Purification and Properties of a Pyridoxal Phosphate and Coenzyme B_{12} Dependent D-α-Ornithine 5,4-Aminomutase†

Ralph Somack‡ and Ralph N. Costilow*

ABSTRACT: The coenzyme B_{12} dependent ornithine mutase from *Clostridium sticklandii* catalyzing the conversion of ornithine to 2,4-diaminopentanoic acid has been purified to homogeneity. A radiochemical assay employing ^{14}C-labeled ornithine and a rapid, coupled spectrophotometric assay employing 2,4-diaminopentanoic acid C_4 dehydrogenase are described. Analysis by gel electrophoresis, sucrose gradient centrifugation, and sodium dodecyl sulfate gel electrophoresis indicated that the mutase has a molecular weight of about 180,000 and consists of two subunits of identical size. The enzyme contains labile sulfhydryl groups and is inhibited by oxygen. The mutase is specific for D-α-ornithine and is inhibited by L-α-ornithine, DL-α-lysine, and β-lysine. Oxidation of catalytically generated 2,4-diaminopentanoic acid with permanganate yielded β-aminobutyrate, which was shown to have the L (3*S*) configuration. Therefore, the C_4 amino group of 2,4-diaminopentanoic acid is in the L (4*S*) configuration. This observation combined with kinetic and inhibitor studies demonstrated that ornithine mutase and C_4 dehydrogenase are directly linked and that pyridoxal phosphate is a cofactor for ornithine mutase. The ornithine mutase reaction is reversible and proceeds to approximately an equal extent in both directions. However, the product, 2,4-diaminopentanoic acid, appeared to inhibit the reverse reaction at concentrations greater than 0.7 mM when present alone. Experiments with ^3H_2O indicated that the reaction proceeds by a mechanism which excludes exchange of hydrogen with the solvent. The absorption spectrum of the mutase measured directly in analytical gels indicated that a substantial amount of native bound cobamide had been converted to inactive hydroxocobalamins. After incubation with coenzyme B_{12} and subsequent dialysis, the spectrum was more typical of bound coenzyme B_{12}.

The initial steps in the fermentation of ornithine and lysine in *Clostridium sticklandii* involve a series of amino group migrations followed by oxidative deaminations which prepare the amino acids for subsequent thiolytic cleavages and conversions to the fatty acid products. The oxidation of ornithine to acetate, carbon dioxide, and ammonia proceeds by an initial coenzyme B_{12} dependent migration of the δ-amino group to C_4 forming 2,4-A_2Pent[1] (Dyer and Costilow, 1968, 1970; Tsuda and Friedmann, 1970; Somack *et al.*, 1971). A subsequent pyridoxal-P-dependent reaction involving a C_4 amino group inversion was next proposed to precede a TPN^+- or DPN^+-linked oxidative deamination forming 2-amino-4-ketopentanoic acid (Tsuda and Friedmann, 1970). The enzyme catalyzing this last step (2,4-diaminopentanoic acid C_4 dehydrogenase) appears to have no further cofactor requirements and has been purified to homogeneity (Somack and Costilow, 1972).

Since the initial demonstration of ornithine mutase activity in crude extracts (Dyer and Costilow, 1970), the enzyme has only been cursorily examined with respect to cofactor requirements using partially purified extracts (Tsuda and Friedmann, 1970). The present paper describes a method for separating ornithine mutase from the 2,4-diaminopentanoic acid C_4 dehydrogenase and purifying the mutase to homogeneity. Evidence is presented which indicates that pyridoxal-P is required in the mutase reaction *per se* and not in a subsequent amino group inversion as previously suggested (Tsuda and Friedmann, 1970).

Materials and Methods

Materials. Acrylamide and *N,N'*-methylbisacrylamide were purchased from Canal Industrial Corp. Coenzyme B_{12} and L-[^{14}C]β-lysine were kindly provided by Dr. H. A. Barker, Department of Biochemistry, University of California, Berkeley. Nonradioactive L-β-lysine and 2,4-A_2Pent were generated with *C. sticklandii* crude extracts and purified by the methods of Costilow *et al.* (1966) and Somack *et al.* (1971), respectively. The concentrations of these diamino acids were estimated by the reduced ninhydrin assay of Moore and Stein (1954). L-[6-^{14}C]Lysine and DL-[1-^{14}C]lysine were obtained from Calbiochem. DL-[5-^{14}C]Ornithine and ^3H_2O were products of New England Nuclear Corp. All other reagents were obtained from commercial sources.

Methods. Culture and cultural methods for growing, harvesting, storing and extracting cells of *C. sticklandii* (ATCC 12662) have been reported previously (Somack *et al.*, 1971).

Preparative and analytical disc gel electrophoresis were performed with a Buchler apparatus employing 6.0-mm (i.d.) diameter glass tubing. The method of Ornstein and Davis (1964) was followed with the gel modifications described by Gilpin and Sadoff (1971). Both stacking gels (0.5 cm) and resolve gels (5.0 cm) contained 10% glycerol (v/v). The buffer used in both the cathode and anode reservoirs contained 3.0 g of Tris, 14.4 g of glycine, and 0.154 g of dithiothreitol per l., pH 8.2–8.4. Analytical gels were stained with Amido

† From the Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823. *Received December 14, 1972.* This investigation was supported by Research Grant No. 1-R01-AI10951-01 from the National Institutes of Health and aided by Grant No. PF-851 from the American Cancer Society. Journal Article No. 6128, Michigan Agricultural Experiment Station. R. S. was a U. S. Public Health Predoctoral trainee, Public Health Training Grant No. GM-01911 of the National Institute of General Medical Sciences. This work was submitted in partial fulfillment of the requirements for the Ph.D. degree.

‡ Present address: Department of Biochemistry, University of California, Berkeley, Calif. 94720.

[1] Abbreviations used are: 2,4-A_2Pent, 2,4-diaminopentanoic acid; coenzyme B_{12}, 5,6-dimethylbenzimidazolylcobamide coenzyme; pyridoxal-P, pyridoxal phosphate.

Schwarz, destained by diffusion and stored in 7% acetic acid.

Subunit analyses were performed by a modification of the sodium dodecyl sulfate electrophoresis technique of Weber and Osborn (1969) using 5% polyacrylamide gels. The following proteins of known molecular weight were employed as markers: glutamate dehydrogenase (53,000), bovine serum albumin (68,000), chymotrypsinogen A (25,700), ovalbumin (45,000), and D-amino acid oxidase (37,000). The markers were added at a level of 5 μg/gel.

