BBA 65702

PROTON RETENTION IN THE γ-ELIMINATION REACTION CATALYSED BY CYSTATHIONINE γ-SYNTHASE

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(Received October 9th, 1967)

SUMMARY

The ability of cystathionine γ-synthase to catalyze both γ-elimination and γ-replacement reactions with the substrate *O*-succinylhomoserine makes it unique among pyridoxal enzymes. The results of carrying out the γ-elimination reaction in 99.5% ^2H_2O show that 80% of the hydrogen which replaces succinate in the γ position originates not in the solvent but in some part of the substrate molecule. The enzyme catalyzes a rapid exchange of hydrogen between cystathionine and water, a finding which precluded studies of the fate of hydrogen during the γ-replacement of succinate by cysteine.

INTRODUCTION

Cystathionine γ-synthase, a pyridoxal phosphate enzyme which has been isolated in pure form from Salmonella[1], catalyzes replacement of the succinate group of *O*-succinylhomoserine by cysteine or, alternatively, in the absence of cysteine, elimination of succinate to form α-ketobutyrate and ammonia[2]. In either case the mechanism may involve a 3,4-unsaturated intermediate (Fig. 1, Reactions 1 and 2) or a more direct displacement of succinate by cysteine or hydrogen (Fig. 1, Reactions 3 and 4). This report describes attempts to test these hypotheses by carrying out the two reactions in ^3H- and ^2H-labeled water.

METHODS

Cystathionine γ-synthase was purified as described previously[1] and assayed by continuous spectrophotometric measurement, with NADH and lactate dehydrogenase, of the α-ketobutyrate produced by γ-elimination (Assay B)[3].

2,4-Dinitrophenyl- (DNP-) hydrazone derivatives of α-ketobutyrate and pyruvate were prepared by adding equimolar DNP-hydrazine to the acidified reaction mixture and collecting the precipitated DNP-hydrazone on a sintered glass filter. The ^3H content of these highly colored derivatives was determined by burning them to CO_2

and H_2O and counting the water in a Packard liquid scintillation spectrometer[4,5]. Alternatively, α-ketoacids could be isolated by applying the reaction mixtures to columns of Dowex 1 (Cl^-) and eluting with a gradient of 0 to 0.2 M HCl. Results obtained by the two methods were in agreement.

Deuterium analyses of DNP-hydrazone derivatives of α-ketoacids, isolated from reaction mixtures in ^2H_2O, were performed by J. NEMETH, 303 West Washington Street, Urbana, Ill., U.S.A.

L-Cystathionine was obtained from the Cyclo Chemical, α-ketobutyrate from Aldrich, lactate dehydrogenase from Boehringer, Mannheim, tritium water from New England Nuclear, heavy water (99.7% ^2H_2O) from Isomet, and deuteroacetone from Merck, Montreal.

RESULTS

If the γ-replacement reaction involves a 3,4-unsaturated intermediate, solvent hydrogen would be incorporated into both α and β positions of cystathionine (Fig. 1, Reaction 1); but if the substitution is more direct (Fig. 1, Reaction 3), solvent hydrogen would be introduced only at the α position. Attempts to answer this question by carrying out the replacement reaction in tritiated water were thwarted by the discovery that the enzyme catalyzed an extremely rapid exchange of ^3H into cystathionine itself (this exchange was first observed by Dr. MARSHAL KAPLAN (personal communication)). With pure enzyme and 2 mM L-cystathionine the initial rate of exchange (assuming no isotope effect) was about 400 μatoms of hydrogen per min per mg protein*, compared with v_{max} values of 100 μmoles per min per mg protein for the γ-replacement reaction

