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The Role of Pyridoxal Phosphate in the B_{12} Coenzyme-Dependent D-α-Lysine Mutase Reaction*

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ABSTRACT: D-α-Lysine mutase catalyzes the interconversion of D-α-lysine and 2,5-diaminohexanoate (2,5-DAH), a reaction involving a shift of the ϵ-amino group of lysine to the adjacent carbon atom. The mutase consists of two dissimilar protein moieties, a cobamide protein (E_1) and a sulfhydryl protein (E_2). Two of the cofactors required for the mutase reaction, B_{12} coenzyme and pyridoxal phosphate (PLP), are bound by the E_1 protein. This protein, in the absence of E_2, catalyzes a PLP- and Mg^{2+}-dependent exchange of hydrogen at position 6 of D-lysine with water. Both the hydrogen-exchange and the overall mutase reaction are inhibited in parallel by known PLP inhibitors. Available evidence suggests that PLP is directly involved in the catalysis of the amino group migration.

The B_{12} coenzyme-dependent D-α-lysine mutase complex that catalyzes reaction 1 exhibits an absolute requirement for pyridoxal phosphate (Morley and Stadtman, 1970). A similar

$$\underset{\text{NH}_2}{\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH}} \rightleftharpoons \underset{\text{NH}_2}{\text{CH}_3\text{CH}}\text{CH}_2\text{CH}_2\underset{\text{NH}_2}{\text{CH}}\text{COOH} \quad (1)$$

reaction catalyzed by L-lysine 2,3-aminomutase (Chirpich *et al.*, 1970), which does not require a B_{12} coenzyme, is activated by pyridoxal phosphate, ferrous iron, and *S*-adenosylmethionine.

Some studies reported in the present communication indicate that in the D-α-lysine mutase reaction pyridoxal phosphate may play a role in the catalytic reaction *per se*. Furthermore, it is suggested that the amino group which replaces the abstracted hydrogen may migrate as a pyridoxal phosphate derivative.

Materials

D-α-Lysine mutase was purified either as the two separated dissimilar proteins, a cobamide protein (E_1) and a sulfhydryl protein (E_2) (Stadtman and Grant, 1971), or as a complex of the two proteins (Morley and Stadtman, 1970).

Pyridoxal 5′-phosphate and pyridoxal were purchased from Nutritional Biochemicals Corp. Pyridoxamine 5′-phosphate and pyridoxine 5′-phosphate were obtained from Dr. M. Flavin. Methyl pyridoxal phosphate was a generous gift from Dr. E. Helmreich, University of Würzburg, Würzburg, Germany. The pyridoxal antagonists, L-1-aminoproline and D-1-aminoproline, were prepared as described by Klosterman *et al.* (1967). Isonicotinic acid hydrazide was purchased from Eastman Kodak. Tritiated water (100 mCi/g) and DL-lysine-6-*t* (8.7 mCi/mmole) were purchased from New England Nuclear.

Methods

Routine assays of D-α-lysine mutase activity were performed as described previously (Morley and Stadtman, 1970). Radioactivity was determined in a Beckman LS-250 liquid scintillation system using 2,5-diphenyloxazole (0.8%) in toluene as counting fluid.

Tritium-Exchange Techniques. Exchange of tritium from H_2O-*t* into amino acids was carried out by incubating the appropriate mixture for 10 hr at 37°, acidifying with $HClO_4$, and passing the supernatant solution, after removal of denatured protein, over a Dowex 50-H^+ × 4 pad (1 × 2 cm). The Dowex was washed with water and the amino acids were eluted with NH_4OH (12%). The eluate was evaporated to dryness and redissolved in 0.5 ml of water, and aliquots were assayed for radioactivity.

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Exchange of tritium from lysine-*6-t* into water was carried out in a similar manner except that the assay mixtures, following incubation, were acidified and frozen in liquid nitrogen. Aliquots (0.1 ml) of water were subsequently distilled off *in vacuo*, collected, and assayed for tritium.

Preparation of Apoenzymes. Apoenzymes (*i.e.*, freed of bound PLP) were prepared by treatment of the proteins with 1 mM hydroxylamine in 0.2 M potassium phosphate buffer (pH 7) for 10 min at room temperature, followed by overnight dialysis at 2° against 500 volumes of 40 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.2).

