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STUDY ON THE MECHANISM OF ACTION OF ADENOSYLCOBALAMIN-DEPENDENT GLYCEROL DEHYDRATASE FROM *AEROBACTER AEROGENES*

II. THE INACTIVATION KINETICS OF GLYCEROL DEHYDRATASE COMPLEXES WITH ADENOSYLCOBALAMIN AND ITS ANALOGS

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Summary

The inactivation kinetics of bacterial glycerol dehydratase (EC 4.2.1.30) in the course of its reaction with adenosylcobalamin (AdoCbl) and its analogs were investigated. It was shown that the inactivation rate of apoenzyme complexes with AdoCbl analogs is determined by the nature of the analogs employed and probably by the rate of their conversion into hydroxycobalamins. A possible inactivation mechanism of glycerol dehydratase is discussed.

Introduction

Some cobamide-dependent enzymes the active centers of which include adenosylcobalamin (AdoCbl) show fast inactivation upon apoenzyme incubation with AdoCbl in the absence of substrates [1,2]. It has been suggested [3] that the inactivation involves the interaction of the apoenzyme with AdoCbl resulting in a Co-C bond dissociation. On the other hand, AdoCbl reacts with oxygen in the absence of substrate, giving rise to hydroxycobalamin, an irrever-

Abbreviations: AdoCbl or I, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-adenosylcobamide; II. Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-(6-exo-*N*-methyl)adenosylcobamide; III, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-(6-exo-*N*-butyl)adenosylcobamide; IV, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-(6-exo-*N*-benzoyl)adenosylcobamide; V, Coα-[α-5,6-dimethylbenzimidazolyl)]-Coβ-inosylcobamide; VI, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-guanosylcobamide; VII, Coα-[α-5,6-dimethylbenzimidazolyl]-Coβ-(1-*N*-oxide)adenosylcobamide; VIII, Coα-[α-3,5,6-trimethylbenzimidazolyl]-Coβ-adenosylcobamide; IX, Coα-[α-3-benzyl-5,6-dimethylbenzimidazolyl]-Coβ-adenosylcobamide; X, Coβ-adenosylcobamide; XI for Coβ-adenosylcobinamide; XII, Coα-[α-5,6-dimethylbenzimidazolyl)-Coβ-adenosyl-a,b,c,d,g-pentaamide cobamic acid; XIII, Coα-(α-5,6-dimethylbenzimidazolyl)-Coβ-adenosyl e-ethoxy-a,b,c,d,g-pentaamide cobamic acid; β-HPA, β-hydroxypropionic aldehyde.

sible inhibitor of all cobamide-dependent enzymes. In turn the Co-C bond dissociation in the holoenzyme molecule is a fundamentally important step in enzymic catalysis. There is experimental evidence of the same mechanism of dissociation in the case of the catalytic reaction and for the interaction of apoenzymes with AdoCbl in the absence of substrate, viz., homolytical cleavage of the Co-C bond affording a Co(II) derivative of AdoCbl [4]. In view of this situation, research concerned with the inactivation mechanism and the properties of catalytically inactive apoenzyme complexes with AdoCbl and its analogs might provide insight into the structure and function of the active sites in AdoCbl-dependent enzymes.

Stroinsky et al. [1] have found that glycerol dehydratase from *Aerobacter aerogenes* PZH 572 undergoes inactivation both in the presence and absence of substrate, providing AdoCbl is included in the reaction.

The present work is concerned with a study of glycerol dehydratase (EC 4.2.1.30) inactivation kinetics in the course of the enzymic reaction of glycerol dehydration and in the glycerol dehydratase reaction with AdoCbl and its analogs in the absence of substrate. AdoCbl analogs, modified at the three main structural components, namely, nucleoside and nucleotide ligands and corrin macrocycle, were used.

Materials and Methods

Reagents

AdoCbl and its freeze-dried analogs were prepared at the Laboratory of Thioorganic Compounds of the All-Union Research Vitamin Institute (Moscow, U.S.S.R.) using the method described earlier [5]. Sephadex G-200 and G-25 were from Pharmacia (Uppsala, Sweden). Other reagents are of Soviet origin.