Molecular Weight Determinations. The molecular weight was estimated by the electrophoretic method of Hedrick and Smith (1968) and by sucrose density gradient centrifugation (Martin and Ames, 1961). The following standards were used in the electrophoretic determination: human transferrin (74,000), beef liver catalase (240,000), alcohol dehydrogenase (314,000), and horse ferritin (monomer) (450,000). The standards were added to gels at a level of 50 μg/gel and the mutase at 150 μg/gel. The relative migration of the mutase was established from the position of the pink band in unstained gels. For the sucrose gradient determination, 500 μg of mutase and 6 μg of lactic dehydrogenase were layered on a 5–20% linear gradient (4.55 ml) in cellulose nitrate tubes. The gradients were centrifuged in a SW39L rotor in a Spinco Model L preparative centrifuge at 48,000 rpm for 6.5 hr at 5°. Six-drop fractions were collected and mutase activity was measured by the spectrophotometric coupled assay (below).

Enzyme Assays. Two procedures were used to assay ornithine mutase; a radiochemical assay employing D-[5-^{14}C]-ornithine as substrate and a rapid and sensitive coupled spectrophotometric assay using 2,4-diaminopentanoic acid C_4 dehydrogenase. The C_4 dehydrogenase was separated from the mutase during the hydroxylapatite chromatography step described later in this paper, and was further purified to homogeneity by preparative disc gel electrophoresis (Somack and Costilow, 1973). The radiochemical assay was carried out anaerobically and protected from direct light in culture tubes as previously described (Costilow and Laycock, 1968). The reaction mixture contained: 20 mM D-ornithine containing 25–50 nCi of DL-[5-^{14}C]ornithine, 0.1 M Tris-Cl (pH 9.0), 10 mM dithiothreitol, 20 μM coenzyme B_{12}, 81 μM pyridoxal-P, and from 0.015 to 0.15 unit of enzyme in a total volume of 50 μl. After adding enzyme, the tubes were gassed with argon for 1 min, stoppered, and incubated for 5 min at 37°. The reaction was initiated by the addition of substrate and terminated after 5 min with 25 μl of 0.4 M formic acid. Denatured protein was precipitated by centrifugation, 20 μl of the supernatant was spotted on Chromar 500 thin-layer silicic acid sheets (Mallinckrodt), and the chromatograms were developed by ascending chromatography with chloroform–methanol–15% NH_4OH (36:46:20). Ornithine and 2,4-A_2Pent were located by spraying with 0.01% ninhydrin in acetone. The spots were excised and the radioactivity was measured using a toluene-based scintillation fluid (Costilow and Laycock, 1968). There is considerable variation between batches of the thin-layer silicic acid sheets which has resulted in deviations reported by several investigators (personal communication from manufacturer). We have noticed with some batches appreciable spreading of radioactivity not restricted to the usual spots comprising the amino acids. When labeled ornithine was eluted from chromatograms and rechromatographed, the distribution of radioactivity was unchanged. This indicates that reaction with the binder may occur which changes the properties of the amino acid, rather than the presence of radioactive substances other than ornithine in the isotope solution. Therefore, it is necessary to chromatograph a sample of unreacted [^{14}C]ornithine to make appropriate corrections. The spectrophotometric assay was performed in the manner described previously (Somack and Costilow, 1973) by measuring the rate of TPN^+ or DPN^+ reduction at 27° using D-ornithine as substrate for the mutase and coupling the reaction with the C_4 dehydrogenase (Tsuda and Friedmann, 1970; Somack and Costilow, 1973). One-centimeter light-path cuvets contained 50 mM sodium pyrophosphate buffer (pH 8.6), 4 mM dithiothreitol, 4 mM TPN^+, 1 μM coenzyme B_{12}, 40.4 μM pyridoxal-P, 20 mM D-ornithine, 0.001–0.002 unit of ornithine mutase, and approximately 1 unit of C_4 dehydrogenase in a volume of 0.25 ml. One unit of enzyme is that amount which catalyzes the formation of 1 μmol of 2,4-A_2Pent/min in the radiochemical assay or 1 μmol of reduced pyridine nucleotide per min in the coupled assay under standard assay conditions. Specific activity is defined as the number of units per milligram of protein. When both assays were performed simultaneously at 30° with 10× the level of mutase in the radiochemical assay than was present in the coupled assay, the specific activity calculated from the coupled system was 60% of that from the radiochemical system.

L-Lysine mutase, D-α-lysine mutase, and β-lysine mutase were assayed radiochemically by the methods described by Chirpich *et al.* (1970), Morley and Stadtman (1970), and Stadtman and Renz (1968), respectively. Lactic acid dehydrogenase was assayed spectrophotometrically with pyruvate and DPNH as substrates.

Analytical Methods. Ornithine and 2,4-A_2Pent in reaction mixtures containing ^3H_2O were adsorbed on small Dowex 50W-X8 (200–400 mesh) H^+ form columns and washed with copious amounts of H_2O. The amino acids were eluted with 3 N NH_4OH and dried under vacuum. They were taken up in small volumes of H_2O and separated on thin-layer silicic acid sheets. The amino acid spots were excised, solubilized in Hyamine hydroxide, and assayed for tritium by scintillation spectrometry after adding a toluene-based fluid (Costilow and Laycock, 1968).

Degradation of 2,4-A_2Pent to β-aminobutyric acid was carried out at 25° for 15 min by the procedure of Strassman and Weinhouse (1953). Unreacted 2,4-A_2Pent was removed by adsorption to Dowex 50W-X8 (200–400 mesh) Na^+ form columns and β-aminobutyrate desalted on Dowex 2-X8 (200–400 mesh), OH^- form columns (Drege *et al.*, 1954). Employing 20 μmol of 2,4-A_2Pent, the yield was 15% β-aminobutyrate estimated by the method of Frame *et al.* (1943). D-Ornithine subjected to the identical procedure resulted in 56% conversion to γ-aminobutyric acid.

Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. All optical absorbance measurements were performed with a Gilford Model 2000 spectrophotometer. Polyacrylamide gel scans were conducted with the same instrument employing a Gilford linear transport attachment. Spectra of solutions were determined with a Coleman, Model 124, double-beam spectrophotometer. All radioactive measurements employed a Packard Tri-Carb, Model 3320, scintillation spectrometer.

Results

Purification of Ornithine Mutase. A summary of the purification is presented in Table I. All operations were carried out at 4° in dim light. In all chromatography steps, buffers contained 10% glycerol (v/v) and 0.5 mM dithiothreitol unless otherwise stated.