In order to selectively inactivate the residual lysine racemase activity which is SH^- dependent and which contaminated highly purified mutase preparations, the protein was alkylated as follows; approximately 4 mg of E_1 protein was treated with 2 mM dithiothreitol in 100 mM potassium phosphate buffer (pH 7.0) for 15 min at 30° under argon. Iodoacetamide (40 mM) was added and the mixture was incubated for 15 min at 30°. Excess iodoacetamide was then removed by overnight dialysis at 2° against 1000 volumes of 40 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.2). This procedure does not affect the activity of the mutase cobamide (E_1) protein (Morley and Stadtman 1970) but effectively inhibits lysine racemase. Enzyme preparations treated in this fashion were used for experiments described in Table VIII and Figure 2 (see later).

TABLE I: Effects of Vitamin B_6 Derivatives on D-α-Lysine Mutase Activity.[a]

B_6 Compound Added	2,5-DAH Formed (nmoles)
None	25
PLP (100 μM)	705
PMP (1 mM)	250
PL (1 mM)	144
PNP (1 mM)	240
Me-PLP (1 mM)	130
PLP (100 μM) + PMP (10 mM)	620
PLP (100 μM) + PL (10 mM)	495
PLP (100 μM) + PNP (10 mM)	620
PLP (100 μM) + Me-PLP (10 mM)	631

[a] The assay mixtures (0.5 ml) contained 40 mM Na_2CO_3–$NaHCO_3$ (pH 9.2), 10 mM D-lysine hydrochloride, 5 mM ATP, 2 mM $MgCl_2$, 8 mM dithiothreitol, 20 μM DMBC, 0.25 mg of apoenzyme complex (treated with NH_2OH), and B_6 derivative, as indicated. All abbreviations are given in the footnotes.

Results and Discussion

Effects of Vitamin B_6 Derivatives on D-α-Lysine Mutase Activity. The "apomutase" (apocomplex or recombined apoproteins) prepared as described in Methods was inactive when assayed in the presence of all of the usual cofactors except a vitamin B_6 derivative but addition of pyridoxal phosphate restored the mutase activity (Table I). The closely related vitamin B_6 derivatives, PMP,[1] PNP, Me-PLP, and PL, were relatively ineffective as cofactors. They were not appreciably inhibitory, even at high concentrations, with the possible exception of PL (Table I). Pyruvate which activates a related enzyme, β-lysine mutase (Stadtman and Renz, 1968), was shown previously to have no effect on D-α-lysine mutase (Morley and Stadtman, 1970).

When each of the D-α-lysine mutase protein components was treated separately with hydroxylamine and then various combinations of treated and untreated proteins were assayed for mutase activity (Table II), it was found that removal of pyridoxal phosphate from the cobamide protein moiety (E_1) was much more effective in reducing the residual catalytic activity observed in the absence of added pyridoxal phosphate than was similar treatment of the sulfhydryl protein moiety (E_2) fraction. When both protein fractions were treated there was no mutase activity observed in the absence of added PLP. These data indicate that pyridoxal phosphate is retained mainly by the cobamide protein moiety during purification of the enzymes.

Although treatment with hydroxylamine renders the mutase completely dependent on added PLP for activity, the enzyme (E_1 protein moiety) still contains bound cobamide compounds which are not removed quantitatively by any of the procedures that resolve several other B_{12} proteins. For this reason spectral binding studies to determine the number of moles of PLP required per mole of E_1 protein for maximal catalytic activity have not been carried out.

Effect of Reduction with Borohydride on D-α-Lysine Mutase Activity. Many PLP-dependent enzymes are known to bind the aldehyde group of pyridoxal phosphate in the form of a Schiff base with the ε-amino group of a lysine residue of the protein. Treatment of the holoenzyme forms of these proteins with $NaBH_4$, at pH 6 or below, reduces the Schiff base linkage forming a catalytically inactive ε-pyridoxyllysine derivative of the enzyme (Anderson and Wang Chang, 1965; Tate and Meister, 1969; Strausbauch and Fischer, 1970). At higher pH values (about 6.5–9) the pyridoxal phosphate is presumed to be bound as a substituted aldimine derivative (Fischer *et al.*, 1958) and this is resistant to reduction with borohydride (Anderson and Wang Chang, 1965).

TABLE II: Identification of the Cobamide (E_1) Protein as the PLP Binding Moiety.[a]

E_1 Protein	E_2 Protein	2,5-DAH Produced (μmoles) −PLP	2,5-DAH Produced (μmoles) +PLP
Untreated	Untreated	560	1180
Untreated	Treated	670	
Treated	Untreated	200	
Treated	Treated	0	960

[a] Treatment of proteins with NH_2OH to remove bound PLP is described in Methods. Assay mixtures are described in the footnote of Table I.

