Studies on the Fermentation of D-α-Lysine. Purification and Properties of an Adenosine Triphosphate Regulated B_{12}-Coenzyme-Dependent D-α-Lysine Mutase Complex from *Clostridium sticklandii**

Colin G. D. Morley† and Thressa C. Stadtman

ABSTRACT: The enzyme system from *Clostridium sticklandii* that catalyzes the migration of the ε-amino group of D-α-lysine to carbon 5, forming 2,5-diaminohexanoate, has been purified to near homogeneity as a complex of two dissimilar proteins, a red cobamide protein (E_1) and a sulfhydryl protein (E_2). The complex is dissociated by acidification to pH 4.0; the precipitate which forms contains E_1 while E_2 remains in the supernatant solution. The combined fractions are catalytically active. The activity of the isolated D-α-lysine mutase complex depends on added pyridoxal phosphate, B_{12}-coenzyme, and a mercaptan. Mutase activity is stimulated by ATP, Mg^{2+}, and a monovalent cation such as K^+ or NH_4^+. ATP, which can be replaced by its phosphonic acid analogs, is an allosteric effector and increases the affinity of the enzyme for the substrate, D-α-lysine. The mutase complex is inactivated by irradiation with visible light or by treatment with alkylating agents.

Analogous amino group migrations are catalyzed by two B_{12}-coenzyme-dependent enzyme systems from *C. sticklandii*: L-β-lysine mutase and ornithine mutase. Preparations of the D-α-lysine mutase have been obtained free of these two activities, showing that the D-α-lysine mutase system is distinct. The D-α-lysine mutase is inhibited by a number of amino acids and related compounds, some of which appear to be competitive inhibitors with respect to the normal substrate, lysine.

On the basis of early isotope studies it was concluded that the fermentation of DL-lysine to acetate, butyrate, and ammonia by *Clostridium sticklandii* involves two separate pathways (Stadtman and White, 1954; Stadtman, 1954). In one pathway, which could be investigated in soluble extracts (Stadtman, 1963), acetate is formed from carbon atoms 1 and 2 of lysine and butyrate is formed from the remainder of the molecule. The primary substrate, L-α-lysine, first undergoes two successive amino group migration reactions (Chirpich *et al.*, 1970; Tsai and Stadtman, 1968; Stadtman and Renz, 1968) and then is oxidatively deaminated to form 3-keto-5-aminohexanoate (Rimerman and Barker, 1968) before eventual cleavage of the carbon chain occurs (Figure 1).

In the other pathway acetate is formed from carbons 5 and 6 of lysine and butyrate is derived from the carboxyl end of the molecule. Soluble extracts fail to carry out this overall series of reactions and, therefore, the reaction sequence is still unknown. However, the recently discovered conversion of D-α-lysine into 2,5-diaminohexanoate (Stadtman and Tsai, 1967) presumably is the first step. (Figure 1). In both lysine pathways there is a B_{12}-coenzyme-mediated migration of the ε-amino group to carbon 5; in the L-lysine pathway this is the second enzymic step and the product is 3,5-diaminohexanoate whereas in the D-lysine pathway it is the first step and the product is 2,5-diaminohexanoate. The two B_{12}-coenzyme-dependent mutases catalyzing these reactions are similar in a number of their properties. Each consists of an acidic orange protein moiety containing bound cobamide coenzyme as its chromophore and a sulfhydryl protein moiety. In addition to B_{12}-coenzyme, ATP, a mercaptan, and a divalent metal ion are required as cofactors by both mutases (Stadtman and Renz, 1968; Stadtman and Tsai, 1967); L-β-lysine mutase activity is further stimulated by pyruvate and a monovalent cation (K^+ or Rb^+). To elucidate the roles of the unusual number of cofactors required for catalysis of these amino group migrations and to gain further insight into the reaction mechanism itself, purification and more detailed study of one of the enzyme systems were undertaken. The present report describes a procedure for isolation of D-α-lysine mutase as a highly purified complex, presents evidence that its cofactor requirements are more complex than at first recognized and elucidates the role of ATP in the system.

Experimental Procedure

Materials. C. sticklandii was cultured and harvested and the cell paste frozen in pellets in liquid N_2 as described elsewhere (Stadtman, 1966), The cells used in this work had been stored at −80° for approximately 6 months.

Synthetic 2,5-diaminohexanoate was prepared by Dr. L. Tsai (unpublished results). Crude L-β-lysine was purchased from the Cyclo Chemical Corp. and further purified by ion-exchange chromatography (Tsai and Stadtman, 1968) prior to use. Other amino acids were obtained from commercial sources. These were checked for purity by silica gel thin-layer chromatography in a chloroform–methanol–12% NH_4OH (40:40:15) solvent system (solvent I). Except for the sample

* From the Laboratory of Biochemistry, National Heart and Lung Institute, Bethesda, Maryland 20014. *Received July 27, 1970.* Part of this work was presented before the American Society of Biological Chemists (Morley and Stadtman, 1970).

† Present address: Department of Medicine and Biology Collegiate Division, University of Chicago, Chicago, Ill.

of DL-δ-hydroxylysine which was contaminated with lysine and two other minor ninhydrin-positive components, the various amino acids and amines used in these studies were virtually free of ninhydrin-positive impurities.

DL-α-Lysine-6-^{14}C and DL-ornithine-2-^{14}C were purchased from Volk Radiochemical Corp; L-β-lysine-6-^{14}C was synthesized enzymically from L-α-lysine-6-^{14}C by Dr. P. Renz.

Other chemicals were purchased from commercial sources as follows: ATP (potassium salt) from P. L. Biochemicals; phosphonic acid analogs of ATP from Miles Laboratories; FAD, *S*-adenosylmethionine, potassium pyruvate, phosphoenolpyruvate, and 1,4-dithiothreitol from Calbiochem; pyridoxamine phosphate, pyridoxine phosphate, and pyridoxal from Sigma Corp.; pyridoxal phosphate from Nutritional Biochemical Corp.; MgK_2EDTA (Magnesium Titriplex) from E. Merck, A.G.; DMBC[1] from Pierrel, Sp.A., Milano, Italy. DEAE-cellulose from Reeve Angel, DEAE-Sephadex and Sephadex-G-200 from Pharmacia, Sweden.

Muscle aldolase was purchased from Worthington Biochemical Corp. and thyroglobulin (porcine) from Mann Research.

Determination of Enzyme Activity. The standard reaction mixture (0.5 ml) containing 100 mM Tris·HCl buffer (pH 8.5–9.0), 5 mM ATP, 2 mM $MgCl_2$, 8 mM dithiothreitol, 20 μM DMBC, 40 μM pyridoxal phosphate, 20 mM D-α-lysine·HCl, enzyme, and 0.1–10 mg of protein (depending on purity) was incubated for 60 min at 37° in 10 × 75 mm stoppered tubes in a helium or argon atmosphere. Samples were protected from light during and after DMBC addition. In some experiments reaction mixtures were further supplemented with 50 mM NH_4Cl. Reactions were terminated by the addition of 0.1 ml of 30% $HClO_3$ and the precipitated proteins removed by centrifugation. The amount of 2,5-DAH formed from D-α-lysine was estimated either by reaction with an acid ninhydrin reagent or by thin-layer chromatography (see below).

Assays of the ornithine mutase (H. C. Friedmann, 1969, personal communication) were carried out using DL-ornithine-2-^{14}C as substrate and measuring the amount of label in the 2,4-diaminopentanoate in a similar manner to that described below for the assay of 2,5-diaminohexanoate.

