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INACTIVATION OF ETHANOLAMINE AMMONIA-LYASE BY 5,5'-DITHIOBIS(2-NITROBENZOIC ACID)

FURTHER EVIDENCE FOR THE INVOLVEMENT OF SULFHYDRYL GROUPS IN ADENOSYLCOBALAMIN-DEPENDENT REARRANGEMENTS

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Summary

Treatment of the adenosylcobalamin-requiring enzyme ethanolamine ammonia-lyase (EC 4.3.1.7) with 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs_2) discloses three classes of -SH groups: a rapidly reacting class (2 -SH groups/enzyme molecule), a slowly reacting class (6 -SH groups/molecule) and a class which does not react unless the enzyme is denatured (7 -SH groups/molecule (Kaplan, B.H. and Stadtman, E.R. (1968) J. Biol. Chem. 243, 1794)). The enzyme is inactivated by Nbs_2 at a rate similar to the rate at which Nbs_2 reacts with the slowly reacting class of -SH groups. Inactivation of the enzyme is retarded by adenosylcobalamin but not by ethanolamine. Once inactivated, the enzyme cannot be reactivated with mercaptoethanol. These observations provide further evidence for the importance of -SH groups in catalysis by adenosylcobalamin-requiring enzymes.

Introduction

Ethanolamine ammonia-lyase (EC 4.3.1.7) is an adenosylcobalamin-dependent enzyme which catalyzes the conversion of ethanolamine to acetaldehyde and ammonia. This enzyme, like most other adenosylcobalamin-dependent enzymes, has been shown to contain essential sulfhydryl groups the blockage of which abolishes catalytic activity [1]. Thus, incubation of the enzyme with *p*-

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chlorophenylmercurisulfonate, a sulfhydryl reagent, destroys the ability of the enzyme to perform its function [2].

In an attempt to understand more completely the role of sulfhydryl groups in the adenosylcobalamin-dependent deamination of ethanolamine, we have carried out a series of studies employing the sulfhydryl reagent 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs_2). The spectral properties of Nbs_2 permit correlations to be made between the activity of the enzyme and the number of enzymatic -SH groups that have combined with the reagent. The results of this study are described in the following report.

Materials and Methods

Ethanolamine ammonia-lyase from *Clostridium* sp. was prepared and resolved from bound cobamides by the method of Kaplan and Stadtman [3]. The concentration of active sites was calculated on the basis of a molecular weight of 520 000 and two sites per enzyme molecule [2,4,5]. Nbs_2, yeast alcohol dehydrogenase and NADH were obtained from Sigma Chemical Co.

Adenosylcobalamin obtained from Calbiochem was purified from contaminating hydroxocobalamin by passage over a 6 ml column of CM-cellulose (H^+), eluting with water. The concentration of adenosylcobalamin was determined spectrophotometrically after photolysis and conversion to the dicyano derivative, using $\epsilon_{mM} = 30.1$ at 367 nm [6]. Enzyme activity was determined spectrophotometrically as described by Kaplan and Stadtman [3]. Protein concentration was determined using the method of Lowry et al. [7].

Titration of the sulfhydryl groups of ethanolamine ammonia-lyase with Nbs_2

Solutions of ethanolamine ammonia-lyase (15.7 μM in 0.02 M potassium phosphate buffer pH 7.4) and Nbs_2 (4 mM, in the same buffer) were each deoxygenated by six cycles of evacuation followed by flushing with nitrogen. 50 μl of each solution were placed in a 0.2 ml microcuvette (path length 1 cm), and the course of the reaction was followed at 412 nm in a Cary 118C recording spectrophotometer in which the cell compartment was constantly purged with nitrogen until no further change in absorbance took place (A_∞). The difference between A_∞ and the absorbance at time t (A_t) was plotted semilogarithmically as a function of time, and rate constants and stoichiometry for the reaction of Nbs_2 with the enzyme were obtained from that plot as described below. Stoichiometry was calculated using a millimolar extinction coefficient (ϵ_{mM}) for 2-nitro-5-thiobenzoic acid of 13.6 [8].