[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; Me-PLP, a derivative of pyridoxal 5′-phosphate in which an *O*-methyl group replaces one of the hydroxyl groups of the phosphate moiety; PMP, pyridoxamine 5′-phosphate; PNP, pyridoxine 5′-phosphate; PL, pyridoxal; 2,5-DAH, 2,5-diaminohexanoate; DMBC, α-(dimethylbenzimidazolyl)-Co-5′-deoxyadenosylcobamide or 5′-deoxyadenosylcobalamin; TNM, tetranitromethane.

TABLE III: Effects of Borohydride on D-α-Lysine Mutase Activity.[a]

Enzyme Prepn	2,5-Diaminohexanoate (μmoles) Produced by: Apoenzyme (1 mg)[b]	Holoenzyme (1 mg)[c]
Untreated	0.27	2.40
$NaBH_4$ treated[d]	0.42	1.30
$NaBH_4$ treated + PLP[e]	2.70	1.70

[a] Enzyme activity was determined as described previously (Morley and Stadtman, 1970). [b] The apoenzyme or D-α-lysine mutase complex free of PLP was prepared as described in Methods. [c] The holoenzyme was prepared by incubating 1 mg of D-α-lysine mutase complex with 160 nmoles of PLP in Tris buffer at pH 9 for 15 min at 30°. [d] To the enzyme complex, in pH 6 potassium phosphate buffer, was added 5 mg of $NaBH_4$ and, after rapid mixing, the solution was held at 0° for 10 min. The enzyme solution then was dialyzed overnight against 500 volumes of 20 mM Tris-HCl buffer (pH 9). [e] The $NaBH_4$-treated enzyme, after dialysis, was incubated with 160 nmoles of PLP per mg of enzyme for 10 min at 30° prior to assay.

TABLE IV: Effect of Tetranitromethane (TNM) on E_1 Protein Activity.

E_1 Protein Treatment[a]	2,5-DAH Produced (μmoles)
Control[b]	2.0
TNM	0.4
TNM + PLP (260 μM)[c]	1.7
TNM + PLP (400 μM) + D-lysine (100 mM)[c]	1.5
TNM + D-lysine (100 mM)[c]	0.4

[a] Nitration was carried out as follows: Apo E_1 protein freed of PLP (500 μg) was incubated at room temperature for 1 hr with 0.7 mM TNM in 50 mM 2-methylimidazole hydrochloride (pH 8.2), 1 mM $MgCl_2$ and 1 M KCl in a total volume of 0.75 ml. The enzyme solution was then dialyzed overnight against 20 mM Tris-HCl buffer, pH 9.0. The samples of E_1 protein were assayed for D-α-lysine mutase activity after addition of E_2 protein and other reagents (see Table I and Morley and Stadtman, 1970). [b] The control contained the complete nitrating mixture, less TNM, and was kept at room temperature for 1 hr and dialyzed overnight as in *a*. [c] Additions to the nitrating mixture.

Treatment of the holoenzyme form of D-α-lysine mutase complex with borohydride resulted in partial destruction of catalytic activity but the inactivation never exceeded 50%. In the experiment of Table III the holoenzyme containing bound PLP was inactivated only 29% by treatment with borohydride at pH 6. At neutral pH no inhibition was observed. No greater inhibition was achieved by treating the separated E_1 protein plus PLP with $NaBH_4$, either in the presence of substrate, D-lysine, or of other cofactors such as ATP and DMBC, or following prior treatment with protein-disrupting agents such as urea or guanidine hydrochloride.