Assay of 2,5-Diaminohexanoate by the Acid Ninhydrin Procedure. An acid ninhydrin reagent (Chinard, 1952) was used to estimate the extent of conversion of α-lysine into 2,5-DAH. The product formed with pure 2,5-DAH is golden in color and adsorbs between 460 and 485 mμ, whereas lysine yields a reddish product of much lower extinction at these wavelengths.

Aliquots of the deproteinized reaction mixtures, usually 20 to 50 μl, containing 0.02–0.1 μmole of 2,5-DAH are diluted to 0.1 ml with water. After addition of 1.5 ml of glacial acetic acid and 1.0 ml of the Chinard acid ninhydrin reagent, the samples are heated in capped tubes in a boiling water bath for 45 min. After cooling and diluting with 4.0 ml of glacial acetic acid, the colored product is estimated in a Klett colorimeter equipped with a No. 47 filter. A zero time sample containing only lysine serves as a blank. Under these conditions, 0.1 μmole of 2,5-DAH is equivalent to a Klett reading of about 148 (A_{470} 0.30). Except where the extent of enzymic reaction is small, this direct ninhydrin assay procedure for 2,5-DAH formation is satisfactory.

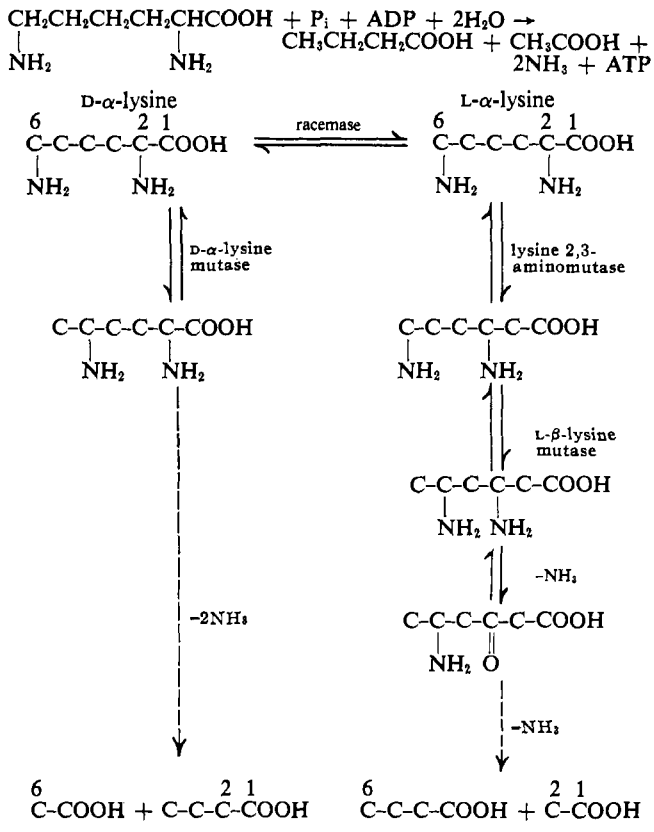

FIGURE 1: Lysine fermentation pathways.

Unlike a similar assay procedure for 3,5-diaminohexanoate (Tsai and Stadtman, 1968), the reaction of 2,5-diaminohexanoate with the acid ninhydrin reagent is not appreciably affected by variations in water content of 0.1–0.2 ml.

This type of colorimetric assay system is not satisfactory for use with the ornithine mutase except when the reaction is extensive enough to measure ornithine disappearance. Ornithine gives a strong red color with the acid ninhydrin reagent and the product of the ornithine mutase reaction, 2,4-diaminopentanoate, gives little, if any, color.

Chromatographic Separation of 2,5-Diaminohexanoate. Aliquots of deproteinized reaction mixtures, after the $HClO_3$ had been neutralized with KOH, were applied to thin-layer silica sheets which were developed in solvent I (see above). Residual α-lysine and the enzyme product, 2,5-diaminohexanoate, detected as purple spots after reaction with ninhydrin, migrate with R_F values of approximately 0.15 and 0.30, respectively. For a sensitive assay of enzyme activity, radioactive lysine was employed as substrate and the amount of ^{14}C in the 2,5-diaminohexanoate product (from bands of the chromatogram not sprayed with ninhydrin) was determined by scintillation spectrometry.

Ornithine mutase activity can be measured by a similar procedure using radioactive ornithine and measuring the amount of ^{14}C in the enzyme product, 2,4-diaminopentanoate, after separation of residual ornithine on thin-layer sheets in

[1] Abbreviations used are: 2,5-DAH, 2,5-diaminohexanoate; PALP, pyridoxal phosphate; and DMBC, dimethylbenzimidazoyl cobamide coenzyme (5′-deoxyadenosylcobalamin); AOPOPCP and AOPCPOP indicate the phosphonic analogs of ATP with a methylene group in place of the appropriate oxygen in the phosphate moiety.

the above solvent. The R_F values of ornithine and 2,4-diaminopentanoate under these conditions are 0.39 and 0.58, respectively.

Other Methods. Protein was estimated in crude enzyme preparations using the biuret procedure. In purified preparations either the ultraviolet absorbancy method (Warburg and Christian, 1941) or, for low protein concentrations, the method of Lowry *et al.* (1951) was employed.

L-β-Lysine mutase activity and the formation of 3,5-diaminohexanoate were assayed as described previously (Stadtman and Renz, 1968).

Radioactivity measurements on amino acids isolated by thin-layer chromatography were made in a scintillation spectrometer (Beckman Model LS-250) using a fluid containing ethanol (2.0 ml) and 0.8% 2,5-diphenyloxazole in toluene (8.0 ml).

Conductivity was measured using a Radiometer CDM type meter.

Results

Purification of the Enzyme Complex. (1) PREPARATION AND DIALYSIS OF THE EXTRACT. French pressure cell extracts were prepared from frozen cells of *C. sticklandii* as described elsewhere (Stadtman, 1966). In a typical experiment the clear cell extract (23 g of protein in 550 ml) obtained from 200 g of cell paste was dialyzed for 18 hr at 4° against 12 l. of 20 mM Tris, pH 8.5–9.0, containing 1 mM MgK_2EDTA.[2]

(2) CHROMATOGRAPHY ONCOARSE DEAE-CELLULOSE. The D-α-lysine mutase enzyme complex can be separated from the major part of the L-β-lysine mutase system (Stadtman and Grant, 1970) and a number of other acidic proteins, including ferredoxin, by rapid passage of the dialyzed cell extract over DEAE-cellulose.

A column of coarse DEAE-cellulose (8 × 27 cm) equilibrated with 20 mM Tris, pH 8.5–9.0, was used. After application of the dialyzed cell extract, the column was washed with the equilibrating buffer until no further protein appeared in the effluent. This fraction (the effluent and the wash), termed fraction I, contains the D-α-lysine mutase activity. Subsequent elution of the column with 0.25 M neutral potassium phosphate buffer removes the L-β-lysine mutase system (Stadtman and Renz, 1968). The entire procedure is carried out at room temperature in dim light.

(3) PROTAMINE SULFATE PRECIPITATION OF THE ENZYME COMPLEX. Two alternative procedures were developed for this step: method b although more tedious is the more reliable and was employed for most enzyme preparations.