Results

Ethanolamine ammonia-lyase contains sulfhydryl groups which are able to react with Nbs_2. When the course of the reaction was monitored spectrophotometrically at 412 nm, it became apparent that the reactive -SH groups could be divided into two classes according to their rate of reaction with Nbs_2 (Fig. 1). The rapidly reacting class contained 2 -SH groups/enzyme molecule, and displayed a pseudo-first-order reaction rate with Nbs_2 of 1.4 min^{-1}. The other class racted at a much slower rate (0.026 min^{-1}), and contained 6 -SH groups/en-

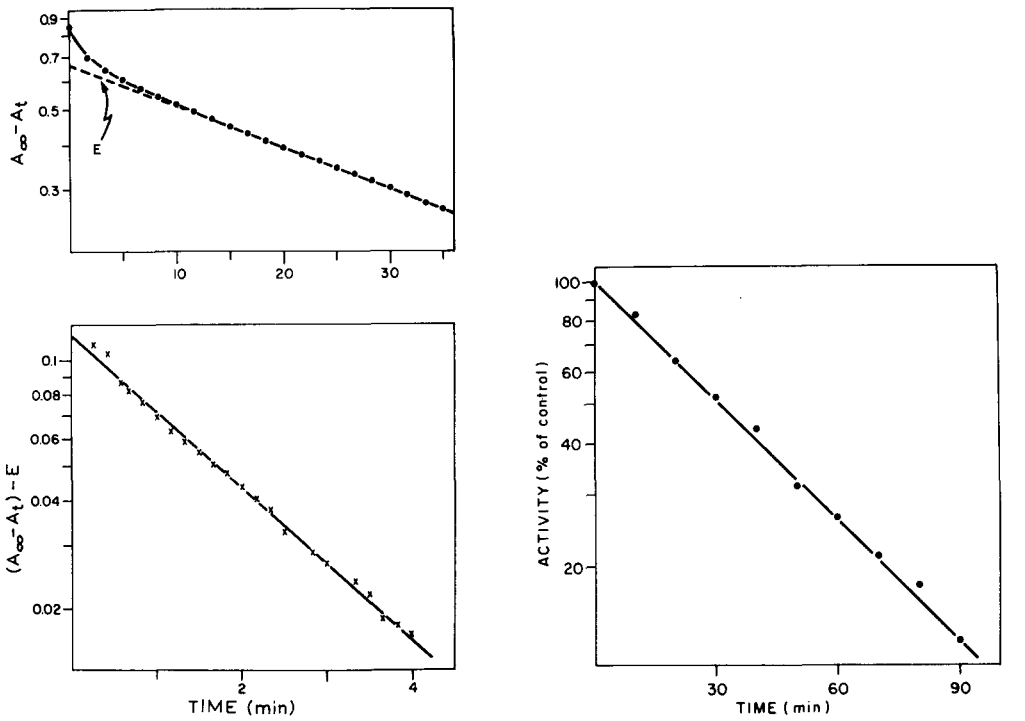


Fig. 1. The reaction of ethanolamine ammonia-lyase with Nbs_2. The experiment was carried out as described in Materials and Methods. Top: The course of the reaction for the first 40 min, plotted as described in Materials and Methods. The biphasic nature of the reaction is shown. The rate constant for the slow phase was calculated from the slope of the linear portion of the curve, and the stoichiometry from the value for $A_0 - A_\infty$ obtained by extrapolating this portion of the curve to $t = 0$ (denoted "E"). Bottom: The rapid phase of the reaction between enzyme and Nbs_2. This plot was prepared by subtracting curve "E" (upper panel) from the total absorbance. The subtracted value represents the contribution of the slow phase to the initial stages of the reaction between enzyme and Nbs_2. The rate constant for the rapid phase of the reaction was calculated from the slope of this plot, and the stoichiometry from the balue of the intercept at $t = 0$.

Fig. 2. Inactivation of enzyme with Nbs_2 as a function of time. The reaction mixture contained 0.084 nmol ethanolamine ammonia-lyase, 40 nmol Nbs_2 and 400 nmol of potassium phosphate buffer (pH 7.4) in a volume of 20 µl. At the times indicated, 1 µl aliquots were withdrawn and the activity determined using the standard assay. The incubation and assays were conducted at 23°C.