INITIAL STEPS. Ornithine mutase was partially purified by

TABLE I: Purification of Ornithine Mutase.[a]

Step	Total Enzyme Act. (Units)	Sp Act. (Units/mg)	-fold Purifcn	Recov (%)
I. Crude extract	61.5	0.061	1	100
II. Streptomycin sulfate and ammonium sulfate (40–70%), dialyzed	44	0.077	1.25	72
III. DEAE-cellulose column	25	0.37	4.8	40
IV. Hydroxylapatite column	11.8	1.51	24.5	19.3
V. Disc gel electrophoresis	7.8	4.38	71	12.8

[a] Conditions of purification are outlined in the text. Activity was measured by the radiochemical assay as described under Materials and Methods.

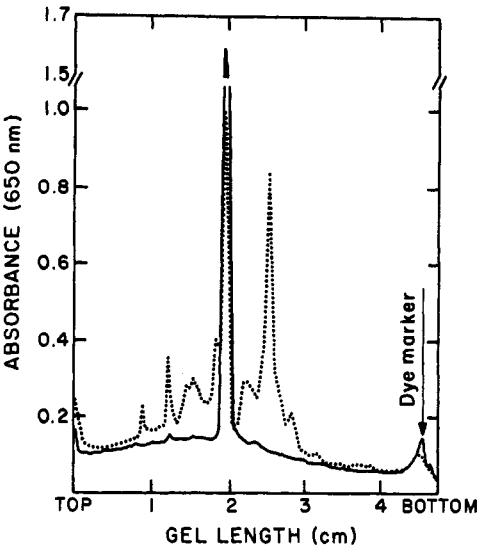

FIGURE 1: Disc gel electrophoresis of highly purified ornithine mutase. Analytical disc gel electrophoresis was performed and gels scanned in electrophoresis buffer at 650 nm as described under materials and Methods. Protein from step 4 (44 μg) was electrophoresed on one gel (-----) and 14 μg of mutase from step 5 on the other (——).

the identical procedure developed for the purification of the next enzyme in the pathway, the 2,4-diaminopentanoic acid C_4 dehydrogenase (Somack and Costilow, 1973). The method involves an initial streptomycin sulfate treatment of crude extract, followed by ammonium sulfate fractionation and then by DEAE-cellulose and hydroxylapatite chromatography. The C_4 dehydrogenase is eluted from the hydroxylapatite column with 0.025 M potassium phosphate buffer (pH 7.5). The column is next washed with 0.035 M buffer and ornithine mutase is eluted by increasing the buffer strength to 0.05 M. The dehydrogenase may be further purified to homogeneity by preparative disc gel electrophoresis (Somack and Costilow, 1973).

DISC GEL ELECTROPHORESIS. The 0.05 M potassium phosphate buffer eluate from hydroxylapatite containing ornithine mutase activity was concentrated by ultrafiltration to 4–5 mg of protein/ml. From 50 to 75 μl containing 250–500 μg of protein were then layered on individual analytical gels which were prepared and electrophoresed in dim light as described in the Materials and Methods section. The electrophoresis unit was maintained at close to 0° with a circulating ice-water bath. With the Buchler apparatus, 12 gels containing as much as 6 mg of total protein could be electrophoresed in a single run. During the run, two protein bands were clearly visible, a pink band containing mutase activity and a faster migrating yellow band. Electrophoresis was terminated when the yellow bands reached the ends of the gels. The pink bands were carefully excised and macerated with a glass Dounce homogenizer in 3.2 ml of a mixture containing: 0.1 M Tris buffer (pH 8.5), 13 μM coenzyme B_{12}, 2 mM dithiothreitol, and 10% glycerol (v/v). The addition of coenzyme B_{12} to the elution buffer significantly increased recovery. The macerate was gently stirred at 0° under argon for 6 hr, the gel pieces were removed by centrifugation, and the supernatant solution was concentrated to 1 ml by ultrafiltration. Routinely, we recovered about two-thirds of the total mutase activity that was placed on the gels, and observed about a 3× increase in the specific activity after this step. Typically, about 13% of the mutase activity observed in the crude extracts was recovered. The specific activity of the purified enzyme was about 70 × that of the crude preparations (Table I), indicating that the mutase accounts for about 1.5% of the total soluble protein in crude extracts.

The mutase appeared essentially homogeneous by the criterion of disc gel electrophoresis. Single major bands developed on both 7 and 5% gels electrophoresed at pH 9.3. If other proteins were present, they were in extremely low levels. Figure 1 shows a scan of a 7% gel containing the highly purified mutase superimposed on a scan of a 7% gel electrophoresed with enzyme from step 4 (hydroxylapatite) of the purification. The faster migrating large peak in the latter gel is the yellow band mentioned above. The mutase from step 5 was found to be free of D-α-lysine mutase, L-α-lysine mutase, and β-lysine mutase activity. (Enzyme, 8.5 μg/reaction, with a specific activity of 1.8 units/mg, was used in these assays.)

Stability. The highly purified mutase lost 35% of its activity in 1 month when stored at a concentration of 0.7 mg/ml in 50-μl aliquots at −20°. Enzyme from step 4 at a concentration of 1.0 mg/ml lost 30% of its activity after 2 days at 4°. By including 10 and 20% glycerol (v/v), the loss at 4° was reduced to 20 and 9%, respectively. The addition of 20 μM coenzyme B_{12} also reduced the loss at 4° to 9%. The inclusion of D- or L-α-ornithine or pyridoxal-P in the buffer had no effect on stability. The protection afforded by glycerol and coenzyme B_{12} was also observed during attempts to purify the mutase on Sepharose 6B columns. A fourfold increase in recovery was achieved by the addition of 10% glycerol and 2 μM coenzyme B_{12} to the buffer.

Molecular Weight and Subunits. The molecular weight of the mutase from step 4 of the purification was estimated by the gel electrophoresis technique of Hedrick and Smith (1968) and by sucrose density gradient centrifugation. The sucrose gradient (Figure 2) utilizing lactic acid dehydrogenase as a reference standard indicated an $s_{20,w}$ value of 8.7 S which corresponds to a molecular weight of approximately 170,000. The molecular weight estimated by the Hedrick–Smith technique indicated a value of 175,000–180,000.