(a) To fraction I (from the coarse DEAE-cellulose column) buffered at pH 8.0–9.0, was added freshly prepared 1% protamine sulfate solution at a level of 0.3 mg of protamine sulfate per mg of protein and the mixture was stirred for 20 min at 0° (Folk and Cole, 1966). If necessary, additional 2 M Tris was added to maintain the pH between 8.0 and 9.0. The reddish colored protamine–protein precipitate, sedimented by centrifugation for 20 min at 16,000*g*, contains the D-α-lysine mutase. The supernatant solution, containing about 40% of the protein but no mutase activity, was discarded. The precipitate was extracted with four successive 20-ml portions of 50 mM ammonium sulfate, pH 5.0 (Folk and Cole, 1966). The final unextracted residue was removed by centrifugation and discarded. In this procedure a considerable quantity of protamine nucleate, which interferes with subsequent purification steps, is extracted along with the enzyme complex. The alternative procedure, b, avoids this difficulty.

(b) Fraction I from the DEAE-cellulose column was diluted to a protein concentration of about 10 mg/ml and adjusted to 0.4 M NaCl by addition of the solid. A freshly prepared 1% solution of protamine sulfate (in 0.4 M NaCl) was then added slowly (no more than 10 ml/min) to a final concentration of 0.2–0.25 mg per mg of protein and the mixture was stirred for 20 min at 0°. The white precipitate, which is principally protamine nucleate, was discarded; at least 50% of the protein of the extract remains in solution. The red colored supernatant solution was brought to 0.8 saturation with ammonium sulfate, stirred for 30 min at 0°, then centrifuged for 2 hr at 16,000*g*. The slimy grey precipitate was redissolved in a minimum volume (about 100 ml) of 20 mM Tris buffer, pH 8.5–9.0, and dialyzed for 24 hr against 12 l. of the same buffer (changed once during this period) and finally for 3 hr against 12 l. of distilled water. During the dialysis a protamine–protein complex containing the D-α-lysine mutase precipitated in the bag. This precipitate (A) was collected by centrifugation for 10 min at 30,000*g* and extracted with 50 mM ammonium sulfate as described above. The supernatant solution from the dialysis bag was treated with protamine sulfate (0.25 mg per mg of protein) and if a precipitate (B) formed this also was collected by centrifugation and extracted with 50 mM ammonium sulfate. The two extracts were assayed for D-α-lysine mutase activity and if both contained significant amounts of the enzyme they were pooled. Generally the extract of A had the higher specific activity.

(4) FRACTIONATION ON DEAE-SEPHADEX PAD. Enzyme preparations from the previous protamine treatment step could be fractionated much more successfully by gradient elution from DEAE-Sephadex columns if they were subjected to preliminary adsorption and then elution from a DEAE-Sephadex pad. If cold buffers are used, this step, which also serves to concentrate the enzyme, can be carried out at room temperature.

The extracts from the previous step were diluted approximately 2-fold with cold distilled water to a conductivity of about 4 mmhos (dilution exceeding 2.5-fold usually results in precipitation of the protamine–enzyme complex and is to be avoided). The diluted extract was poured over an 8 × 3 cm pad of DEAE-Sephadex A-50 (40–120 μ) previously equilibrated with 20 mM Tris, pH 8.5 (conductivity 2.0 mmhos), and the pad was washed with the same buffer until no more protein came off. This effluent and wash fraction, containing 20–30% of the protein applied to the pad, was discarded. Elution of the pad with 0.2 M NaCl, pH 6–7, removed another 10–15% of the protein which also was discarded. The D-α-lysine mutase activity was then eluted as a reddish colored fraction (20–30% of the total protein) with 0.33 M NaCl. The remaining red protein on the pad could be eluted with 1 M NaCl, but it exhibited no D-α-lysine mutase activity.

(5) GRADIENT ELUTION FROM DEAE-SEPHADEX. This column procedure was carried out at 5–10°. The 0.33 M NaCl fraction from step 4 was diluted or dialyzed until its conductivity was

[2] Unless otherwise noted all buffers used for enzyme purification contained 1 mM MgK_2EDTA.

TABLE I: Purification of the Enzyme Complex.

Step	Vol (ml)	Total Protein (mg)	Total Activity (Units × 10^{-6})[a]	Specific Activity (Units/mg)	Yield (%)
1 Cell extract	570	26,000	7.5	290	
2 Dialyzed cell extract	620	18,600	8.4	450	100[b]
3 DEAE-cellulose (fraction I)	1440	16,000	4.35	270	52
4 Protamine sulfate precipitation (extracted proteins)	75	410	2.1	5,100	25
5 DEAE-Sephadex pad; 0.33 M NaCl eluate	82	89	1.89	21,300	22
6 DEAE-Sephadex gradient	15	58	1.74	30,000	21
7 Sephadex G-200					
Peak 1	2	10	0.45	45,000	5
Peak 2	4	12	0.78	64,800	9

[a] A unit of enzyme activity is defined as the amount required to produce 1.0 mμmole of 2,5-diaminohexanoate per hr under the standard assay conditions. [b] Assays of enzyme activity are unreliable in the crude cell extract because of the presence of interfering compounds which may either activate or inhibit. Calculations of yield, therefore, are based on the dialyzed cell extract.

1–2 mmhos and absorbed on a 2 × 15 cm column of DEAE-Sephadex A-50 (40–120 μ) previously equilibrated with 20 mM Tris (pH 8.5). The sample was applied to the column with a Sigmamotor pump at the rate of 20 ml/hr and the column also was run at this rate with a linear gradient (0.02–0.6 M NaCl, pH 6.0) being applied through the pump. The elution pattern of the proteins under these conditions is shown in Figure 2. The highest specific activity material, tubes 20–25 inclusive in Figure 2, were pooled, diluted until the conductivity was about 2 mmhos, and concentrated by rapidly adsorbing the protein on a pad of DEAE-Sephadex (2 × 0.5 cm) and eluting with 0.5 M NaCl (pH 6.0).

(6) MOLECULAR SIEVE CHROMATOGRAPHY ON SEPHADEX G-200. Providing the flow rate of the column is adequate, at least 12 ml/hr, the gel filtration step may be performed at room temperature. A 3 × 36.5 cm column of Sephadex G-200 (140–400 μ) equilibrated with 20 mM Tris, pH 9.0, was prepared and calibrated in two separate runs with thyroglobulin (porcine) (10 mg) and muscle aldolase (18 mg) in the equilibrating buffer. The elution profiles of these two proteins are shown in Figure 3. The D-α-lysine mutase sample from the preceeding purification step (50–60 mg of protein in 10 ml) was then introduced and eluted with 20 mM Tris. Fractions of 6.0 ml were collected automatically and assayed for protein content (absorbancy at 280 mμ) and enzyme activity. In a typical experiment shown in Figure 3, two major peaks of D-α-lysine mutase activity were detected. Peak 1, tubes 10–12, and peak 2, tubes 13–16, were pooled separately and concentrated on small DEAE-Sephadex pads (2.5 × 0.5 cm). The adsorbed proteins were eluted with 1 M NaCl, pH 6.0–7.0. Although the enzyme complex is relatively stable at this pH it is preferable to store it in 20 mM Tris buffer, pH 8.5–9.0, at a protein concentration of no less than 3 mg/ml. Under these conditions 60–80% of the original activity of the complex remained after storage at −15° for 1 month.

