °C of apoenzyme, holoenzyme, and substrate analog ternary complex systems. The following mixture was preincubated at 37 °C for the time indicated. (1) Apoenzyme (○); 0.074 unit of apoenzyme, 50 μmoles of KCl, 25 μmoles of potassium phosphate buffer (pH 8.0). Total volume, 0.8 ml. (2) Holoenzyme (●): (1) *plus* 15 nmoles of coenzyme B_{12}. Total volume, 0.9 ml. (3) 1,2-Butanediol ternary complex (□): (2) *plus* 50 μmoles of 1,2-butanediol. Total volume, 0.9 ml. (4) Styrene glycol ternary complex (■): (2) *plus* 255 μmoles of styrene glycol. Total volume, 0.9 ml. After preincubation, the activity was assayed by adding 200 μmoles of 1,2-propanediol and/or 15 nmoles of coenzyme B_{12}.

Fig. 4. Time-course change of activity of various forms of the enzyme at 0 °C. The following mixture was allowed to stand at 0 °C for the time indicated. (1) Apoenzyme (○—○): 0.090 unit of apoenzyme, 50 μmoles of KCl, 25 μmoles of potassium phosphate buffer (pH 8.0). (2) Apoenzyme + 1,2-propanediol (– – –): (1) *plus* 10 μmoles of 1,2-propanediol. (3) Apoenzyme + 1,2-butanediol (– – –): (1) *plus* 50 μmoles of 1,2-butanediol. (4) Apoenzyme + styrene glycol (– – –): (1) *plus* 255 μmoles of styrene glycol. Volumes of (1)–(4): 0.8 ml. (5) Apoenzyme + coenzyme B_{12} (×—×): (1) *plus* 15 nmoles of coenzyme B_{12}, in a volume of 0.9 ml. (6) Holoenzyme (●—●), (7) reacting holoenzyme (– – –), (8) 1,2-butanediol ternary complex (□—□), and (9) styrene glycol ternary complex (– – –) were formed by incubation at 37 °C for 3 min of the mixture (0.9 ml) containing the same constituents as (5) and 10 μmoles of 1,2-propanediol or its analog (50 μmoles of 1,2-butanediol or 255 μmoles of styrene glycol). Activity was assayed by adding 200 μmoles of 1,2-propanediol and/or 15 nmoles of coenzyme B_{12}.

somewhat unstable when diluted, whereas added 1,2-diols such as 1,2-propanediol, 1,2-butanediol and styrene glycol effectively stabilized the apoenzyme. This indicates that substrate analogs, like the substrate, interacted with the apoenzyme. In contrast to the lability of holoenzyme even at 0 °C, ternary complexes were stable as expected. Fig. 5 depicts the comparison of heat stability of ternary complexes with those of the apoenzyme and holoenzyme at different temperatures. Contrary to the thermolability of apoenzyme over 40 °C, the apoenzyme–coenzyme–styrene glycol complex was more stable to heat and retained about 60% of the activity after heating at 50 °C for 5 min. These results may suggest that this ternary complex is structually more rigid than the apoenzyme. The extraordinarily large inactivation rate of the holoenzyme seems to be due to oxygen inactivation rather than heat denaturation. To obtain information about the state of the cobalt–carbon bond of the coenzyme moiety of the complexes, photostabilities of the complexes were compared with free coenzyme B_{12} and apoenzyme, as shown in Fig. 6. Complexes were formed at 37 °C by incubating the