The number and molecular weight of subunits was investigated by sodium dodecyl sulfate gel electrophoresis of the mutase from step 5. In two experiments using as reference standards either ovalbumin, D-amino acid oxidase, and chymotrypsinogen A or glutamate dehydrogenase, ovalbumin,

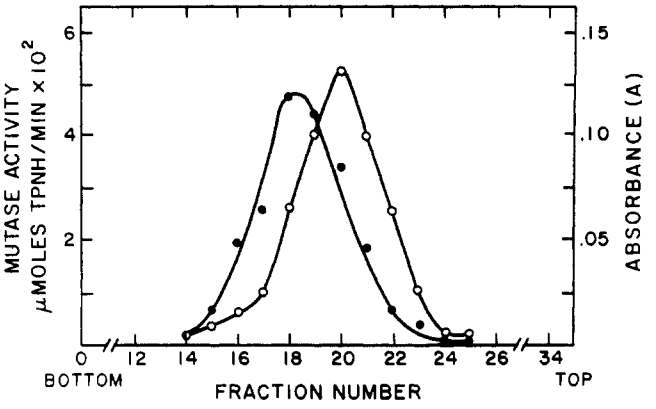

FIGURE 2: Sucrose density gradient centrifugation of ornithine mutase. A 5–20% linear sucrose gradient (4.55 ml) was layered with 500 μg of protein from step 4 and 6 μg of lactic acid dehydrogenase in a volume of 0.15 ml containing 1 mM dithiothreitol and 0.1 M Tris (pH 8.5) (see Materials and Methods for experimental details and enzyme assays): (●) ornithine mutase; (○) lactate dehydrogenase ($-\Delta A_{340}$/min).

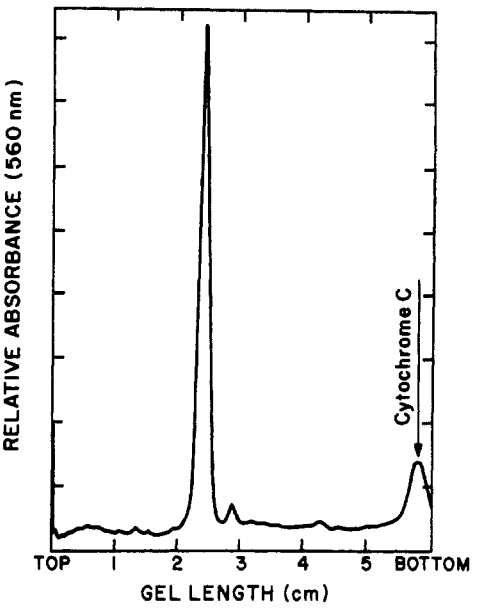

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of highly purified ornithine mutase. Ten micrograms of mutase from step 5 were applied to the gel which was electrophoresed at 20° as described under Materials and Methods.

and bovine serum albumin, one major band with a molecular weight of 95,000–98,000 and a minor component with a molecular weight of approximately 83,000 was observed. A scan of the mutase gel (Figure 3) shows that the minor component represented only approximately 2% of the total protein and therefore probably was a breakdown product or minor contaminant rather than a subunit of the enzyme. These data and the results obtained with the intact enzyme indicate that ornithine mutase has a molecular weight of about 180,000 and consists of two polypeptide chains of equal size.

Temperature, pH Optimum, and Protein Concentration. The optimum temperature for the mutase reaction under standard radiochemical assay conditions was approximately 37°, with one-half maximal activities occurring at 23 and 49°. Enzyme from step 4 (22 μg/reaction) with a specific activity of 1.0 unit/mg was used in this experiment. Other experiments demonstrated that the mutase is rapidly inactivated at temperatures exceeding 45°.

The optimum pH was found to be 9 with one-half maximal activities occurring at approximately 7.4 and 9.7. The pH curve (Figure 4) indicates that potassium phosphate buffer is inhibitory. We also observed that the addition of potassium chloride at a concentration of 1 mM to reactions in Tris buffer decreased activity.

With the radiochemical assay, a plot of activity *vs.* protein concentration was linear in the range of 10–90 μg of protein/reaction and extrapolated through the origin. Enzyme from step 4 with a specific activity of 1.2 units/mg was used in this experiment.

Substrate Specificity and Analogs. The enzyme from DEAE-cellulose columns utilized either D- or L-ornithine as substrate. However, following hydroxylapatite treatment, no activity was detectable with L-ornithine as substrate when tested with either assay. Therefore, D-ornithine is the true substrate for the ornithine mutase reaction and it is likely that an ornithine racemase was removed by step 4 of the purification.

The apparent K_m for D-ornithine obtained by the radiochemical assay using mutase from step 5 with a specific activity of 2.8 units/mg (8.5 μg/reaction) was 6.7 mM. The K_m observed using the coupled assay with 0.85 μg of mutase/reaction was 0.44 mM. This large difference in observed K_m values may have resulted from the large differences in the protein concentrations utilized in the two assay systems and/or the differences in temperature and buffers used. It was not possible to determine the K_m by both procedures under the same conditions because of differences in the sensitivities of the assays and the optimum conditions for the C_4 dehydrogenase activity.

A number of amino acids and compounds structurally related to ornithine were examined for their effect on mutase activity. The radiochemical assay was employed with 8.5 μg of enzyme from step 5 and the compounds tested present with D-α-ornithine at equimolar levels (20 mM). The per cent inhibition observed with these compounds was as follows: L-α-ornithine, 57%; L-α-lysine, 26%; D,L-α-lysine, 62%; β-L-lysine, 56%; and δ-amino-*n*-valeric acid, 9%. No inhibition was detected with cadaverine, putrescine, or *N*-α-acetyl-L-ornithine. Tsuda and Friedmann (1970) reported that L-α-lysine did not act as an inhibitor when tested using a partially purified extract.

Configuration of 2,4-A_2Pent. Tsuda and Friedmann (1970) previously established that the α-amino group of 2,4-A_2Pent is in the D configuration. In the present study the configuration of the amino group at C_4 was determined as follows. A sample of 2,4-A_2Pent·2HCl purified from crude extracts and active as substrate with the C_4 dehydrogenase was oxidized with permanganate as described under Materials and Methods. The resulting β-aminobutyrate was reacted with an excess of L-glutamyl-*N*-carboxyanhydride by the procedure of Manning and Moore (1968). The product was resolved by the method of Hong and Barker (1973) using an amino acid analyzer with L-glutamyl derivatives of authentic D-(3*R*)- and L-(3*S*)-β-amino-*n*-butyrate employed as standards. The dipeptide generated from 2,4-A_2Pent eluted as a single peak coincident with L-(3*S*)-β-amino-*n*-butyrate, indicating that the product of the ornithine mutase reaction is D-*threo*-2,4-A_2Pent ((2*R*,4*S*)-A_2Pent).