Table I gives a summary of the entire purification procedure. The final specific activity of the complex can vary from 20,000 to 64,000 mμmoles of 2,5-diaminohexanoate produced

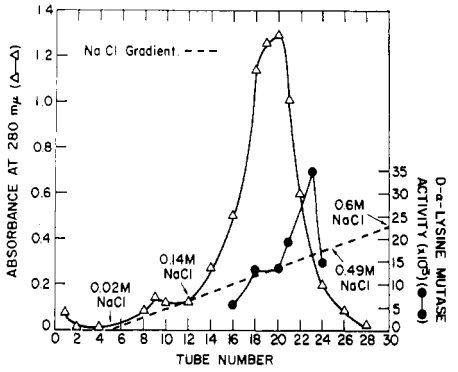

FIGURE 2: Elution of D-α-lysine mutase complex from DEAE-Sephadex with a NaCl gradient. The sample, 80–100 mg of protein in 20 mM Tris buffer (pH 9.0), was applied to a column of DEAE-Sephadex (2 × 15 cm) and a linear gradient of 0.02–0.6 M NaCl (pH 6.0) applied. The flow rate of 20 ml/hr was controlled using a Sigma motor pump and samples of 6.5 ml were collected.

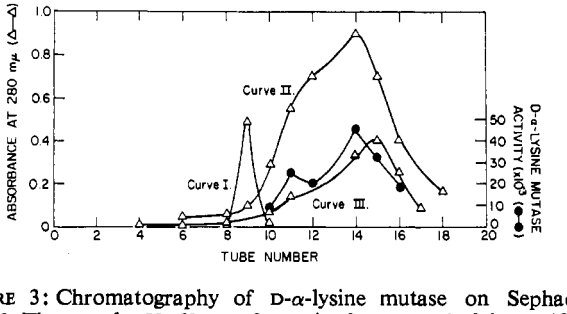

FIGURE 3: Chromatography of D-α-lysine mutase on Sephadex G-200. The sample, 50–60 mg of protein, from step 6 of the purification procedure, was applied to a column of Sephadex G-200 (3 × 36.5 cm), equilibrated with 20 mM Tris (pH 9.0), and fractions of 6.0 ml were collected at room temperature at a rate of 12–20 ml/hr (curve I, thyroglobulin; curve II, D-α-lysine mutase sample; curve III, aldolase.

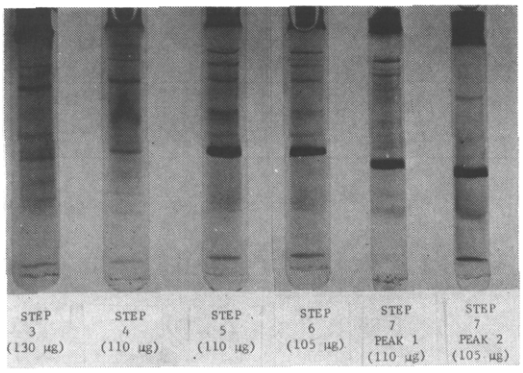

FIGURE 4: Analytical disc gels of samples from various stages of purification of the enzyme complex. The gels were run at pH 8.3 in Tris–glycine buffer for about 1.5 hr at 4 mA/gel at 4° (Ornstein and Davis, 1964). All the gels shown are 7.5% acrylamide. Following electrophoresis gels were stained in Amido Schwarz dye in 10% AcOH for 1 hr followed by destaining.

TABLE II: Reconstitution of D-α-Lysine Mutase System from Separated Cobamide Protein (E_1) and Sulfhydryl Protein (E_2) Fractions.

Protein Fractions Added[b] (mg)		2,5-DAH Formed[a] (Total Δ at 485 mμ)	
E_1	E_2	Expt 1	Expt 2
3.5	0	0.365	1.48
3.5	0.50	10.3	11.9
3.5	0.75	*c*	19.5
3.5	1.0	16.6	*c*
0	1.0	0.073	*c*

[a] Estimated by ninhydrin assay procedure as described in Methods. [b] E_1 and E_2 were partially purified from a D-α-lysine mutase preparation (Stadtman and Grant, 1970). [c] Not tested.

per milligram of protein per hour, depending on the batch of cells used and the efficiency of the purification.

The fact that the higher specific activity D-α-lysine mutase complex of peak 2 (Figure 3) was eluted from the Sephadex G-200, slightly ahead of the muscle aldolase (mol wt 160,000) but much later than thyroglobulin (mol wt 670,000) allowed an estimation of a molecular weight of 250,000 for the complex.

Analysis of Purified Complex by Disc Gel Electrophoresis. The highly purified D-α-lysine mutase complex (peak 2 from the Sephadex G-200 column, Figure 3) exhibited only one major protein band when analyzed by disc gel electrophoresis (Figure 4). Prior to staining, a reddish orange colored band was visible at the same position in the gel. When this colored protein band was sliced from a parallel gel, extracted with pH 9.0 Tris buffer, and assayed for D-α-lysine mutase 75% of the applied enzyme units were recovered. The lower specific activity enzyme from peak 1 of the Sephadex G-200 column exhibited a higher proportion of slower moving, presumably higher molecular weight, contaminating protein bands (Figure 4). For comparison the disc gel profiles of the enzyme complex at earlier stages of purification are also shown in Figure 4.

Dissociation of D-α-Lysine Mutase into Two Dissimilar Protein Moieties. Earlier studies with the D-α-lysine mutase of *C. sticklandii* indicated that the enzyme from step 3 of Table I, freed of nucleic acids by treatment with protamine sulfate and then subjected to exhaustive dialysis, could be separated into a very acidic red-orange cobamide protein moiety (E_1) and a less acidic colorless protein moiety (E_2) by chromatography on microgranular DEAE-cellulose or DEAE-Sephadex (Stadtman and Grant, 1970). The experiments of Table II show that these protein fractions (E_1 and E_2) have to be recombined in order to convert D-α-lysine into 2,5-diaminohexanoate.

Separation of D-α-lysine mutase into its two protein components can also be achieved by acidification of the purified complex. A solution of the highly active complex (from step 7 of Table I) was adjusted to pH 4.0 at 0° by the slow addition of cold 0.1 N hydrochloric acid. After an additional 5 min of stirring at 0° the precipitated protein was removed by centrifugation for 5–10 min at 16,000*g*. The supernatant solution was neutralized at once with 0.5 N KOH and 8 mM dithiothreitol was added to stabilize the sulfhydryl enzyme. The reddish precipitate was dissolved in 20 mM Tris·HCl, pH 9.0. Neither fraction alone exhibited D-α-lysine mutase activity but when recombined 80% of the original mutase activity was recovered. The supernatant solution from this pH step exhibited E_2 activity and, when subjected to disc gel electrophoresis in 7.5% polyacrylamide gel, previously cleared of potassium persulfate with thiolglycollic acid (W. B. Jakoby, 1970, personal communication) a protein band that migrated appreciably faster than the original complex was found. This faster moving band, extracted from an unstained gel, was shown to exhibit E_2 activity. The advantages of the acid precipitation procedure are that the mutase is more stable when purified as a complex and upon subsequent dissociation, each of the two components is highly purified.

Effect of Iodoacetamide Treatment on the Activity of D-α-Lysine Mutase. One of the two dissimilar protein moieties of the L-β-lysine mutase system is a sulfhydryl protein that is readily inactivated by alkylation (Stadtman and Renz, 1968). Since this protein resembles in many of its properties the E_2 moiety of the D-α-lysine mutase, the effect of iodoacetamide on D-α-lysine mutase activity was investigated.

Catalytic activity was lost when either the reconstituted D-α-lysine mutase (E_1 and E_2 proteins mixed) or E_2 alone (samples 4 and 5, respectively, of Table III) was treated with iodoacetamide. In similar experiments not shown here the purified D-α-lysine mutase, isolated as the complex, was also inactivated by iodoacetamide but in this case a somewhat higher concentration of the alkylating agent was required to give complete inhibition.