Apo-glycerol dehydratase preparations. The procedure for obtaining partially purified apoenzyme preparations is described by Yakusheva et al. (accompanying paper).

Determination of the enzyme activity. Glycerol dehydratase activity was determined on the basis of the β-HPA quantity formed from glycerol upon its incubation with enzyme in the presence of AdoCbl. 1 ml of the reaction mixture contains 100 μmol of glycerol, 40 μmol of potassium phosphate buffer, pH 8.0, 20 nmol of AdoCbl and 2—3 apoenzyme units; the reaction was initiated by AdoCbl addition. The samples were incubated for 10 min at 37° C. The β-HPA quantity formed in the course of incubation was determined using Smiles-Sobolov's calorimetric method [6] from the solution absorbance measured on a SP-4A spectrometer at 555 nm. The synthetical β-HPA was employed for calibration. The enzyme quantity catalyzing 1 μmol of β-HPA for 10 min under the standard conditions was taken as a unit of enzyme activity.

Study of enzyme inactivation. Glycerol dehydratase inactivation was investigated in the absence of substrate in thermostated cuvettes with 1 ml of 2—3 units of apoenzyme free of glycerol, 40 μmol of potassium-phosphate buffer (pH 8.0) and 20 nmol of AdoCbl. The reaction was started with the addition of AdoCbl (or one of its analogs). The mixture was incubated at 37° C. At definite intervals, the residual enzymic activity was determined by taking 0.9-ml samples and placing them in a test tube with 0.1 ml of a solution containing 20 nmol of

AdoCbl and 100 μl of glycerol (AdoCbl was added in a 10—50 fold excess with respect to its analog). The samples were incubated for 10 min at 37°C, after which the quantity of β-HPA formed was determined.

Results

The kinetic curve of β-HPA formation in the course of glycerol dehydration in the presence of glycerol dehydratase is shown in Fig. 1A (curve 1). It can be seen from the figure that the rate of reaction product formation decreases with time and that after 10 min, it is practically zero. The inhibition observed cannot be explained by substrate deficiency, since the quantity of β-HPA formed in the course of the reaction (2 μmol) is substantially smaller than the glycerol amount used in the test (100 μmol). The inactivation is not due to reaction product accumulation, since β-HPA addition (6—20 mmol) did not affect the inactivation rate. New portions of AdoCbl added to the reaction mixture resulted in no additional product formation and only fresh apoenzyme addition could restart the reaction. Therefore, the decrease in reaction rate with time is a result of enzyme inactivation.

Kinetic analysis showed the inactivation to be a monomolecular reaction. Earlier [7], we found that the quantity of product formed during time t is proportional to the initial reaction rate, i.e. to the active enzyme concentration which can be determined from the reaction product amount formed during a fixed time. In our further experiments, we employed the β-HPA quantity formed during a 10 min incubation (P_{10}). Thus, the exponential equation describing the inactivation process may be written in the form:

$$P_t = P_\infty(1 - e^{-kt}),$$

where P_t is the quantity of product formed for time t, P_∞ is the limiting quantity of product formed in the course of reaction when $t \to \infty$, k is the inactivation rate constant.

From this equation, we have the relationship

$$\ln \frac{P_\infty}{P_\infty - P_t} = kt$$

the plot of which gives the inactivation rate constant k in reaction with glycerol

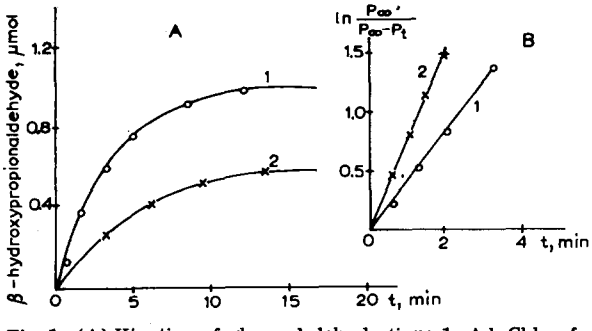

Fig. 1. (A) Kinetics of glycerol dehydration: 1, AdoCbl cofactor; 2, analog V cofactor. (B) Inactivation rate constant.

(Fig. 1 B, line 1). This value is equal to 0.35 min^{-1}.

