Studies on the Fermentation of D-α-Lysine. On the Hydrogen Shift Catalyzed by the B_{12} Coenzyme Dependent D-α-Lysine Mutase*

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ABSTRACT: The B_{12} coenzyme dependent D-α-lysine mutase of *Clostridium sticklandii* catalyzes the interconversion of D-α-lysine and 2,5-diaminohexanoate. In this reaction a hydrogen, abstracted from C-5 of lysine, replaces the amino group which migrates from C-6 to C-5. Using amino acids and B_{12} coenzyme suitably labeled with tritium, it was shown that B_{12} coenzyme serves as the carrier of the hydrogen that is transferred.

As in other B_{12} coenzyme mediated reactions, hydrogen is transferred to and from the 5′-carbon of the 5′-deoxyadenosine moiety covalently linked to the cobalt of the coenzyme molecule.

The B_{12} coenzyme dependent D-α-lysine mutase catalyzes the migration of the terminal amino group of lysine as shown in reaction 1. This is analogous to the amino group migration

$$\underset{\text{α-lysine}}{CH_2CH_2CH_2CH(NH_2)COOH} \rightleftharpoons \underset{\text{2,5-DAH}}{CH_3CH(NH_2)CH_2CH_2CH(NH_2)COOH} \quad (1)$$

catalyzed by β-lysine mutase (Stadtman and Renz, 1968; Tsai and Stadtman, 1968) which utilizes B_{12} coenzyme as an intermediate carrier in the stereospecific transfer of hydrogen from C-5 of β-lysine to C-6 of 3,5-diaminohexanoate (Retey *et al.*, 1969). Furthermore, as in several other B_{12} coenzyme mediated processes (Frey *et al.*, 1967; Barker, 1967), this hydrogen is transferred to and from the 5′-methylene carbon of the 5′-deoxyadenosyl moiety covalently linked to the cobalt atom of the coenzyme. Although B_{12} coenzyme could be presumed to play the same role in the D-α-lysine mutase catalyzed reaction, it seemed advisable to establish this point experimentally. Such a study became especially important when it was discovered that in the presence of pyridoxal phosphate, an essential cofactor for D-α-lysine mutase activity (Morley and Stadtman 1970), the enzyme catalyzes a slow exchange of the hydrogen of the terminal methylene group of D-lysine with that of water (C. G. D. Morley, unpublished results). This conceivably could influence the hydrogen migration reaction, at least in a quantitative manner.

The availability of the two protein moieties of D-α-lysine mutase, E_1 (the cobamide protein that also binds pyridoxal phosphate) and E_2 (the sulfhydryl protein) in highly purified form (Stadtman and Grant, 1971) made it possible to study the effects of varying ratios of the enzymes and B_{12} coenzyme on the hydrogen-transfer process. The results of the experiments using either tritiated α-lysine, 2,5-diaminohexanoate, or B_{12} coenzyme (DMBC)[1] are described in this paper.

Experimental Section

Materials. D-α-Lysine mutase was isolated from extracts of *Clostridium sticklandii* and purified (separated E_1 and E_2 proteins as described previously (Stadtman, 1966; Stadtman and Grant, 1971).

DMBC-*5′-t* (2.7×10^6 cpm/μmole) was the gift of Dr. H. P. C. Hogenkamp, DL-lysine-4,5-*t* (9.55×10^6 cpm/μmole) was supplied by Volk Radiochemical, and DL-lysine-6-*t* (1.9×10^7 cpm/μmole) by New England Nuclear. 2,5-DAH-*6-t* (1.27×10^6 cpm/μmole) and 2,5-DAH-*2*-^{14}C were prepared enzymically from DL-lysine-*6-t* and DL-lysine-*2*-^{14}C, respectively, and purified as described in the following section. Other materials were supplied as described elsewhere (Morley and Stadtman, 1970).

