A COBAMIDE COENZYME DEPENDENT MIGRATION OF THE ε-AMINO GROUP OF D-LYSINE

by

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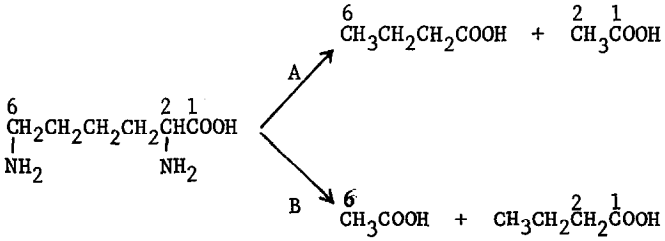
Clostridium sticklandii and certain other amino acid fermenting clostridia convert DL-lysine to a mole each of acetate and butyrate and two moles of ammonia (Stadtman and White, 1954). Whereas intact cells cleave a 2-carbon moiety from either end of the carbon chain with equal facility to yield a mixture of the products shown in Scheme I, soluble enzyme preparations, in general, produce acetate only from carbons 1 and 2 of lysine (Stadtman, 1955, 1963). The first intermediate in the



Scheme I

type A cleavage process was shown by Costilow et al. (1966) to be L-β-lysine formed by migration of the α-amino group to the β-position. The next reaction step, a cobamide coenzyme dependent migration of the amino group on carbon 6 of β-lysine to carbon 5 shown in equation 1, results in the formation of 3,5-diaminohexanoate[1,2] (Stadtman and Renz, 1967). The latter, in the presence of the additional cofactors required

1. Tsai, L. and Stadtman, T.C. Unpublished experiments.
2. Barker, H.A. Personal communication.

$$\begin{matrix}CH_2CH_2CH_2CHCH_2COOH \\ \quad\quad\quad\quad\quad\quad | \\ NH_2 \quad\quad\quad NH_2\end{matrix} \rightarrow \begin{matrix}CH_3CHCH_2CHCH_2COOH \\ \quad\quad | \quad\quad | \\ NH_2 \quad NH_2\end{matrix} \quad (1)$$

for the final steps leading to fatty acid formation, is converted to acetate and butyrate according to cleavage A of Scheme I[3]. Still a different diamino acid, tentatively identified as 2,5-diaminohexanoate, is formed from D-α-lysine. This new product is accumulated by a partially purified enzyme fraction from C. sticklandii. Again the migration of the amino group from carbon 6 to carbon 5 requires cobamide coenzyme. Thus there exist in C. sticklandii two different cobamide coenzyme dependent enzyme systems which catalyze the migration of the ε-amino group of a diaminohexanoate to the adjacent carbon atom 5. The substrate for one of these is L-β-lysine (L-3,6-diaminohexanoate) as shown in equation 1 whereas the cobamide coenzyme dependent system which is the subject of the present report acts on D-α-lysine and forms 2,5-diaminohexanoate (equation 2). It is possible that the latter

$$\begin{matrix}CH_2CH_2CH_2CH_2CHCOOH \\ \quad\quad\quad\quad\quad\quad\quad | \\ NH_2 \quad\quad\quad\quad NH_2\end{matrix} \rightarrow \begin{matrix}CH_3CHCH_2CH_2CHCOOH \\ \quad\quad | \quad\quad\quad | \\ NH_2 \quad\quad NH_2\end{matrix} \quad (2)$$

represent the first step in the B type cleavage process shown in Scheme I.

EXPERIMENTAL

Methods of distinguishing α-lysine and the three diaminohexanoic acids formed from it by C. sticklandii enzyme preparations are shown in Table I. The thin layer chromatographic procedure is reliable for separation of α-lysine, 2,5-diaminohexanoate and 3,5-diaminohexanoate. Although α-lysine and β-lysine (3,6-diaminohexanoate) exhibit similar mobilities in this as well as a number of other solvent systems they can be readily separated electrophoretically. An authentic sample of 2,5-diaminohexanoate[4] synthesized by Takagi (1957, 1959) served as

3. Stadtman, T.C. and Renz, P. Unpublished experiments.

4. The dipicrate, a generous gift from Dr. M. Mangyo (1964), was converted to the free amino acid by adsorption on Dowex-50-H^+ and elution with NH_4OH.

