Mechanism of Reactivation of Coenzyme B_{12}-Dependent Diol Dehydratase by a Molecular Chaperone-like Reactivating Factor†

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ABSTRACT: The mechanism of reactivation of diol dehydratase by its reactivating factor was investigated in vitro by using enzyme·cyanocobalamin complex as a model for inactivated holoenzyme. The factor mediated the exchange of the enzyme-bound, adenine-lacking cobalamins for free, adenine-containing cobalamins through intermediate formation of apoenzyme. The factor showed extremely low but distinct ATP-hydrolyzing activity. It formed a tight complex with apoenzyme in the presence of ADP but not at all in the presence of ATP. Incubation of the enzyme·cyanocobalamin complex with the reactivating factor in the presence of ADP brought about release of the enzyme-bound cobalamin, leaving the tight apoenzyme-reactivating factor complex. Although the resulting complex was inactive even in the presence of added adenosylcobalamin, it dissociated by incubation with ATP, forming the apoenzyme, which was reconstitutable into active holoenzyme with added coenzyme. Thus, it was established that the reactivation of the inactivated holoenzyme by the factor in the presence of ATP and Mg^{2+} takes place in two steps: ADP-dependent cobalamin release and ATP-dependent dissociation of the apoenzyme·factor complex. ATP plays dual roles as a precursor of ADP in the first step and as an effector to change the factor into the low-affinity form for diol dehydratase. The enzyme-bound adenosylcobalamin was also susceptible to exchange with free adeninylpentylcobalamin, although to a much lesser degree. The mechanism for discrimination of adenine-containing cobalamins from adenine-lacking cobalamins was explained in terms of formation equilibrium constants of the cobalamin·enzyme·reactivating factor ternary complexes. We propose that the reactivating factor is a new type of molecular chaperone that participates in reactivation of the inactivated enzymes.

Diol dehydratase is an adenosylcobalamin (AdoCbl)[1] dependent enzyme that catalyzes the conversion of 1,2-propanediol, 1,2-ethanediol, and glycerol to the corresponding aldehydes (*1*, *2*). Although diol dehydratase substitutes glycerol dehydratase, an isofunctional enzyme, for the essential role in the anaerobic dissimilation of glycerol by *Klebsiella oxytoca* ATCC8724 (*2*−*5*) through the so-called dihydroxyacetone (dha) pathway, both enzymes undergo suicide inactivation by glycerol (*2*, *6*−*8*). Since glycerol is a growth substrate for the bacteria that produce these enzymes, their inactivation by glycerol is enigmatic. This enigma was solved by our finding that the glycerol-inactivated holoenzymes of diol and glycerol dehydratases in *Klebsiella pneumoniae* and *K. oxytoca* undergo rapid reactivation in situ in the presence of AdoCbl, ATP, and Mg^{2+} (or Mn^{2+}) (*9*, *10*). Recently, we identified two genes, designated as *ddrAB*, in the 3′-flanking region of the diol dehydratase genes as the genes encoding a putative reactivating factor for diol dehydratase (*11*, *12*). The DdrA and DdrB proteins purified from an overexpressing *Escherichia coli* strain were confirmed to exist as a tight complex that actually functions in vitro as the reactivating factor for diol dehydratase (*13*).

It was highly suggested in vitro as well as in situ that the reactivation takes place by the replacement of the enzyme-bound, modified coenzyme with free AdoCbl (*11*−*13*). The reactivating factor was shown to mediate the exchange of the enzyme-bound, adenine-lacking cobalamins for free, adenine-containing cobalamins (*13*) (Figure 1). However, the mechanism of the reactivation has remained largely not understood.

In the present paper, we report intermediary formation of apoenzyme in the reactivating factor-mediated cobalamin exchanges as well as the role of ATP in the reactivation process. The molecular basis on which adenine-containing cobalamins are discriminated from adenine-lacking cobalamins is also proposed here.

EXPERIMENTAL PROCEDURES

Materials. Crystalline AdoCbl was a gift from Eisai Co. Ltd. (Tokyo, Japan). CN-Cbl was obtained from Glaxo Research Laboratories (Greenford, U.K.). aqCbl was prepared by photolysis of methylcobalamin. AdePeCbl (Figure

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[1] Abbreviations: AdoCbl, adenosylcobalamin or coenzyme B_{12}; CN-Cbl, cyanocobalamin; AdePeCbl, 5-adeninylpentylcobalamin; OH-Cbl, hydroxocobalamin; aqCbl, aquacobalamin; PAGE, polyacrylamide gel electrophoresis; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMPPNP, 5′-adenylyl imidodiphosphate; AMPPCP, 5′-adenylyl methylenediphosphonate.

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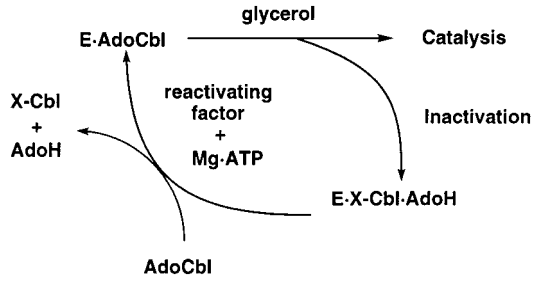

FIGURE 1: Reactivation of glycerol-inactivated holodiol dehydratase by the cobalamin exchange mechanism. E, apodiol dehydratase; AdoH, 5′-deoxyadenosine; X-Cbl, unidentified cobalamin formed upon suicide inactivation by glycerol.

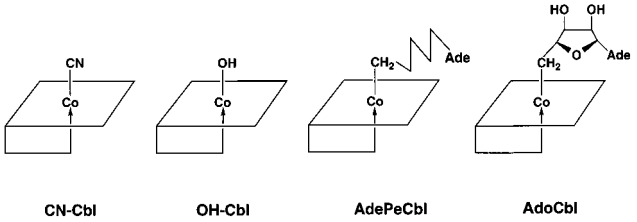

FIGURE 2: Partial structures of the cobalamins used in this study.

2) was synthesized by the published procedures (*14*). ATPγS, AMPPNP, and AMPPCP were purchased from Boehringer Mannheim GmbH. [γ-^{32}P]ATP and [^{57}Co]CN-Cbl were obtained from New England Nuclear and Eastman Kodak Co. Ltd., respectively. All other chemicals were commercial products of the highest grade available and were used without further purification. *K. oxytoca* diol dehydratase and its reactivating factor were purified to homogeneity from overexpressing *E. coli* JM109 harboring expression plasmids pUSI2E(DD) (*15*) and pUSI2ENd(6/5b) (*13*), respectively, as reported previously (*13*, *16*).