Solvents used in chromatography were (1) $CHCl_3$–CH_3OH–NH_4OH (12%) (40:40:15, v/v), (2) *sec*-butyl alcohol–glacial acetic acid–water (100:1:50, v/v), (3) *sec*-butyl alcohol–water–NH_4OH (25%) (100:30:14, v/v), and (4) *n*-butyl alcohol–glacial acetic acid–water (5:2:3, v/v).

Methods

Determination of Mutase Activity. Enzyme activity was measured by determining the amount of 2,5-diaminohexanoate formed under standard assay conditions (Morley and Stadtman, 1970).

Procedure for the Isolation of Amino Acids from Incubation Mixtures Containing DMBC-5′-t. The incubation mixtures (0.5 ml), containing DMBC-*5′-t*, were treated with 0.1 ml of $HClO_4$ (30%) and denatured protein removed by centrifugation. The acid supernatant solution together with a 0.3-ml water wash of the protein precipitate was adjusted to pH 5.0 and treated with a little Norit A charcoal. After vigorous shaking, the charcoal was precipitated by centrifugation at 20,000*g* for 30 min. The supernatant solution was treated with charcoal a second time and the pooled charcoal layers were washed with 1 ml of water. The above steps were carried out in dim light to prevent photolysis of the B_{12} coenzyme. The combined supernatant solution and water wash from the charcoal step contained greater than 98% of the amino acids originally present in the incubation mixture. This solution was then adjusted to pH 10 with concentrated KOH, centri-

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[1] Abbreviations used are: 2,5-DAH, 2,5-diaminohexanoate; DMBC, α-(5,6-dimethylbenzimidazolyl)cobamide coenzyme (5′-deoxyadenosylcobalamin); PALP, pyridoxal phosphate.

fuged to remove potassium perchlorate, and poured over a small Dowex 1 (OH^-)-X2 (200–400 mesh) column (1 × 2 cm). The Dowex was washed thoroughly with water, to remove any remaining traces of DMBC, and the amino acids were eluted from the column with 1 N HCl. After evaporation to dryness the amino acids were redissolved in a minimum volume of water, streaked on thin-layer silica gel sheets and separated using solvent 1 (see Materials). For determination of radioactivity the amino acids were eluted from the silica with CH_3OH–NH_4OH (12%) (4:1, v/v), evaporated to dryness and redissolved in water. Aliquots were counted using the scintillation mixtures described previously (Morley and Stadtman, 1970).

Procedure for the Isolation of DMBC from Incubation Mixtures Containing Tritiated Amino Acids. Following the precipitation of proteins with $HClO_4$ (as above), the supernatant solution was adjusted to pH 10 with concentrated KOH and poured over a Dowex 1 (OH^-)-X2 (200–400 mesh) column 1 × 2 cm). The column was washed with water to elute the DMBC (amino acids, retained by the resin at this pH, were subsequently eluted as described above). The DMBC solution was adjusted to pH 3 and passed over a 1 × 1 cm pad of Dowex 50 (H^+)-X4 (200–400 mesh). The Dowex pad containing the adsorbed DMBC was washed with water and then the DMBC was eluted with 12% NH_4OH. After evaporation to dryness the coenzyme was taken up in a minimum volume of water and streaked on thin-layer silica gel sheets which were developed with solvent 1 (see Materials). The region of the chromatograms containing the B_{12} compound was cut out and the DMBC was eluted with CH_3OH–NH_4OH (12%) (4:1, v/v), evaporated to dryness and the residue dissolved in the minimum of water and streaked on a second thin-layer silica gel sheet. This chromatogram was developed in solvent 2 (see Materials). After elution of the DMBC from the silica as before, aliquots were taken for counting.

The two thin-layer chromatographic steps were necessary in order to remove traces of labeled contaminants derived from the labeled amino acids.