reference standard for this compound. Mixtures of this synthetic compound and the enzyme product described in the present communication migrated as a single spot electrophoretically and chromatographically in the systems of Table I. At pH 3.5 α-lysine and 2,5-diaminohexanoate exhibit almost identical electrophoretic mobilities and also are eluted as a single peak with pH 5.28 citrate buffer from a long ion exchange column in the Spinco amino acid analyzer. Their differences in adsorption on silicic acid, however, could be exploited for development of a preparative scale column separation method that allowed the ready isolation of material produced enzymically from α-lysine. The amino acids, separated from deproteinized reaction mixtures by adsorption on Dowex-50-H^+ and elution with ammonia emerged as discrete peaks from columns of Merck AG silica (0.05-0.20 mm particle size) developed with the

TABLE I

CHROMATOGRAPHIC AND ELECTROPHORETIC SEPARATIONS OF DIAMINOHEXANOATES

Compound	Ninhydrin product color[1]	R_f on silica TLC[2]	Electrophoretic mobility[3] at pH 3.5
			cm → cathode
α-lysine (2,6-diaminohexanoate)	Purple	0.15	51 - 52
2,5-diaminohexanoate	Purple	0.30	50
3,6-diaminohexanoate	Purple	0.15	59 - 59.5
3,5-diaminohexanoate	Orange	0.36	56 - 58

1. Detected as spots on chromatograms at neutral to alkaline pH.
2. Silica thin layer chromatograms Eastman Type K (301 R) on plasticback developed with $CHCl_3$ (40) + CH_3OH (40) + 15% NH_4OH (15).
3. High voltage electrophoresis on Whatman No 3 paper in approximately 0.5 M pH 3.5 pyridine-acetate buffer (2 hours at 3.5 to 4 Kvolts). Values are for distance from the origin to the front of the spot (0.25 to 1 μmole of amino acid per spot). In a mixture, α-lysine and 2,5-diaminohexanoate do not separate; 3,5-diaminohexanoate partly overlaps 3,6-diaminohexanoate but is clearly visible because of color difference after reaction with ninhydrin.

same solvent mixture used for thin layer chromatograms (Table I). The 2,5-diaminohexanoate preceeded the residual α-lysine substrate. A sample of labeled 2,5-diaminohexanoate produced from 6-C^{14}-α-lysine and isolated in this fashion was subjected to oxidation with CrO_3 in H_2SO_4 in a modified Kuhn-Roth procedure in order to demonstrate the presence of a terminal methyl group. As expected, a radioactive steam volatile acid was recovered from the oxidation mixture. The Duclaux distillation constants of this radioactive product were identical with those of known C^{14}-acetic acid determined under the same conditions. Very little over oxidation to $C^{14}O_2$, either of the labeled 2,5-diaminohexanoate or of known 2-C^{14}-acetate, occurred with the acid chromate mixtures employed. A control containing 6-C^{14}-α-lysine yielded negligible $C^{14}O_2$ and no labeled steam volatile acid. The data of these oxidation experiments thus confirm the presence of a terminal methyl group in the amino acid produced from α-lysine. The fact that the product is radioactive when formed either from 1-C^{14} or from 6-C^{14}-α-lysine suggests that it also is a hexanoate. Its electrophoretic mobility at pH 3.5, identical with that of α-lysine and authentic 2,5-diaminohexanoate, indicates that the compound is considerably more basic than various ω-monoamino acids such as ε-amino caproic acid, δ-aminovaleric acid, γ-amino-butyric acid or β-alanine all of which migrate more slowly at this pH. These observations, together with the identical Rf values of the enzyme product and authentic 2,5-diaminohexanoate on silica thin layer chromatograms, all point to the likelihood that the new amino acid is indeed 2,5-diaminohexanoate.