Enzyme Assays. The amount of aldehydic products formed by diol dehydratase reaction was determined by the 3-methyl-2-benzothiazolinone hydrazone method (*17*). One unit of diol dehydratase is defined as the amount of enzyme activity that catalyzes the formation of 1 μmol of propionaldehyde/min at 37 °C. Activity of the reactivating factor was routinely assayed in vitro by its capability to activate the inactive diol dehydratase·CN-Cbl complex with 1,2-propanediol as substrate, because this capability was shown to be well correlated with its capability to reactivate glycerol-inactivated or O_2-inactivated holoenzymes (*13*).

ATPase Activity. The products formed by the hydrolysis of ATP with the reactivating factor was identified by TLC. The reactivating factor (59 μg) was incubated at 37 °C for the indicated time periods with 3 mM ATP in 40 mM potassium phosphate buffer (pH 8.0) containing 3 mM $MgCl_2$, in a total volume of 25 μL. The reaction was terminated by adding 25 μL of ethanol. After 30 min on ice, the precipitated protein was removed by centrifugation. Aliquots (1 μL) of the supernatant were chromatographed on a poly(ethylene imine)−cellulose F TLC plate (Merck) with 2 M formic acid containing 0.5 M LiCl as a solvent system (*18*). The adenine nucleotides on TLC plates were located under UV light at 254 nm.

ATP-hydrolyzing activity of the reactivating factor was quantitatively determined by the release of [^{32}P]P_i from [γ-^{32}P]ATP, as described by Schnebli and Abrams (*19*) with some modifications. The reactivating factor was incubated at 37 °C for 30 min with 3 mM [γ-^{32}P]ATP (approximately 8.7×10^5 dpm) in 20 mM potassium phosphate buffer (pH 8.0) containing 3 mM $MgCl_2$, in a total volume of 100 μL. The reaction was terminated by adding 0.5 mL of ice-cold suspension of 6% (w/v) charcoal in 5 mM NaH_2PO_4/8% (w/v) trichloroacetic acid. After vigorous mixing for 10 min, the charcoal was removed by centrifugation. The same ice-cold charcoal suspension (0.7 mL) was then added again to 0.3 mL of the supernatant. After removal of the charcoal by centrifugation, the amount of radioactivity in 0.5 mL of the supernatant was determined by liquid scintillation counting. ATPase activity was obtained by subtracting the radioactivity of a minus reactivating-factor control.

PAGE. PAGE was performed under nondenaturing conditions as described by Davis (*20*) or under denaturing conditions as described by Laemmli (*21*). To keep the diol dehydratase subunits associated, nondenaturing PAGE of this enzyme was performed in the presence of 0.1 M 1,2-propanediol (*22*). In some experiments, ATP or ADP was added with $MgCl_2$ (1 mM each) to gels and electrode buffer. Protein was stained with Coomassie Brilliant Blue R-250.

RESULTS

Direct Evidence for Cobalamin Exchange in Reactivation. The reactivating factor, free AdoCbl, ATP, and Mg^{2+} were absolutely required for both reactivation of the glycerol-inactivated or O_2-inactivated holodiol dehydratase and activation of the inactive complex of diol dehydratase with CN-Cbl (*13*). It was therefore suggested strongly that the reactivating factor reactivates the inactivated holoenzymes and activates the enzyme·CN-Cbl complex by mediating exchange of the enzyme-bound, modified coenzymes and CN-Cbl, respectively, for free intact AdoCbl. Spectral evidence for the exchange of the enzyme-bound CN-Cbl with free AdePeCbl was obtained (*13*), but it does not rule out the possibility that only an upper ligand undergoes the exchange under the conditions.

To examine this possibility, the enzyme·[^{57}Co]CN-Cbl complex was incubated with and without the reactivating factor in the presence of AdePeCbl and ATP/Mg^{2+}, followed by ultrafiltration to remove unbound cobalamins. CN-Cbl and AdePeCbl were used as models for the modified coenzyme and AdoCbl, respectively (Figure 2). The use of AdoCbl itself was avoided, because the enzyme·AdoCbl complex (holoenzyme) is catalytically active and undergoes gradual inactivation during catalysis or rapid inactivation by oxygen in the absence of substrate. As shown in Table 1, the ^{57}Co radioactivity corresponding to about 2 mol of CN-Cbl/mol of enzyme remained bound to diol dehydratase in the absence of the reactivating factor, whereas negligible amount of the radioactivity was retained in the protein fraction in the presence of the reactivating factor. This result, together with the spectroscopic observation (*13*), indicates that the entire molecule of the enzyme-bound CN-Cbl underwent the exchange for free AdePeCbl in the presence of reactivating factor. Thus, it can finally be concluded that the reactivating factor mediates the exchange of the enzyme-bound, adenine-lacking cobalamins for free, adenine-containing cobalamins.

Intermediary Formation of Apodiol Dehydratase. The reactivating factor-mediated cobalamin exchange may pro-

Table 1: Reactivating Factor-Mediated Exchange of Diol Dehydratase-Bound CN-Cbl for Free AdePeCbl[a]

reactivating factor	protein-bound ^{57}Co (cpm)	CN-Cbl bound to diol dehydratase (mol/mol)
+	31	0.03
−	1,930	1.96

[a] Enzyme·CN-Cbl complex was formed by incubation of apoenzyme (10 units) with 15 μM [^{57}Co]CN-Cbl (5870 cpm) at 37 °C for 30 min in 70 μL of 0.04 M potassium phosphate buffer (pH 8.0). To 30 μL of enzyme·[^{57}Co]CN-Cbl complex was added 0.14 mg or none of reactivating factor in 35 mM potassium phosphate buffer (pH 8.0) containing 15 mM ATP/15 mM $MgCl_2$ and 5 μM AdePeCbl, in a total volume of 90 μL. The mixtures were incubated at 37 °C for 1 h and then treated by ultrafiltration on a Microcon-10 microconcentrator (Amicon) to remove unbound cobalamins. The protein fraction retained on the filter was washed twice with 90 μL of 30 mM potassium phosphate buffer (pH 8.0) containing 16 mM ATP/16 mM $MgCl_2$. The radioactivity in the protein fraction was determined by a γ-counter. The similar experiment without the proteins was performed as a control.

ceed not directly but through intermediary formation of apoenzyme. To test this possibility, the enzyme·CN-Cbl complex was incubated with the reactivating factor in the presence of ATP/Mg^{2+}, followed by dialysis to remove unbound cobalamin. The spectrum of the dialysate, however, indicated that no significant dissociation of CN-Cbl took place. Instead of enzyme·CN-Cbl complex, the glycerol-inactivated holoenzyme or enzyme·OH-Cbl·5′-deoxyadenosine complex was used, and SO_3^{2-} was added to the above-mentioned dialysis buffer to prevent the dissociated cobalamin from recombination with apoenzyme. SO_3^{2-} is known to react with aqCbl, forming sulfitocobalamin (*23*), which is bound to diol dehydratase only loosely (*24*). We reported previously that OH-Cbl in the enzyme·OH-Cbl·5′-deoxyadenosine complex and the modified cobalamin in the glycerol-inactivated or O_2-inactivated holoenzyme does not react with SO_3^{2-}, possibly because of the spatial block of the Coβ position by the enzyme-bound 5′-deoxyadenosine (*24*). As shown in Figure 3, both the glycerol-inactivated holoenzyme and the enzyme·OH-Cbl·5′-deoxyadenosine complex underwent dissociation of cobalamins in the presence of reactivating factor but not in its absence. The resulting enzyme in the dialysate was reconsititutable into catalytically active holoenzyme with added AdoCbl (data not shown). Thus, it is evident that the cobalamin exchange in the reactivation takes place through intermediary formation of apoenzyme.