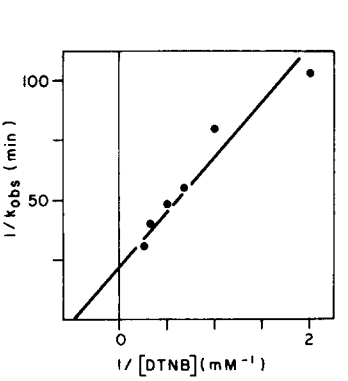
zyme molecule. Kaplan and Stadtman also reported the presence of a class of -SH groups on ethanolamine ammonia-lyase which reacted rapidly with Nbs_2 [2], although their studies revealed only 1 -SH group/enzyme molecule in that class. Unlike the present findings, they noted no further reaction with Nbs_2 unless the enzyme was denatured with SDS; it is likely, however, that the difference between their results and ours reflects differences in experimental conditions, since much lower concentrations of Nbs_2 were employed in their experiments than in ours.

Ethanolamine ammonia-lyase is inactivated by Nbs_2. The time course of this process is shown in Fig. 2. The inactivation is strictly pseudo-first-order, with a rate constant of 0.022 min^{-1}. The rate of inactivation of enzyme is thus similar to the slower of the two rates observed for the reaction of enzyme -SH groups with Nbs_2.

Determination of the rate of inactivation as a function of Nbs_2 concentration revealed saturation kinetics (Fig. 3). From the reciprocal plot, the K_m for Nbs_2 is approximately 2 mM. This finding, which is similar to observations made in other studies of enzyme inhibition by sulfhydryl reagents [19], suggests that a reversible interaction between Nbs_2 and enzyme, possibly involving the non-covalent binding of the reagent to the enzyme, takes place prior to inactivation.

With other adenosylcobalamin-dependent enzymes, it has been shown that inactivation by sulfhydryl reagents is retarded or prevented in the presence of the cofactor. Similar results were obtained with ethanolamine ammonia-lyase (Table I). In the presence of adenosylcobalamin, the rate of inactivation of the enzyme by Nbs_2 was substantially reduced. Calculations from the data in the table, assuming that the binding to the enzyme of adenosylcobalamin and Nbs_2 are mutually exclusive, indicate that the K_m for adenosylcobalamin in terms of its ability to retard the inactivation of the enzyme by the sulfhydryl reagent is 1.7 μM. This value is in excellent agreement with the K_m of 1.5 μM for adenosylcobalamin as a cofactor for the deamination of ethanolamine [3], and suggests that protection against inactivation involves the binding of the cofactor to the active site of the enzyme. Unlike adenosylcobalamin, the substrate ethanolamine appears to have no effect on the inactivation of the enzyme by Nbs_2.

Because the inactivation of the enzyme is presumably associated with the formation of mixed disulfide bonds between enzyme -SH groups and 2-nitro-5-thiobenzoic acid, an attempt was made to reactivate the enzyme by displacing the enzyme bound thiophenol residues with a competing sulfhydryl compound.



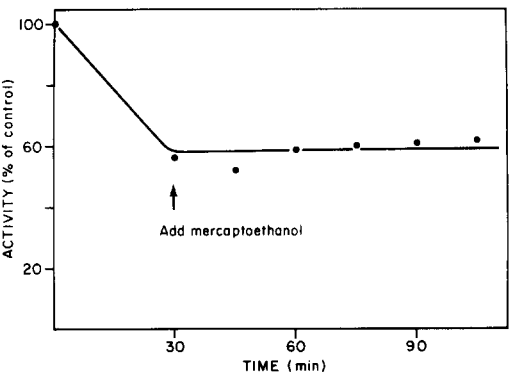


Fig. 3. The effect of Nbs_2 concentration on the pseudo-first-order rate constant for enzyme inactivation. Ethanolamine ammonia-lyase (4.2 μM) was incubated at 23°C with 0.5—4 mM Nbs_2 in 0.02 M potassium phosphate buffer, pH 7.4. Aliquots of the incubation mixtures were withdrawn at 10-min intervals and assayed for residual activity as described in Materials and Methods. Pseudo-first-order rate constants for inactivation were calculated from semilogarithmic plots of the data (see Fig. 2). DTNB, 5,5'dithiobis(2-nitrobenzoic acid).