The E_2 protein preparation used for the experiments of Table III also effectively reconstituted L-β-lysine mutase activity when added to a separated L-β-lysine mutase cobamide protein but failed to do so after treatment with iodoacetamide.

Assay of the Purified Complex for the Other B_{12}-Coenzyme-Dependent Amino Acid Mutases. The highly purified D-α-lysine mutase complex and the crude cell extract were compared with respect to ability to catalyze the related amino

TABLE III: Effect of Iodoacetamide on the Reconstituted D-α-Lysine Mutase.

Sample	Type of Treatment[a]	Total 2,5-Diaminohexanoate Formed (mμmoles)
1	Mixed E_1 and E_2 proteins not pretreated with $R(SH)_2$; no iodoacetamide added	315
2	As 1 but iodoacetamide and $R(SH)_2$ mixed and present in the assay mixture.	285
3	Mixed E_1 and E_2 proteins pretreated with $R(SH)_2$, no iodoacetamide added	315
4	Mixed E_1 and E_2 proteins pretreated with $R(SH)_2$ and iodoacetamide added	40
5[b]	E_2 protein only pretreated with $R(SH)_2$ and iodoacetamide added	20
6[b]	E_1 protein pretreated with $R(SH)_2$ and iodoacetamide added	240

[a] The initial treatment with $R(SH)_2$ to reduce proteins consisted of incubation in 0.5 M potassium phosphate buffer (pH 7.1), with 1.0 mM dithiothreitol for 15 min at 30° under argon. Then 10 mM iodoacetamide was added and the samples were held at 30° again for 15 min. Next, residual iodoacetamide was destroyed by reaction with a fourfold excess of dithiothreitol (20 mM) for 15 min at 30°. Finally the remaining reactants of the usual assay mixture were added and samples were incubated at 37° for 45 min; 600 μg of E_2 protein and 100 μg of E_1 protein were used in each experiment. [b] In these experiments untreated E_1 or E_2 protein at the levels added in expt 1–4 were added to the appropriate tubes before assaying.

TABLE IV: Efficiency of Removal of Contaminating Enzyme Activities from D-α-Lysine Mutase During the Purification Procedure.

Enzyme System	Specific Activity (μmoles of Product[a]/mg of Protein)		Purification (-fold)
	Cell Extract	Purified complex	
D-α-Lysine mutase	0.48	65.0	135
L-β-Lysine mutase (+E_2)[b]	1.96	6.9	3.5
L-β-Lysine mutase (−E_2)	1.96	0[c]	
Ornithine mutase (+E_2)[b]	0.125	0	

[a] The products formed in the D-α-lysine mutase, L-β-lysine mutase, and ornithine mutase systems are 2,5-diaminohexanoate, 3,5-diaminohexanoate (Stadtman and Renz, 1968), and 2,4-diaminopentanoate (Tsuda and Friedmann, 1970), respectively. The assays for the latter reactions are given in methods. [b] Supplemented with a partially purified E_2 fraction. [c] 0 indicates a level of activity too low to detect either by the standard ninhydrin assay or by using radioactive substrate (see Methods).

group migrations (L-β-lysine mutase and ornithine mutase reactions). As shown by the data in Table IV, the purification procedure removed all detectable ornithine mutase activity and most of the L-β-lysine mutase activity originally present in the *C. sticklandii* extract. Although L-β-lysine mutase activity was detected only when the complex was supplemented with a preparation of E_2 protein, there was no effect of this component on D-α-lysine mutase activity unless the complex had been stored for several days in dilute solution or subjected to repeated freezing and thawing. The loss in activity that occurred as the enzyme complex aged could be partially reversed by addition of purified E_2 protein. This suggests that the complex slowly dissociates on storage with concomitant inactivation of the more labile E_2 protein component and that, although more of this is added, the conditions necessary for regeneration of the highly active complex are not achieved.

Visible and Ultraviolet Absorption Spectra of the Complex. Spectra of the purified D-α-lysine mutase complex from peak 2 of the Sephadex G-200 column (Figure 3) were recorded in the ultraviolet and visible regions and are shown in Figure 5. The absorption maximum in the ultraviolet (Figure 5A) is at 278 mμ. The absorption spectrum in the visible range (Figure 5B) is typical of a B_{12} chromophore. The relatively high absorbancy at 355 mμ suggests that a considerable portion of the chromophore is a hydroxycobamide. Pyridoxal phosphate is also bound to the complex (see below) but the extent of its contribution to this spectrum is not apparent.

Inactivation of the Complex by Visible Light. A preparation of the D-α-lysine mutase that exhibited partial dependence on added DMBC for activity was irradiated with visible light in an attempt to inactivate the residual bound cobamide

TABLE V: Light Inactivation of the Mutase Complex.

DMBC Added (μM)	2,5-Diaminohexanoate Produced (mμmoles)	
	Control Enzyme	Irradiated Enzyme[a]
0	273	162
8	630	207
40	663	459

[a] A sample of the protein complex (750 μg) in ice, was irradiated with a 600-W tungsten lamp at a distance of 6 cm for 6 hr. As a control, a similar protein sample was kept in ice in the dark for this period of time. Aliquots (containing 250 μg of protein) of the irradiated and control samples were then incubated at 37° for 30 min with 8 μM DMBC, with 40 μM DMBC, or with no addition. They were then assayed with all reactants including pyridoxal phosphate but with no additional DMBC.

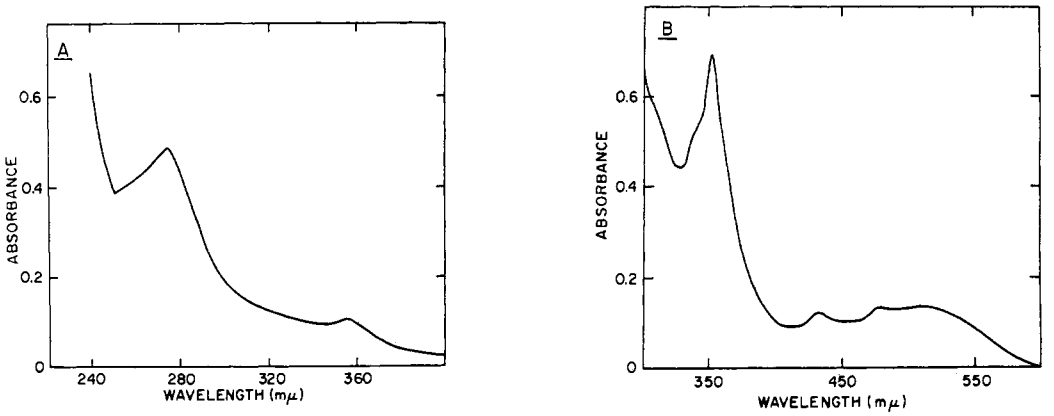

FIGURE 5: Ultraviolet spectrum (A) and visible spectrum (B) of the mutase complex. The spectra were measured in 0.1 M NaCl–potassium phosphate buffer (pH 6.0) against a buffer blank in 1-cm quartz cuvets using a Cary 15 spectrophotometer.

coenzyme. When assayed in the absence of added DMBC, the irradiated enzyme was 40% less active than the control (Table V) and required treatment with a higher than usual concentration of DMBC (40 μM rather than 8 μM) for maximal activity. However, incubation with DMBC, even at the higher concentration, did not fully reactivate the irradiated enzyme. Similar irreversible light inactivation of other cobamide coenzyme dependent enzymes has been observed (Yamane *et al.*, 1966).