ATP-Hydrolyzing Activity of the Reactivating Factor. ATP was absolutely required for both reactivation of the inactivated holoenzymes or activation of the enzyme·CN-Cbl complex by the reactivating factor. AMPPCP, a β,γ-nonhydrolyzable analogue of ATP, was not able to replace ATP in the in situ reactivation of the glycerol-inactivated holoenzyme (*9*, *11*), suggesting that the hydrolysis of ATP is obligatory for the reactivation by the reactivating factor. To confirm that the reactivating factor hydrolyzes ATP, ATP was incubated with the reactivating factor, and the products were analyzed by poly(ethylene imine)−cellulose TLC (Figure 4). In the presence of reactivating factor, a decrease of ATP and time-dependent formation of ADP were observed (lanes 4−7). When [γ-^{32}P]ATP was incubated with the reactivating factor, release of [^{32}P]P_i was observed. These results indicate that the reactivating factor hydrolyzes ATP to ADP and P_i. The rate of ATP hydrolysis was determined

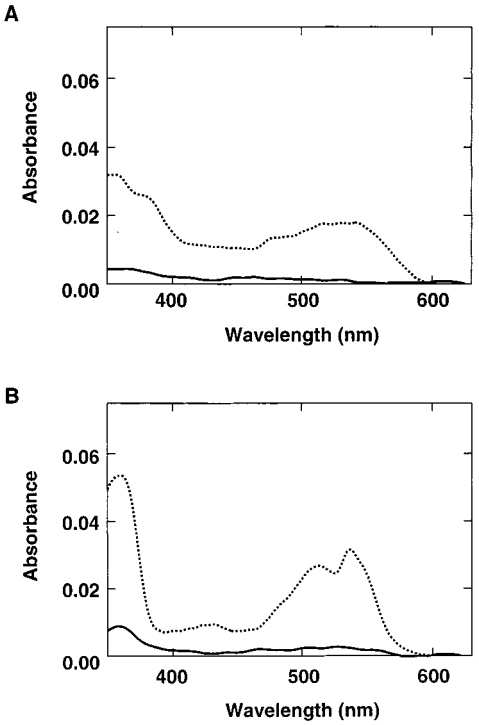

FIGURE 3: Intermediary formation of apodiol dehydratase in the reactivation of glycerol-inactivated holoenzyme and enzyme·OH-Cbl·5′-deoxyadenosine complex by the reactivating factor in the presence of ATP, Mg^{2+}, and SO_3^{2-}. Glycerol-inactivated holoenzyme (A) was prepared by incubation of apoenzyme (120 units) with 97 μM AdoCbl at 37 °C for 30 min in 0.65 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 2 M glycerol. The enzyme·OH-Cbl·5′-deoxyadenosine complex (B) was prepared by preincubation of apoenzyme (120 units) with 0.9 mM 5′-deoxyadenosine at 37 °C for 30 min in 0.65 mL of 0.05 M potassium phosphate buffer (pH 8.0) containing 0.5 M glycerol, followed by incubation at 37 °C for additional 30 min with 150 μM aqCbl in a total volume of 0.7 mL and subsequent dialysis at 4 °C for 2 days against 1500 volumes of the same buffer containing 0.3 M 1,2-propanediol with a buffer change. To aliquots of dialysates containing 50 units of inactive complexes were added 1.25 mg (—) or none (- - -) of reactivating factor in a volume of 0.4 mL. The mixtures were dialyzed again at 4 °C for 18 h against 250 volumes of 0.01 M potassium phosphate buffer containing 0.3 M 1,2-propanediol, 20 mM ATP, 20 mM $MgCl_2$, and 50 mM K_2SO_3 (pH 8.0) and for an additional 2 days against 2500 volumes of 0.01 M potassium phosphate buffer (pH 8.0) containing 0.3 M 1,2-propanediol with a buffer change. The spectra of the dialysates were measured with a minus cobalamin control as reference and corrected for dilution.

with [γ-^{32}P]ATP under various conditions (Table 2). The rate was constant within at least 60 min (data not shown) and not significantly affected by the presence of diol dehydratase or AdoCbl. Moreover, ATP-hydrolyzing activity of the reactivating factor during the glycerol dehydration (reactivating conditions) was essentially the same as that during the 1,2-propanediol dehydration (nonreactivating conditions). Diol dehydratase itself did not hydrolyze ATP at all. Therefore, it is evident that the ATP hydrolysis by the reactivating factor is not directly linked to the reactivation of the inactivated holoenzyme. The time required for hydrolysis of 1 mol of ATP/mol of the reactivating factor was calculated to be ca. 43 s, indicating that this factor is an ATPase with extremely low activity.

Complex Formation between Diol Dehydratase and the Reactivating Factor. Complex formation between the reac-

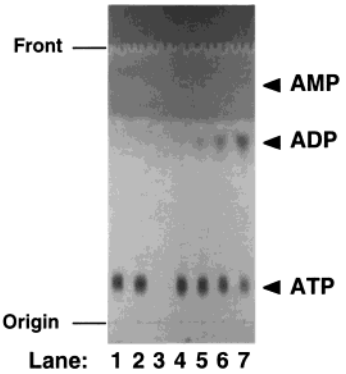

FIGURE 4: TLC analysis of the products upon hydrolysis of ATP by the reactivating factor. ATP was incubated at 37 °C with the factor for the indicated time periods, and aliquots were chromatographed on a poly(ethylene imine)−cellulose F TLC plate as described under Experimental Procedures. Lane 1, ATP; lane 2, ATP (60 min at 37 °C); lane 3, reactivating factor; lanes 4−7, ATP plus reactivating factor (0, 10, 30, and 60 min at 37 °C, respectively).