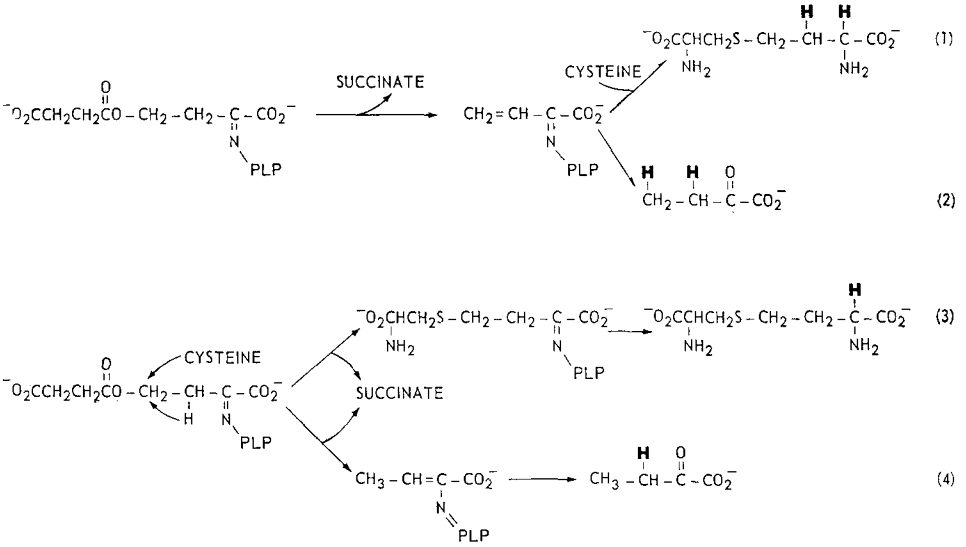

Fig. 1. Mechanisms of γ-replacement (Reactions 1 and 3) and γ-elimination (Reactions 2 and 4). PLP = pyridoxal phosphate. The steps of Schiff base formation and α-hydrogen labilization are not shown. Hydrogens originating in the solvent are shown in bold face type.

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* S. GUGGENHEIM AND M. FLAVIN, unpublished observations.

and 20 µmoles per min per mg protein for the γ-elimination reaction. Even more surprising was the finding that at equilibrium a total of 4 hydrogens of cystathionine had exchanged with water.

Like the replacement reaction, the elimination reaction might also either involve a 3,4-unsaturated intermediate[6] (Fig. 1, Reaction 2); or the separation of the leaving group might, in the absence of cysteine, become coupled with a β to γ hydride transfer[7] (Fig. 1, Reaction 4). In the former case solvent hydrogen would be incorporated into both β and γ positions of α-ketobutyrate; whereas the hydride transfer would introduce solvent hydrogen only at the β position.

The γ-elimination reaction was first studied in tritiated water[5] (S. GUGGENHEIM AND M. FLAVIN, unpublished observations). The enzymically formed α-ketobutyrate was found to have acquired 0.20 ±0.03 atom solvent hydrogen per mole assuming no isotope discrimination. (This value was obtained after subtracting 0.06 ±0.03 atom solvent hydrogen per mole incorporated in control experiments when the same amount of enzyme was incubated with α-ketobutyrate and the other reaction products.) Since high isotope discrimination against ^{3}H has been observed in similar reactions[8,9] it was not possible to say whether the incorporated ^{3}H was in one or more than one position.

O-Succinyl-L-serine*, although not a naturally occurring substrate, was found to undergo cystathionine γ-synthase catalysed β-elimination. When this reaction was carried out in tritiated water the resulting pyruvate contained 0.20 atom solvent hydrogen per mole, the same amount as found in the α-ketobutyrate formed by γ-elimination. Since β-elimination could introduce ^{3}H at only one position, it seemed possible that the ^{3}H introduced by γ-elimination might also be in one position (Fig. 1, Reaction 4).

To test this possibility the γ-elimination reaction was carried out in pure ^2H_2O, thus overcoming the problem of isotope discrimination. At 37° and pH 8.2 (or p^{2}H 8.2)

TABLE I

INCORPORATION OF DEUTERIUM INTO α-KETOBUTYRATE DURING CYSTATHIONINE γ-SYNTHASE CATALYSED γ-ELIMINATION IN ^2H_2O

The reactions were carried out in stoppered tubes at 35° containing in 2 ml of ^2H_2O: potassium pyrophosphate, 100 µmoles (pH 8.2); cystathionine γ-synthase, 0.05 mg; and the reactants listed below. After 1 h the reactions were stopped by adding 0.2 ml of 1.5 M trichloroacetic acid. After centrifugation carrier α-ketobutyrate was added and all the ketoacids were precipitated with DNP-hydrazine. The derivatives were reprecipitated from acetone and analysed for ^{2}H. A direct analysis of a dilution of Reaction mixture 1 showed that the ^{2}H content was 99.5%.

Reaction mixture	*Additions (µmoles)*	*α-Ketobutyrate treated with DNP-hydrazine*		*Atom % excess ^{2}H corrected for dilution*	*Atoms ^{2}H per mole of α-ketobutyrate DNP-hydrazone*
		Recovered (µmoles)	*Carrier (µmoles)*		
1	*O*-Succinyl-L-homoserine, 40	28	249	13.3	1.33
2	α-Ketobutyrate, 44; NH_4Cl, 40	44	396	3.4	0.34

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* This compound has been prepared recently in our laboratory from carbobenzoxy-L-serine (Cyclo Chemical) by the method previously used to synthesize succinylhomoserine[10].