Following chromatography of crude C. sticklandii extracts on DEAE-cellulose, removal of nucleic acids with protamine and fractionation with ammonium sulfate, preparations of D-α-lysine mutase are obtained which utilize L-α-lysine only very poorly as substrate whereas D-α-lysine is readily converted to 2,5-diaminohexanoate. A lysine

racemase[5] initially present enables either optical isomer to be used with equal facility but upon repeated fractionation with ammonium sulfate this activity is gradually separated from the α-lysine mutase. Typical assay conditions employed for the enzyme at this state of purity are illustrated in Table II where it is seen that the formation of 2,5-diaminohexanoate is proportional to time and to enzyme concentration over appreciable ranges. The data of Table III show that α-lysine mutase is completely inhibited by the glycoprotein intrinsic factor and that this inhibition is markedly reversed by the addition of dimethylbenzimidazolylcobamide coenzyme. In fact the enzyme preparation

TABLE II

PROPORTIONALITY OF 2,5-DIAMINOHEXANOATE FORMATION TO TIME AND TO ENZYME CONCENTRATION

Expt. No.	Incubation time min.	Enzyme added, mg. protein	2,5-diaminohexanoate formed cts./min.
1	0	5.3	0
	30	"	11,900
	60	"	19,200
	90	"	27,800
	120	"	36,450
2	120	0	0
	"	1.77	17,000
	"	3.54	30,650
	"	5.3	36,450
	"	7.1	43,700

Reaction mixtures (0.66 ml) incubated under helium at 30 C contained 2-methyl-imidazole-Cl buffer, pH 8, 50 μmoles; ATP, 10 μmoles; 1,4-dimercaptothreitol, 4 μmoles; $MgCl_2$, 5 μmoles; cobamide coenzyme, 0.003 μmole; FAD, 0.02 μmole; D-α-lysine·HCl, 20 μmoles; 1-C^{14}-DL-α-lysine, 0.4 μcurie and the indicated amount of partially purified enzyme prepared as described in the text. The reaction product, separated from the deproteinized incubation mixture by adsorption on and elution from small Dowex-50-H^+ columns followed by chromatography on thin layer silica sheets was detected after spraying with ninhydrin reagent. Unreacted bands from the chromatograms were assayed for radioactivity in a scintillation counter. In this experiment, a micromole of the 2,5-diaminohexanoate product is equivalent to about 8,000 cts./min.

5. Assayed directly in a manometric assay by coupling with L-lysine decarboxylase of Bacterium cadaveris (Gale, 1945).

employed was activated 2-fold by the addition of the cobamide coenzyme in the absence of any inhibitor. Other cofactor requirements for the partially resolved enzyme system are illustrated in Table IV. These

TABLE III

INHIBITION OF D-α-LYSINE MUTASE BY INTRINSIC FACTOR AND REACTIVATION WITH COBAMIDE COENZYME

Cobamide coenzyme added, mμmoles	2,5-diaminohexanoate formed, cts./min.	
	Intrinsic factor[1] added	No intrinsic factor added
0	0	14,600
0.5	8,070	--
1.0	9,700	19,150
2.0	18,300	28,750
3.0	16,500	30,650

1). The amount of intrinsic factor[6] (2.5 mg. per sample) was sufficient to bind 0.95 mμmole of cobamide.

The composition of the reaction mixtures and conditions are the legend of Table II. The 3.54 mg level of enzyme was employed.

TABLE IV

REQUIREMENTS FOR D-α-LYSINE MUTASE REACTION

Omission	Addition	2,5-diaminohexanoate formed, cts./min.
None		35,500
ATP		3,330
$R(SH)_2$		5,770
Mg^{++}		5,720
B_{12}-coenzyme		19,000
"	$OH\text{-}B_{12}$	23,400
FAD		30,400
B_{12}-coenzyme + FAD		12,050
Enzyme	Heated enzyme	0

The composition of the complete reaction mixture is given in the legend of Table II. Hydroxycobalamin (0.003 μmole) was substituted for cobamide coenzyme where indicated. The same enzyme preparation, 3.54 mg protein, was employed. Incubation, 120 min; sample volume, 0.56 ml.

6. Generously supplied by Dr. L. Ellenbogen.

are ATP, a mercaptan such as 1,4-dimercaptothreitol and magnesium ion. Hydroxycobalamin replaces the cobamide coenzyme only partially suggesting that ability to resynthesize the coenzyme is limiting. Activation by FAD, although slight with the enzyme preparation shown in Table IV, is magnified by repeated ammonium sulfate fractionation steps. These same cofactors, plus pyruvate, are required for the cobamide coenzyme dependent amino group migration reaction catalyzed by β-lysine mutase (equation 1).

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