Physicochemical Characterization of Renal Dipeptidase†

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ABSTRACT: Renal dipeptidase, purified from porcine kidney cortex, was analyzed by means of a number of physicochemical methods. The value of $s_{20,w}$ for the enzyme extrapolated to zero protein concentration was 5.42. The apparent diffusion coefficient determined at a protein concentration of 7.15 mg/ml was 5.74×10^{-7} cm²/sec. The isoelectric point of the enzyme measured by isoelectric focusing was 4.89. Molecular weight averages obtained by equilibrium ultracentrifugation at pH 7.70 ranged from 94,000 to 96,000. The protein was observed to dissociate as the pH was lowered to 2.26. Elevation of the pH from 2.26 to 7.70 produced an apparently nonspecific association as evidenced by the presence of high molecular weight components without restoration of biological activity. The zinc content of the enzyme was 1.42 μg/mg which corresponds to 2.04 g-atoms of zinc per mole of enzyme of molecular weight 94,000. An apoenzyme of the peptidase was prepared which contained 12% of the original zinc content and less than 1% of the enzyme activity of the native enzyme. The apoenzyme exhibited an apparent sedimentation coefficient of 5.33 S. Reconstitution of zinc renal dipeptidase was achieved by the addition of 2 g-atoms of zinc/mole of enzyme, and complete restoration of peptidase activity to the reconstituted enzyme was observed. The addition of the first gram-atom of zinc/mole of enzyme restored 24% of the original activity, and the second gram-atom restored the remaining 76% of the specific activity. A cobalt renal dipeptidase was prepared which contained 2 g-atoms of cobalt/mole of enzyme and which exhibited 42% of the specific activity of the native zinc enzyme.

Renal dipeptidase, an enzyme located in the microsomal fraction of procine kidney cortex, acts upon a variety of dipeptides but not upon esters, tripeptides, or proteins (Campbell, 1970). Previous work has led to a partial characterization of some of the physical–chemical properties of the enzyme. Sedimentation velocity measurements of an earlier preparation revealed the presence of only one symmetrical peak after 60 min at 59,780 rpm, but insufficient material was available to complete a determination of the sedimentation coefficient (Campbell *et al.*, 1966). In the same study the molecular weight of the peptidase was estimated by the Archibald approach-to-equilibrium method to be 47,200. Reexamination of the molecular weight of the enzyme using Sephadex gel filtration with appropriate molecular weight standards led to the assignment of a molecular weight of 90,000 (Harper *et al.*, 1971). The diffusion coefficient and the isoelectric point of the purified enzyme have not been previously reported.

Also in previous investigations (Campbell *et al.*, 1966; Harper *et al.*, 1971) the metal content of the peptidase and kinetic analyses with various inhibitors have suggested that renal dipeptidase is a metalloenzyme. However, the quantitative relationship between the zinc content of the peptidase and its biological activity has not been previously established; nor has the preparation of the apoenzyme of renal dipeptidase been reported.

In the present investigation preparative polyacrylamide gel electrophoresis was employed to obtain renal dipeptidase of adequate purity for physical–chemical characterization. The sedimentation coefficient, diffusion coefficient, molecular weight, and isoelectric point of the purified peptidase were obtained. Furthermore, preparation of the apoenzyme of renal dipeptidase was achieved, and restoration of the biological activity of the peptidase by reconstitution with zinc and cobalt was quantitatively related to the metal content of the enzyme.

Experimental Section

Preparation of Renal Dipeptidase. Partial purification of renal dipeptidase from hog kidney cortex was carried out by means of solubilization with 1-butanol, isoelectric precipitation, and $(NH_4)_2SO_4$ precipitation. Details regarding these techniques have been previously reported (Campbell *et al.*, 1966; René and Campbell, 1969). In the present procedures concentration of dilute enzyme solutions was accomplished using an Amicon TCF-10 ultrafiltration apparatus equipped with an XM-50 membrane. The solutions were filtered at 4° and 45 psi. Following concentration all solubilized preparations were clarified by centrifugation at 25,000*g* for 30 min. The final purification step for the enzyme employed preparative polyacrylamide gel electrophoresis, a procedure not previously reported for the isolation of renal dipeptidase. The Buchler Poly-Prep electrophoresis apparatus was operated according to the method described by Jovin *et al.* (1964). Concentrating and separating gels were made as described by Ornstein and Davis (1964). The lower gel and the gel plug buffers were solutions of 28.8 g of glycine and 6.0 g of Tris per liter. The upper gel buffer consisted of a 1:20 dilution of the lower buffer. Elution was performed using a 0.1 M Tris-HCl buffer at pH 7.6. The entire Poly-Prep apparatus was maintained at 0° by circulation of coolant from a refrigerated water bath. In a typical experiment 170 mg of protein in 15 ml of 0.1 M Tris-HCl buffer at pH 7.6 was layered on the top of the concentrating gel. In order to avoid mixing of sample solution and upper buffer, sucrose was dissolved in the sample solution to a concentration of approximately 5% (w/v). The current was adjusted to 50 mA providing a voltage of 700–900 V during the course of the experiment. Flow rate was regulated at 1 ml/min by means of a peristaltic pump, and