Time, Temperature, and pH Optimum for D-α-*Lysine Mutase Activity.* A lag period of 10–15 min before attainment of maximal D-α-lysine mutase activity is observed at 30° whereas at 37° this lag is not apparent (Figure 6). The enzyme complex preparation used for these experiments (500 μg of protein per ml of reaction mixture) was partially dependent on both ATP and pyridoxal phosphate for full activity. The amount of D-α-lysine converted, ultimately, into 2,5-diaminohexanoate (approximately 5 μmoles from 20 μmoles of added substrate) was the same at both incubation temperatures. For routine studies incubations were carried out at 37°. When a linear rate of reaction was required, a suitable adjustment of either enzyme concentration or time of incubation was made.

The pH optimum for the reaction in 20 mM Tris·HCl buffer is in the range of 9.0–9.2 (Figure 7) and there is appreciable reaction even at pH 10.6. Below pH 7.5 D-α-lysine mutase activity is very low. With other buffers such as 2-methylimidazole·HCl (pH 9.0) or potassium Tricine (pH 8.7) both at a concentration of 20 mM, D-α-lysine mutase activity was 54 and 62% lower, respectively, than with Tris at pH 9.0.

Cofactor Requirements. Crude D-α-lysine mutase preparations studied earlier required the addition of ATP, DMBC, a mercaptan, and magnesium ion for maximal activity (Stadtman and Tsai, 1967). The highly purified D-α-lysine mutase complex is activated by the same cofactors and additionally exhibits almost complete dependence on pyridoxal phosphate (Table VI). The amount of bound pyridoxal phosphate varies from one preparation of the complex to another (Table VI, line 6). However, it has been possible to obtain mutase preparations free of bound pyridoxal phosphate and these are completely inactive (unpublished observation). Full catalytic activity is restored when pyridoxal phosphate is added. Pyruvate does not replace pyridoxal phosphate (Table VI) nor do pyridoxamine phosphate, pyridoxine phosphate, or pyridoxal (Table VII). Although the mutase complex employed for the experiment of Table VII also

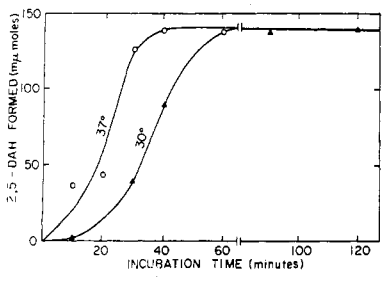

FIGURE 6: Time and temperature dependence of the mutase reaction. Assays were carried out as described in the text. Enzyme used was 500 μg of purified complex per ml of reaction mixture.

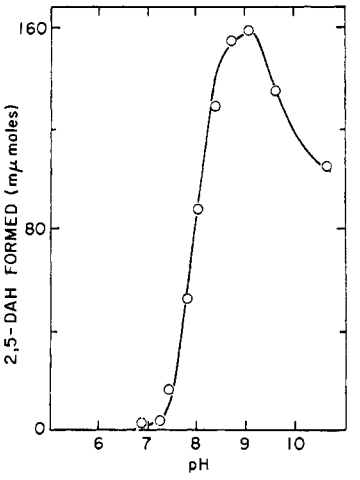

FIGURE 7: pH optimum for the mutase reaction. Assays were carried out as described in the text. The same enzyme preparation used for the experiments of Figure 6 was employed. The buffer was potassium phosphate in the pH range 6–7.5 and Tris in the range 7.5–10.5.

TABLE VI: Cofactor Requirements for the D-α-Lysine Mutase.

Omission	Addition	2,5-Diaminohexanoate Formed (mμmoles) Expt 1[a]	Expt 2[a]
None[b]		648	608
ATP		225	
$MgCl_2$		426	
$R(SH)_2$		75	
DMBC		186	
PALP		15	150
PALP	Pyruvate[c]		150
None	FAD[c]		630

[a] Different enzyme complex preparations were used for experiments 1 and 2. [b] The complete assay system as described in Methods except that 20 mM Tris (pH 9.0) was used for experiments 1 and 2 in place of the usual 100 mM Tris, pH 9.0. [c] In experiment 2 only, potassium pyruvate (100 mM) and FAD (40 μM) were added as indicated.

exhibited activity on the L isomer of α-lysine the requirement for pyridoxal phosphate was not limited to activation of a racemase since the utilization of both isomers was stimulated to about the same extent by pyridoxal phosphate. FAD, which was frequently observed to stimulate crude preparations of the mutase, is not required for maximal catalytic activity of the complex (Table VI). The effects of increasing concentrations of three of the cofactors, DMBC, pyridoxal phosphate, and ATP, on activity of the purified mutase complex are shown in Figure 8A, 8B, and 8C, respectively.

TABLE VII: Comparison of Pyridoxal Derivatives as Cofactors for the Mutase.

Substrate	Omission[a]	Addition	2,5-Diaminohexanoate Formed (mμmoles)
D-α-Lysine	None	None	870
D-α-Lysine	PALP	None	258
D-α-Lysine	PALP	Pyridoxamine phosphate[b]	150 (60) 130 (200)
D-α-Lysine	PALP	Pyridoxal[b]	81 (60) 102 (200)
D-α-Lysine	PALP	Pyridoxine phosphate[b]	100 (60) 96 (200)
L-α-Lysine	None	None	620
	PALP	None	150

[a] The complete system was as described in Methods with 40 μM pyridoxal phosphate (PALP) and either D-α-lysine (20 mM) or L-α-lysine (20 mM) as indicated. [b] The final concentrations (μM) of pyridoxamine phosphate, pyridoxal, and pyridoxine phosphate tested are shown in parentheses in column 4.

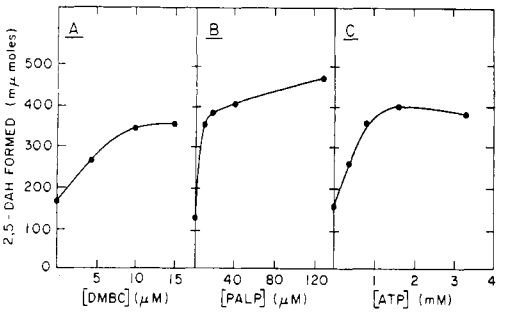

FIGURE 8: The dependence of the mutase reaction on (A) DMBC, (B) PALP, and (C) ATP. The assays were carried out as described in the text. The enzyme complex (900 μg of protein) was incubated as usual with saturating levels of all the assay reagents except the one under investigation, which was added as indicated.

The complex as isolated contains tightly bound cobamide coenzyme and therefore is only partly dependent on added DMBC.

ATP as Allosteric Activator of D-α-*Lysine Mutase.* Although crude enzyme preparations, in general, exhibit little D-α-lysine mutase activity in the absence of added ATP, purified preparations are less dependent on this nucleotide and characteristically are activated as shown in Figure 8C. This presumably is a reflection of the rapid rate of decomposition of ATP observed with many crude enzyme preparations as contrasted to its stability in reaction mixtures containing the purified mutase. Since the purified mutase catalyzed no measureable ATP hydrolysis concomitant with the conversion of D-α-lysine into 2,5-diaminohexanoate, it seemed unlikely that the nucleotide participated directly in the catalytic reaction. In fact, as shown in Figure 9, kinetic studies in the presence and absence of ATP, indicated that ATP serves instead as an allosteric effector in the system. In the absence of ATP the substrate saturation curve is markedly sigmoidal whereas in the presence of ATP the curve is nearly hyperbolic. The apparent K_m for lysine and the V_{max} for the reaction, change from 20 mM and 8 μmoles of 2,5-DAH formed/min in the absence of ATP to 0.3 mM and 15 μmoles of 2,5-DAH/min in the presence of ATP.