For efficient reduction of the muscle phosphorylase–PLP complex (Fischer *et al.*, 1958) it was necessary to treat with borohydride either at lower pH (pH 4.5) or in the alkaline pH range (above pH 9.5). Therefore, cyanoborohydride ($NaBH_3CN$), a reagent which is stable in acid down to pH about 3 (Borch *et al.*, 1971), was also employed in further attempts to fully inactivate the D-α-lysine mutase E_1 protein. However treatment with 50 mM $NaBH_3CN$ for 1 hr at pH 5.4 in potassium dimethylglutarate buffer (1.5 mg of E_1 protein per ml incubated initially with 200 μM PLP) resulted in only slight inactivation. The effect of lower pH could not be tested because the enzyme precipitates at about pH 5. No inactivation was observed at pH 9.2 even with much longer exposure to 200 mM $NaBH_3CN$. Unfortunately, the cobamide chromophore still tightly bound to the E_1 protein moiety of D-α-lysine mutase (Morley and Stadtman, 1970) prevents detection of the spectral shifts characteristically exhibited by PLP enzymes as a function of changes in pH (Kent *et al.*, 1958). Hence decreases in the 420-nm absorption maximum characteristic of PLP–Schiff bases cannot be used to monitor the course of reduction of E_1 protein with borohydride as has been done with glutamate decarboxylase (Strausbauch and Fischer, 1970). Although the quantitative aspects of the experiments just described are not satisfactory, the fact that the apoenzyme is unaffected by treatment with borohydride (Table III) whereas the holoenzyme containing bound PLP is partially inhibited in an irreversible fashion suggests that PLP is bound in Schiff base form to D-α-lysine mutase. Final decision on this point awaits direct demonstration of an ε-pyridoxyllysine derivative of the protein following reduction with borohydride.

Effects of Tetranitromethane and Acetylation on E_1 Protein Activity. Studies with aspartate aminotransferase (Turano *et al.*, 1968; Fasella and Turano, 1970; Christen and Riordan, 1970) provide one example of an enzyme where a tyrosine residue of the protein, in addition to a lysine, is involved in the PLP binding site. In order to see if this might be the case with the E_1 protein moiety of D-α-lysine mutase, the effects of treatment with two reagents, tetranitromethane (TNM) and *N*-acetylimidazole, that modify tyrosine residues (Sokolovsky *et al.*, 1966) were studied.

Preliminary experiments with TNM showed that this reagent inhibited the enzyme. For maximal inhibition (20% residual activity) about 0.5 mM TNM was required under the experimental conditions described in Table IV. Marked protection against inactivation of E_1 protein by TNM was afforded by prior addition of a high level of PLP (260 to 400 μM) whereas the substrate (lysine) did not protect (Table IV).

Treatment with *N*-acetylimidazole inhibited the E_1 protein activity 70% (Table V). In this case PLP, even at high concentrations, was unable to protect against the effect of the modifying reagent. Lysine partially protected the enzyme but the specificity of its effect has not been investigated. Inactivation of the E_1 protein by *N*-acetylimidazole was reversed by treatment with hydroxylamine (Table V), suggesting that a catalytically important site on the enzyme had been inactivated by acetylation and that subsequent cleavage of this acetyl derivative by hydroxylamine regenerated the active species. Although both tetranitromethane and *N*-acetylimidazole also react with cysteine residues in proteins, the insensitivity of the apo-E_1 protein to treatment with iodo-

TABLE V: Inactivation of E_1 Protein by Treatment with *N*-Acetylimidazole.[a]

Treatment of E_1 Protein	2,5-Diaminohexanoate Formed (μmoles)
Control[b]	2.80
N-Acetylimidazole	0.80
N-Acetylimidazole + PLP (400 μM)	0.96
N-Acetylimidazole + PLP (400 μM) + lysine (100 mM)	2.30
N-Acetylimidazole + lysine (100 mM)	2.0
N-Acetylimidazole followed by NH_2OH (10 mM)[c]	2.2

[a] Acetylation of E_1 protein was carried out as follows; 500 μg of the apoenzyme form of E_1 (freed of pyridoxal phosphate as described in Methods) in 100 mM imidazole hydrochloride + 1 mM $MgCl_2$, pH 7 buffer, was treated for 1 hr at room temperature with 1.3 mM *N*-acetylimidazole (freshly dissolved in 50 mM 2-methylimidazole hydrochloride, pH 7.6) in a total volume of 0.75 ml as described by Cimino *et al.* (1970). The mixture was then dialyzed for 4 hr against 4 l. of 20 mM Tris-HCl buffer, pH 9.0. [b] The control was treated as in *a* except that no *N*-acetylimidazole was included in the buffer mixture. [c] After treatment with *N*-acetylimidazole and dialysis as in *a*, the enzyme sample then was dialyzed against 10 mM hydroxylamine in 40 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.2) overnight. The various E_1 protein preparations were assayed for mutase activity after addition of E_2 protein, 240 μM PLP, and the other usual reaction mixture components (see Table I footnote *a*). The higher concentration of PLP was used in these experiments to overcome the inhibitory effect of the hydroxylamine carried over with the enzyme in *c*.