Pyridoxal Phosphate Requirement. The enzyme from step 5 was almost completely dependent on pyridoxal-P. The deletion of this cofactor from the radiochemical assay mixture (8.5 μg of mutase, specific activity of 1.8 units/mg) resulted in an 85% loss of activity, and the addition of either isoniazid or hydroxylamine at a concentration of 20 mM completely inhibited

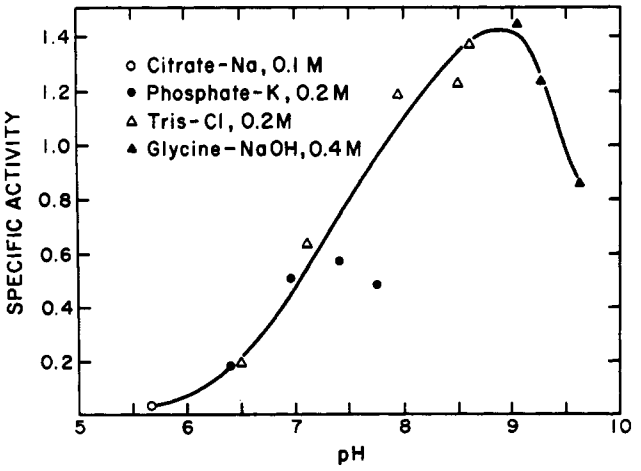

FIGURE 4: pH optimum of ornithine mutase. Standard radiochemical assays were conducted with 22 μg of protein from step 4 (1.4 units/mg). The final pH was measured from scaled up, control reaction mixtures.

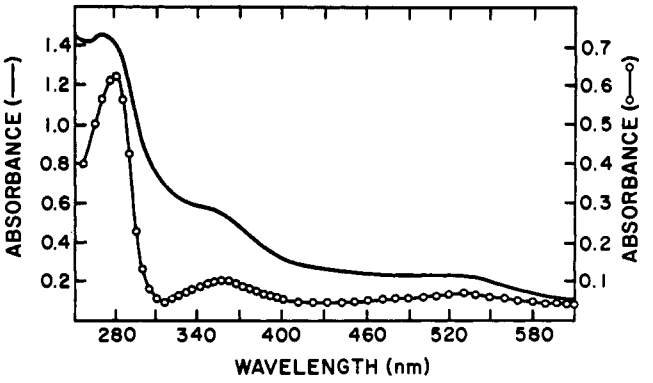

FIGURE 5: Evidence for mutase-bound cobamide. Extract from step 4 (250 μg of protein) was applied to a 7% analytical polyacrylamide gel and electrophoresed with a gel containing no protein for 2.5 hr as described under Materials and Methods. Readings at the indicated wavelengths were made using the Gilford linear transport apparatus by focusing on the blank gel in electrophoresis buffer and repeated after focusing on the pink mutase band in the gel electrophoresed with protein. The spectrum is derived from the difference at each wavelength between absorbance of protein and blank gel (O). For the other spectrum mutase from step 5 (0.7 mg/ml, 3.5 units/mg) was dialyzed against 0.05 M potassium phosphate buffer (pH 8.0), containing 0.1 M KCl. The spectrum (trace line without points) was determined using 1-cm quartz cuvets with the dialysis buffer as a blank.

the reaction. These results provide evidence for enzyme bound pyridoxal-P or a pyridoxyl-P analog. Pyridoxal-P was previously shown to stimulate the conversion of ornithine to 2-amino-4-ketopentanoic acid in ammonium sulfate fractionated extracts containing both ornithine mutase and C_4 dehydrogenase (Tsuda and Friedmann, 1970). However, it was proposed that pyridoxal-P functioned as a cofactor in a reaction following ornithine mutase which catalyzed the inversion of the C_4 amino group of 2,4-A_2Pent prior to the oxidative deamination by the dehydrogenase. The K_m for pyridoxal-P reported by Tsuda and Friedmann using a partially purified extract containing both mutase and C_4 dehydrogenase was 1.3×10^{-6} M. The apparent K_m which we obtained employing the coupled assay and enzyme from step 5 with a specific activity of 3.8 units/mg (0.85 μg/reaction) was 3.6×10^{-7} M. The value obtained using the radiochemical assay with 8.5 μg of protein/reaction was 1.8×10^{-6} M. Again, the differences in assay conditions may have resulted in the differences in the apparent K_m observed.

Absorption Spectra of the Enzyme. The marked stimulation of mutase activity in crude or partially purified extracts by coenzyme B_{12}, and the inhibition resulting from the addition of intrinsic factor, previously established the participation of a B_{12} coenzyme (Dyer and Costilow, 1970; Tsuda and Friedmann, 1970). In the present study, very little activity was detected in the absence of coenzyme B_{12} with enzyme preparations following the hydroxylapatite step of purification. The addition of coenzyme B_{12} to the extraction buffer to stabilize the mutase during the final step of purification (step 5) precluded the spectral investigation of the nature of the native bound corrinoid. Therefore, a spectrum was derived directly from the pink band constituting ornithine mutase activity in gels electrophoresed with enzyme from step 4 of purification. This was accomplished with a linear transport gel scanner attached to the Gilford spectrophotometer as described in detail in Figure 5. The spectrum in the uv region shows an absorbance maximum at 280 nm and a 280:260 ratio of 1.34. The spectrum in the visible region is typical of a corrinoid compound with a peak at 355–360 nm and a smaller absorption peak at 520–540 nm. The former peak indicates that a substantial amount of bound coenzyme is in the hydroxocobalamin form. The spectrum of enzyme from step 5, dialyzed to remove the coenzyme B_{12} added to stabilize the enzyme, is shown in the same figure. The broad absorption band in the visible region with shoulders in the neighborhood of 355 and 520–530 nm and the high absorbance in the 260-nm region is indicative of the coenzyme B_{12}. Even after exposure at 4° to a 100-W tungsten filament lamp at 12 cm for 4 hr, the spectrum was essentially unchanged.

Extent of Conversion of Ornithine to 2,4-A_2Pent. The conversion of ornithine to 2,4-A_2Pent approached 45% using either enzyme from step 4 or from step 5 of the purification. Equilibrium was usually attained within 40 min employing D-[^{14}C]ornithine at a concentration of 20 mM. Attempts to measure the extent of the reverse reaction using similar levels of [^{14}C]-2,4-A_2Pent resulted in little, if any, accumulation of ornithine. Performing assays with much lower concentrations of product to test for possible inhibition would have required excessively high specific activity radioactive 2,4-A_2Pent. Therefore, the extent of the reverse reaction using low levels of 2,4-A_2Pent was estimated by employing the C_4 dehydrogenase reaction modified as a substrate assay (see legend of Figure 6). Levels of 2,4-A_2Pent in excess of approximately 0.7 mM led to progressively less utilization. The extent of conversion in both directions was measured in the manner indicated above employing 20 mM D-ornithine or 0.7 mM 2,4-A_2Pent. The results presented in Figure 6 demonstrate that the reaction is readily reversible and proceeds to approximately the same extent in both directions.