Table 2: Relationship between ATP Hydrolysis and Reactivation of Diol Dehydratase by the Reactivating Factor[a]

reaction conditions				
reactivating factor	diol dehydratase	substrate	AdoCbl	rate of ATP hydrolysis (min^{-1})
+	+	glycerol	+	1.4
+	+	1,2-propanediol	+	1.3
+	+	glycerol	−	1.3
−	+	glycerol	+	0.0
+	−	glycerol	+	1.5

[a] The amount of ATP hydrolyzed was measured in the presence and absence of reactivating factor (70 μg) and/or diol dehydratase (2.9 units) in 20 mM potassium phosphate buffer (pH 8.0) containing 1.2 M glycerol or 1,2-propanediol, 250 μM AdoCbl, 3 mM [γ-^{32}P]ATP, and 3 mM $MgCl_2$, in a total volume of 100 μL.

tivating factor and the enzyme was analyzed by nondenaturing PAGE in the presence and absence of ATP or ADP (Figure 5). When apoenzyme was incubated with the reactivating factor in the presence of ADP or absence of adenine nucleotides, density of the bands corresponding to diol dehydratase and the reactivating factor was markedly reduced, and new two major bands appeared above them (Figure 5A, lanes 5 and 19). To determine the subunit compositions of the new bands, lane 19 was excised and developed in the second dimension by SDS−PAGE (Figure 5B) on 13.5% and 9.0% gels. At least three new bands contained the α, β, and γ subunits of diol dehydratase and the A and B subunits of the reactivating factor. Thus, it was shown that these bands are complexes between diol dehydratase and the reactivating factor. These forms of the complexes seems different in the assembly of these oligomeric proteins, although the exact molar ratio of diol dehydratase to the reactivating factor in these complexes was not determined. Similar new bands observed in the absence of adenine nucleotides (lane 5) were also shown by two-dimensional PAGE to be composed of both diol dehydratase and the reactivating factor (data not shown). Formation of the apodiol dehydratase·reactivating factor complexes was more efficient in the presence of ADP than in its absence. On the contrary, the apoenzyme·reactivating factor complexes were not detectable in the presence of ATP (Figure 5A, lane 12). From these results, it can be concluded that the ADP and ATP forms of reactivating factor are the high- and low-affinity forms for apodiol dehydratase, respectively.

When similar experiments were performed with the enzyme·cobalamin complexes, the enzyme·reactivating factor complex was not formed at all from the enzyme·AdePeCbl complex in the presence of ATP (lane 8) nor in its absence (lane 1). Even in the presence of ADP, only a small amount of the enzyme·reactivating factor complex was formed from the enzyme·AdePeCbl complex (Figure 5A, lane 15). In contrast, a major part of the enzyme·CN-Cbl complex formed the enzyme·reactivating factor complexes only in the presence of ADP (Figure 5A, lane 17). In the presence of ATP, however, the enzyme·reactivating factor complex was not formed at all from the enzyme·CN-Cbl complex (Figure 5A, lane 10).

Release of Enzyme-Bound Cobalamin upon Complex Formation between Enzyme and the Reactivating Factor. The above-mentioned observations led us to examine whether the enzyme·reactivating factor complexes formed from the enzyme·CN-Cbl complex in the presence of ADP still binds CN-Cbl or not. The enzyme·CN-Cbl and enzyme·AdePeCbl complexes were incubated with and without the reactivating factor in the presence and absence of ATP or ADP, followed by ultrafiltration to remove unbound cobalamins. As shown in Figure 6A, spectra of the protein fractions indicate that essentially all the enzyme-bound CN-Cbl was released from the enzyme by the reactivating factor in the presence of either ATP or ADP. Such release did not occur at all without the reactivating factor. Thus, it is evident that the reactivating factor causes release of the enzyme-bound CN-Cbl in the presence of ATP or ADP. In contrast, release of the enzyme-bound AdePeCbl was not observed even in the presence of reactivating factor with ATP or ADP (Figure 6B). It is thus likely that the release of the diol dehydratase-bound, adenine-lacking cobalamins from the enzyme takes place through the complex formation with the reactivating factor.

Inhibition by Complex Formation between Apoenzyme and the Reactivating Factor and Reversal by ATP. We have previously reported that ADP is not able to replace ATP in the in situ reactivation of the inactivated holoenzyme and the activation of the enzyme·CN-Cbl complex (*11*). When the enzyme·CN-Cbl complex was incubated with the reactivating factor in the presence of ADP, the dissociation of the enzyme-bound CN-Cbl took place (Figure 6A), but the resulting "apoenzyme" was not reconstitutable into catalytically active holoenzyme with added AdoCbl. Therefore, we assumed that this is because of the formation of tight complexes (Figure 5) between apoenzyme and the ADP form (high-affinity form) of reactivating factor and that ATP is required for dissociation of the resulting inactive apoenzyme·reactivating factor complexes. To examine these assumptions, apoenzyme was incubated with the reactivating factor in the presence and absence of ATP or ADP, and remaining activity was measured by adding AdoCbl with and without ATP. As shown in Figure 7, when incubated with the reactivating factor in the presence of ADP, the enzyme activity decreased markedly in a time-dependent fashion. ADP alone did not inhibit the enzyme activity (data not shown). Even in the absence of adenine nucleotides, the enzyme activity was strongly inhibited by the reactivating factor. On the contrary, inhibition was not observed at all in the presence of ATP. The extents of inhibition of enzyme activity by the reactivat-