In the study of glycerol dehydratase inactivation in the absence of glycerol, apoenzyme was incubated with AdoCbl for a specific time. Then the residual enzymic activity (a) was determined by adding a saturating quantity of AdoCbl and glycerol (Fig. 2A, curve 1). As can be seen from Fig. 2, in the absence of substrate, glycerol dehydratase is rapidly inactivated and after 5 min preincubation its activity decreases by 75%. It should be noted that in the absence of AdoCbl the apoenzyme itself is stable after 15 min of incubation (Fig. 2A, line 2).

The rate of glycerol dehydratase inactivation in the absence of substrate obeys an exponential equation: $\ln P_0/P_t = k't$, where P_0 is the product initial concentration at $t = 0$, P_t is the quantity of product formed for time t and k' is the inactivation rate constant.

From this equation one may graphically determine k' (Fig. 2B). Thus the computed glycerol dehydratase inactivation rate constant in the absence of substrate was equal to 0.42 min^{-1}.

The enzyme inactivation rate constant is independent of AdoCbl concentration in the $2 \cdot 10^{-4}$—$2 \cdot 10^{-7}$ M range at which all apoenzyme is bound since K_m for AdoCbl equals $2.0 \cdot 10^{-8}$ M [8].

Thus, glycerol dehydratase inactivation is a monomolecular reaction and the apoenzyme · AdoCbl complex (holoenzyme) undergoes inactivation. One may suggest that a similar inactivation of the binary holoenzyme complex occurs in the catalytic reaction as well. It is, however, quite feasible that in the enzymic reaction the ternary holoenzyme · substrate complex is inactivated in the enzymic reaction, this being the cause of the observed time-dependent inhibition of reaction. Additional investigation is necessary for determining which of the two complexes (binary or ternary) is inactivated. The enzymic inactivation rate constant in glycerol dehydration is somewhat below the rate constant of holoenzyme inactivation in the absence of substrate (0.35 and 0.42 min^{-1}, respectively). Where 1,2-propanediol and ethyleneglycol were used as substrates, the inactivation rate constants were much lower (0.046 and 0.195 min^{-1}, respectively) than that of the apoenzyme · AdoCbl complex [7]. This prompts the conclusion that all substrates exert a protective effect on the holo-

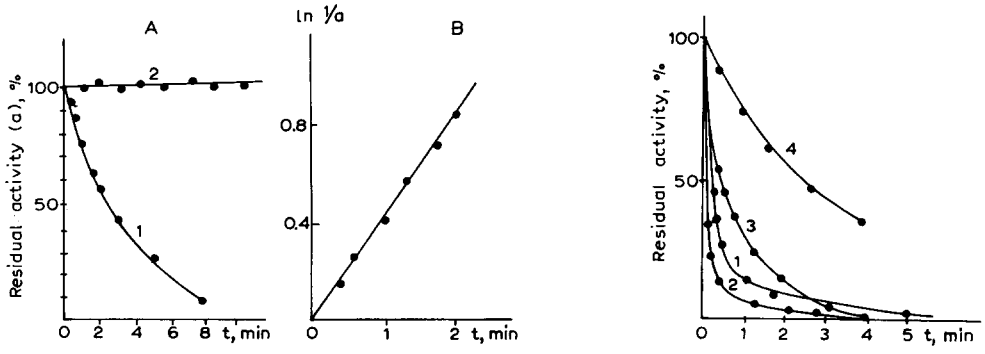

Fig. 2. Glycerol dehydratase residual activity vs. preincubation time of apoenzyme with AdoCbl without substrate (A): 1, apoenzyme + AdoCbl; 2, apoenzyme without AdoCbl. (B) Inactivation rate constant.

Fig. 3. Glycerol dehydratase residual activity vs. preincubation time of apoenzyme with AdoCbl analogs: 1, analogs II and III; 2, analogs IV and VIII; 3, analog V; 4, analog VI.

enzyme, the extent of which depends on the nature of the substrate. However, the mechanism of the substrate protective effect remains obscure.

