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Elucidation of the coenzyme binding mode of further B_{12}-dependent enzymes using a base-off analogue of coenzyme B_{12}

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Abstract

(Coβ-5′-Deoxyadenosin-5′-yl)-(*p*-cresyl)cobamide (Ado-PCC), a base-off analogue of coenzyme B_{12} (Ado-Cbl), was used to elucidate the coenzyme B_{12} binding mode of glutamate mutase, 2-methyleneglutarate mutase and ethanolamine ammonia–lyase (EAL). Ado-PCC functions as excellent coenzyme for the carbon skeleton rearrangements with apparent K_m values of 200 and 10 nM for glutamate and 2-methyleneglutarate mutases, respectively. The corresponding values for Ado-Cbl are 60 and 54 nM, respectively. In contrast, Ado-PCC showed no coenzyme activity with EAL but was a competitive inhibitor with respect to Ado-Cbl. The K_i value for Ado-PCC was 26 nM, the apparent K_m value for Ado-Cbl was 30 nM. These results are in agreement with the notion that in glutamate and 2-methyleneglutarate mutases, a conserved histidine residue of the protein is coordinated to the cobalt atom of coenzyme B_{12}, whereas in EAL the dimethylbenzimidazole residue of the coenzyme itself serves as ligand. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: (Coβ-5′-deoxyadenosin-5′-yl)-(*p*-cresyl)cobamide; Base-off analogue of coenzyme B_{12}; Ethanolamine ammonia lyase (*Salmonella typhimurium*); Glutamate mutase (*Clostridium cochlearium*); 2-Methyleneglutarate mutase (*Clostridium barkeri*)

1. Introduction

Coenzyme B_{12}-dependent enzymes, as are methyltransferases and carbon skeleton mutases, bind their cofactor via a conserved histidine residue of the protein, which is coordinated to the cobalt atom of coenzyme B_{12}, and the dimethylbenzimidazole residue of the coenzyme is attached to another site [1,2]. On the other hand, it has been shown for dioldehydratases

Abbreviations: HO-Cbl, aquacobalamin (vitamin B_{12a}); Ado-PCC, (Coβ-5′-deoxyadenosin-5′-yl)-(*p*-cresyl)cobamide

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that upon binding of the coenzyme to the apoenzyme, the dimethylbenzimidazole ligand remains coordinated to the cobalt and is not exchanged by histidine residue from the protein [6–9]. This difference in coenzyme binding has been termed ''base off'' and ''base on'', respectively. Since the imidazole part of histidine is also a base, the designation ''base exchange'' rather than ''base off'' is more appropriate.

Until now three methods have been published to distinguish between these binding modes: X-ray crystallography [2,9,10], EPR spectroscopy using ^{15}N-labelled enzymes and/or coenzymes [3–7] and using the base-off analogue of coenzyme B_{12}, Ado-PCC, which behaves either as excellent coenzyme or an inhibitor [8] depending on the binding mode.

The first X-ray structure showing a cobalamin coordinated to a histidine residue of the protein was elucidated by Drennan et al. [1] with the methylcobalamin-dependent methionine synthase. More recently, Mancia et al. [2] and Reitzer et al. [10] identified the same kind of binding in the coenzyme B_{12}-dependent methylmalonyl CoA-mutase and glutamate mutase, respectively.

The EPR method is based on the superhyperfine splitting of the spectrum of cob(II)alamin by an axial nitrogen ligand. Interaction of unpaired electron with the ^{59}Co nucleus [$I(^{59}Co) = 7/2$] [11,12] leads to an octet (hyperfine splitting) while each member thereof is further split to triplets, reflecting additional interaction with the axial nitrogen ligand. Substitution of the latter by the isotope ^{15}N [$I(^{15}N) = 1/2$] results in an octet of doublets. On the basis of this phenomenon, it has been shown using ^{15}N-labelled enzymes that those rearranging carbon skeletons replace the original dimethylbenzimidazole ligand by the imidazole of a histidine residue of the protein.