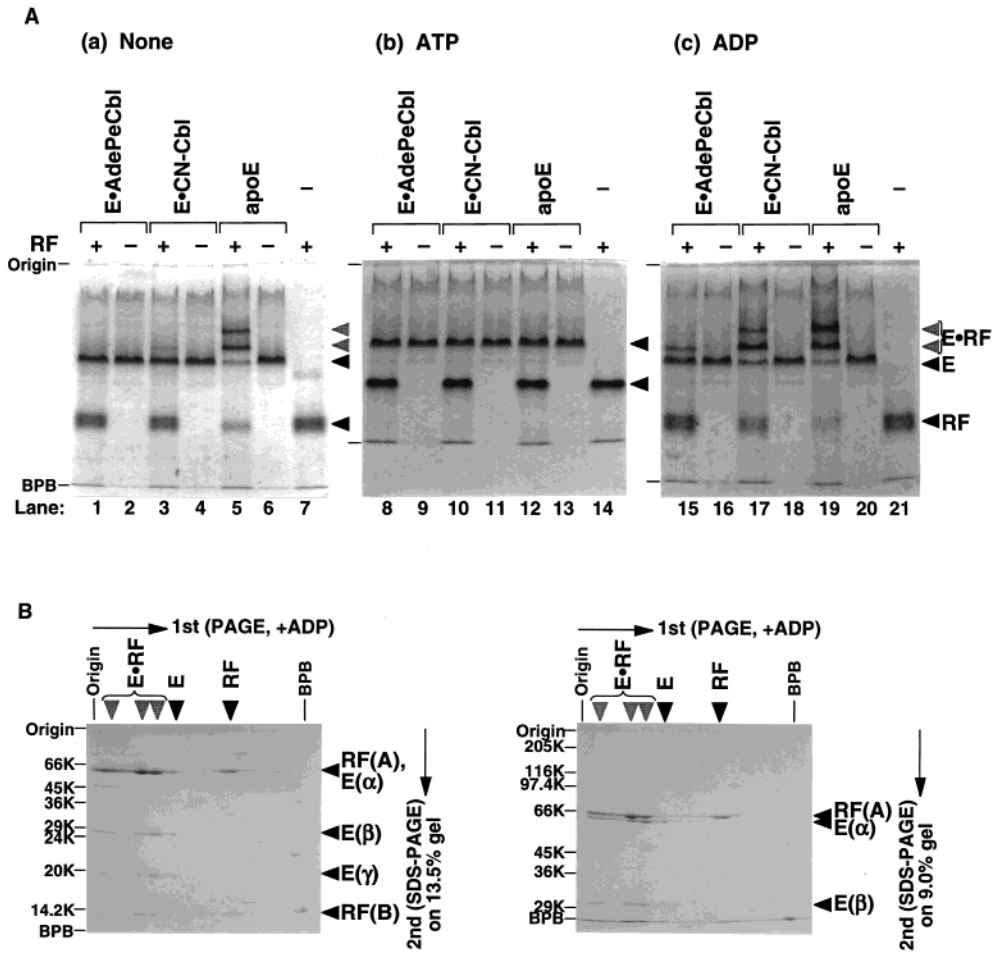

FIGURE 5: Analyses of interactions of the reactivating factor with diol dehydratase by PAGE. (A) Enzyme·AdePeCbl (E·AdePeCbl) and enzyme·CN-Cbl (E·CN-Cbl) complexes were formed by incubation of apoenzyme (apoE) (0.89 unit) with 15 μM AdePeCbl and CN-Cbl, respectively, at 37 °C for 30 min in 6 μL of 45 mM potassium phosphate buffer (pH 8.0). To 2.5 μL of the resulting mixtures were added 12 μg or none of reactivating factor in the absence (a) and presence of 16 mM ATP/16 mM $MgCl_2$ (b) or 16 mM ADP/16 mM $MgCl_2$ (c) in 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 7.5 μL. After 1.5 h at 37 °C, the mixtures were subjected to PAGE (6% gel) under nondenaturing conditions in the absence (a) and presence of 1 mM ATP/1 mM $MgCl_2$ (b) or 1 mM ADP/1 mM $MgCl_2$ (c). Positions of diol dehydratase (E), reactivating factor (RF), and their complexes (E·RF) are indicated with arrowheads to the right of the gels. (B) Lane 19 in panel A was excised and subjected to SDS−PAGE on 13.5% and 9% gels. Positions of diol dehydratase (E), reactivating factor (RF), and their complexes (E·RF) in the first dimension and positions of their subunits in the second dimension are indicated with arrowheads to the top and the right of the gels, respectively.

ing factor under the conditions seem to correspond to the extents of complex formation between apoenzyme and the reactivating factor (Figure 5A). Therefore, it was concluded that the apodiol dehydratase·reactivating factor complexes cannot be reconstituted into active holoenzyme with added AdoCbl alone. When ATP was added together with AdoCbl, inhibition of enzyme activity was completely reversed (Figure 7). This result indicates that the inactive apoenzyme·reactivating factor complexes were dissociated and thus activated by ATP. Dissociation of the apoenzyme·reactivating factor complexes into free apoenzyme and the reactivating factor upon incubation with ATP was confirmed by nondenaturing PAGE (data not shown). Such an effect of ATP seems reasonable, because the ATP form of reactivating factor is the low-affinity form for diol dehydratase (Figure 5A).

Differential Effects of ADP and ATP on Activation of Inactive Enzyme·Cobalamin Complexes. From the above-mentioned results, it was highly suggested that the reactivation of the inactivated holoenzymes and activation of the enzyme·CN-Cbl complex by the reactivating factor takes place in two steps: cobalamin release in the first step and dissociation of the resulting apoenzyme·reactivating factor complexes in the second step. To confirm this two-step mechanism of reactivation, the enzyme·CN-Cbl complex was preincubated with the reactivating factor in the presence of ATP or ADP to release the enzyme-bound CN-Cbl. After dilution, enzyme activity was measured by adding AdoCbl alone or AdoCbl plus ATP (Table 3, run 1). When the enzyme·CN-Cbl complex was preincubated with the reactivating factor in the presence of ATP, ca. 50% of the enzyme activity was recovered when assayed with AdoCbl, irrespective of the addition of ATP to the diluted mixture. In contrast, the enzyme·CN-Cbl complex was not activated at all by preincubation with the reactivating factor in the presence of ADP, when assayed with added AdoCbl alone after dilution. However, when assayed with added AdoCbl plus ATP, ca. 60% of the activity was restored. From these results, the above-mentioned two-step mechanism was proved very likely in the activation of the enzyme·CN-Cbl complex. Similar experiments were performed with the complex of enzyme with AdePeCbl, an adenine-containing analogue (Table 3,

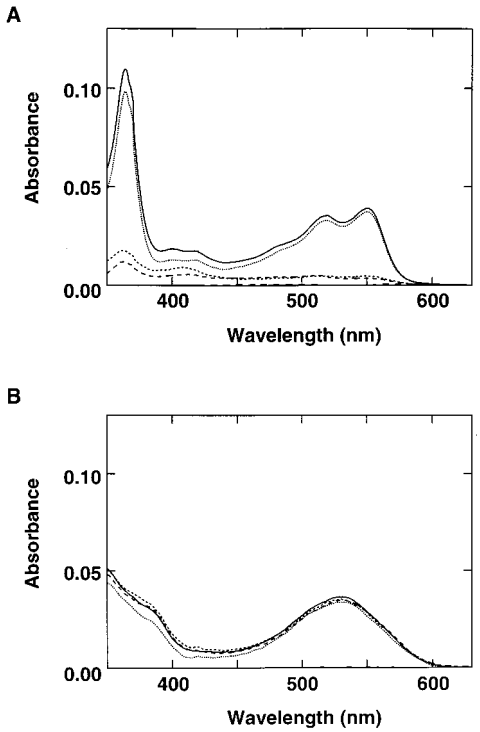

FIGURE 6: Release of the diol dehydratase-bound cobalamin by the reactivating factor. Enzyme·CN-Cbl (A) and enzyme·AdePeCbl (B) complexes were formed by incubation of apoenzyme (21 units) with 15 μM CN-Cbl and AdePeCbl, respectively, at 37 °C for 30 min in 140 μL of 45 mM potassium phosphate buffer (pH 8.0). To 30 μL of the resulting mixtures was added none (—) or 0.14 mg of reactivating factor in the absence (···) and presence of 16 mM ATP/16 mM $MgCl_2$ (— — —) or 16 mM ADP/16 mM $MgCl_2$ (- - -) in 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 90 μL. The mixtures were incubated at 37 °C for 1.5 h and then treated by ultrafiltration, as described in the footnote to Table 1. The protein fraction was washed twice with 90 μL of 35 mM potassium phosphate buffer (pH 8.0) with and without 16 mM ATP/16 mM $MgCl_2$ or 16 mM ADP/16 mM $MgCl_2$. The spectrum of the protein fraction was measured and corrected with a minus cobalamin control.

