The Mechanism of Action of Ethanolamine Ammonia-lyase, an Adenosylcobalamin-dependent Enzyme

THE SOURCE OF THE THIRD METHYL HYDROGEN IN THE 5'-DEOXYADENOSINE GENERATED FROM THE COFACTOR DURING CATALYSIS*

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Ethanolamine ammonia-lyase is an adenosylcobalamin-dependent enzyme which catalyzes the conversion of ethanolamine and propanolamine to ammonia and the corresponding aldehydes. A mechanism has been proposed for this and other adenosylcobalamin-dependent reactions which involves cleavage of the carbon-cobalt bond of the cofactor followed by abstraction of a substrate hydrogen atom by the adenosyl fragment to form 5'-deoxyadenosine. In support of this proposal, a previous study demonstrated that the deamination of propanolamine by ethanolamine ammonia-lyase is accompanied by the reversible cleavage of the carbon-cobalt bond of the cofactor, with the production of 5'-deoxyadenosine (Babior, B. M., Carty, T. J., and Abeles, R. H. (1974) J. Biol. Chem. 249, 1689-1695). The present study is concerned with the origin of the third hydrogen atom on the methyl group of the 5'-deoxyadenosine produced in that reaction. The 5'-deoxyadenosine isolated from an incubation mixture initially containing enzyme, [5',5'-D]adenosylcobalamin, and [l,l-D]propanolamine was chemically degraded so that the 4' and 5' carbon atoms were, respectively, converted to the carboxyl and methyl carbons of acetaldehyde. Analysis of the p-nitrophenylhydrazone of the acetaldehyde by gas-liquid chromatography-mass spectroscopy revealed 3 deuterium atoms/molecule, indicating that two of the methyl hydrogens originated from adenosylcobalamin and the third was donated by substrate. This observation provides further support for the participation of 5'-deoxyadenosine in the mechanism of adenosylcobalamin-dependent reactions.

Ethanolamine ammonia-lyase (EC 4.3.1.7) catalyzes the adenosylcobalamin-dependent conversion of 2-aminoethanol and 2-aminopropanol to ammonia and the respective aldehydes (2, 3). In the course of this reaction, a hydrogen originally attached to the carbinol carbon of substrate is transferred to the adjacent carbon atom, appearing in the methyl carbon of acetaldehyde or the methylene carbon of propionaldehyde, depending on the starting material (3, 4).

H  H
C=C=O = C=CH

In this process the cofactor participates as an intermediate hydrogen carrier, accepting the migrating hydrogen from the substrate and conveying it in a subsequent step to the product (5). The position on the cofactor to which the migrating hydrogen atom is transferred is the adenosine carbon atom that is bonded to the cobalt of the corrin ring (hereinafter referred to as the C-5' carbon). Much evidence has suggested that the mechanism of hydrogen transfer to the cofactor involves homolysis of the carbon-cobalt bond followed by a free radical hydrogen abstraction from the substrate (6–9). In the first step, cob(I)alamin and the 5'-deoxyadenosyl-5'-yl radical are formed, and in the second step the radical abstracts a hydrogen atom from the substrate to form 5'-deoxyadenosine and substrate radical. 5'-Deoxyadenosine is a crucial intermediate in this...
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that amount of deuterium. Since only limited amounts of coenzyme were used previously, 1.6 atoms of deuterium were incorporated into the were available, no deuterium analysis was carried out. Other reagents terminated.

propanol as substrate; therefore, no estimate can be made as to the known about the kinetics of this exchange reaction with 2-amino-

D,]propanol, and 10 amol of potassium phosphate buffer (pH 7.4) in a nmol of ethanolamine ammonia-lyase, 78 nmol of [5',5'-D,,U-

reaction mixtures were prepared. Reaction mixture A contained 40 tion. except that it contained only 67 nmol of [U-14C]adenosyl-

"ClAdenosylcobalamin (8), L-2-amino-[l,l-D,]propanol (7), and 5'-

incubating ["Cladenosylcobalamin in D,O with ribonucleotide reduc-

"Cladenosylcobalamin was purchased from Calbiochem. Ribonucleotide reductase shown to possess two active sites per molecule (18). Adenosyl-

coenzyme during catalysis (8, 9) and that several other adenosylcobalamin-dependent enzymes, including diol dehy-

drase (10), methylmalonyl-CoA mutase (11), and P-lysine mutase (12), generate the nucleoside under other conditions. The reversibility of the formation of 5'-deoxyadenosine from adenylcobalamin has also been shown (9). With diol dehy-

drase, arguments have been made, based on stereochemical and kinetic results (13, 14), which suggest that a compound with three equivalent hydrogens (two derived from the coen-

zyme and one from the substrate) must be an intermediate in the catalytic process; 5'-deoxyadenosine is a logical choice for this intermediate. Kinetic evidence for the intermediate in-

volvement of 5'-deoxyadenosine has also been presented for glutamate mutase (15) and methylmalonyl-CoA mutase (16).