TABLE VI: Exchange of C_6 Hydrogen of Lysine with Water.

Additions	Omissions[a]	% of Total ^3H from Lys-6-*t* Released to Water
Boiled holo complex (E_1 + E_2)	All other reagents	2.04
Apo-E_1	PLP	6.46
Apo-E_1	None	12.37
Apo-E_1, intrinsic factor[b]	DMBC	12.61
Apo-E_1 + Apo-E_2	PLP	6.33
Apo-E_1 + Apo-E_2	None	11.62

[a] Incubation mixtures (0.5 ml) contained DL-α-lysine-6-*t* (1.5×10^6 cpm; 8.7 mCi/mmole) and other reagents as listed in the legend to Table I. 1.0 mg of apo-E_1 protein and 1.0 mg of apo-E_2 protein were used. After incubation for 10 hr at 37° the reaction was stopped by addition of $HClO_4$ and the water from the reaction mixture isolated and analyzed for tritium content as described in Methods. [b] Intrinsic factor, 2 mg (binding capacity 3.8×10^{-4} μmole of vitamin B_{12} per mg), was added.

acetamide suggests that in this case a tyrosine group rather than a cysteine may have been modified. If this interpretation is correct then D-α-lysine mutase and aspartate aminotransferase are indeed similar in that a tyrosine residue in both proteins is important for the binding of PLP.

Exchange of Hydrogen at C-6 of Lysine with Water. Among the possible roles of pyridoxal phosphate in the D-α-lysine mutase reaction is direct participation in the amino group migration process by formation of Schiff base intermediates with the ω-amino groups of the substrates. Thus during catalysis Schiff base formation with a substrate amino group rather than a protein lysine amino group would occur. Since formation of aldimine intermediates and their subsequent tautomerization to ketimines are readily reversible processes, the exchange of protons of the solvent with hydrogen on the α-carbon of the amine component of the imine generally results (Snell and DiMari, 1970). Labilization of the hydrogen on this α-carbon of the imine intermediate can occur independently of the overall reaction catalyzed by the enzyme (Jenkins, 1964).

In preliminary experiments using nominally DL-lysine-6-t_2 as substrate, the E_1 protein did indeed catalyze the slow release of tritium to the solvent (Table VI and Morley and Stadtman, 1971). This tritium transfer was stimulated by pyridoxal phosphate, but not by DMBC. The E_2 protein moiety of the mutase was not required. Additional studies with H_2O-*t* established that in the reverse reaction tritium incorporation into lysine requires only active E_1 protein, PLP, and Mg^{2+} (Table VII), in contrast to the mutase reaction which requires both E_1 and E_2 proteins, PLP, Mg^{2+}, ATP, dithiothreitol, and B_{12} coenzyme (Morley and Stadtman,

TABLE VII: Enzyme and Cofactor Requirements for Tritium Incorporation into Lysine from H_2O-*t*.

Addition	Omission	^3H Incorpd (μatom/μmole of Lys)[b]
Apo-E_1 + Apo-E_2	None[a]	0.53
Boiled Apo-E_1	None	0.05
Apo-E_2	None	0.05
Apo-E_1	None	0.80
Apo-E_1	PLP	0.20
Apo-E_1	ATP	1.01
Apo-E_1 + EDTA·K (100 mM)	$MgCl_2$	0.56
Apo-E_1 + intrinsic factor[c]	DMBC	1.07

[a] Incubation mixtures (0.25 ml) contained 2.5 mM D-lysine hydrochloride, 100 μM PLP, H_2O-*t* (6 μl, 0.6 mCi), and other reactants listed in the footnote to Table I. The apo-E_1, 1.0 mg of protein, and the apo-E_2, 1.3 mg of protein, prepared as described in Methods, were added as indicated. [b] These values are those actually observed and have not been corrected for the contribution (about 30%) due to the presence of lysine racemase (see Table VIII). [c] Intrinsic factor (2 mg) (see Table VI legend) was added to bind endogenous B_{12} compounds on the apo-E_1 protein. The intrinsic factor preparation itself did not catalyze tritium incorporation into lysine.