Results

Tritium Transfer from Labeled DMBC to Amino Acids. If the reaction catalyzed by D-α-lysine mutase is analogous to several other B_{12} coenzyme dependent reactions, the hydrogen that migrates from C-5 to C-6 of lysine to replace the amino group should first be transferred to the 5′-methylene carbon of the 5′-deoxyadenosyl moiety of DMBC. To test the possibility that hydrogen from this position on the coenzyme also can be transferred to the amino acid product during the mutase reaction, coenzyme specifically labeled on the 5′-carbon with tritium (synthetic DMBC-*5′-t*) was added to the usual incubation mixtures and the reaction product and residual substrate examined for radioactivity. As shown in Table I there was a transfer of tritium from labeled coenzyme to 2,5-DAH, the product of the mutase reaction, and both mutase protein components were required for this transfer to occur.

The amount of tritium transferred increased with the amount of enzyme when a weight ratio of approximately 1:2 for E_1/E_2 was maintained (expt 3 and 6, Table I).

If the amount of E_1 protein was kept constant at an intermediate level and increasing amounts of E_2 protein were added, the tritium transfer was dependent on the amount of E_2 protein only as long as increasing amounts of substrate were converted into product (expt 4 and 5, Table I). Once apparent equilibrium for the mutase reaction had been reached (*i.e.*, about 36% conversion of lysine into 2,5-DAH), further addition of E_2 protein had no additional effect on the tritium transfer (expt 5 and 6, Table I).

In contrast, the amount of tritium transferred from DMBC-*t* to amino acids was greater when the E_1 or cobamide binding protein moiety was increased beyond the level required to produce the maximal yield of product (Table I, expt 6 and 7).

Effect of Incubation Time on the Tritium Transfer from Coenzyme to Amino Acids. Tritium incorporation from DMBC-*t* into amino acids was not increased by prolonged incubation with the mutase. In a time course study using levels of enzymes similar to those employed in expt 5 and 6 of Table I that catalyzed the maximal conversion of α-lysine into 2,5-DAH within 1 hr at 37°, (*i.e.*, apparent equilibrium had been reached) the total tritium incorporation into amino acids during the same period was 25–28%. The amount incorporated after incubation for 2.5 and 4 hr was 26 and 29%, respectively. Even allowing for an increased loss of some incorporated tritium to water due to the pyridoxal phosphate dependent exchange reaction (C. G. D. Morley, unpublished results), it is evident that little additional tritium incorporation occurred after the reaction was complete.

Effect of Varying the Level of DMBC-t on the Tritium Transfer to Amino Acids. Table II shows the results of an experiment in which the enzyme level ($E_1 + E_2$) was kept constant and the concentration of tritiated coenzyme was varied from 10 to 250 μM. In expt 1 (Table II) the mutase converted only 20% of the substrate to product but in expt 2 and 3 apparent equilibrium had been reached. Although the ratios of tritium incorporated into lysine and 2,5-DAH were different in the two experiments, the overall tritium incorporation was the same, even though the level of DMBC-*t* was increased 5-fold in expt 3. It will be noticed in expt 2 (Table II) that the tritium incorporated into amino acids is about 20%, whereas at the higher level of DMBC (250 μM) in expt 3, only 3.7% of the tritium was transferred.

Location of Tritium Incorporated from DMBC into 2,5-DAH. To establish that the tritium transferred from tritium-labeled coenzyme to 2,5-DAH by D-α-lysine mutase (Tables I and II) is in the terminal methyl group as would be predicted from eq 1, the isolated labeled 2,5-DAH was subjected to a Kuhn–Roth degradation (Wiesenberger, 1948) to convert C-5 and -6 of the amino acid into C-1 and -2, respectively, of acetate. Accordingly, a mixture containing 2,5-DAH-*t* (0.25 μmole, 2.5 × 10^4 cpm), 1.6 ml of 5% CrO_3, 0.4 ml of concentrated H_2SO_4, and 25 μl of 2 N CH_3COOH (as carrier) was heated at 140° for 2 hr in a sealed ampoule in a sand bath. The contents of the ampoule then were steam distilled and the distillate, after neutralization to phenolphthalein and evaporation, was redissolved in water and aliquots were counted. The recovery of tritium in the steam volatile fraction was 89%; the labeled steam volatile acid was identified as acetic acid by Duclaux distillation (Barker, 1957). These results show that the major portion of the tritium that was transferred from tritiated DMBC to 2,5-DAH was located in the terminal methyl group of the mutase reaction product.