Despite the evidence described above, which strongly supports a catalytic role for 5'-deoxyadenosine in adenylcobalamin-

dependent rearrangements, it has not yet been directly shown that the hydrogens on the methyl group of 5'-deoxyadenosine isolated when the catalytic process is interrupted are derived solely from coenzyme and substrate, as required by the proposed mechanism. Alternative origins for 5'-deoxyadeno-

sine can be envisioned, as for example its production if an adenosyl radical were formed which abstracts a hydrogen from the environment (protein or solvent) during the process of isolation. We have therefore investigated the origin of the methyl hydrogen atoms of the 5'-deoxyadenosine generated during the ethanolamine ammonia-lyase-catalyzed deamina-

tion of propanolamine. MATERIALS AND METHODS

Ethanolamine ammonia-lyase from Clostridium sp. was prepared and resolved of bound cobamides by the method of Kaplan and Stadtman (2). Enzyme concentration was calculated on the basis of a molecular weight of 520,000 (17). The enzyme had previously been shown to possess two active sites per molecule (18). Adenyl-

cobalamin was purchased from Calbiochem. Ribonucleotide reductase was prepared by the method of Hogenkamp et al. (19). [U-

-14C]Adenosylcobalamin (8), L-2-arginine-[1,1-D,]propanol (7), and 5'-

deca deoxyadenosine (10) were prepared as previously described (8,9,5-D,]-U-14C]Adenosylcobalamin (4.8 x 10^10 cpm/μmol) was prepared by incubating [14C]adenosylcobalamin in D,O with ribonucleotide reductase in the presence of a reducing agent (19, 20). When this procedure was used previously, 1.6 atoms of deuterium were incorporated into the coenzyme. We therefore assume that the coenzyme obtained contained that amount of deuterium. Since only limited amounts of coenzyme were available, no deuterium analysis was carried out. Other reagents were the best grade commercially available, and were used without further purification.

Isolation and Degradation of 5'-Deoxyadenosine—The following two reaction mixtures were prepared. Reaction mixture A contained 40 nmol of ethanolamine ammonia-lyase, 78 nmol of [5',5'-D,,U-

-14C]adenosylcobalamin (3.8 x 10^10 cpm), 10 μmol of L-2-arginine-[1,1-

D,]propanol, and 10 μmol of potassium phosphate buffer (pH 7.4) in a total volume of 1.3 ml. Reaction mixture B was identical in composition, except that it contained only 65 nmol of [U-14C]adenosyl-

cobalamin (3.9 x 10^10 cpm), and neither the coenzyme nor L-2-amino-

propanol contained deuterium. The same procedure was followed with each of the reaction mixtures. The incubations were begun with addition of adenosylcobalamin (in order to prevent the enzyme- dependent rearrangements of the hydrogen donor by products, which proceed in the dark at room temperature for 5 min. Trichloroacetic acid (20%, w/v), 0.3 ml, was then added, the precipitated protein removed by centrifugation and washed with 1 ml of 3% (w/v) trichloroacetic acid, and the supernatant and washings were pooled. The pooled fluids were combined and extracted four times with 10-ml portions of ether. The aqueous layer was then brought to pH 8 to 9 with NaOH and concentrated to dryness on a rotary evaporator. The dry residue was extracted with 0.2 ml of methanol and then with 0.1 ml of methanol, and the combined extracts streaked on Whatman No. 3MM paper which had been prewashed sequentially with 12.4 m pyridine, 0.1 m NaOH, and ethanolamine. L-arginine and ethanolamine were detected with 1% NH₄OH at 400 V for 6.5 hours. After electrophoresis, the major radioactive area was located by scanning with a Tracelab 4X scanner and eluted with water. The eluate was concentrated and subjected to ascending paper chromatography for 14 hours on Whatman No. 3MM paper, developing with water-saturated 2-butanol. Scanning revealed a peak of radioactivity with an Rs identical with that of 5'-deoxy-