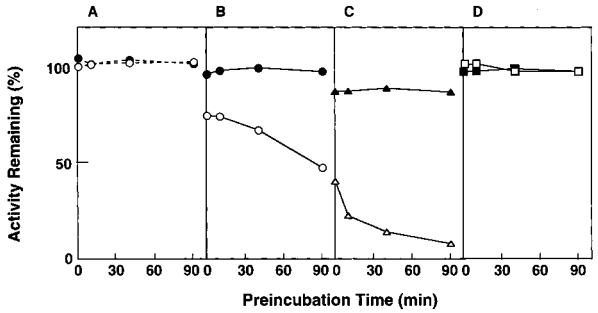

FIGURE 7: Inhibition of apodiol dehydratase by the reactivating factor and its reversal in the presence of ATP. Apoenzyme (0.74 unit) was incubated with (—) (B—D) or without (- - -) (A) 23 mg of reactivating factor in the absence (○, ●) (A and B) and presence of 16 mM ATP/16 mM $MgCl_2$ (□, ■) (D) or 16 mM ADP/16 mM $MgCl_2$ (△, ▲) (C) in 35 mM potassium phosphate buffer (pH 8.0) containing 1.2 M 1,2-propanediol, in a total volume of 15 μL. The mixtures were incubated at 37 °C for the indicated time periods. AdoCbl (30 μM) was then added with (●, ▲, ■) or without (○, △, □) additional ATP and $MgCl_2$ (10 mM each), in a total volume of 25 μL. After incubation at 37 °C for 10 min, the reaction was terminated by adding an equal volume of 0.1 M potassium citrate buffer (pH 3.6). After removal of precipitate by centrifugation, the reaction mixture was appropriately diluted and determined for the amount of propionaldehyde formed.

run 1), but this complex was not activatable by the reactivating factor under any conditions.

ATP-Replacing Activities of ADP and ATP Analogues. Since the two-step mechanism was established, it was examined whether ATP can be replaced by ADP or ATP analogues in each step. When the enzyme·CN-Cbl complex was preincubated with the reactivating factor in the presence of ATP, ADP, or ATP analogues, followed by the assay for enzyme activity with added AdoCbl plus ATP, none of the ATP analogues tested were able to substitute ATP significantly (Table 3, run 2). Except ATP, only ADP was effective for the first step, i.e., the release of CN-Cbl from the enzyme. The reactivating factor-bound ATP itself is hydrolyzed to ADP. Thus, it is strongly suggested that formation of the tight complexes between diol dehydratase and the ADP form (high-affinity form) of reactivating factor effects dissociation of the enzyme·CN-Cbl complex. In this sense, the binding of cobalamin and the binding of the reactivating factor to diol dehydratase can be considered "competitive".

To investigate the ATP-replacing effects of ADP and the ATP analogues on the second step, i.e., dissociation of the inactive apoenzyme·reactivating factor complexes, the enzyme·CN-Cbl complex was preincubated with the reactivating factor in the presence of ADP, and enzyme activity was then measured with added AdoCbl plus ATP, ADP, or ATP analogues (Table 4). Significant enzyme activity was restored by the presence of ATP, ATPγS, or AMPPNP in the assay mixtures, but not all in the presence of ADP or AMPPCP. It was confirmed that neither ATPγS, AMPPNP, nor AMPPCP was hydrolyzed by the reactivating factor. Therefore, it was concluded that ATP is involved in the second step (dissociation of the inactive apoenzyme·reactivating factor complexes) not as a high-energy compound but as an effector for converting the reactivating factor to the low-affinity form for the enzyme.

Inhibition of the Reacting Holoenzyme by Free AdePeCbl in the Presence of Reactivating Factor. As shown in Figure 5A, a small amount of the enzyme·reactivating factor complex was formed even from the enzyme·AdePeCbl complex in the presence of ADP. This result suggests that even the complex of diol dehydratase with adenine-containing cobalamins may be susceptible to cobalamin release by the reactivating factor plus ADP. To test this possibility, the reacting holoenzyme was subjected to incubation with the reactivating factor, ATP, ADP, and AdePeCbl in various combinations. As shown in Figure 8, even without the reactivating factor, activity of the reacting holoenzyme was slightly inhibited by AdePeCbl, probably because this inactive coenzyme analogue possesses higher affinity for the enzyme than AdoCbl. Furthermore, with the reactivating factor plus ATP or ADP, AdePeCbl showed stronger inhibition. These results indicate that even the enzyme-bound AdoCbl, an adenine-containing cobalamin, is susceptible to exchange with free AdePeCbl, another adenine-containing cobalamin, to a small extent under the conditions. In other words, the reactivating factor interacts even with the complexes between diol dehydratase and adenine-containing cobalamins (e.g., AdePeCbl and AdoCbl) and causes their dissociation, although the extent was much smaller than that with the complexes with adenine-lacking cobalamins (e.g., CN-Cbl, OH-Cbl, and the modified coenzymes in the

Table 3: ATP-Replacing Effects of ADP and ATP Analogues on Cobalamin Release[a]

run no.	diol dehydratase	preincubation: reactivating factor	preincubation: addition	ATP in the assay mixture	activity recovered (%)
1	E·CN-Cbl	+	none	+	2
		+	none	−	1
		+	ATP	+	48
		+	ATP	−	49
		+	ADP	+	61
		+	ADP	−	5
	E·AdePeCbl	+	none	+	1
		+	none	−	0
		+	ATP	+	2
		+	ATP	−	2
		+	ADP	+	6
		+	ADP	−	0
	apoenzyme	−	none	+	102
	control	−	none	−	100
2	E·CN-Cbl	+	none	+	1
		+	none	−	1
		+	ATP	+	56
		+	ATP	−	57
		+	ATPγS	+	2
		+	ATPγS	−	2
		+	AMPPCP	+	9
		+	AMPPCP	−	0
		+	AMPPNP	+	2
		+	AMPPNP	−	1
		+	ADP	+	60
		+	ADP	−	2
		−	none	+	2
		−	none	−	0
	apoenzyme	−	none	+	102
	control	−	none	−	100

[a] Enzyme·AdePeCbl (E·AdePeCbl) and enzyme·CN-Cbl (E·CN-Cbl) complexes were formed by incubation of apoenzyme (6.5 units) with 15 μM AdePeCbl and CN-Cbl, respectively, at 37 °C for 30 min in 44 μL of 45 mM potassium phosphate buffer (pH 8.0). To 5 μL of the resulting mixtures was added 23 μg or none of reactivating factor in the presence and absence of 16 mM ATP, ADP, or ATP analogues and 16 mM $MgCl_2$ in 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 15 μL. After 1.5 h at 37 °C, the reaction mixtures were appropriately diluted with 50 mM potassium phosphate buffer (pH 8.0), and the enzyme activity was measured by adding 15 μM AdoCbl with or without 3 mM ATP/3 mM $MgCl_2$.