Transfer of Tritium from α-Lysine-4,5-t to DMBC. Lysine, nominally tritiated on C-4 and -5 (highly radioactive commercial product) was used in studies to demonstrate tritium transfer from substrate to coenzyme. The coenzyme concentration was kept at an intermediate level but the enzyme concentration was increased roughly 4-fold, to increase

TABLE I: Transfer of ^3H from DMBC-*5'-t* to Amino Acids.[a]

Expt	E_1 Protein (mg)	E_2 Protein (mg)	Conversion of Substrate into 2,5-DAH[b] (%)	Fraction of the Total Tritium Recovered[c] in α-Lysine (%)	2,5-DAH (%)	DMBC (%)
1	1.1	0	1	0.25	0.41	96
2	0	1.9	2	0.19	0.63	95
3	0.55	0.95	24	3.7	8.0	83
4	1.1	0.48	26	3.8	11	80
5	1.1	0.95	36	4.8	25	64
6	1.1	1.9	38	5.0	26	66
7	2.2	1.9	39	5.3	36	54

[a] Incubation mixtures (0.5 ml) contained 20 mM Tris-HCl buffer (pH 9.0), 5 mM ATP, 2 mM $MgCl_2$, 8 mM dithiothreitol, 40 μM PALP, 2.5 mM D-α-lysine·HCl, enzyme proteins as shown in the table and 50 μM DMBC-*5'-t* (2.7×10^6 cpm/μmole). After 1 hr at 37° the reaction was stopped by addition of 0.1 ml of 30% $HClO_4$. [b] 2,5-DAH was assayed by (1) using the acid ninhydrin technique described previously (Morley and Stadtman, 1970) and (2) by use of ^{14}C-labeled lysine and determination of ^{14}C in the product after chromatographic separation. [c] Separation of amino acids and DMBC was as described in Methods.

the amount of enzyme bound DMBC and thus increase the incorporation of label into the coenzyme.

DL-α-Lysine-*4,5-t* was diluted with carrier DL-lysine to a final specific activity of 7.5×10^5 cpm/μmole. In a reaction mixture containing 1.29 μmoles of DL-α-lysine-*4,5-t* and 150 mμmoles of DMBC, if there was complete equilibration of one hydrogen from C-5 of lysine with the two coenzyme hydrogens during conversion of 44% of the lysine into 2,5-DAH then 3.8% of the total tritium should be found in the coenzyme. The results shown in Table III indicate that less than one hydrogen of lysine equilibrates with the hydrogens on the coenzyme (2.2% of tritium transferred). The above calculations assume the presence of the lysine racemase (Stadtman and Tsai, 1967) which is found as a contaminant of most lysine mutase preparations.

Tritium Transfer from 2,5-DAH-6-t to DMBC. A series of experiments using 2,5-DAH-*6-t* were carried out in order to demonstrate that (1) the overall mutase reaction is reversible and (2) to confirm that the hydrogens on C-6 of 2,5-DAH can exchange with those on the 5'-methylene carbon of DMBC.

2,5-DAH-*6-t* (1.27×10^6 cpm/μmole) was synthesized enzymically from DL-α-lysine-*6-t* using D-α-lysine mutase. The product was checked by a Kuhn–Roth oxidation (see above) and shown to contain 90% of its radioactivity in the C-6 position.

2,5-DAH-*6-t* (1.35 μmoles) and DMBC (200 mμmoles) were used in the experiment and high levels of enzyme (almost 18-mg total). In a parallel reaction mixture containing 2,5-DAH-*2-*^{14}C, 25% of the 2,5-DAH was converted into lysine. Assuming complete equilibration of one hydrogen from C-6 of 25% of the 2,5-DAH with the two coenzyme hydrogens, then 4.5% of the total tritium should be recovered in the coenzyme. As shown in Table IV, 2.8% of the tritium was transferred or a total of 48,000 cpm.