adenosine, and a minor area of radioactivity (3 to 5%) which coincided with adenosylcobalamin. Up to this point, all operations were carried out in the dark. The major area was eluted with water. The amount of 5'-deoxyadenosine recovered was determined by the total amount of radioactivity as measured by liquid scintillation counting. From reaction mixture A, 49 nmol (2.4 x 10^10 cpm) of 5'-deoxyadenosine were recovered, while 75 nmol (3.6 x 10^10 cpm) were recovered from reaction mixture B. To each reaction mixture 0.37 μmol of 5'-deoxyadenosine was added, and the eluate brought to dryness on a rotary evaporator. The dry residue was dissolved in 2.0 ml of 0.1 N HCl, and the solution sealed in a tube under vacuum and heated for 1 hour at 100° to hydrolyze the 5'-deoxyadenosine. The hydrolysate was applied to a column of Dowex 50-X2 (H+) (1.1 x 8.0 cm). The column was eluted with 0.1 N HCl, which washed the 5'-deoxyribose but not the adenine from the column, and the fractions containing radioactivity were combined and lyopholized. Water (0.5 ml) was added to the dry residue and the solution again lyopholized. To oxidize the sugar, the residue was taken up in 2 ml of cold 0.075 M NaIO₄, pH 6.5, and allowed to stand in the refrigerator for 25 hours. The solution was then subjected to bulb to bulb distillation in vacuum. The acetaldehyde formed in this oxidative reaction was derivatized by adding to the distillate 4.0 ml of 0.25% p-nitrophenylhydrazine in redistilled methanol. After standing overnight in the refrigerator, the solution was brought to dryness in a rotary evaporator. The residue was dissolved in 2 to 3 ml of benzene and introduced onto a dry alumina (Alcoa) column (0.6 cm x 5 cm). A column of benzene was used to wash the column free of the benzene eluate was then concentrated. The benzene eluate was then concentrated to dryness under a stream of N₂, and the residue was dissolved in 0.2 ml of ethanol and subjected to ascending paper chromatography. For chromatography, Whatman No. 3MM, prewashed as described above, was impregnated with 30% formamide in absolute ethanol. The sample was streaked on the paper and chromatography was carried out with cyclohexane/benzene/formamide (250/200/15, (v/v)). The material with a mobility corresponding to that of authentic acetaldehyde p-nitrophenylhydrazine was eluted with ethanol and concentrated (approximately to 0.2 ml) under N₂. It was then taken up in 1 ml of H₂O and passed through a small column of Amberlite XAD-2 to remove formamide. The column was washed with 15 ml of water, and the hydrazone eluted with ethanol. At this point, 12.5 μg of hydrazide were recovered from Experiment A and 8.5 μg from Experiment B. The eluate was concentrated to dryness under a stream of N₂.

Mass Spectrometry—Gas-liquid chromatography-mass spectrometry was performed on an LKB 9000 combined instrument. The samples of acetaldehyde p-nitrophenylhydrazine were dissolved in 90 ml of CH₄Cl₄. Portions of 35 μl were absorbed on small cylinders of stainless steel gauge (21) and allowed to dry. The dry samples were injected in the flash heater (275°). The sample was impregnated on Gas-Chrom Q; 1.8 x 4 mm (iner diameter) was maintained at 168-170°, the helium carrier gas flow at 27 ml/min. Mass spectra were taken at 13 eV.

RESULTS AND DISCUSSION

In this study, an incubation containing equivalent amounts of ethanolamine ammonia-lyase and [5',5'-D,]adenosyl-