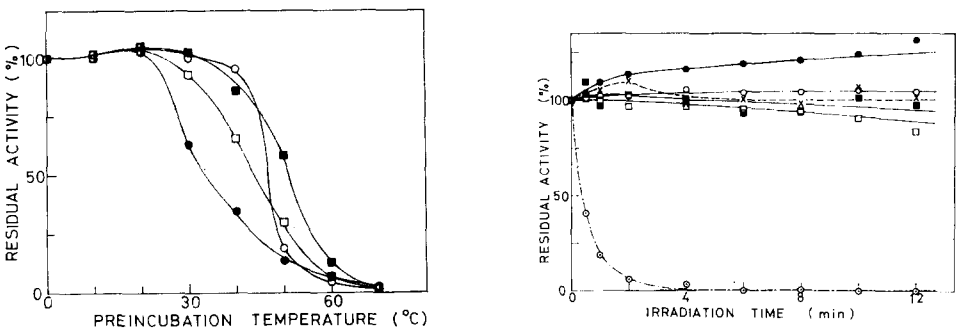


Fig. 5. Thermal stabilities of apoenzyme, holoenzyme and substrate analog ternary complex systems. The mixture and experimental procedure were identical with those described in Fig. 3 except that the preincubation was conducted for 5 min at the temperature indicated. Apoenzyme (○); holoenzyme (●); 1,2-butanediol ternary complex (□); styrene glycol ternary complex (■).

Fig. 6. Photostabilities of apoenzyme, holoenzyme, reacting holoenzyme and substrate analog ternary complexes. Holoenzyme (●—●); reacting holoenzyme (×---×); 1,2-butanediol ternary complex (□—□) and styrene glycol ternary complex (■—■) were formed and subjected to Sephadex G-25 gel filtration as described in Expt I of Table V, in order to remove excess unbound coenzyme B_{12}. After irradiation at 0 °C for the indicated time with light from a 300 W tungsten lamp at a distance of 40 cm, activity was assayed by adding 200 μmoles of 1,2-propanediol. The tubes containing 0.083 unit of apoenzyme (○—○) or 2 nmoles of free coenzyme B_{12} (⊙-·-⊙) were also irradiated as references. Coenzyme activity remaining after irradiation was assayed according to the procedure of Abeles and Lee[1].

apoenzyme and corresponding ligands under a nitrogen atmosphere, and were submitted to irradiation after the removal of excess, unbound coenzyme B_{12} by gel filtration on Sephadex G-25. Photolysis of free coenzyme B_{12} leads to homolytic cleavage of its cobalt–carbon σ bond, resulting in the formation of adenosine-5′-aldehyde under aerobic conditions[11] or 5′,8-cycloadenosine under anaerobic conditions[12]. Two apoenzyme–coenzyme–substrate analog ternary complexes, as well as holoenzyme and reacting holoenzyme, were photostable under the conditions where free coenzyme B_{12} was rapidly photolyzed.

Behavior to sulfhydryl inhibitors

Although the propanediol dehydratase apoenzyme is readily inactivated when incubated with *p*-hydroxymercuribenzoate (PHMB), the holoenzyme once formed by incubating the apoenzyme with coenzyme B_{12} resists inactivation by PHMB[2]. Table II summarizes the effects of substrate and coenzyme additions on *p*-chloromercuribenzoate (PCMB) inactivation of the apoenzyme, and PCMB susceptibility of the reacting holoenzyme. Neither substrate nor free coenzyme B_{12} protected the apoenzyme against PCMB inactivation, while the reacting holoenzyme which was formed by preincubation of the complete reaction system was insensitive to PCMB, indicating that PCMB reacts with the apoenzyme before the coenzyme binding occurs. Revers-

TABLE II

EFFECTS OF SUBSTRATE AND COENZYME ADDITIONS ON PCMB INHIBITION OF ENZYME

The enzyme was treated with PCMB as follows. The untreated enzyme served as controls. I and II: The reaction mixture (0.8 ml) contained 0.046 unit of apoenzyme, 50 μmoles of KCl, 30 μmoles of potassium phosphate buffer (pH 8.0), 200 μmoles of 1,2-propanediol where indicated, and varying amounts of PCMB. After incubation at 37 °C for 5 min, activity was assayed by adding 15 nmoles of coenzyme B_{12} and 200 μmoles of 1,2-propanediol (I) or coenzyme B_{12} (II). III: Varying amounts of PCMB were added at 0 °C to the complete system, and the activity was assayed. IV: The complete system (0.9 ml) was preincubated at 37 °C for 4 min. Varying amounts of PCMB were added to the system, and the activity was assayed.