Since incorporation of tritium into DMBC can be demonstrated in these experiments when both mutase proteins are present, and some formation of α-lysine from 2,5-DAH was also observed, it can be concluded that the overall reaction is reversible; the maximum conversion of 2,5-DAH into α-lysine was 25% under the conditions used (Table IV).

Location of the Tritium Label in Coenzyme Tritiated from Amino Acids. Evidence from the experiments using DMBC-*5'-t* indicate that, as expected (Frey *et al.*, 1967) it is the 5'-methylene hydrogens of DMBC which participate in the

TABLE II: Effect of Increasing Tritiated Coenzyme Concentration on the Tritium Transfer to Amino Acids.[a]

Expt	Substrate Conversion (%)	DMBC-*5'-t* Added (μM)	Radioactivity Recovered in α-Lysine (cpm)	Radioactivity Recovered in 2,5-DAH (cpm)	Total ^3H Incorp into Amino Acids (%)
1	20	10	2600	3,200	43.0
2	35	50	1400	11,330	18.9
3	36	250	4400	8,240	3.7

[a] Incubation mixtures were as described in the legend of Table I except that 3 mg of enzyme (equal amounts of E_1 and E_2 proteins) and the indicated levels of DMBC-*5'-t* were employed.

TABLE III: Incorporation of Tritium from α-Lysine-*4,5-t* into DMBC.[a]

E_1 Protein (mg)	E_2 Protein (mg)	Substrate Conversion (%)	Fraction of Total ^3H in DMBC (%)
5.0	0	1	0.1
0	3.8	4	0.2
5.0	3.8	44	2.2

[a] Experiments were carried out as described in the Methods. The incubation mixture was as in legend to Table I, except that 2.58 mM DL-α-lysine-*4,5-t* (7.5×10^5 cpm/μmole) and 300 μM unlabeled DMBC were used.

TABLE IV: Tritium Transfer from 2,5-DAH-*6-t* to DMBC.[a]

E_1 Protein (mg)	E_2 Protein (mg)	Substrate Conversion[b] (%)	Fraction of Total Tritium in DMBC (%)
10	0	0	0.15
0	7.3	0	0.30
10	7.3	25	2.8

[a] Incubation mixtures were as described in the legend to Table I, but using 2.7 mM 2,5-DAH-*6-t* (1.27×10^6 cpm/μmole) as substrate and 400 μM unlabeled DMBC. [b] The conversion of 2,5-DAH into α-lysine was calculated from separate experiments, run under identical conditions, using 2,5-DAH-*2-*^{14}*C* as substrate (Stadtman and Tsai, 1967).

hydrogen transfer. As confirmation of this, the DMBC-*t* isolated from incubation mixtures containing unlabeled DMBC and α-lysine-*4,5-t* or 2,5-DAH-*6-t*, was degraded to locate the label.

The procedure adopted for this makes use of the fact that DMBC, when photolyzed, yields aquocobalamin and adenosine 5′-aldehyde (aerobically) or cyclic adenosine (anaerobically) or a mixture of both nucleosides under partially anaerobic conditions (Hogenkamp, 1964).

A sample of DMBC-*t* (4.3×10^4 cpm/μmole) was photolyzed in dilute solution (10^{-4} M) in a stoppered tube, with a 600-W tungsten lamp, at a distance of 6 cm at pH 6.0 and 0° for 3 hr. The solution was then evaporated to a small volume and either of the following two procedures was adopted for isolation and assay of the photolysis products.

PROCEDURE I. Aliquots of the concentrated solution were spotted on thin-layer silica gel and developed in solvent 3 (see Methods). The nucleosides (R_F 0.60) formed by photolysis of DMBC are well separated from aquocobalamin (R_F 0.05) in this system. The nucleoside regions of the chromatogram were located by ultraviolet quenching and cut out. The nucleosides were eluted from the gel with ethanol–NH_4OH (12%) (4:1, v/v) and aliquots counted. Samples of the aquocobalamin were also eluted and counted.