Fig. 4. Failure of β-mercaptoethanol to restore the activity of enzyme inactivated with Nbs_2. Ethanolamine ammonia-lyase (3.2 μM) was incubated at 23°C with 2 mM Nbs_2 in 0.02 M potassium phosphate buffer, pH 7.4 (reaction volume 50 μl). After 30 min, 1 μl of 0.71 M β-mercaptoethanol was added to the reaction mixture, giving a final concentration of 12 mM β-mercaptoethanol. At the times indicated, 1 μl aliquots of the reaction mixture were withdrawn and assayed for activity as described in Materials and Methods. "Control" was the activity at zero time.

TABLE I

Effect of adenosylcobalamin and ethanolamine on the inactivation of enzyme by Nbs_2. Ethanolamine ammonia-lyase (4.2 μM) was incubated at 23°C with either 2 mM Nbs_2 (for experiments with adenosylcobalamin) or 1 mM Nbs_2 (for experiments with ethanolamine) in 20 mM potassium phosphate buffer, pH 7.4. Adenosylcobalamin or ethanolamine were present as indicated. At various times (within the first hour if adenosylcobalamin was used, so the slow inactivation of enzyme in the presence of cofactor [2] would not affect the results), 1-μl aliquots were withdrawn from the incubation mixtures and the activity determined as described in Materials and Methods. The pseudo-first-order rate constants of inactivation were calculated as described in the legend to Fig. 3. The k_{inact} shown is the average of two determinations.

Addition (μM)	k_{inact} (min^{-1})
Adenosylcobalamin	
0	0.023
2	0.014
5	0.010
Ethanolamine	
0	0.012
100	0.012

In the experiment, catalytic activity was followed with time in an enzyme-containing reaction mixture to wich Nbs_2 was added to zero time and β-mercaptoethanol 30 min later. The results (Fig. 4) show that, while the process of inactivation ceased upon addition of mercaptoethanol, probably owing to the destruction of Nbs_2 by the new thiol, enzyme inactivated before the addition of mercaptoethanol did not recover its activity.

Discussion

The sulfhydryl groups of ethanolamine ammonia-lyase seem to fall into three categories: those which react rapidly with Nbs_2, those which react slowly with Nbs_2, and those which do not react with Nbs_2 unless the enzyme is denatured (this last category is defined by the observation [2] that the number of -SH groups reacting with Nbs_2 in the presence of SDS exceeds by 7 the maximum number measured in our titration, which was carried out in the absence of a denaturing agent). Reaction of the second category of -SH groups with Nbs_2 is accompanied by the inactivation of the enzyme, a process which is not reversed when a competing thiol is added to the incubation mixture in an attempt to displace Nbs_2 from the enzyme. Inactivation of ethanolamine ammonia-lyase by Nbs_2 suggests that an -SH group at the active site is essential for its function, such that when this group is blocked the enzyme is no longer able to catalyze the deamination of substrate. Inactivation by spontaneous denaturation of the enzyme after reaction with the sulfhydryl reagent, however, has not been ruled out.

The ethanolamine ammonia-lyase apoenzyme, like other adenosylcobalamin-requiring apoenzymes, is protected against Nbs_2 inactivation by the cofactor adenosylcobalamin. Protection is generally ascribed to a steric effect wherein adenosylcobalamin blocks access of the sulfhydryl reagent to the essential -SH group at the active site. Unlike the cofactor, the substrate is unable to protect the enzyme against Nbs_2 even at concentrations exceeding its K_m.

The present observations add to the evidence that -SH groups participate in adenosylcobalamin-requiring reactions. Their involvement as electron carriers in the conversion of ribonucleotide triphosphates to deoxyribonucleotide triphosphates by the adenosylcobalamin-requiring ribonucleotide reductase from *Lactobacillus leichmannii* is well documented [10]. The nature of their participation in adenosylcobalamin-dependent rearrangements, however, is not understood.

Acknowledgements

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