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6-ml fractions were collected. The absorbance of each tube was measured at 280 mμ, and the enzyme activity was determined by the peptidase assay described below. In this technique care must be taken to remove a polyacrylamide polymer which sometimes leaks from the 7.5% gel. This polymer is not retained by the XM-50 membrane employed in the ultrafiltration procedure, and it can therefore be removed by washing and filtration.

Assay of Renal Dipeptidase. A spectrophotometric peptidase assay was employed in which the rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide, glycyldehydrophenylalanine, was measured by observing the fall in absorbance at 275 mμ of a solution of 5.00×10^{-5} M glycyldehydrophenylalanine as reported previously (René and Campbell, 1969). Protein concentrations of impure protein fractions were estimated by the method of Warburg and Christian (1942). Protein concentrations of purified fractions were determined from the $E^{1\,cm}_{1\%}$ of 8.96 (René and Campbell, 1969). The term specific activity refers to μmol of substrate hydrolyzed per min per mg of enzyme times 100, when the substrate concentration is 5.00×10^{-5} M.

Biophysical Characterization of Renal Dipeptidase. Analytical polyacrylamide gel electrophoresis was carried out as described by Ornstein and Davis (1964). The isoelectric point of renal dipeptidase was determined using an LKB 8101 electrofocusing column. Coolant circulating through the jacketed column maintained the temperature at 0°. A linear (5–20%) sucrose gradient was applied to the column together with the appropriate LKB Ampholines and the renal dipeptidase sample. Isoelectric focusing gradients were established over a pH range of 3–10 and over the range of pH 3–6. The voltage during a 48-hr experiment was maintained at approximately 700 V. At the end of the experiment the column was carefully drained, and the effluent collected in 2-ml fractions. The optical density of the fractions at 280 mμ was measured, and the pH was determined using a Radiometer Model 26 pH meter with a combination electrode. Residual enzyme activity was estimated using the spectrophotometric assay described above.

The method employed to measure the diffusion coefficient of the enzyme was that of Gosting (1956) using the Spinco Model E analytical ultracentrifuge. A synthetic boundary centerpiece was used in the standard interference cell. In the right-hand sector was placed 0.15 ml of protein solution (7.15 mg/ml), and the left-hand sector contained the dialysate buffer (0.1 M NaCl–0.035 M K_2HPO_4–0.004 M KH_2PO_4 (pH 7.70)). The cell and interference counterbalance were rotated in the AN-J rotor to an acceleration of 5000 rpm. Immediately after the layering process was complete a picture was taken and subsequent pictures were taken at 8-min intervals. Peak areas and heights were analyzed using a Nikon comparator. Density increments for the buffer solutes employed in the diffusion experiments and in the following sedimentation analyses were estimated from data given in the Handbook of Chemistry and Physics by the method of Svedberg and Peterson (1940). The diffusion coefficient was calculated from the equation

$$D_a = (A^2/X^2)(1/4\pi t) \quad (1)$$

where D_a is the apparent diffusion coefficient, A is the area of the schlieren peak (cm^2), X is the height of the peak (cm), and t is the time (sec). The apparent diffusion coefficient was then corrected to $D_{20,w}$ by the method described by Chervenka (1969).

All analytical ultracentrifugation experiments were carried out with a Spinco Model E analytical ultracentrifuge with the temperature controlled at 20° by the Rotor Temperature Increment Control (RTIC) unit. In the sedimentation velocity studies the schlieren optical system was used, and protein concentrations ranged from 3 to 10 mg/ml. A single-sector cell was used with an AN-D rotor at a speed of 59,780 rpm. Pictures taken on Kodak metallographic photographic plates were analyzed with the Nikon comparator. The sedimentation coefficients were calculated from the rate of displacement of the maximum ordinate of the refractive index gradient curve as described by Schachman (1959) and all sedimentation coefficients were corrected to $s_{20,w}$.