PCMB concn (M)	*Inhibition (%)*			
	Apoenzyme (I)	*Apoenzyme + substrate (II)*	*Apoenzyme + substrate + coenzyme B_{12} (III)*	*Reacting holoenzyme (IV)*
0	(0)	(0)	(0)	(0)
$1 \cdot 10^{-7}$	8	2	1	0
$1 \cdot 10^{-6}$	48	20	17	0
$1 \cdot 10^{-5}$	95	94	82	0
$1 \cdot 10^{-4}$	96	96	89	0
$1 \cdot 10^{-3}$	96	97	97	1

ibility of PCMB inactivation of the apoenzyme was studied (see Table III). Both incubation at 37 °C with mercaptoethanol and dialysis against mercaptoethanol-containing buffer partially reversed the PCMB inactivation. Enzyme activity was also restored by treatment of PCMB-inactivated apoenzyme with dithiothreitol. These results clearly indicate that PCMB reacts with the sulfhydryl groups of the enzyme essential for the catalytic activity. Table IV shows the susceptibility of the apoenzyme to other sulfhydryl inhibitors. Complete inhibition was caused by treating the apoenzyme at 37 °C with 10^{-3} M iodoacetamide or 10^{-5} M *N*-ethylmaleimide. On the other hand, arsenite at 10^{-3} M did not inhibit the enzyme activity, suggesting that sulfhydryl groups, but not vicinal ones, may be involved at the active site of the enzyme. In contrast to the case of inactivation by PCMB, the substrate protected the apoenzyme against alkylation by iodoacetamide or by *N*-ethylmaleimide. This is of interest and might suggest that PCMB is a stronger sulfhydryl-modifying agent. In Fig. 7, PCMB-susceptibility of apoenzyme–coenzyme–substrate analog ternary complexes are compared with those of the apoenzyme, holoenzyme and reacting holoenzyme at different

TABLE III

REVERSAL OF PCMB INHIBITION WITH MERCAPTOETHANOL

The apoenzyme, 0.50 unit, was treated at 37 °C for 7 min with 40 nmoles of PCMB, in a volume of 2.8 ml. The mixture was then either incubated at 37 °C or 15 min with 56 μmoles of mercaptoethanol, in a volume of 5.6 ml, or dialyzed at 3 °C for 3 h against 1 l of the indicated buffers with continuous stirring. Native apoenzyme was treated with mercaptoethanol in a similar manner as control in each experiment.

Expt No.	*Type of treatment*	*Specific activity (activity recovered (%)) (units/mg)*	
		PCMB-treated apoenzyme	*Native apoenzyme*
1	Untreated	0.00 (0)	0.59 (100)
2	Incubated with 10 mM mercaptoethanol	0.31 (57)	0.54 (100)
3	Dialyzed against		
a	0.05 M potassium phosphate buffer (pH 8.0)	0.02 (4)	0.47 (100)
b	0.05 M potassium phosphate buffer (pH 8.0) *plus* 20 mM mercaptoethanol	0.26 (46)	0.56 (100)

TABLE IV

INHIBITION BY SULFHYDRYL REAGENTS

The apoenzyme, 0.10 unit, was incubated at 37 °C for 15 min with varying amounts of each sulfhydryl inhibitor in the presence or absence of 10 μmoles of 1,2-propanediol, and then the activity was assayed as described in Table II. The unalkylated enzyme served as control.