By contrast, using Ado-Cbl labelled with ^{15}N in the dimethylbenzimidazole ligand, it has been shown that dioldehydratase [6] and ribonucleotide reductase [13] bind the cofactor without base exchange. The same conclusion has been

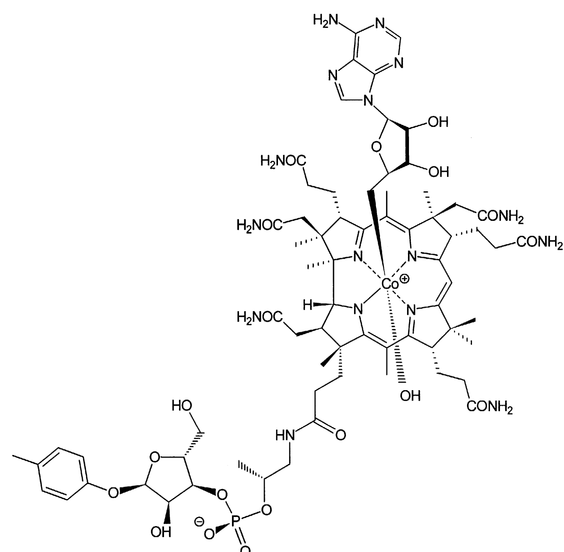

Fig. 1. Structure of (Coβ-5′-deoxyadenosin-5′-yl)-(*p*-cresyl)-cobamide (Ado-PCC).

drawn using an ^{15}N-labelled artificial coenzyme B_{12} analogue [7].

The third method to differentiate between the two binding modes is the use of base-off analogues of coenzyme B_{12}. In a previous paper, we have shown that Ado-PCC served as a coenzyme in the methylmalonyl–CoA mutase reaction, while it was an inhibitor for dioldehydratase and glycerol dehydratase [8].

Here we present further results confirming the usefulness of Ado-PCC (Fig. 1) for differentiating between the two coenzyme binding modes.

2. Experimental

2.1. Materials

Adenosine, coenzyme B_{12} and sodium tetrahydroborate were obtained from Fluka Chemie. Other biochemicals were purchased from Boehringer Mannheim. (Coβ-5′-Deoxyadenosin-5′-yl)-(*p*-cresyl)cobamide (Ado-PCC) was synthesized and purified as previously described [9].

2.2. Enzymes

Alcohol dehydrogenase from yeast (EC 1.1.1.1) was obtained from Boehringer Mannheim. Glutamate mutase (EC 5.4.99.1) from *Clostridium cochlearium* [14] and 2-methyleneglutarate mutase (EC 5.4.99.4) from *C. barkeri* [16] were overproduced in *Escherichia coli* and isolated as previously described. Methylaspartate ammonia lyase (EC 4.3.1.2) and 3-methylitaconate isomerase (EC 5.3.3.6) were isolated from *C. barkeri* [14–18]. The *E. coli* strain CAG626, which overexpresses the genes coding for ethanolamine ammonia lyase (EC 4.3.1.7) from *Salmonella typhimurium* (plasmid pE AL31/50) [19], was generously provided by Drs. B. Babior [19] and C.B. Grissom [20].

2.2.1. Isolation of ethanolamine ammonia lyase (EAL)

2.2.1.1. Cell cultivation and harvesting. Luria–Bertani-agar plates (containing ampicillin, 60 mg/l) were inoculated with the *E. coli* strain and incubated at 37°C with shaking at 250 rpm for 16 h. This culture was added to 1 l of the above LB media and incubated at 37°C with shaking at 250 rpm for 3 h (until the A_{600} reached about 1.0) and then (130 mg of isopropyl β-D-thiogalactoside was added. The culture was shaken at 37°C for further 4 h. Finally the cells were harvested by centrifugation at $4500 \times g$ for 10 min and the pellet was washed with potassium phosphate buffer (20 mM, pH 7.5).

2.2.1.2. Enzyme isolation. The pellet (3.5 g wet paste) was suspended in 10 ml 20 mM potassium phosphate, pH 7.5, which contained 40 U benzonase (highly active nuclease, Merck/Darmstadt), 5 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride, and the cells were disrupted by sonification (Branson, Model 450, output power 140 W, 70% power setting) at 4–8°C for 10 min. The resulting slurry was centrifuged at $30{,}000 \times g$ and 0°C for 30 min. To the supernatant 16.4% ammonium sulfate was added and the solution was stirred at 4°C for 30 min and centrifuged at $30{,}000 \times g$ and 0°C for 30 min. The pellet was dissolved in 30 ml 10 mM potassium phosphate, pH 7.4, 1 mM ethanolamine hydrochloride, 10 mM potassium chloride, 5 mM dithiothreitol and 10 vol.% glycerol. The solution was clarified by dialysis against the same buffer at 4°C overnight and applied to a 30×16 mm Resource Q column (Pharmacia) equipped with a 25×16 mm HiTrap Q (Pharmacia) precolumn 20°C, 6 ml/min. The enzyme was eluted with a linear gradient of solvent B in solvent A; solvent A: 10 mM potassium phosphate, pH 7.4, 10 mM potassium chloride, 10 mM ethanolamine hydrochloride and 5 mM dithiothreitol; solvent B: 10 mM potassium phosphate, pH 7.4, 1.5 M potassium chloride, 10 mM ethanolamine hydrochloride and 5 mM dithiothreitol. The active fractions of EAL were eluted between 20% and 30% of solvent B. The active fractions were combined and applied to a 600×26 mm HiLoad 26/600 Superdex 200 column (Pharmacia) (4°C, 1.5 ml/min, elution with 20 mM potassium phosphate, pH 7.5, 0.5 M potassium chloride, 10 mM ethanolamine hydrochloride and 5 mM dithiothreitol). The fractions showing enzyme activity (100–130 min) were combined to yield 34.6 mg of protein with a specific activity of 36 U/mg. After the solution was kept at 4°C overnight, glycerol was added to a final concentration of 50% (by vol.) and the mixture was frozen at −20°C. Under these conditions, the specific activity of 28.8 U/mg remained unchanged after storage for 6 months.