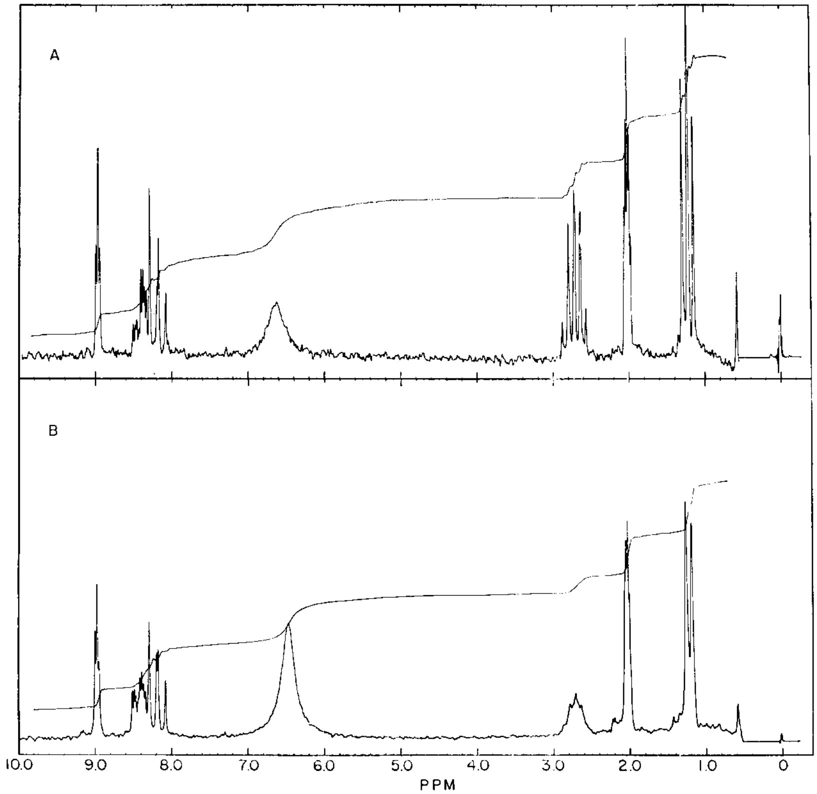

Fig. 2. 100 megacycle field sweep nuclear magnetic resonance spectra of non-deuterated (A) and deuterated (B) α-ketobutyrate DNP-hydrazones in deuteroacetone. The concentration of the derivatives was 75 mg/ml. The correspondence of protons to signal position is as follows: 3-H of aromatic ring, 9.0 parts per million; 5-H and 6-H of aromatic ring, 8.3 parts per million; exchangeable protons and contaminating H_2O, 6.5 parts per million; methylene protons, 2.7 parts per million; residual protons of deuteroacetone, 2.0 parts per million; methyl protons, 1.2 parts per million, spinning sideband of tetramethylsilane, 0.3 part per million; tetramethylsilane internal reference, 0.0 part per million. The step shaped curves in each spectrum are the cumulative integrals of the spectra themselves.

$k_H/k_{^2H}$ for this reaction was 1.25. Table I shows the results of an experiment in which α-ketobutyrate was formed enzymically in 99.5% ^2H_2O. The DNP-hydrazone of the α-ketobutyrate formed contained 1.33 atoms ^2H per mole compared with 0.34 atom ^2H per mole in the DNP-hydrazone of α-ketobutyrate from a control incubation of enzyme, ammonia and α-ketobutyrate. Slightly less ^2H was incorporated when the reaction was carried out in 96% ^2H_2O. The enzymically formed α-ketobutyrate contained 1.12 atoms ^2H per mole and the α-ketobutyrate from the control incubation contained 0.16 atom ^2H per mole. Subtraction of the control values is probably not justified for two reasons. The ^2H incorporated into α-ketobutyrate by exchange may go into the same position as the ^2H introduced in the γ-elimination reaction; in this case exchange would not increase the net ^2H content of the α-ketobutyrate formed by γ-elimination. Furthermore the α-ketobutyrate formed by γ-elimination is not in contact with the enzyme for as long, on the average, as the added α-ketobutyrate in the control experiment.

TABLE II

RELATIVE INTENSITY OF SPECIFIC PROTON SIGNALS IN DNP-HYDRAZONES OF α-KETOBUTYRATE

10 integral spectra of each sample were obtained and the integral changes due to each signal were measured and averaged over the 10 determinations. For the non-deuterated sample the relative intensity was calculated relative to the average intensity of all 8 protons. For the deuterated sample the intensity was calculated relative to the average intensity of the 3 aromatic ring protons.