Sulfhydryl inhibitor	*Concentration (M)*	*Inhibition (%)*	
		Apoenzyme	*Apoenzyme + substrate*
None		(0)	(0)
Iodoacetamide	$1 \cdot 10^{-6}$	0	0
	$1 \cdot 10^{-5}$	5	4
	$1 \cdot 10^{-4}$	29	7
	$1 \cdot 10^{-3}$	82	17
N-Ethylmaleimide	$1 \cdot 10^{-6}$	0	0
	$1 \cdot 10^{-5}$	96	25
	$1 \cdot 10^{-4}$	97	36
	$1 \cdot 10^{-3}$	100	38
Arsenite	$1 \cdot 10^{-3}$	0	

concentrations of PCMB. In clear contrast with the complete inactivation of the apoenzyme by $5 \cdot 10^{-6}$ M PCMB, reacting holoenzyme was not affected even by 10^{-3} M PCMB (Table II) and holoenzyme also was insensitive to 10^{-5} M PCMB. On the other hand, both of the two analog ternary complexes were comparatively susceptible to PCMB inactivation. The unexpected behavior of these ternary complexes to PCMB may be explained by presuming that they have distorted structures compared to the holoenzyme and reacting holoenzyme. The distortion of the enzyme may be caused by forming the complex with more bulky substrate analogs, resulting in the partial exposure of mercurial-sensitive sulfhydryl groups essential for the activity.

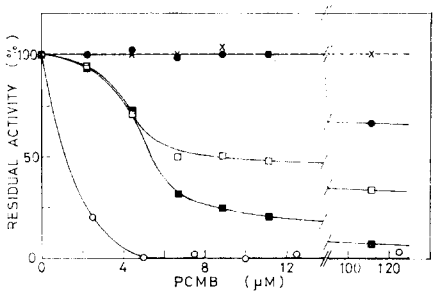


Fig. 7. PCMB-susceptibilities of apoenzyme, holoenzyme, reacting holoenzyme, and substrate analog ternary complex systems. The mixture, identical to those described in Fig. 3, was pre-incubated at 37 °C for 5 min under a nitrogen atmosphere, and then the indicated amount of PCMB was added to these systems. After re-incubation at 37 °C for 4 min, activity was assayed by adding 200 μmoles of 1,2-propanediol and/or 15 nmoles of coenzyme B_{12}. Apoenzyme (○—○); holoenzyme (●—●); reacting holoenzyme (×---×); 1,2-butanediol ternary complex (□—□); styrene glycol ternary complex (■—■).

Dissociation of coenzyme B_{12} from ternary complex

To estimate the tightness of coenzyme binding, the extents of coenzyme dissociation from the holoenzyme, reacting holoenzyme, and apoenzyme–coenzyme–substrate analog ternary complexes upon Sephadex G-25 gel filtration were compared (shown in Table V). In Expt 1, complexes were formed at 37 °C by incubating the apoenzyme and corresponding ligands under a nitrogen atmosphere, and were subjected to gel filtration in buffers containing the substrate or its analog. In Expt II, the reacting holoenzyme formed was subjected to gel filtration in buffers identical to those used in Expt I. In the latter case, the analog ternary complexes were formed during gel filtration by the replacement of the initially bound substrate with its analogs

TABLE V

DISSOCIATION OF COENZYME B_{12} FROM HOLOENZYME, REACTING HOLOENZYME AND SUBSTRATE ANALOG TERNARY COMPLEXES UPON GEL FILTRATION

In Expt I, complexes were formed by anaerobic incubation at 37 °C for 10 min of a mixture (3.0 ml) containing 6.0 units of apoenzyme, 300 μmoles of KCl, 75 μmoles of potassium phosphate buffer (pH 8.0), 180 nmoles of coenzyme B_{12}, and 750 μmoles of 1,2-propanediol or its analog (180 μmoles of 1,2-butanediol or 750 μmoles of styrene glycol) where necessary, and were then subjected to gel filtration on the column (1.6 cm × 18 cm) of Sephadex G-25 (fine) as described earlier[4]. The following buffers were used (pH 8.0); 0.05 M potassium phosphate–0.05 M KCl for holoenzyme; 0.05 M potassium phosphate–0.05 M KCl–0.10 M 1,2-propanediol for reacting holoenzyme, 0.05 M potassium phosphate–0.05 M KCl–0.05 M 1,2-butanediol for 1,2-butanediol ternary complex; 0.05 M potassium phosphate–0.05 M KCl–0.25 M styrene glycol for styrene glycol ternary complex. In Expt II, the 3.0 units of reacting holoenzyme formed as in Expt I were subjected to gel filtration using the four buffers used in Expt I. The degree of coenzyme dissociation was determined by duplicate assays of activity in the protein-containing fraction in the presence and absence of exogenously added coenzyme B_{12} (ref. 4).