PROCEDURE II. The concentrated solution from photolysis of DMBC was treated with water-saturated phenol. Aquocobalamin is extracted into the phenol (Gleason and Hogenkamp, 1969) and thereby separated from the nucleosides that remain in the aqueous layer; the latter fraction was then desalted, after adjustment to pH 1.0, by passage over a pad of Dowex 50 (H^+). Following a water wash, the adsorbed nucleosides were eluted with 12% NH_4OH and aliquots of the eluent were counted. Aquocobalamin was back-extracted from phenol into water and also examined for radioactivity. From the results of these experiments (Table V) it can be seen that after cleavage by photolysis the major portion of the label associated with the coenzyme is found in the nucleoside moiety. To determine more precisely the location of the tritium the adenosine 5′-aldehyde was separated from 5,8-cyclic adenosine and the specific radioactivity of each measured. The solution containing the two nucleosides from the photolysis of DMBC, extracted by procedure II, was spotted on Whatman No. 1 paper pretreated with $NaHSO_3$ and developed in solvent 4 (Morley *et al.*, 1968). The $NaHSO_3$ complexes with the aldehydic function of adenosine 5′-aldehyde and retards its migration (R_F 0.16) thus facilitating its separation from the cyclic nucleoside (R_F 0.52). The two nucleosides, located by ultraviolet quenching and by reference to authentic samples run simultaneously, were cut out of the paper and eluted with ethanol–NH_4OH (12%) (4:1, v/v) and their specific radioactivity determined. The original DMBC-*t* contained 4.3×10^4 cpm/μmole, the adenosine 5′-aldehyde, 1.68×10^4 cpm/μmole, and the 5,8-cyclic adenosine, 3.28×10^4 cpm/μmole. These values are in agreement with those obtained when authentic DMBC-*5′-t* is photolyzed (Frey *et al.*, 1967), *i.e.*, adenosine 5′-aldehyde, has half the specific activity of the original DMBC-*t* and cyclic adenosine has the same specific activity. Hence it can be concluded that tritium incorporated into DMBC during the course of the α-lysine mutase reaction was indeed in the 5′-methylene position of the coenzyme.

TABLE V: Location of the Tritium Label in Coenzyme, Labeled from Amino Acids.

Expt	Photolysis of DMBC Followed by	^{3}H in DMBC before Photolysis (cpm)	^{3}H in Nucleoside (cpm)	^{3}H in Aquocobalamin (cpm)
1	Procedure I[a]	2.1×10^4	1.45×10^4	8×10^2
2	Procedure II[a]	2.1×10^4	1.2×10^4	1.2×10^2

[a] Procedures I and II are described in the text.

TABLE VI: Release of ^{3}H from α-Lysine-*4,5-t* to Water.[a]

Expt	E_1 Protein (mg)	E_2 Protein (mg)	Fraction of the Total ^{3}H Released to Water (%)	Conversion of Substrate into 2,5-DAH (%)
1	0	0.95	0	3[b]
2	1.1	0.48	1.6	26
3	1.1	0.95	4.9	38

[a] The incubation mixture was as described in the legend to Table I, except that 2.5 mM DL-α-lysine-HCl-*4,5-t* (1.18×10^6 cpm/μmole) was used in place of unlabeled lysine and the DMBC was not labeled. After 1 hr at 37° the reaction was stopped by the addition of 0.1 ml of 30% $HClO_4$; the acid supernatant solution, after removal of precipitated protein, was poured over a small pad of Dowex 50 (H^+)-X4 (200–400 mesh) and washed thoroughly with water. The combined effluent and wash from the Dowex was diluted to 5 ml and suitable aliquots were counted; identical reaction mixtures that were not incubated served as controls. The adsorbed amino acids were then eluted from the Dowex with 12% NH_4OH and assayed for 2,5-DAH as a measure of the extent of the mutase reaction (Morley and Stadtman, 1970). [b] The E_2 protein fraction used was not completely resolved in E_1 protein and hence catalyzed the overall reaction to a slight extent.