The ability of the phosphonic acid analogs of ATP to

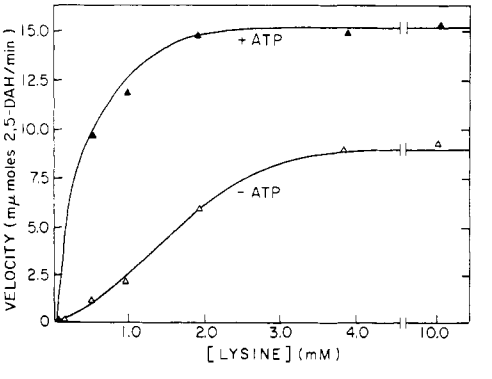

FIGURE 9: Substrate saturation curves in absence and presence of ATP. Incubations at 37° for 20 min were carried out as described in Methods using the radioactive assay and 500 μg of purified enzyme.

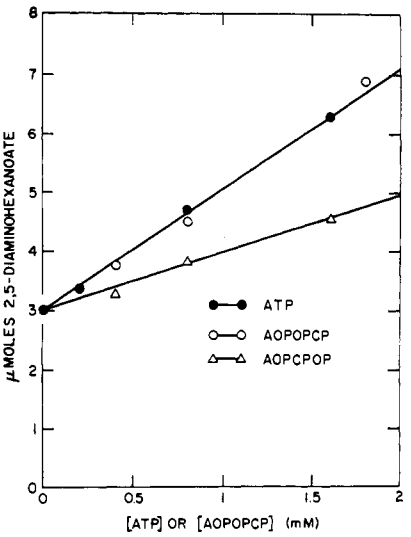

FIGURE 10: Effect of ATP and its methylenephosphonic analogs on the mutase. Incubations at 37° for 45 min were carried out as described in Methods using 1 mg of purified mutase complex.

TABLE VIII: Effects of Monovalent Cations on D-α-Lysine Mutase Activity.

Additions[a,b]	2,5-Diaminohexanoate Formed (mμmoles)
None	608
LiCl	480
NaCl	540
KCl	900
RbCl	890
NH_4Cl	1040

[a] The reaction mixtures contained the components described in Methods except that the Tris salt of ATP was used. The enzyme preparation added at a concentration of 0.5 mg/ml, was peak 1 material from the Sephadex G-200 column (Table I). [b] All test salts added at a final concentration of 50 mM.

replace the latter in the D-α-lysine mutase system (Figure 10) further supports the view that ATP serves merely as an activator. The β,γ-methylene analog of ATP is equally as effective as ATP; the α,β-methylene analog also activates appreciably.

A number of other naturally occurring nucleotides and some compounds metabolically related to ATP were tested for their ability to replace the latter as activator. A preparation of the complex maximally activated (2.3-fold) by 2 mM ATP was neither activated nor inhibited by similar concentrations of 2′-deoxy-ATP, AMP, or GTP and only slightly activated by CTP and ADP (also tested at 2 mM concentration). Phosphoenolpyruvate and *S*-adenosylmethionine failed to replace ATP as activator.

Effects of Monovalent Cations. The activity of D-α-lysine mutase is appreciably stimulated by certain monovalent cations. As shown by the data in Table VIII, ammonium, potassium and rubidium ions are effective activators of the mutase, whereas sodium and lithium are slightly inhibitory.

Inhibitors of D-α-Lysine Mutase. A large number of amino acids, particularly those structurally related to lysine, significantly inhibit the activity of D-α-lysine mutase (Table IX). Among the most potent inhibitors are L-β-lysine and ornithine, the substrates of the two mutases analogous to D-α-lysine mutase. The effective concentration ranges of these amino acids, and also of citrulline and 1,4-diaminobutane (putrescine), are shown for comparison in Figure 11.

Discussion

The chief advantages of the purification procedure for the mutase complex, described here, are (1) the enzyme system so isolated has 10–50 times the specific activity of the recombined proteins isolated by other techniques, (2) after purification the complex may be split by acid treatment to yield purified E_1 and E_2 proteins directly, and (3) most preparations of the purified complex are free of the two analogous enzyme systems, L-β-lysine mutase and ornithine mutase.

In contrast, in other experiments where the D-α-lysine mutase complex was resolved at an early stage in the purification procedure, into its two dissimilar protein moieties, it was virtually impossible to obtain the D-α-lysine cobamide protein moiety free of contaminating L-β-lysine cobamide protein (Stadtman and Grant, 1970).

It can be seen from Figure 4 that the purified D-α-lysine mutase complex is remarkably homogeneous and probably accounts for more than 90% of the protein in the sample. Recent work has indicated that most of the minor bands appearing on the disc gels can be attributed to the presence of small amounts of the subunits of the complex; further studies on this aspect of the mutase are in progress.

The significant time lag of the D-α-lysine mutase reaction at 30° (Figure 6) is reminiscent of that observed with L-β-lysine mutase (Stadtman and Renz, 1968). With the latter system the lag period can be eliminated by addition of high levels of DMBC which presumably displace inhibitory hydroxycobamide residues left on the protein as the result of destruction of the natural coenzyme during the isolation (M. A. Grant and T. C. Stadtman, 1969, unpublished experiments). This cannot be the explanation of the lag in the D-α-lysine mutase reaction since the initial reaction rate at 30° remains low even at high DMBC concentrations. The finding that incubation at 37° eliminates the lag suggests that, instead, it may be a reflection of an essential conformational change that takes place much more rapidly at the higher temperature.

As already pointed out the ability of the phosphonic acid analogs of ATP to replace the natural nucleotide as activator of D-α-lysine mutase supports the conclusion that ATP is not consumed in the catalytic reaction. The α,β-methylene analog is not as effective as the β,γ-methylene analog in replacing ATP and this difference is probably a reflection of a lower affinity of the α,β analog for the enzyme. These same analog preparations, tested with *S*-adenosylmethionine synthetase proved to be potent inhibitors of the enzyme and the β,γ analog was the more effective of the two (H. S. Mudd, 1970, personal communication).

Originally ATP was reported to be a requirement of the B_{12}-

TABLE IX: Survey of Inhibitors of D-α-Lysine Mutase.

Inhibitor[a]	Maximum Inhibition (%)	Approximate Concentration Needed to give Max Inhibition[b] (mM)
L-β-Lysine	78	18
3,5-Diaminohexanoate	63	50
ε-Amino-*n*-caproic acid	14	50
DL-δ-Hydroxylysine	63	50
DL-ε-*N*-Acetyllysine[c]	90	40
S-Aminoethylcysteine	60	30
DL-Citrulline	38	25
L-Ornithine	63	50
α-Amino-*n*-valeric acid	50	50
DL-Proline	64	50
1,4-Diaminobutane	63	30
L-Isoleucine	27	50
α-Aminoisobutyrate	22	50
β-Aminoisobutyrate	58	50
n-Caproic acid	47	50
Butyrate	10	30
Acetate	14	50
α-Amino-*n*-butyrate	57	50
β-Amino-*n*-butyrate	47	50
γ-Amino-*n*-butyrate	47	50
L-2,4-Diamino-*n*-butyrate	44	50

[a] The enzyme was preincubated at 0° for at least 10 min with various concentrations of the test substances before addition of the usual assay mixture and subsequent incubation at 37°. [b] In the presence of 20 mM D-α-lysine added with the assay mixture. [c] DL-α-*N*-acetyllysine was neither an inhibitor nor substrate for the mutase.