Complex	*Coenzyme dissociation (%)*	
	Expt I	*Expt II*
Reacting holoenzyme	0	0
Holoenzyme	34	29
1,2-Butanediol ternary complex	51	43
Styrene glycol ternary complex	58	44

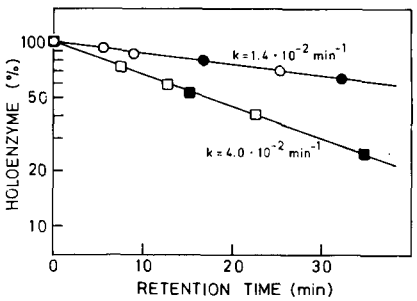
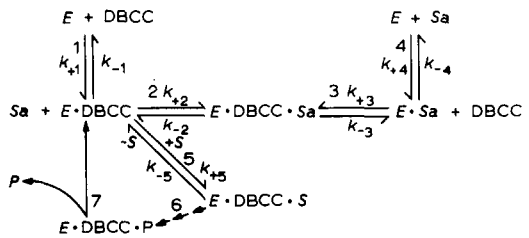


Fig. 8. Kinetics of dissociation of coenzyme B_{12} from holoenzyme (○ and ●) and 1,2-butanediol ternary complex (□ and ■) upon gel filtration. The experimental procedure was identical with that of Expt. II of Table V except that the mixture was applied to a column, 1.6 cm × 10 cm (○ and □) or 1.6 cm × 20 cm (● and ■), of Sephadex G-25 (fine) and was eluted over a period of the indicated retention time.

which were contained in the eluting buffers. Upon gel filtration, no dissociation of coenzyme B_{12} from the reacting holoenzyme occurred, whereas the holoenzyme was partially resolved into the apo- and coenzyme in the absence of the substrate, in agreement with our previous data[4]. More significant coenzyme dissociation from the apoenzyme–coenzyme–substrate analog complexes was observed. The kinetics of dissociation of coenzyme B_{12} upon gel filtration was studied to compare the tightness of coenzyme binding in the ternary complex with that in the holoenzyme. As shown in Fig. 8, a plot of logarithm of the percentage of undissociated complex against the retention time on the gel column is found to be linear. This indicates that the coenzyme dissociation from both the holoenzyme and the 1,2-butanediol ternary complex proceeds with first-order kinetics, and was independent of the bed volume of gel column. The rate constant for the coenzyme dissociation at 0 °C are calculated to be $1.4 \cdot 10^{-2}$ min^{-1} and $4.0 \cdot 10^{-2}$ min^{-1}, respectively. Thus, it can be concluded that the dissociation of coenzyme B_{12} from the apoenzyme–coenzyme–substrate analog ternary complex occurs more rapidly than from the holoenzyme by a factor of about three, suggesting that coenzyme binding in the ternary complex is less tight than that in the holoenzyme. This conclusion may present further evidence for the distorted structures of the ternary complexes compared with the holoenzyme and reacting holoenzyme.

DISCUSSION

The results described in this paper would be reasonably explained by Scheme I.



Scheme I. Proposed reaction sequences. E, apoenzyme; S, substrate; P, product; Sa, substrate analog; DBCC, coenzyme B_{12}.