Extent of Exchange of Hydrogen of Lysine-4,5′-t with Water During Course of Mutase Reaction. The data of Table I show that 95% or more of the tritium from tritiated DMBC was

recovered in amino acids and coenzyme at the end of every experiment and therefore less than 5% could have exchanged with water. However, in view of the fact that the E_1 protein moiety of D-α-lysine mutase catalyzes a slow pyridoxal phosphate mediated exchange of hydrogen on C-6 of lysine or C-5 of 2,5-DAH with water (C. G. D. Morley, unpublished results), it was of interest to determine, directly, whether a loss of tritium to water sufficient to account for the small discrepancy observed might have occurred under the experimental conditions employed. Tritiated lysine-4,5-*t*, which in the complete mutase system yields 2,5-DAH still containing one tritium on C-5 and, eventually, lysine labeled also on C-6, was used as substrate. The results of such a study shown in Table VI, indicate that under conditions where extensive conversion of lysine into 2,5-DAH occurred (expt 3), the loss of tritium to water was approximately 5%. With a lower level of E_2 protein or with the E_2 fraction alone, very little tritium was found in the water.

Discussion

The results of the studies with tritium-labeled amino acids and B_{12} coenzyme reported here show that, as in several other B_{12} coenzyme linked reactions, DMBC serves as the hydrogen carrier in the reaction catalyzed by D-α-lysine mutase. The experiments with DMBC-*5-t* (synthetic) show that hydrogens attached to the 5′-methylene carbon of the deoxyadenosyl moiety of the coenzyme are transferred to the amino acid substrate and product. The mutase product (2,5-DAH) tritiated from DMBC-*5′-t* during the mutase reaction was labeled almost exclusively in the terminal methyl group (C-6) of the molecule as would be predicted if DMBC is the transfer agent for the hydrogen migration from C_5 to C_6. Complementary experiments showed that tritiated coenzyme was formed both from α-lysine-*4,5-t* and 2,5-DAH-*6-t* and that the tritium incorporated enzymically was located in the 5′-methylene group of the deoxyadenosyl moiety attached to the cobalt of DMBC.

The fraction of tritium transferred from labeled coenzyme to product (and substrate) depends on the concentration of coenzyme, the amount of mutase enzyme, the ratio of coenzyme to enzyme and the amount of substrate that reacts. When the concentrations of tritium-labeled coenzyme and the substrate are kept constant, the fraction of the total tritium transferred is particularly dependent on the level of E_1 or cobamide binding protein. At higher E_1 protein levels, a larger fraction of coenzyme tritium is transferred to product even after equilibrium for the mutase reaction has been reached. The fraction of total tritium transferred when enzyme and substrate are kept constant does not increase with increasing coenzyme concentrations except at subsaturation levels.

Under optimal conditions less than 50% of the tritium is transferred to amino acids. Possible explanations of the low tritium incorporation found with this enzyme are (1) there may be a marked isotope selection mechanism in operation or (2) the high affinity of DMBC for the enzyme means that little exchange between free and bound DMBC-*t* occurs and the bound DMBC-*t* undergoes many catalytic events, the tritium label being rapidly diluted out. A slow rate of exchange of free and enzyme-bound DMBC has also been observed with glutamate mutase (Switzer *et al.*, 1969). In contrast, there seems to be a more rapid rate of exchange of free and bound DMBC relative to the rate of the catalytic reaction with L-β-lysine mutase (Rétey *et al.*, 1969) and α-methylene glutarate mutase (H. F. Kung, unpublished results).

The reversibility of the reaction was demonstrated by incorporation of tritium from 2,5-DAH-*6-t* into DMBC, from labeled coenzyme into D-α-lysine, and by the measureable formation of α-lysine-^{14}C from 2,5-DAH-^{14}C.

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