2.3. Enzyme assays

2.3.1. Glutamate mutase

The assay was based on the monitoring of the absorption of mesaconate ($\lambda_{max} = 240$ nm) [16,17]. The assay mixture (total volume 1 ml) contained: 10 mM glutamate, 1 mM mercaptoethanol, 47 mM Tris/HCl pH 8.3, 9.4 mM KCl, 0.94 mM $MgCl_2$, 3 U methylaspartase,

0.25 μM component E and 3.1 μM component S of glutamate mutase. Component S was incubated with mercaptoethanol for 3 min at 37°C. The reaction was started by addition of the coenzyme or coenzyme analogue [21].

2.3.2. 2-Methyleneglutarate mutase

The assay was conducted by monitoring of the absorption of dimethylmaleinate at 256 nm [18]. The assay mixture (total volume 1 ml) contained: 10 mM 2-methyleneglutarate, 100 mM potassium phosphate buffer (pH 7.4), 0.3 U 3-methylitaconate isomerase, and 0.3 μM 2-methyleneglutarate mutase. After incubation of the assay mixture at 37°C for 5 min, the reaction was started by addition of the coenzyme or coenzyme analogue.

2.3.3. EAL

The assay was carried out using the established yeast alcohol dehydrogenase–NADH coupled method [22] with minor modifications. In a cuvette, 50 mM potassium phosphate buffer, pH 7.5, 10 mM ethanolamine hydrochloride and 0.2 mM of β-NADH, 5 U yeast alcohol dehydrogenase and 20 μl EAL solution (1:3 dilution from the above glycerol stock with 50 mM potassium phosphate, pH 7.5) were mixed and the resulting solution was incubated at 37°C for 3 min. A blank was determined without addition of coenzyme B_{12}. The enzymatic reaction was started by addition of 0.001–1 mM coenzyme B_{12} solution and the decrease of absorbance at 340 nm was recorded for several min at 37°C. The rate of reaction was calculated from the change of absorption at 340 nm.

2.4. Kinetic investigations

The kinetic constants for coenzyme B_{12} and Ado-PCC with the three enzymes were measured using the spectrophotometric assays described above. The K_m and V_{max} values were determined with 1 nM–1 mM coenzyme B_{12} or Ado-PCC at nine data points. The standard linearisation method by Lineweaver–Burk, Hanes or Eadie–Hofstee were used.

The apparent Michaelis constants for coenzyme B_{12} and Ado-PCC are given in Table 1. It should be noted that the determined apparent K_m values for the cobamides are dependent on the concentration of the enzyme in the assay. In the simple Michealis–Menten equation used in this work, a basic assumption is a great excess of substrate over enzyme. Since here the enzyme and coenzyme concentrations were in the same range, these constants should be regarded as apparent relative values.

The apparent inhibition constant (K_i) for Ado-PCC was determined by the so-called ''parallel'' method [23]. To the assay mixture coenzyme B_{12} and Ado-PCC were added simultaneously. The inhibitor concentrations were varied (6–8 data points) at three different coenzyme B_{12} concentrations (0.5–2.5 μM). Inhibition constants were calculated from the equa-

Table 1
Kinetic properties of Ado-PCC with 2-methyleneglutarate mutase, glutamate mutase and EAL
Apparent K_m constants were measured as described under ''Kinetic investigations''. Apparent inhibition constant K_i was estimated by the ''parallel'' method [23]. Enzymes: EAL of *S. typhimurium* overproduced in *E. coli* ($V_{max} = 35 \pm 3$ nmol/min with coenzyme B_{12}); 2-methyleneglutarate mutase from *C. barkeri* overproduced in *E. coli* ($V_{max} = 129 \pm 7$ nmol/min with coenzyme B_{12}); glutamate mutase from *C. cochlearium* overproduced in *E. coli* ($V_{max} = 3760 \pm 120$ nmol/min with coenzyme B_{12}).