Essential Sulfhydryl Groups and Effect of Oxygen. The activity of the mutase is markedly decreased in the presence of 0.2 mM *p*-chloromercuribenzoate or *N*-ethylmaleimide and moderately affected by iodoacetate at a level of 2 mM (Table II). (Since the inhibitors were added to the enzyme in the presence of coenzyme B_{12} and pyridoxal-P, any protection these cofactors may have afforded would not have been observed.) This suggests that ornithine mutase contains sulfhydryl groups essential for activity. The failure of arsenite to cause inhibition suggests that the essential sulfhydryl groups are not vicinal.

The effect of dithiothreitol and oxygen are presented in Table III. Ornithine mutase exhibited little activity under

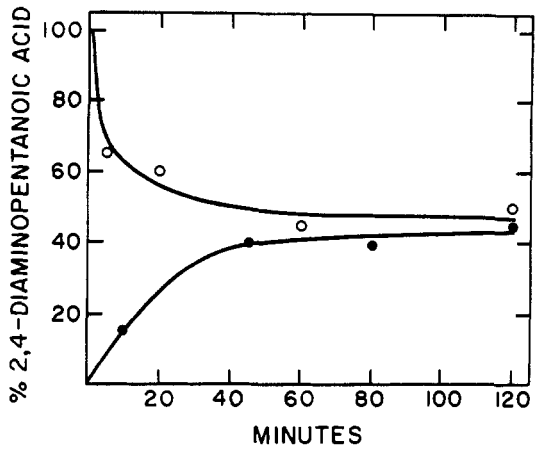

FIGURE 6: Extent of conversion of ornithine to 2,4-A_2Pent. The standard radiochemical assay was empolyed with 17 μg of mutase from step 5 (specific activity 3.0 units/mg) and 20 mM D-ornithine (●), or 8.5 μg of enzyme and 0.7 mM 2,4-A_2Pent (○). At the indicated times, aliquots were boiled, diluted as necessary, and added to cuvets containing the components of the coupled assay with 0.005 unit of pure C_4 dehydrogenase. The percentage 2,4-A_2Pent generated or lost was estimated from the initial rate of TPN^+ reduction relative to a standard curve constructed from a control reaction mixture containing 2,4-A_2Pent at various concentrations but no mutase.

aerobic conditions either in the presence or absence of a mercaptan. However, about 75% of maximal activity was recovered in the absence of dithiothreitol if oxygen was depleted by gassing with argon. Dithiothreitol completely replaced the anaerobic requirement achieved with argon. Presumably, the mercaptan functions to keep essential, labile sulfhydryl groups reduced and protected from oxygen, or also protects coenzyme B_{12} from oxidation during the catalytic process.

Hydrogen Exchange. A standard reaction employing ornithine mutase from step 5 (8.5 μg, specific activity of 2 units/mg) was carried out in the presence of a level of ^3H_2O providing a total of 5×10^8 or 9×10^6 cpm per μatom of hydrogen. The reaction mixture was incubated for 1 hr and the diamino acids were separated and analyzed for the incorporation of tritium as described under Methods. There was no incorporation of tritium into nonexchangeable positions of either 2,4-A_2Pent or ornithine. An identical reaction employing D-[^{14}C]ornithine as substrate indicated the formation of 0.12 μmol of 2,4-A_2Pent. If exchange had occurred to the extent of 1 μatom of hydrogen/μmol of substrate converted and there was no isotope selection, approximately 3.2×10^5 cpm would have been detected in the 2,4-A_2Pent analyzed.

TABLE II: Effect of Sulfhydryl Inhibitors on Mutase Activity.[a]

Additions	Concn (mM)	Act. (Units × 10^2)	Inhibn (%)
None		32.2	
pCl-HgBzO	0.2	3.9	88
	0.02	22.5	30
Ethylmaleimide	0.2	11.0	66
	0.02	31.0	4
Iodoacetate	2.0	20.3	37
	0.2	25.7	20
Arsenite	2.0	33.5	0

[a] Radiochemical assays were performed as described under Materials and Methods. Reactions contained 15 μg of protein from step 4 which was dialyzed against 100 mM Tris-Cl (pH 8.0) containing 10% glycerol (v/v) to remove dithiothreitol. The inhibitors were added at the indicated concentrations before the addition of enzyme.

TABLE III: Effect of Dithiothreitol and Oxygen on Mutase Activity.[a]

Addition[b]	Gas Phase[c]	Act. (Units × 10^2)	Rel Act.
(1) Dithiothreitol	Argon	21.1	100
(2) None	Argon	16.2	77
(3) Dithiothreitol	Air	21.2	101
(4) None	Air	4.0	19
(5) Dithiothreitol	O_2	9.4	45
(6) None	O_2	3.0	14
(7) Dithiothreitol	O_2 → argon	19.9	94
(8) None	O_2 → argon	9.2	44

[a] The enzyme preparation free of dithiothreitol used for the experiments reported in Table II and the standard radiochemical assay were employed. [b] When present, the mercaptan was added to reactions prior to enzyme at a final concentration of 10 mM. [c] Gasing with oxygen (1 min) and/or argon (2 min) was initiated after the addition of enzyme.

Discussion

Molecular weight estimations using sucrose gradient centrifugation and gel electrophoresis combined with sodium dodecyl sulfate gel electrophoresis indicate that ornithine mutase has a molecular weight of approximately 180,000 and is composed of two subunits of identical size. The enzyme appears to be physically quite different from the two enzymes in the lysine pathway catalyzing analogous amino group migrations. The β-lysine mutase (Stadtman and Renz, 1968) and the D-α-lysine mutase (Morley and Stadtman, 1970) are physically indistinguishable and exist as complexes of two distinct protein molecules, an acidic protein with a molecular weight of 150,000 containing tightly bound cobamide, and a labile sulfhydryl protein of mol wt 60,000. The two proteins of the β-lysine mutase are resolved during purification but resolution of the D-α-lysine mutase has been achieved only after acidification to pH 4.0 where the cobamide protein is rendered insoluble. When crude dialyzed extracts containing ornithine mutase were treated with acid at pH 5.0, the activity recovered (50%) was found only in the resulting precipitate.