A monovalent cation, *e.g.* K^+, included in the system plays an important role in the binding of coenzyme B_{12} to the apoenzyme[4]. However, the role of K^+ is not shown in this scheme in order to simplify the expression, since all these experiments were performed in the presence of a sufficient amount of K^+. When both coenzyme and substrate analog are present in amounts sufficient to saturate the apoenzyme in the system, the Equilibria 1, 2, and 3 lie far towards the ternary complex (E·DBCC·*Sa*) formation between apoenzyme, coenzyme, and substrate analog. Upon gel filtration of this ternary complex in the presence of a sufficient amount of the substrate analog, absence of coenzyme B_{12} shifts Equilibrium 3 to the right dissociating coenzyme B_{12} from the complex, since the concentration of coenzyme is kept very low in the protein fraction during gel filtration and recombination between the apoenzyme and coenzyme released does not significantly occur at 0–4 °C. Gel filtration in the absence of both coenzyme B_{12} and substrate analog leads to a shift of Equilibrium 2 to the left and to further displacement of Equilibrium 1 to the dissociation of *E*·DBCC into the active apoenzyme and coenzyme B_{12}. In both cases, coenzyme dissociation proceeded according to first-order kinetics despite a difference in their rate constants for dissociation (k_{-3} and k_{-1}). This result supports the above hypothesis. On the other hand, when the true substrate is added in excessive amounts to the *E*·DBCC·*Sa* system, the initially bound substrate analog is displaced by the substrate which has higher affinity for the enzyme. Thus, the enzyme reaction proceeds normally in such a manner as $5 \rightarrow 6 \rightarrow 7 \rightarrow 5 \rightarrow\rightarrow$. The reason why no resolution of reacting holoenzyme occurred upon gel filtration in the presence of the substrate has been discussed in our earlier paper[4].

For the protection of the holoenzyme against oxygen inactivation, styrene glycol was apparently more effective than 1,2-butanediol. Two explanations may be offered for this finding. First, the styrene glycol ternary complex may be thermodynamically more stable to oxygen than the 1,2-butanediol complex. The bulky π-electron system of the phenyl group of styrene glycol might cover the oxygen-sensitive active site where the activated cobalt–carbon bond of coenzyme B_{12} seems to be involved. A second and perhaps more likely explanation can be provided based on the assumption that oxygen inactivation of the holoenzyme formed kinetically from the ternary complex is more feasible than that of the ternary complex itself; it should then be observed that the 1,2-butanediol ternary complex system undergoes inactivation at a faster rate if the oxygen-sensitive holoenzyme has a higher steady-state concentration in the 1,2-butanediol ternary complex system than in the styrene glycol complex system.

The apoenzyme–coenzyme–substrate analog ternary complexes were as stable to irradiation as both the holoenzyme and reacting holoenzyme. The photostability of the reacting holoenzyme is in agreement with the data of Yamane *et al.*[13]. It has been postulated that the mechanism of several coenzyme B_{12}-dependent reactions involves dissociation of the cobalt–carbon bond of the coenzyme[10,14–19]. Thus, the cobalt–carbon bond of both the holoenzyme and substrate analog ternary complexes, like the reacting holoenzyme, might be in an activated or dissociated state. The binding of coenzyme B_{12} to the active site of the enzyme in the presence of monovalent cations might inevitably result in the dissociation of the cobalt–carbon bond, irrespective of the presence of the substrate. This hypothesis is compatible with the observation that the electron paramagnetic resonance signals resembling those of anaerobically

photolyzed coenzyme B_{12} are exhibited by both irradiated and unirradiated enzyme–coenzyme B_{12} complexes of ethanolamine deaminase[20]. The possibility that photolysis of the holoenzyme might cause the homolytic cleavage of the cobalt–carbon σ bond, followed by immediate recombination of the two fragments produced must also be considered. Another alternative explanation that the enzyme prevents the homolytic cleavage of the cobalt–carbon bond by light is difficult to reconcile with the following observation; irradiation increased the activity of the holoenzyme to some extent. This might be comparable to the finding that irradiation of the holoenzyme, under anaerobic conditions, elicits a two-fold increase in its activity[13].