Next, our studies were concerned with the effect of various AdoCbl modifications on glycerol dehydratase inactivation kinetics. These were concerned with that of nucleoside ligand modifications; in view of existing concepts, this AdoCbl ligand is directly involved in the enzymic catalysis. Among the available AdoCbl analogs were the compounds possessing coenzyme activity and those analogs functioning as competitive inhibitors with respect to AdoCbl. The first group includes analogs II, III and V; the second group embraces analogs IV, VI and VII.

For investigating the kinetics of apoenzyme interaction with these AdoCbl analogs in the absence of substrate, the apoenzyme was incubated with each analog for a definite time period, followed by the measuring of the residual enzyme activity by the addition of saturating quantities of AdoCbl and glycerol. Fig. 3 shows the dependence of glycerol dehydratase residual activities on the time of apoenzyme preincubation with AdoCbl purine analogs. Fig. 3 shows that in the absence of substrate, glycerol dehydratase inactivation is very fast. Thus, after a 30 s period of apoenzyme incubation with analogs II and III, glycerol dehydratase loses 77—80% of its activity (curve 1) and 85—88% with the analogs IV and VII (curve 2). The complex of apoenzyme with analog V is completely inactivated after a 5 min preincubation (curve 3). Analog VI provides an exception (curve 4): its apoenzyme complex inactivation rate is comparable to that of the glycerol dehydratase · AdoCbl complex. The inactivation rate constants of glycerol dehydratase complexes with AdoCbl analogs were determined graphically from the above-mentioned equation [5], as in the case of true holoenzyme and the values found are given in Table I.

The glycerol dehydratase complexes of analogs II, III and VII are inactivated almost instantaneously, so that it is impossible to determine the numerical value of the constant by conventional procedures. Accordingly (Table I) the inactivation constants of the enzyme complexes with these analogs are listed as exceeding 1.0 min^{-1}. In Table I, the rate constants of inactivation of the enzyme

TABLE I

EFFECT OF NUCLEOSIDE LIGAND MODIFICATIONS ON THE RATE OF INACTIVATION OF THE APOENZYME COMPLEXES WITH THE ANALOGS IN THE ABSENCE OF SUBSTRATE AND IN THE ENZYMIC REACTION

AdoCbl and its purine analogs	Inactivation rate constants of complexes (min^{-1})		
	In the course of the reaction	In the absence of substrate Analog concentration (M)	
		$3 \cdot 10^{-6}$	$3 \cdot 10^{-5}$
I	0.37	0.41	0.44
V	0.63	0.68	0.73
VI	—	0.43	0.45
II	—	>1.0	—
III	—	>1.0	—
IV	—	>1.0	—
VII	—	>1.0	—

complexes with AdoCbl analogs are given for two analog concentrations differing by one order of magnitude. At either concentration, the entire apoenzyme is bound, since the concentrations in question exceed the K_m or K_i for these analogs by at least an order of magnitude. That these constants are analog concentration-independent is consistent with the monomolecular mechanism of inactivation. Hence, as in the case of holoenzyme, inactivation affects the apoenzyme-analog complexes.

As can be seen from the data of Table I, the investigated analogs may be divided into two groups. The first includes AdoCbl analogs for which the inactivation rates of their glycerol dehydratase complexes are comparable to that of the true holoenzyme (analogs V and VI). The other group includes the analogs for which the inactivation rate constant of their apoenzyme complexes exceeds 1 min^{-1}. The data obtained indicate that the inactivation rates of apoenzyme complexes of AdoCbl analogs is defined by the chemical nature of the analog.

In the case of analog V as cofactor, the rate of glycerol dehydratase inactivation in the enzymic reaction was investigated. Fig. 1A (curve 2) presents the kinetics of β-HPA formation in reaction with V. It can be seen from the figure that the rate of product formation in the case of this analog decreases still faster than with AdoCbl (curve 1). The enzyme inactivation rate constant with the analog is 0.63 min^{-1} (Fig. 1B, curve 2). From the results obtained, it follows that the rate of enzyme inactivation is determined by the chemical nature of the cofactor.

To elucidate the role of the nucleotide ligand and corrin ring of AdoCbl in glycerol dehydratase inactivation, we studied the kinetics of apoenzyme reaction with AdoCbl analog modified in these moieties.