Table 4: ATP-Replacing Effects of ADP and ATP Analogues on Dissociation of the Apodiol Dehydratase−Reactivating Factor Complexes[a]

diol dehydratase	preincubation: reactivating factor	preincubation: addition	addition to the assay mixture	activity recovered (%)
E·CN-Cbl	+	ADP	none	1
	+	ADP	ATP	49
	+	ADP	ATPγS	32
	+	ADP	AMPPNP	22
	+	ADP	AMPPCP	1
	+	ADP	ADP	2
	−	ADP	none	0
apoenzyme control	−	ADP	none	100

[a] Enzyme·CN-Cbl complex (E·CN-Cbl) was formed as described in the footnote to Table 3. To 9 μL of the resulting mixtures was added 42 μg or none of reactivating factor in the presence and absence of 16 mM ADP/16 mM $MgCl_2$ in 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 27 μL. After 1.5 h at 37 °C, to 4 μL of the mixtures was added 26 mM potassium phosphate buffer (pH 8) containing 0.22 M 1,2-propanediol with or without 20 mM ATP, ADP, or ATP analogues and 20 mM $MgCl_2$, in a total volume of 45 μL, followed by incubation at 37 °C for 15 min. AdoCbl (15 μM) was then added to a total volume of 50 μL. After incubation at 37 °C for 10 min, the reaction was terminated by adding 0.1 M potassium citrate buffer (pH 3.6). After removal of precipitate by centrifugation, the reaction mixture was appropriately diluted and determined for the amount of propionaldehyde formed.

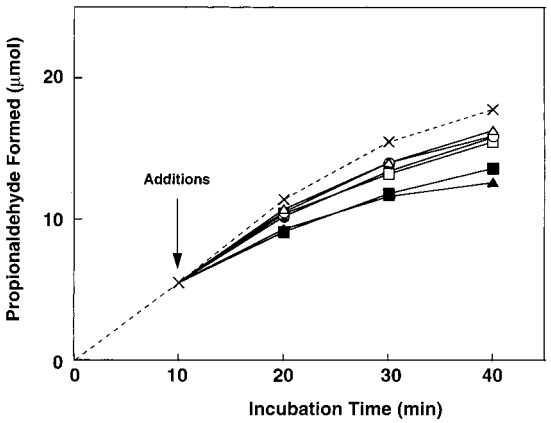

FIGURE 8: Inhibition of reacting holodiol dehydratase by AdePeCbl in the presence of reactivating factor. Reacting holoenzyme was formed by incubation of apoenzyme (0.74 unit) with 7.5 μM AdoCbl at 37 °C for 10 min in 15 μL of 22 mM potassium phosphate buffer (pH 8.0) containing 2 M 1,2-propanediol. To the holoenzyme was added 23 μg (●, ▲, ■) or none (○, △, □) of reactivating factor with (—) or without (×- - -×) 4.5 μM AdePeCbl in the absence (○, ●) and presence of 10 mM ATP/10 mM $MgCl_2$ (□, ■) or 10 mM ADP/10 mM $MgCl_2$ (△, ▲) in 25 mM potassium phosphate buffer (pH 8.0), in a total volume of 25 μL. After incubation at 37 °C for the indicated time periods, the reaction was terminated by adding 0.1 M potassium citrate buffer (pH 3.6). After removal of precipitate by centrifugation, the reaction mixture was appropriately diluted and determined for the amount of propionaldehyde formed.

glycerol-inactivated or O_2-inactivated holoenzymes).

DISCUSSION

The reactivating factor showed low but distinct ATPase activity. The hydrolysis of ATP was absolutely required for the reactivation of the inactivated holoenzymes by the reactivating factor. However, we were unable to demonstrate direct linkage between the ATP hydrolysis and the reactivation of the glycerol-inactivated holoenzyme. Therefore, it is

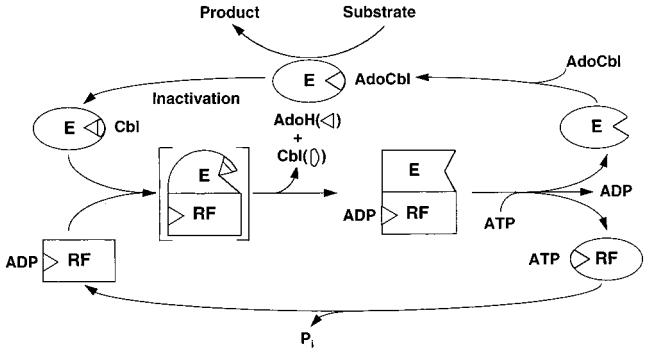

FIGURE 9: Proposed mechanism for reactivation of inactivated holodiol dehydratase by the reactivating factor. E, apodiol dehydratase; RF, reactivating factor; AdoH, 5′-deoxyadenosine; Cbl, cobalamin.

likely that the ATP hydrolysis and the reactivation is not coupled or coupled only loosely. Such phenomenon is not unusual in nature. For example, although the ATP-hydrolyzing activity of ribulose bisphosphate carboxylase/oxygenase activase is absolutely required for activation of an inactive complex between ribulose bisphosphate carboxylase/oxygenase and ribulose bisphosphate, the rate of ATP hydrolysis is not coupled to the activation (*25*). Hydrolysis of ATP and folding of polypeptides by chaperonine are also not directly coupled (*26*).