Unlike holoenzyme and reacting holoenzyme, the substrate analog ternary complexes showed a considerable sensitivity to PCMB. Dissociation of coenzyme B_{12} from the ternary complexes occurred more readily than from the holoenzyme, suggesting the less tight binding of coenzyme in the ternary complexes. These lines of evidence may suggest distorted structures for the analog ternary complexes compared with the holoenzyme and reacting holoenzyme. The ineffectiveness of these analogs as substrates might be concerned with the presumed distorted structures of the apoenzyme–coenzyme–substrate analog ternary complexes. The distortion might be induced by the bulky side chains of the bound analogs as compared with the substrates, 1,2-propanediol and 1,2-ethanediol. This important distortion discussed here might be analogous to the findings of Levitzki *et al.*[21] that the conformation of the 6-diazo-5-oxonorleucine-labeled cytidine 5′-triphosphate synthetase is significantly different from that of the enzyme in the presence of saturating glutamine. In any event, the apoenzyme–coenzyme–substrate analog ternary complex reported here would be a suitable, stable model for the so-called Michaelis complex (intermediate enzyme–substrate complex) and would provide a useful means of investigating the reaction mechanism.

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REFERENCES

1 R. H. Abeles and H. A. Lee, Jr, *J. Biol. Chem.*, 236 (1961) PC 1.
2 H. A. Lee, Jr and R. H. Abeles, *J. Biol. Chem.*, 238 (1963) 2367.
3 T. Toraya, Y. Sugimoto, Y. Tamao, S. Shimizu and S. Fukui, *Biochem. Biophys. Res. Commun.*, 41 (1970) 1314.
4 T. Toraya, Y. Sugimoto, Y. Tamao, S. Shimizu and S. Fukui, *Biochemistry*, 10 (1971) 3475.
5 T. Toraya, M. Kondo, Y. Isemura and S. Fukui, *Biochemistry*, 11 (1972) 2599.
6 T. Toraya and S. Fukui, *Biochem. Biophys. Res. Commun.*, 36 (1969) 469.
7 V. Prelog, M. Wilhelm and D. B. Bright, *Helv. Chim. Acta*, 37 (1954) 221.
8 H. Moureu and M. Dodé, *Bull. Soc. Chim. France*, (5) 4 (1937) 281.
9 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
10 O. W. Wagner, H. A. Lee, Jr, P. A. Frey and R. H. Abeles, *J. Biol. Chem.*, 241 (1966) 1751.
11 H. P. C. Hogenkamp, J. N. Ladd and H. A. Barker, *J. Biol. Chem.*, 237 (1962) 1950.
12 H. P. C. Hogenkamp, *J. Biol. Chem.*, 238 (1963) 477.
13 T. Yamane, S. Shimizu and S. Fukui, *Biochim. Biophys. Acta*, 110 (1965) 616.
14 W. W. Miller and J. Richards, *J. Am. Chem. Soc.*, 91 (1969) 1498.
15 B. M. Babior, *J. Biol. Chem.*, 245 (1970) 1755.

16 B. M. Babior, *J. Biol. Chem.*, 245 (1970) 6125.
17 T. J. Carty, B. M. Babior and R. H. Abeles, *J. Biol. Chem.*, 246 (1971) 6313.
18 R. Yamada, Y. Tamao and R. L. Blakley, *Biochemistry*, 10 (1971) 3959.
19 A. Stroinski, Z. Schneider and J. Pawelkiewicz, *Bull. Acad. Pol. Sci.*, 15 (1967) 727.
20 B. M. Babior, H. Kon and H. Lecar, *Biochemistry*, 8 (1969) 2662.
21 A. Levitzki, W. B. Stallcup and D. E. Koshland, Jr, *Biochemistry*, 10 (1971) 3371.

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