Enzyme	Coenzyme B_{12}		Ado-PCC		
	K_m [nM]	k_{cat} [s^{-1}]	K_m [nM]	k_{cat} [s^{-1}]	K_i [nM]
EAL	30 ± 6		no activity		25 ± 6
2-Methyleneglutarate mutase	54 ± 4	2.9 ± 0.2	10 ± 1	2.2 ± 0.2	no inhibition
Glutamate mutase	100 ± 20	9.0 ± 1	200 ± 20	0.9 ± 0.1	no inhibition[a]

[a]At higher concentrations (> 300 nM) Ado-PCC caused some inhibition.

tion: $K_i = (\text{slope} \times [\text{coenzyme B}_{12}] \times V_{max})/K_m$, where K_i, K_m and [coenzyme B_{12}] are given in nM, V_{max} in nmol/min; the slopes were taken from the linear regressions in Dixon plots (1/V vs. [Ado-PCC]); the final was K_i taken as an average of the particular K_i values at different coenzyme B_{12} concentrations.

3. Results and discussion

Ado-PCC, a base-off analogue of coenzyme B_{12} [8], was probed as a coenzyme and/or inhibitor of the coenzyme B_{12} dependent recombinant enzymes, EAL, 2-methyleneglutarate mutase and glutamate mutase. The kinetic constants of Ado-PCC with the three enzymes are listed in Table 1. For EAL, Ado-PCC acts only as an inhibitor and the corresponding inhibition constant (K_i) is similar to the relative K_m for coenzyme B_{12} (30 nM).

For the two carbon skeleton rearranging enzymes 2-methyleneglutarate mutase and glutamate mutase, Ado-PCC serves as coenzyme. In the case of 2-methyleneglutarate mutase, the relative K_m value for the Ado-PCC was found about five times lower than that for coenzyme B_{12}. Moreover, while there was a lag phase of 1–2 min when the reaction was started with the natural coenzyme, this was not observed with Ado-PCC. On the other hand, the K_m value for glutamate mutase was about twice as high with Ado-PCC as with coenzyme B_{12}. At higher concentrations (> 300 nM) Ado-PCC caused some inhibition.

Ado-PCC, a base-off analogue of coenzyme B_{12}, turned out to be an excellent probe for testing the binding mode of Ado-Cbl dependent enzymes. For those enzymes binding the coenzyme in the base-exchange mode, Ado-PCC is an excellent coenzyme, whereas for the others it acts as an inhibitor [8]. Recently, we suggested that the planar hydrophobic *p*-cresyl group may occupy the same hydrophobic binding pocket of the base-off enzymes as the dimethylbenzimidazolyl group of coenzyme B_{12} [8]. This idea is supported by the high apparent affinity, i.e. low K_m value, of Ado-PCC for these enzymes. In the case of Ado-PCC, the removal of the original base from the co-ordination sphere of the cobalt is not necessary, which may facilitate the binding process. Support for this interpretation comes from kinetic results with 2-methyleneglutarate mutase. As mentioned above, the lag phase upon initiating the reaction with coenzyme B_{12} is abolished when Ado-PCC is used as coenzyme. In other words, the energy barrier to remove the dimethylbenzimidazole base from the co-ordination sphere of the cobalt may cause the lag phase observed with the natural coenzyme.

4. Conclusions

The kinetic results with three further coenzyme B_{12}-dependent enzymes confirmed the utility of Ado-PCC to demonstrate the binding mode of the coenzyme to the enzyme protein. For glutamate mutase, the base-exchange binding mode has been shown by EPR spectroscopy using ^{15}N-labelled enzymes [4]. Glutamate mutase, 2-methyleneglutarate mutase and methylmalonyl–CoA mutase possess a consensus sequence in the B_{12}-binding region with a conserved histidine that substitutes for the displaced dimethylbenzimidazole as axial cobalt ligand [2,14,15,24–27]. Such a consensus sequence is lacking in diol and glycerol dehydratases as well as in EAL [11,28,29]. Our results with Ado-PCC are consistent with expectations derived from the amino acid sequences of these enzymes.

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