The evidence presented argues against the proposal by Tsuda and Friedmann (1970) that a pyridoxal-P-dependent epimerase catalyzing the inversion of the C_4 amino group of 2,4-A_2Pent follows the ornithine mutase reaction in the conversion of ornithine to 2-amino-4-ketopentanoic acid. The almost complete dependence of the mutase on pyridoxal-P with a very low apparent K_m (4×10^{-7} to 2×10^{-6} M) and the complete inhibition of activity by isoniazid and hydroxylamine, indicate that pyridoxal-P functions as a cofactor for ornithine mutase. The possibility of contamination of the highly purified mutase by a C_4 epimerase is further discounted by the complete lack of hydrogen exchange noted during the mutase reaction. Highly purified preparations of D-α-lysine mutase are contaminated by a pyridoxal-P-dependent lysine racemase (Morley and Stadtman, 1972) which catalyzes the

incorporation of hydrogen from water into the α position of both isomers of lysine. The possibility exists, however, that either isotope selection or shielding by the enzyme prevents detection of labile hydrogens. Alternatively, a C_4 epimerase may operate by a mechanism which excludes pyridoxal-P dependency. However, the result that only the D-threo epimer was obtained from crude extracts and was active as substrate for highly purified C_4 dehydrogenase strongly suggests that no epimerase is present.

The ornithine mutase has a number of features in common with the lysine aminomutases. Thus, ornithine mutase, D-α-lysine mutase, and L-α-lysine mutase are pyridoxal-P-dependent whereas pyruvate may serve the function of pyridoxal-P in the β-lysine mutase reaction. Morley and Stadtman (1972) have demonstrated that the cobamide moiety of the D-α-lysine mutase complex contains bound pyridoxal-P and in the presence of Mg^{2+} catalyzes a slow pyridoxal-P-dependent exchange of a C_6 methylene hydrogen of D-lysine with the solvent. This suggests that pyridoxal-P may be the carrier involved in these amino group migrations through enzyme-directed pyridoxal-P–substrate amino group Schiff base intermediates.

Like the D-α-lysine and β-lysine mutases, purified ornithine mutase contains tightly bound cobamide or degradation products thereof and activity is stimulated by the addition of coenzyme B_{12}. The native bound coenzyme is nevertheless readily exchangeable with coenzyme B_{12} which subsequently is resistant to photolysis. Since free coenzyme B_{12} is readily inactivated by light, bound coenzyme is presumably protected in such a way as to prevent the photolytic reaction. This result is reminiscent of the binding of a benzimidazole-containing cobamide coenzyme to sheep methylmalonyl coenzyme A mutase (Cannata *et al.*, 1965). The indication that a substantial amount of native bound cobamide appears in the hydroxocobalamin form after purification (Figure 5) suggests that either a photolytically susceptible bound cobamide other than coenzyme B_{12} is the native prosthetic group (*C. sticklandii* synthesizes adenylcobamide coenzyme, Stadtman, 1960), or a significant amount of hydroxocobalamin from photolytic decomposition in extracts is subsequently bound to the enzyme.

Presumably, the cobamide functions as the carrier of the ornithine C_4 hydrogen which exchanges for the amino group on C_5. This hydrogen carrier function has been demonstrated in the analogous L-β-lysine mutase (Retey *et al.*, 1969), the D-α-lysine mutase (Morley and Stadtman, 1971), and a number of other coenzyme B_{12} dependent reactions (Abeles, 1971; Barker, 1972). In all cases, the hydrogen migration occurs without exchange with solvent hydrogen. This is apparently also true for the ornithine mutase reaction, since tritium uptake could not be detected when the reaction was carried out in the presence of ^3H_2O.

Ornithine mutase contains sulfhydryl groups which must be kept reduced either by the exclusion of oxygen or the addition of a mercaptan. This is a common feature of the lysine aminomutases which also exhibit little activity in the absence of added mercaptans under anaerobic conditions (Stadtman and Renz, 1968; Morley and Stadtman, 1970; Chirpich *et al.*, 1970).

The D-α-lysine mutase is inhibited by a number of diamino acids including, L-β-lysine, 3,5-diaminohexanoate, and L-ornithine (Morley and Stadtman, 1970). It is interesting to note that the substrates of the lysine aminomutases, D-α-lysine, L-α-lysine, and β-lysine also inhibit ornithine mutase.

The precise equilibrium positions of the D-α-lysine and β-lysine mutase reactions are unknown. Both proceed in the forward direction to the extent of approximately 45–50% and in the reverse direction to about 25–30% (Stadtman, 1972). The formation of addition products between pyruvate and 3,5-diaminohexanoate probably inhibit the β-lysine mutase reaction in the reverse direction (Stadtman and Renz, 1968). With respect to the ornithine mutase reaction, the inhibition observed in the reverse direction with 2,4-A_2Pent concentrations in excess of 0.7 mM is undoubtedly a different phenomenon unless adduct formation with pyridoxal-P prevents the cofactor from participation in the reaction.

Acknowledgments

We are indebted to Barbara Baltimore for performing the amino acid analysis and to Dr. H. A. Barker for valuable advice during the course of this investigation.

Added in Proof

A detailed paper by Baker *et al.* (1973) on the properties of β-lysine mutase published after the present manuscript was completed reports that β-lysine mutase in a tetramer (mol wt 170,000), rather than a complex of two dissimilar proteins as previously reported (Statdtman and Renz, 1968), and demonstrates the absolute dependence of the mutase on pyridoxal-P. Furthermore the product, 3,5-diaminohexanoate, inactivates the enzyme, presumably by catalyzing the cleavage of the carbon–cobalt bond of enzyme-bound coenzyme B_{12}.