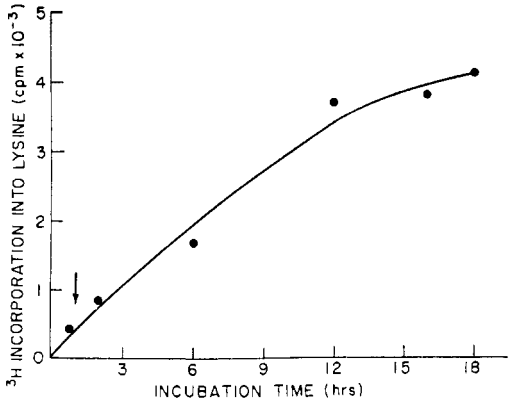

FIGURE 1: Equilibration time for tritium exchange. Experiments were performed using incubation mixtures and conditions described in the footnote to Table VII.

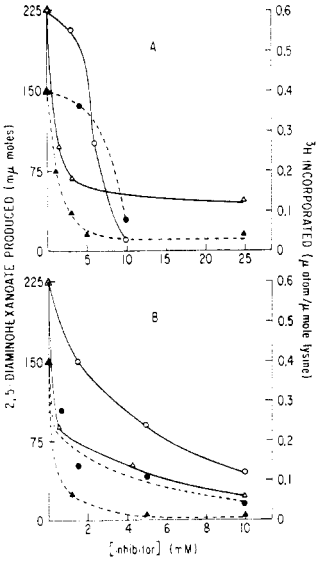

FIGURE 2: Inhibition of mutase activity and tritium exchange by PLP inhibitors. Assays were performed as described in the legends to Tables I and VII except PLP was omitted from the incubation mixtures. A preparation of E_1–E_2 complex, not resolved of PLP (0.8 mg of protein), was used for the mutase reaction and alkylated E_1 protein containing bound PLP (1 mg of protein) was used for the tritium-exchange experiments. 1-Amino-D-proline is the active constituent of linatine, the naturally occurring vitamin B_6 antagonist from flaxseed (*Linium usitatissimum*) and linseed. The diastereoisomer of linatine which contains 1-amino-L-proline is equally toxic for chicks (Klosterman *et al.*, 1967). In both A and B the solid lines show 2,5-diaminohexanoate formation and the broken lines show ^{3}H incorporation into D-lysine. (A) (O–O and (●)----●, + isonicotinic acid hydrazide. (△)—(△) and (▲)----▲, + NH_2OH. (B) O—O and ●----●, + 1-amino-L-proline. (△)—△ and (▲)----▲, 1-amino-D-proline.

1970). In fact, the hydrogen-exchange reaction with solvent appeared to be slightly inhibited by DMBC and ATP. Although dithiothreitol was in the incubation mixtures of Table VII, the experiments of Table VIII show it was not required. Monovalent cations, which stimulate the mutase reaction (Morley and Stadtman, 1970), have no effect on the hydrogen exchange with solvent.

The time course for tritium incorporation into lysine from H_2O-*t* is shown in Figure 1. The arrow indicates the time required, under identical conditions, to reach apparent equilibrium for the mutase reaction. It can be seen that the reaction involving hydrogen exchange with solvent takes 12–15 times longer to reach equilibrium than does the overall mutase reaction. Solvent hydrogen does not participate in the faster mutase reaction which involves the transfer of a hydrogen from C-5 of lysine to C-6 *via* B_{12} coenzyme. The slow hydrogen exchange with water (Figure 1), although indicative of the mode of interaction of the pyridoxal phosphate cofactor and the amino acid substrates, probably represents an independent activity of the E_1 component that is not part of the normal catalytic reaction.

Substrate Specificity of the Hydrogen-Exchange Reaction. Although the enzyme preparations used in the experiments of Table VII and Figure 1 were highly purified, they were not entirely free of lysine racemase which can catalyze the incorporation of hydrogen from water into the α position of both the D and the L isomers of lysine. To determine how much of the total tritium incorporated into lysine from H_2O-*t* was due to racemase activity, additional exchange experiments were performed with E_1 protein preparations that had been treated with iodoacetamide to selectively inactivate the racemase (see Methods). As shown in Table VIII, prior to alkylation E_1 protein catalyzed the incorporation of tritium into D-α-lysine and also L-α-lysine but the alkylated protein was virtually inactive on the L isomer. Tritium incorporation into D-α-lysine was reduced one-third by alkylation of the E_1 protein indicating that about 30% of the total tritium incorporation by untreated enzyme could be attributed to racemase activity and therefore must be located at the α position rather than in the ε position. A derivative of DL-lysine blocked in the ε-amino position, ε-*N*-acetyl-DL-lysine, which is an effective inhibitor of D-α-lysine mutase activity and thus must bind to the mutase (Morley and Stadtman, 1970), did not serve as substrate for the hydrogen-exchange reaction with alkylated E_1 protein (Table VIII). These results, together with the demonstration that the E_1 protein catalyzes the release of tritium from DL-α-lysine-6-*t* to the solvent, indicate that the hydrogen exchange catalyzed by the alkylated E_1 preparation is exclusively with hydrogen at the ε- rather than at the α-amino position. Furthermore, in agreement with the stereospecificity of the overall mutase reaction (Morley and Stadtman, 1970), the substrate for the hydrogen exchange is D-lysine and not L-lysine.