Signal	*Chemical shift (parts per million)*	*Number $H + {}^2H$ per position*	*Relative signal intensity*	
			Non-deuterated sample	*Deuterated sample*
Aromatic ring 3-H	9.0	1	1.00 ± 0.02*	0.98 ± 0.03
Aromatic ring 5-H + 6-H	8.3	2	2.01 ± 0.02	2.04 ± 0.04
Methylene $-CH_2-$	2.7	2	1.97 ± 0.02	0.95 ± 0.03
Methyl CH_3-	1.2	3	3.05 ± 0.03	2.80 ± 0.05

* Standard deviation.

In order to determine the position of the incorporated ^2H, 175 μmoles of α-ketobutyrate were prepared enzymically as in Reaction mixture 1 of Table I and precipitated as the DNP-hydrazone without adding any carrier. The 100 megacycle nuclear magnetic resonance spectrum of the resulting derivative is shown in Fig. 2B. For comparison, Fig. 2A shows the spectrum of the DNP-hydrazone derivative of a non-deuterated sample of α-ketobutyrate. The deuterated compound (Fig. 2B) appears to have only 1 hydrogen at the methylene carbon (the β-carbon of α-ketobutyrate) as shown by the smaller integral at 2.7 parts per million and the presence of a doublet instead of a triplet in the signal of the methyl bound hydrogens (the γ-carbon of α-ketobutyrate) at 1.2 parts per million. (The methylene hydrogen signal in the control compound, Fig. 2A, is a quintet because of *cis–trans* isomerism about the N=C double bond in the DNP-hydrazone derivatives.) Repeated determination of the integral curves with greater signal amplification allowed accurate calculations of the relative intensities of specific proton signals. The results shown in Table II indicate that 1.05 atoms of ^2H per mole are on the methylene carbon (the β-carbon) and 0.20 atom of ^2H per mole are on the methyl carbon (the γ-carbon). The total of 1.25 atoms ^2H per mole is in agreement with the independent analytic value of 1.33 atoms ^2H per mole. Some ^2H may be present in the exchangeable positions on the hydrazine and carboxyl groups of the α-ketobutyrate DNP-hydrazone.

DISCUSSION

The succinate lost from *O*-succinylhomoserine during γ-elimination is replaced by a hydrogen atom. The results of carrying out this reaction in ^2H_2O show that 80% of the hydrogen introduced at the γ position originates not in the solvent but presumably in some other part of the substrate molecule. This γ-hydrogen originally may have been the α-hydrogen of *O*-succinylhomoserine which is lost or one of the β-hydrogens which turn over in both Mechanisms 2 and 4 of Fig. 1. The problem could be resolved by studies with *O*-succinylhomoserine specifically deuterated in the α or β position.

Since exclusion of ^2H from the γ position was not complete, a hydride transfer mechanism (Fig. 1, Reaction 4) is unlikely. The results favor a protolytic mechanism (Fig. 1, Reaction 2) in which the enzyme protects the itinerant proton from undergoing complete exchange with the solvent. Several other cases of this kind have been discussed in a recent review[11].

Presumably the β-hydrogen which does originate in the solvent is introduced late in the course of the reaction (Fig. 1, Reaction 2). Indeed this protonation has been assumed to occur in a spontaneous step until recently when some evidence for enzymic catalysis has been reported[4,5].

The enzymic reaction most closely related to the γ-elimination and γ-replacement reactions of Fig. 1 is that in which *O*-phosphorylhomoserine yields threonine[12], a γ-elimination reaction coupled to introduction of a new substituent at carbon-3. When this reaction was carried out in ^2H_2O, exactly 1 atom of ^2H per mole was found in the γ position of threonine; $k_H/k_{^2H}$ for this reaction was 6.7 (see ref. 9). These results, and also the 40 to 1 discrimination against ^3H in the γ protonation[9], have been taken to mean that the γ protonation is rate limiting in the threonine synthase reaction[13]. In the γ-elimination reaction ^2H_2O inhibits relatively little ($k_H/k_{^2H} = 1.25$). However, since ^2H is largely excluded from the γ position of α-ketobutyrate, γ protonation might be rate limiting in this reaction also.

The failure of the migrating proton to exchange with ^2H_2O in Reaction 2 of Fig. 1 could be due to partial exclusion of water from the active site of the enzyme; water must, however, have access to the corresponding site of threonine synthase because the attacking nucleophile is a hydroxyl group. Other explanations have been proposed in relation to the aconitase reaction, which involves an elimination and addition of water, where solvent exchanges with the hydroxyl group but not with the proton[14].

ACKNOWLEDGEMENTS

We wish to thank Dr. C. PORANSKI, JR. of the U.S. Naval Research Laboratory, Washington D.C. who operated the 100 megacycle nuclear magnetic resonance spectrometer, and Dr. EDWIN BECKER of the National Institute of Arthritis and Metabolic Diseases who advised us in the interpretation of the spectra.

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