Sedimentation equilibrium analyses were performed using the high-speed equilibrium centrifugation method of Yphantis (1964). The standard double-sector cell with quartz windows was used in all runs. The channels were filled with 0.01 ml of FC-32 fluorocarbon (Beckman) and 0.1 ml of protein solution or dialysate. Protein concentrations were 0.1–1.0 mg/ml, and the Rayleigh interference optical system was employed. The experiments were carried out with an AN-D rotor at 17,000 rpm for the high pH runs, and at 21,000 rpm for the low pH runs. The actual rpm for the experiments was determined by the revolution count during the time course of each experiment. The Rayleigh patterns were photographed on Kodak II G photographic plates. The data from each experiment were reduced and analyzed on the IBM 360 computor. The Fortran program PAS 001C (Beckman) was used for the calculation of various molecular weights and for the plotting of these molecular weights as a function of concentration and position in the cell according to the procedures of Yphantis (1964). The partial specific volume used in all calculations was 0.725 cm^3/g. The sedimentation equilibrium runs at pH 7.70 were carried out in 0.1 M NaCl–0.035 M K_2HPO_4–0.004 M KH_2PO_4 buffer; experiments at pH 4.39 were performed in 0.1 M NaCl and 0.037 M KH_2PO_4; experiments at pH 2.26 were carried out in 0.1 M NaCl and 0.0007 M H_3PO_4. No density corrections due to protein were made.

Zn^{2+} and Co^{2+} Analyses. The metal content of the enzyme was measured by atomic absorption spectroscopy with the use of a Perkin-Elmer Model 303 spectrometer. The analyses were carried out by two methods; the first was that described by René and Campbell (1969), and the second was that reported by Fuwa *et al.* (1963). Zinc was determined at 214 mμ, and cobalt was analyzed at 241 mμ.

Apoenzyme Preparation. Two methods were employed to produce the apoenzyme of renal dipeptidase. The first method was a modification of the procedure described by Vallee *et al.* (1960). In this method the purified native peptidase at a concentration of 1 mg/ml was dialyzed at 4° against a buffer of 2 mM *o*-phenanthroline in 2 mM metal-free Tris-HCl at pH 6.7 for 36 hr with one change of the dialyzing buffer. The *o*-phenanthroline was then removed by dialyzing the protein solution against 2 mM metal-free Tris-HCl at pH 7.6 for 2.5 days with four changes of the dialyzing buffer.

The second method of apoenzyme preparation was a modification of the procedure reported by Lazdunski *et al.* (1969). Zinc was again removed from the peptidase by dialyzing against the above metal-free, *o*-phenanthroline buffer. The peptidase solution (2 mg/ml) was dialyzed for 16 hr at 4° followed by dialysis for 6 hr at 25° with two changes of dialyzing buffer at pH 7.6. After dialysis, removal of *o*-phenanthroline was achieved by passing the enzyme solution through a Sephadex G-25 column (24 × 1.5 cm) which had been equilibrated with 2 mM Tris-HCl buffer at pH 7.6.

Reconstitution of Zn^{2+} and Co^{2+} Renal Dipeptidases. Resto-

TABLE I: Purification of Renal Dipeptidase by Polyacrylamide Gel Electrophoresis.

Fraction	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
50–75% ammonium sulfate	126	18,560	148
1st electrophoretic step	26.1	15,100	576
2nd electrophoretic step	10.3	8,400	815

ration of metal ions to the apoenzyme was accomplished either by dialysis of the apoenzyme for 24 hr against a 10^{-4} M pure metal salt dissolved in metal-free Tris-HCl buffer at pH 7.6 followed by dialysis of the enzyme solution against metal-free buffer to removed excess metal ion, or by addition of a measured amount of metal ion directly to the apoenzyme solution followed by immediate assay (within 3 min) for peptidase activity.

Spectral Analysis of Co^{2+} Renal Dipeptidase. The visible spectra of the cobalt enzyme, the apoenzyme, and buffer containing $Co(NO_3)_2$ were determined using 5-cm path-length microcells in the Carl Zeiss PMQ II spectrophotometer. The absorption of the $Co(NO_3)_2$ solution and of the apoenzyme solution at the appropriate concentrations were subtracted from the absorption due to the cobalt enzyme over the wavelength range from 400 to 700 mμ to produce the spectrum of cobalt renal dipeptidase.