In experiments similar to those of Table VIII, tritium

TABLE VIII: Substrate Specificity of ^{3}H Incorporation from H_2O-*t* by E_1 Protein.[a]

Enzyme	^{3}H Incorpd (μatom/μmole of Substrate)		
	D-α-Lys	L-α-Lys	ε-*N*-Ac-DL-Lys
Apo-E_1	1.20	1.10	
Alkylated apo-E_1	0.80	0.10	0.15
Boiled alkylated apo-E_1	0.08		

[a] The incubation mixtures (0.25 ml) contained 40 mM sodium carbonate buffer (pH 9.2), 2 mM $MgCl_2$, 100 μM PLP, 2.5 mM lysine derivative as indicated, and enzyme (1.0 mg), as shown in the table. Preparation of the alkylated apo-E_1 is described in Methods.

incorporation from water into the other mutase substrate, 2,5-diaminohexanoate, likewise was shown to be catalyzed by E_1 protein in the presence of PLP and magnesium ion. This exchange (also slow) presumably involves the hydrogen on C-5.

Effects of PLP Inhibitors on Mutase and Hydrogen-Exchange Activities. To further characterize the roles of PLP in the overall mutase reaction and the slow hydrogen-exchange reaction with solvent, certain known inhibitors and antagonists of pyridoxal were tested for their effects on these processes. The holoenzyme form of D-α-lysine mutase was used to catalyze the overall reaction and E_1 protein alkylated with iodoacetamide to free it of lysine racemase activity (see Methods) was used for the measurement of tritium incorporation from H_2O-*t* into D-α-lysine. As shown in Figure 2a,b, hydroxylamine, isonicotinic acid hydrazide, and 1-aminoproline (the D and L forms) caused parallel losses of mutase and exchange activities. Hydrazine (not shown in the figure) inhibited both activities in a fashion similar to hydroxylamine except that higher concentrations were required to achieve equivalent inhibition. The results with these inhibitors, together with the data presented and discussed above, all point to the essential nature of PLP, and in particular to the aldehyde group of this cofactor, in both the mutase and hydrogen-exchange reactions. On the basis of the information available it seems likely that the mutase and hydrogen-exchange activities are related and that both involve Schiff base formation between PLP and the ω-amino group of the amino acid substrates. In this respect the D-lysine mutase resembles several other PLP-dependent enzymes that also react with amino groups other than those α to a carboxyl group; *e.g.*, the L-lysine aminotransferase of *Achromobacter liquidium* which transfers the ϵ-amino group of L-lysine and also the δ-amino group of L-ornithine to α-ketoglutarate (Soda and Misona, 1968), D-ornithine transaminase (Strecker, 1965), diamine transaminase (Kim, 1964), and γ-aminobutyrate aminotransferase (Waksman and Roberts, 1965).

It is noteworthy that carbonyl compounds are cofactors for amino group transfers in all three of the lysine mutase reactions studied. Both the B_{12} coenzyme-dependent D-lysine mutase studied here and the B_{12} coenzyme-independent L-lysine 2,3-aminomutase studied by Chirpich *et al.* (1970) require the presence of PLP, whereas the B_{12} coenzyme-dependent β-lysine mutase requires pyruvate rather than PLP (Stadtman and Renz, 1968). Furthermore, studies with ^{15}N-labeled substrates have demonstrated the amino group migrations catalyzed by the β-lysine mutase and the lysine 2,3-aminomutase to occur with complete retention of nitrogen (Bray and Stadtman, 1968). These observations and the similarities between these various reactions invite the consideration that in all three instances migration of the amino group occurs while it is bound to a carbonyl group on the enzyme.

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