Figure 9 shows a mechanism that we propose for the reactivation of glycerol-inactivated holodiol dehydratase. The O_2-inactivated holoenzyme and the enzyme·CN-Cbl complex can be considered (re)activated by the same mechanism. ATP plays dual roles in the reactivation of the inactivated holoenzyme. One of the roles of ATP is played by hydrolysis to ADP. It is likely that the hydrolysis of ATP in the reactivating factor induces its conformational change from a low-affinity ATP form to a high-affinity ADP form. The ADP form of the reactivating factor forms a tight complex with the apoenzyme, resulting in release of the modified coenzyme. This is probably because the apoenzyme becomes energetically more stabilized by complex formation with the reactivating factor (ADP form) than by complex formation with adenine-lacking cobalamins. Nonhydrolyzable ATP analogues were not effective for overall reactivation, since they did not form ADP. The resulting apoenzyme·reactivating factor (ADP form) complex is inactive even when assayed with added AdoCbl, because this form of apoenzyme cannot be reconstitutable into active holoenzyme with the coenzyme. The other role of ATP is to dissociate this apoenzyme·reactivating factor (ADP form) complex into free apoenzyme and the reactivating factor. Some of the nonhydrolyzable ATP analogues were able to replace ATP in this role, indicating that ATP participates as an effector for the dissociation. The exchange of the reactivating factor-bound ADP for free ATP would induce the conformational change of the reactivating factor from the high-affinity ADP form to the low-affinity ATP form. This results in dissociation of the complex into free apoenzyme and the reactivating factor (ATP form). The resulting free apoenzyme binds free intact AdoCbl to reconstitute catalytically active holoenzyme, and the reactivating factor returns to the reactivation cycle by the hydrolysis of ATP. The reactivating factor for diol dehydratase hydrolyzes ATP at a rate of 1.4 min^{-1}. This value is in almost the same range as the rate of suicide inactivation of the holodiol dehydratase during the glycerol dehydration reaction [1.3 min^{-1} (*6*)]. As a result, the inactivation of diol dehydratase by glycerol and its reactivation by the reactivating factor can be roughly synchronized, although the ratio of cellular concentrations of the enzyme to the reactivating factor in *K. oxytoca* is not yet known.

It is likely that the modified coenzyme released from the inactivated holoenzyme is back converted to AdoCbl by reductive adenosylation with reducing agent(s) and ATP. The activities of enzymes catalyzing this conversion, i.e., cobalamin reductases and an adenosylating enzyme, were detected in cell-free extracts of *K. oxytoca* (data not shown).

Recently, we have reported that a reactivating factor for AdoCbl-dependent glycerol dehydratase is encoded by two genes with unknown functions in the *dha* regulon of *K. pneumoniae* (*27*). The amino acid sequences of the subunits of the reactivating factor for glycerol dehydratase showed high homologies to those for diol dehydratase (*12*). Therefore, each AdoCbl-dependent enzyme may have its own reactivating factor that plays a role in keeping the enzyme active. This seems reasonable, because in general, holoenzymes of AdoCbl-dependent enzymes tend to undergo inactivation during catalysis or by oxygen in the absence of substrate. Stadtman and co-workers (*28*) reported that AdoCbl-dependent β-lysine mutase undergoes concomitant inactivation during catalysis that accompanies irreversible cleavage of the coenzyme's Co−C bond, forming 5′-deoxyadenosine. They described that this inactivation is prevented by the addition of a sulfhydryl protein and ATP. However, there might be possibilities that this phenomenon is not due to prevention of the inactivation but due to reactivation of the inactivated holoenzyme and that this sulfhydryl protein may be a kind of reactivating factor for β-lysine mutase.

Another important question to be answered is how the reactivating factor distinguishes the adenine-lacking cobalamins from the adenine-containing cobalamins. One possible mechanism is as follows. Although both complexes between the enzyme and adenine-lacking or adenine-containing cobalamins form transient complexes with the ADP form of reactivating factor and undergo conformational change to a form with low affinity for cobalamins, adenine-containing cobalamins still retain some interaction with the enzyme through the adenine moiety, whereas the adenine-lacking cobalamins completely dissociates from the enzyme. If this mechanism is correct, the interaction between the enzyme and the adenine moiety of the adenine-containing cobalamins in the transient complexes would be weakened by added adenosine. However, no dissociation was observed when the enzyme-bound AdePeCbl was incubated with the reactivating factor in the presence of adenosine, Mg^{2+}, and ATP or ADP (data not shown). Thus, this mechanism of discrimination between the enzyme-bound adenine-lacking and adenine-containing cobalamins seems not likely.

Another possible mechanism of the discrimination that we propose is illustrated in Figure 10. Important assumptions are as follows. (i) There is an equilibrium: enzyme·cobalamin complex + reactivating factor (ADP form) $\rightleftarrows$ cobalamin·enzyme·reactivating factor (ADP form) complex, and (ii) release of cobalamins takes place from this ternary complex. If the diol dehydratase-bound cobalamin is an

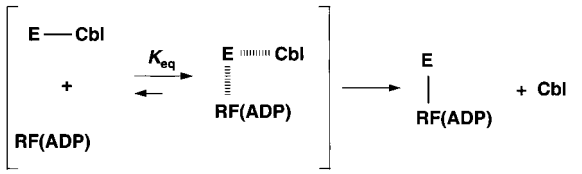

FIGURE 10: Possible mechanism of discrimination of the enzyme-bound adenine-lacking cobalamins from adenine-containing cobalamins. E, apodiol dehydratase; RF, reactivating factor; Cbl, cobalamin.

adenine-lacking cobalamin, the equilibrium shown in the brackets shifts toward the right, and the cobalamin is finally released from the enzyme. This would be because the enzyme gets a larger binding energy upon binding with the reactivating factor (ADP form) than with an adenine-lacking cobalamin. In contrast, if the enzyme-bound cobalamin is an adenine-containing cobalamin, the equilibrium shifts toward the left, and the cobalamin is not released or released to a much lesser degree from the enzyme. The reason for this difference would be explained by a larger binding energy obtained upon binding with an adenine-containing cobalamin than that with an adenine-lacking cobalamin. We have estimated $\Delta G°$ upon binding of the adenine moiety of the coenzyme to diol dehydratase to be approximately -6 kcal/mol at 37 °C from the K_D value for adenine (*29*). This value of $\Delta\Delta G°$ corresponds to a decrease in K_{eq} by a magnitude of 1.7×10^4. From such estimation, the molecular basis of discrimination between the enzyme-bound adenine-lacking and adenine-containing cobalamins shown in Figure 10 seems reasonable.

It was concluded that the affinity of the reactivating factor for diol dehydratase is controlled by the conformational change between the ATP and ADP forms. This conformational change is assured by the hydrolysis of bound ATP to ADP and the exchange of bound ADP for free ATP. Moreover, the reactivating factor induces conformational change of diol dehydratase through complex formation with it, resulting in release of the modified coenzyme. However, it does not become a constituent of the final product (apoenzyme). Such a mechanism of action of the reactivating factor is quite analogous to those of molecular chaperones. Molecular chaperones mediate folding of the nascent polypeptides, refolding of the denatured proteins, targeting proteins to specific organella, and so on (*26*). The rate of ATP hydrolysis by the reactivating factor (1.4 min^{-1}) is very low as compared to regular ATPases, but such a low ATPase activity is not unusual for molecular chaperones (*26*). Parts of the deduced amino acid sequence of the A subunit of the reactivating factor show high fragmentary homology with those of the DnaK protein and the other Hsp70 group of molecular chaperones (*12*, *30*). This homologous regions include amino acid residues corresponding to the ATP-binding site of Hsp70 (*31*, *32*). Therefore, the reactivating factor for diol dehydratase can be considered as a new type of molecular chaperone that is involved in reactivation of the inactivated enzymes.

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