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cobalamin was terminated with trichloroacetic acid while the enzyme was engaged in catalyzing the deamination of 2-amino-[1,1-D₂]propanol. The 5'-deoxyadenosine that has been shown to be formed reversibly at the active site under these conditions was isolated, and the deuterium content of its methyl group was analyzed by gas-liquid chromatography-mass spectrometry after suitable derivatization. The presence of 3 deuterium atoms in the methyl group would indicate that the hydrogens in this position were derived solely from coenzyme and substrate as predicted by the proposed mechanism (8, 9). The incubation with undeuterated material served as control. Under the conditions of chromatography, authentic acetaldehyde p-nitrophenylhydrazone emerged from the column as a single peak with a retention time of 9 min (Fig. 1). Mass spectrometry of this peak revealed a prominent molecular ion at m/e 179. This was the ion used for determination of the deuterium content of the experimental samples. The relative heights of the mass spectrometry peaks in the vicinity of the molecular ion are given in Table I. Data from the gas chromatograph detector showed that the experimental samples were heavily contaminated with extraneous material. With both experimental samples, however, the only compound emerging from the column in the first 18 min that gave a mass spectrometry peak at m/e 179 displayed chromatographic properties virtually identical with those of authentic acetaldehyde p-nitrophenylhydrazone (Fig. 2). From these findings we conclude that this mass spectrometry peak corresponds to the undeuterated hydrazone in the experimental material.

The mass spectra of the sample obtained from the experiment with deuterated substrate and cofactor are presented in Fig. 2 (right). In this figure, the size of the peaks at m/e 179, 181, and 182 (i.e. M, M + 2, and M + 3) are plotted against the retention time. The peak heights are corrected for 13C, assuming a molecular formula of C₉H₈₋₅DₐNₐO₈ (n = 0, 2, 3) for the various species represented. Relative proportions of the three species were determined by experimental integration of the three curves in Fig. 2 (right), a procedure accomplished by cutting out and weighing Xerox copies of the curves. The predominant species are those corresponding to M and M + 3, comprising 91.1% and 7.2% of the total, respectively, while M + 2 only accounts for 1.7% of the total. The results obtained from mass spectrometry thus show that 8.9% of the molecules present contain deuterium. From the original reaction, 49 nmol of 5'-deoxyadenosine were isolated and diluted with 370 nmol of unlabeled 5'-deoxyadenosine. Therefore, 11.7% of the 5'-deoxyadenosine molecules should be deuterated, if one assumes that the original cofactor was completely deuterated. This number is in reasonable agreement with the percentage determined independently by gas-liquid chromatography-mass spectrometry. The data show that 81% of the deuterated acetaldehyde methyl groups contained 3 deuterium atoms and 19% contained 2 deuterium atoms. Thus, of the methyl groups of 5'-deoxyadenosine, 81% contained 3 deuterium atoms, indicating that the third hydrogen atom which is required to convert the 5'-methylene group of the coenzyme to a methyl group is primarily derived from the substrate, not from the environment (protein or solvent). The deuterated species could have arisen from a number of sources. (a) Some loss from the methyl group of acetaldehyde could have occurred during the process of isolation. (b) Dideuterated acetaldehyde could have appeared because the coenzyme used was not completely deuterated (see above). For reasons indicated elsewhere, it is difficult to estimate how much hydrogen this would contribute to the methyl group. (c) The 5'-deoxyadenosyl radical, a species which we believe participates in the catalytic process, may well be present under steady state conditions and could be converted to 5'-deoxyadenosine by abstraction of a hydrogen atom from the environment during isolation.

According to the mechanism which we have proposed, an important and sometimes disputed step in the sequence of reactions catalyzed by ethanolamine ammonia-lyase is the abstraction of a hydrogen atom from the substrate by a radical derived from the adenosyl moiety of the coenzyme to form 5'-deoxyadenosine. We believe that the evidence now available leaves little doubt concerning the involvement of 5'-deoxyadenosine in the catalytic process with ethanolamine ammonia-lyase, and (by analogy) supports its participation in other adenosylcobalamin-dependent rearrangements such as those catalyzed by diol dehydrase (10, 13, 14), glutamate mutase (15), and methymalonyl-CoA mutase (11, 16).

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![Fig. 1. Gas-liquid chromatography of authentic acetaldehyde p-nitrophenylhydrazone. The hydrazone (12 nmol) was chromatographed as described in the text. The sample was injected at the time indicated by the arrow.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>m/e</th>
<th>Relative peak height</th>
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<td>177</td>
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<tr>
<td>178</td>
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</tr>
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<td>182</td>
<td>0</td>
</tr>
<tr>
<td>183</td>
<td>0</td>
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* Molar ion.

*No ion was detected at m/e 180 (M + 1).*
REFERENCES

The mechanism of action of ethanolamine ammonia-lyase, an adenosylcobalamin-dependent enzyme. The source of the third methyl hydrogen in the 5'-deoxyadenosine generated from the cofactor during catalysis.

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