References

Abeles, R. H. (1971), *Enzymes, 3rd Ed., 5*, 481.
Baker, J. J., van der Drift, C., and Stadtman, T. C. (1973), *Biochemistry 12, 1054.*
Barker, H. A. (1972), *Enzymes, 3rd Ed., 6*, 509.
Cannata, J. J. B., Focesi, A., Mazumder, R., Warner, R. C., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3249.
Chirpich, T. P., Zappia, V., Costilow, R. N., and Barker, H. A. (1970), *J. Biol. Chem. 245*, 1778.
Costilow, R. N., and Laycock, L. (1968), *J. Bacteriol. 96*, 1011.
Costilow, R. N., Rochovansky, O. M., and Barker, H. A. (1966), *J. Biol. Chem. 241*, 1573.
Drege, A., Moore, S., and Bigwood, E. J. (1954), *Anal. Chim. Acta 11*, 554.
Dyer, J. K., and Costilow, R. N. (1968), *J. Bacteriol. 96*, 1617.
Dyer, J. K., and Costilow, R. N. (1970), *J. Bacteriol. 101*, 77.
Frame, E. G., Russell, J. A., and Wilhelmi, A. E. (1943), *J. Biol. Chem. 149*, 255.
Gilpin, R. W., and Sadoff, H. L. (1971), *J. Biol. Chem. 246*, 1475.
Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.
Hong, S. L., and Barker, H. A. (1973), *J. Biol. Chem. 248*, 41.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Manning, J. M., and Moore, S. (1968), *J. Biol. Chem. 243*, 5591.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Morley, C. G. D., and Stadtman, T. C. (1970), *Biochemistry 9*, 4890.
Morley, C. G. D., and Stadtman, T. C. (1971), *Biochemistry 10*, 2325.

Morley, C. G. D., and Stadtman, T. C. (1972), *Biochemistry 11*, 600.
Ornstein, L., and Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 305.
Retey, J., Kunz, F., Stadtman, T. C., and Arigoni, D. (1969), *Experientia 25*, 801.
Somack, R. L., Bing, D. H., and Costilow, R. N. (1971), *Anal. Biochem. 41*, 132.
Somack, R., and Costilow, R. N. (1973), *J. Biol. Chem. 247*, 385.
Stadtman, T. C. (1960), *J. Bacteriol. 79*, 904.
Stadtman, T. C. (1972), *Enzymes, 3rd Ed., 6*, 539.
Stadtman, T. C., and Renz, P. (1968), *Arch. Biochem. Biophys. 125*, 226.
Strassman, M., and Weinhouse, S. (1953), *J. Amer. Chem. Soc. 75*, 1680.
Tsuda, Y., and Friedmann, H. C. (1970), *J. Biol. Chem. 245*, 5914.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Adenosine 3′,5′-Monophosphate and Protein Kinase Dependent Phosphorylation of Ribosomal Protein†

Gordon M. Walton and Gordon N. Gill*·‡

ABSTRACT: Cyclic adenosine 3′,5′-monophosphate (cAMP) stimulated phosphorylation of adrenal cortical ribosomal protein *in vitro*. The phosphorylation reaction is catalyzed by cAMP-dependent protein kinase (EC 2.7.1.37) purified from the cytosol or by endogenous activity remaining on 0.5 M KCl washed ribosomes. cAMP-dependent phosphorylation of the same ribosomal protein bands is observed using either exogenous or endogenous protein kinase activity. When salt washing is carried out prior to phosphorylation, 53% of the total ribosomal sites for ^{32}P incorporation remain with the ribosome. When phosphorylation is carried out prior to salt washing, only 22% of the total phosphorylated sites remain with the ribosome suggesting that phosphorylation has altered the affinity of these substrate proteins for the ribosomal structure. Phosphorylated proteins from isolated 60S and 40S subunits have been resolved by gel electrophoresis into six–eight labeled protein bands from the large subunit and a single radioactive band from the small subunit. Complete phosphorylation of the subunits resulted in a total of 10 and 2 mol of phosphate incorporated/mol of 60S and 40S subunits, respectively, in the *in vitro* catalyzed reaction. It is postulated that ribosomal protein phosphorylation is regulated through cAMP-dependent protein kinase; this provides one mechanism for hormonal control of ribosome function.

Mammalian ribosomes contain phosphoproteins. The phosphate groups esterified to serine and threonine residues turn over in reticulocyte ribosomes at a rate of approximately 3%/min (Kabat, 1972). The phosphate groups are present on only a limited number of ribosomal proteins (Kabat, 1970, 1971; Eil and Wool, 1971; Blat and Loeb, 1971; Bitte and Kabat, 1972; Traugh *et al.*, 1973). Because different methodology has been used for ribosomal protein separation, it is uncertain whether the same ribosomal proteins are phosphorylated in different tissues. However, when the same gel system was used the patterns of ribosomal protein phosphorylation in mouse sarcoma 180 cells and rabbit reticulocytes were found to be identical (Bitte and Kabat, 1972). The hormonal state appears to influence the phosphorylation of ribosomal protein. Treatment of rats with glucagon resulted in increased phosphorylation of ribosomal proteins (Blat and Loeb, 1971). Phosphorylation of one ribosomal protein band was increased two- to threefold (Blat and Loeb, 1971). Thyroidectomy resulted in a 35% decrease in the phosphate content of ribosomal proteins; 3,5,3′-triiodothyrionine administration restored the phosphate content to control levels (Correze *et al.*, 1972).

One mechanism for hormonal control of ribosomal protein phosphorylation is through cAMP[1]-dependent protein kinase. cAMP-dependent protein kinase (EC 2.7.1.37) originally described in skeletal muscle by Walsh *et al.* (1968) is the major effector of cAMP action in eukaryotic cells presently identified. cAMP-dependent protein kinase exists as a molecular complex consisting of regulatory receptor and catalytic kinase subunits; binding of cAMP to receptor results in dissociation of the inhibitory receptor and full activation of the catalytic kinase subunit (Gill and Garren, 1970, 1971; Tao *et al.*, 1970; Kuman *et al.*, 1970; Reimann *et al.*, 1971; Erlichman *et al.*, 1971).

A number of substrates for cAMP-dependent protein kinase have been identified. cAMP-dependent protein kinase catalyzed phosphorylation of phosphorylase kinase and of glycogen synthetase results in marked alteration of enzyme activity with activation of the first and inactivation of the second enzyme (Soderling *et al.*, 1970). Similar cAMP-depen-

† From the Department of Medicine, Division of Endocrinology, University of California, San Diego, School of Medicine, La Jolla, California 92037. *Received October 31, 1972.* Supported by U. S. Public Health Service Research Grant No. AM13149-05 from the Institute of Arthritis and Metabolic Diseases.

‡ U. S. Public Health Service Research Career Development awardee from the Institute of Arthritis and Metabolic Diseases.

[1] cAMP, cyclic adenosine 3′,5′-monophosphate; ACTH, adrenocorticotropic hormone; TKM buffer, 50 mM Tris-HCl (pH 7.5)–25 mM KCl–5 mM $MgCl_2$; TMD buffer, 2 mM Tris-HCl (pH 7.5)–0.5 mM $MgCl_2$–0.1 mM dithiothreitol; TM buffer, 10 mM Tris-HCl (pH 7.5)–2 mM $MgCl_2$.