Materials. In all operations, care was taken to avoid metal contamination. Metal-free buffers were prepared by repeated extractions with dithizone (Vallee *et al.*, 1960). Glassware and containers were cleaned and washed as described by Thiers (1957). Dialysis tubing was prepared free of metals as described by Klotz and Hughes (1956). Wherever possible solutions were prepared and stored in polyethylene ware. Metal solutions were prepared from spectroscopically pure metals or metal salts (Johnson Matthey Company, Ltd., London). Glycyldehydrophenylalanine was synthesized by methods previously described (Campbell *et al.*, 1963) and recrystallized from demineralized water.

Results

Preparation of Renal Dipeptidase. The elution profile of renal dipeptidase that had been fractionated through the ammonium sulfate step and purified by preparative polyacrylamide gel electrophoresis is presented in Figure 1. Preparative electrophoresis was performed twice upon this material, and the data reported in Figure 1 represent the second electrophoretic purification. A summary of the purification of renal dipeptidase prepared in this manner is reported in Table I. These values are the averages of two independent preparations and demonstrate the highest specific activities thus far reported for the enzyme. The schlieren photographs of the sedimentation velocity analyses of this preparation exhibited only one symmetrical peak after 60 min at 59,780 rpm, and polyacrylamide disc electrophoresis of the purified enzyme at pH 8.3 resulted in the appearance of only one protein band. These results are the same as those reported for earlier preparations using the same techniques (Campbell *et al.*, 1966; René and Campbell, 1969).

Biophysical Characterization of Renal Dipeptidase. The data obtained from isoelectric focusing of purified renal dipeptidase over the pH range 3–6 indicated that the isoelectric point of

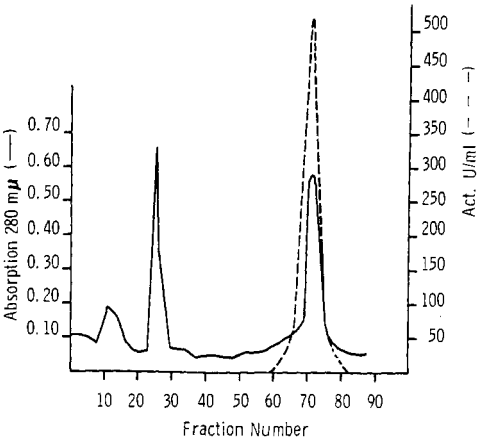

FIGURE 1: Preparative electrophoresis of renal dipeptidase. The protein was applied and eluted using a 0.1 M Tris-HCl buffer at pH 7.6. The volume of the concentrating gel was 25 ml, and that of the resolving gel was 50 ml. The elution flow rate was 0.75 ml/min, and 6-ml fractions were collected.

the enzyme was 4.89. When the experiment was performed over the wider pH range of 3–10 only one adsorbance peak was obtained which demonstrated a p*I* of 4.90. Approximately 10% of the original activity was observed in these peaks, and no enzyme activity was detected in the remaining fractions. In the determination of the diffusion coefficient of the enzyme no significant differences in the areas of the schlieren peaks were observed during the time course of the experiment. A plot of A^2/X^2 *vs.* t using data obtained during the course of the diffusion measurements was employed to calculate a value of 5.74×10^{-7} cm²/sec for the $D_{20,w}$ of renal dipeptidase. The sedimentation coefficient, $s_{20,w}$, was determined as a function of protein concentration, and extrapolation of the $s_{20,w}$ to zero protein concentration gave a value of 5.42. When the apoenzyme of renal dipeptidase which contained 12% of the original zinc content and less than 1% of the original enzyme activity was analyzed by the sedimentation velocity method, a $s_{20,w}$ value of 5.33 was obtained for a protein concentration of 1.40 mg/ml at pH 7.7.