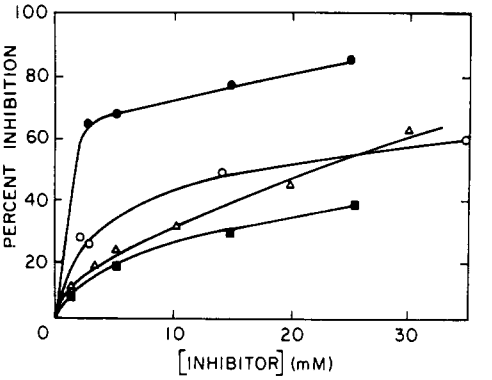

FIGURE 11: The effects of varying inhibitor concentrations on the D-α-lysine mutase activity. Assays using ^{14}C-labeled substrate were performed as described in the text with the appropriate levels of inhibitor added prior to assay. D-α-Lysine (20 mM) was used as substrate: (●—●) L-β-lysine, (○—○) L-ornithine, (△—△) 1,4-diaminobutane, (■—■) DL-citrulline.

dependent methyl transfer reaction catalyzed by methionine synthetase but upon purification of the enzyme it was found that the real activator is *S*-adenosylmethionine. Also, the first amino group migration reaction in the L-lysine fermentation pathway, catalyzed by lysine-2,3-aminomutase, is activated by *S*-adenosylmethionine (Chirpich *et al.*, 1970). In the D-α-lysine mutase system no activating effect of *S*-adenosylmethionine has been noted either with pure or impure enzyme preparations. The ability of the phosphonic acid analogs of ATP to replace ATP as activator further excludes the possibility that the nucleotide merely serves as an adenosyl source for the generation of *S*-adenosylmethionine. As noted above, both the α,β and the β,γ analogs are inhibitors of *S*-adenosylmethionine synthetase and neither serve as substrate even though the primary cleavage of ATP in this reaction gives rise to tripolyphosphate (H. S. Mudd, 1970, personal communication).

The finding that ATP plays the role of activator of D-α-lysine mutase affords an explanation of the otherwise problematical requirement for this nucleotide. It is likely that ATP has a similar function in the L-β-lysine mutase system where it also is required. If this is the case then it is an example of an enzyme that is activated by one of the end products of the overall series of reactions, since it is known from balance studies (Stadtman, 1963) that concomitant with the fermentation of L-lysine to fatty acids and ammonia there is the net synthesis of 1 equiv of ATP from ADP and orthophosphate. Although the overall stoichiometry for the series of reactions from D-α-lysine to fatty acids and ammonia (Figure 1) is not known it is presumed that there is a similar net synthesis of ATP.

The requirement for pyridoxal phosphate as an obligatory cofactor for D-α-lysine conversion into 2,5-diaminohexanoate affords yet another similarity between the three ω-amino group migration reactions catalyzed by *C. sticklandii*. Whereas the carbonyl compound that serves as activator of L-β-lysine mutase is pyruvate, ornithine mutase (Tsuda and Friedmann, 1970) and D-α-lysine mutase instead require pyridoxal phosphate.

The pyridoxal phosphate requirement of D-α-lysine mutase can be made absolute by treatment of the complex with hydroxylamine hydrochloride followed by dialysis and it appears that pyridoxal phosphate binds only to the cobamide protein (E_1) moiety (C. G. D. Morley, unpublished results). Although lysine racemase, presumably also a pyridoxal phosphate linked enzyme, is a contaminant of the purified complex, pyridoxal phosphate is required for D-α-lysine mutase activity *per se* as judged by the fact that it is needed with either D- or L-α-lysine as substrate (Table VII). Experiments in progress show, furthermore, that there is a pyridoxal phosphate dependent, enzyme-mediated, interaction with the ε-amino end of the substrate molecule.

Like several other B_{12}-coenzyme-linked enzymes the D-α-lysine mutase, (1) consists of two dissimilar protein moieties, one of which is a sulfhydryl protein, (2) depends on added mercaptan for catalytic activity, and (3) requires a monovalent cation such as NH_4^+ or Rb^+.

Evidence to be presented in another paper of this series will show that B_{12}-coenzyme functions as a hydrogen carrier in this reaction as it does in the L-β-lysine mutase reaction (Retey *et al.*, 1969) and in a number of other B_{12}-coenzyme linked enzymic reactions (Barker, 1967).

The metabolic significance of the large number of com-

pounds that serve as inhibitors of D-α-lysine mutase (Table IX) is as yet difficult to assess.

All of the inhibitors tested, with the possible exception of *n*-caproic acid and D-proline show saturation type curves similar to those of Figure 11 in plots of per cent inhibition *vs.* inhibitor concentration. Certain diamino acids closely related in structure to lysine are among the more powerful inhibitors. *S*-aminoethylcysteine is a good inhibitor of the mutase and inhibits by reason of being a nonmetabolizable substrate analog. At present there appears to be little correlation between structure and effectiveness of the inhibitors tested.

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Biological and Immunological Activity of Fructose 1,6-Diphosphatase. Application of a Quantitative Displacement Radioimmunoassay*

Helmut J. Kolb and Gerold M. Grodsky†

ABSTRACT: A displacement radioimmunologic assay of fructose 1,6-diphosphatase (FDPase) from rabbit liver was developed. Enzyme was purified and a high titer anti-FDPase serum was obtained from guinea pigs. The catalytic activity of FDPase was partially but not completely inhibited by the antiserum. Of the iodination methods tested, radioiodinated enzyme with high specific activity was best obtained by the chloramine-T procedure. Since the enzyme proved sensitive to exposure to the oxidant, an incubation time of 7–10 sec was chosen for the iodination. A constant amount of labeled FDPase was incubated in presence of varying standard amounts of unlabeled enzyme with antiserum, and preferential sodium sulfate precipitation was used for separating the bound and free FDPase. The displacement radioimmunoassay was sensitive to an enzyme concentration of 0.08 μg or 0.01 enzyme unit. The substrate and AMP did not quantitatively interfere with the assay up to concentrations of 5×10^{-3} M. During various steps in the purification of FDPase, the biologic activity per unit immunologic activity remained constant. Native enzyme was estimated quantitatively in crude 100,000*g* supernatants of tissue homogenate. The ratio of biologic to immunologic activity was the same in supernatants of rabbit liver and kidney. Supernatants of rabbit muscle, brain, heart, and spleen were immunologically inactive. Hepatic supernatants of the rat, sheep, cow, and pig did not cross-react, despite demonstrable biologic activity. Studies indicate the quantitative displacement radioimmunoassay in conjunction with biologic assays is useful to evaluate the level and structural characteristics of an enzyme in a biologic environment.

Displacement radioimmunological assays have been derived and are routinely used for the measurement of various peptide hormones including insulin (Yalow and Berson, 1960; Grodsky and Forsham, 1960), ACTH[1] (Bersen and Yalow, 1968a), glucagon (Unger *et al.*, 1959; Nonaka and Foa, 1969), growth hormone (Utiger *et al.*, 1962), vasopressin (Millrod and

* From the Department of Biochemistry and Biophysics and the Metabolic Research Unit, University of California Medical Center, San Francisco, California 94122. *Received March 25, 1970.* Supported by U. S. Public Health Service Grants AM 06115 and AM 01410.

† To whom to address correspondence.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1964), are: ACTH, adrenocorticotropic hormone; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; FDPase, fructose 1,6-diphosphatase.