We found the nucleotide ligand and the corrin macrocycle to be the moieties which contribute most to AdoCbl binding with apoenzyme (cf. the accompanying paper, by M.I. Yakusheva et al.). The inactivation rate constants for apoenzyme complexes with analogs XII (e-propionamide group of the corrin cycle is replaced by the propionic acid residue), XIII (the same group is replaced by ethyl propionate), VIII, X (eliminated 5,6-dimethyl-benzimidazol) and XI (completely eliminated nucleotide) in the absence of substrate are shown in Table II. It can be seen that the inactivation rate of these complexes

TABLE II

EFFECT OF MODIFICATIONS IN THE NUCLEOTIDE LIGAND AND CORRIN MACROCYCLE OF AdoCbl ON THE INACTIVATION RATE OF APOENZYME COMPLEXES WITH THE ANALOGS IN THE ABSENCE OF SUBSTRATE AND IN THE COURSE OF GLYCEROL DEHYDRATION

AdoCbl and its analogs	Inactivation rate constants of complexes (min^{-1})	
	Without substrate	In the course of reaction
I	0.41	0.37
XIII	0.29	0.26
VIII	0.25	0.22
XII	0.08	0.07
X	0.02	—
XI	0.0	—

is lower than that of the true holoenzyme. Thus, a modification of functional groups in the corrin macrocycle and nucleotide ligand produces structural changes in apoenzyme complexes with the analogs, whereby the complexes are more stable towards inactivation. Investigations on the kinetics of apoenzyme interaction with analog XI showed the complex thus formed to escape inactivation; the enzyme catalytic activity is completely retained even after a 15 min period of incubating the apoenzyme with this analog.

Analogs VIII, XII and XIII which possess the properties of coenzymes, were used as cofactors in the study of enzyme inactivation kinetics in the course of enzymic glycerol dehydration. It was shown that the glycerol dehydratase inactivation rate constants in the absence of substrate are lower than that of enzyme inactivation with AdoCbl as cofactor (Table II). Thus the apoenzyme complexes with the analogs modified at the binding points of AdoCbl with apoenzyme are more resistant to inactivation. At the same time, these complexes are catalytically less active with respect to holoenzyme in the enzymic reaction.

Discussion

Investigation of the kinetics of glycerol dehydratase inactivation in the course of apoenzyme interaction with AdoCbl, in the absence of substrate revealed that this reaction is monomolecular and that the complex, enzyme · AdoCbl, is subject to inactivation. This conclusion appears to be valid in the case of AdoCbl analogs. The rate of the inactivation process is dependent on the chemical nature of the analog. On the basis of Wagner and Abeles' hypothesis [3], one may assume that the different inactivation rates of the glycerol dehydratase · analog complexes are governed by dissimilar rates of AdoCbl analog conversions into their respective hydroxy forms. This suggestion is favoured by the fast transformation of AdoCbl analogs modified at the adenine moiety into hydroxycobalamin [9]. On the other hand, the analogs modified at the nucleotide ligand undergo conversion into their hydroxy forms at a substantially slower rate. In our experiments, the enzyme complexes with analogs modified at the nucleoside moiety were inactivated almost instantaneously or at a rate comparable with true holoenzyme inactivation (Table I). Moreover, in view of the homolytic mechanism of Co-C bond cleavage in the molecule of associated AdoCbl and the formation of the adenosyl radical, it is quite feasible that chemical modification of the adenosine moiety would minimize the life-time of this radical and thus accelerate glycerol dehydratase inactivation.

AdoCbl analogs modified at the binding points of coenzyme with the protein (corrin macrocycle and nucleotide ligand) yielded the apoenzyme complexes which display greater stability towards oxygen. For this group of analogs, some correlation was observed viz. the analogs producing apoenzyme complexes more resistant to inactivation which were found to be less active in the catalytic reaction. Similar results were observed by Fukui et al. [10] in the experiments with 5′-deoxyadenosyl-10-Cl-cobalamin in the diol dehydratase proposed that conformational changes may be involved in the inactivation. In any case, to elucidate the precise mechanism of inactivation of the AdoCbl-dependent enzymes, further research relying on direct physical methods appears to be necessary.

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