An Yphantis plot of ln C *vs.* r^2 for the sedimentation equilibrium analysis of renal dipeptidase at 0.13 mg/ml concentration was linear in form, and the molecular weight calculated from the slope of the plot was 94,900 ± 300. The weight average molecular weight, $\bar{M}_w$, was 94,400 ± 500, and the z-average molecular weight, $\bar{M}_z$, was 95,700 ± 1500 at the same protein concentration. There was no significant change in these values when the protein concentration was increased to 1.13 mg/ml. The effect of lowering the pH upon the molecular weight of the enzyme is demonstrated in Figure 2. In these plots the weight average molecular weights calculated from a consecutive short series of points between the arrows are indicated for experiments carried out at pH 4.39, at pH 2.26, and for enzyme which had been maintained at pH 2.26 for 24 hr and then readjusted to pH 7.70. Enzyme assays of the peptidase at pH 7.6 in the presence of excess zinc following the sedimentation experiments gave specific activities of 292 units/mg for the material sedimented at pH 4.39; 7 units/mg for that sedimented at 2.26; and 2 units/mg for the protein reassociated at pH 7.70.

Zn^{2+} and Co^{2+} Binding to Renal Dipeptidase. The relationship between zinc content and enzyme activity for purified renal dipeptidase is shown in Figure 3. The zinc content of the native enzyme was determined to be 1.42 μg of Zn/mg of enzyme, which corresponds to 2.04 g-atoms of zinc per mole

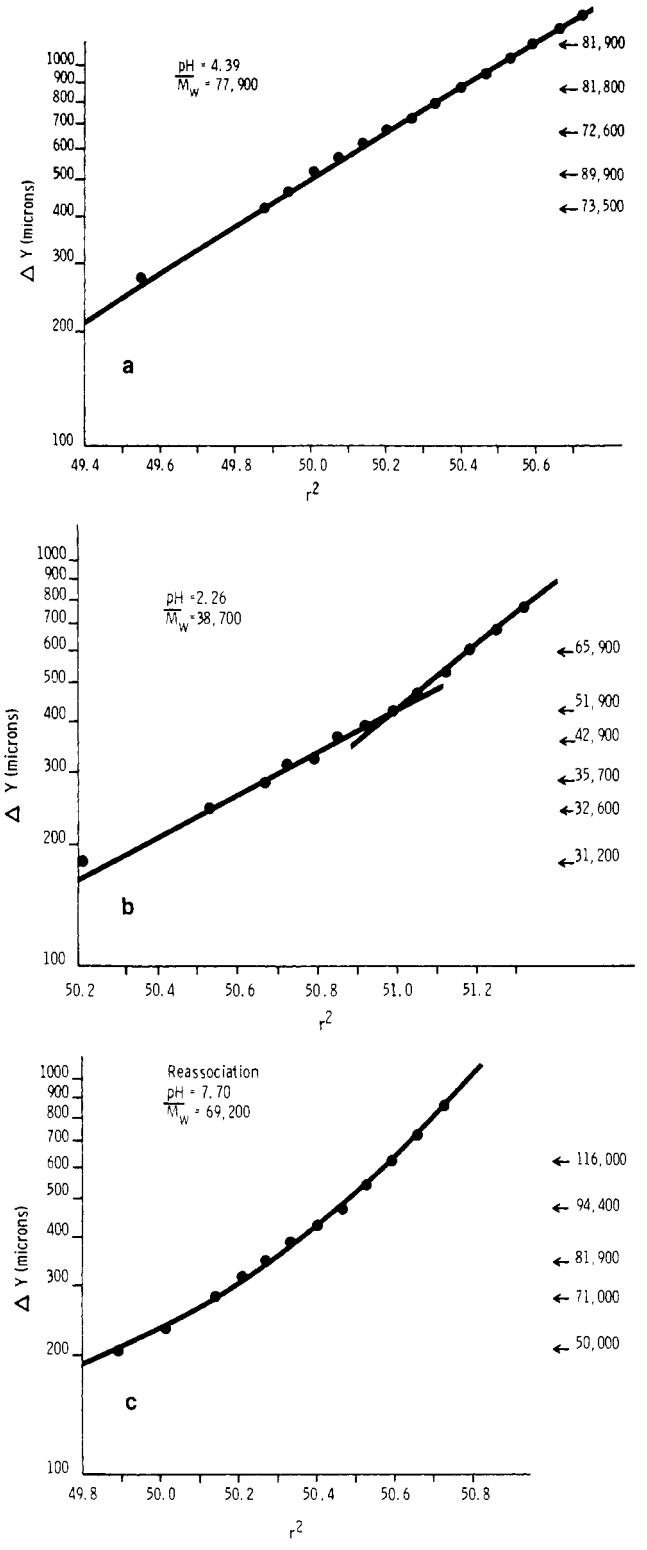

FIGURE 2: Effect of pH upon sedimentation equilibrium. The curves are semilogarithmic plots of the fringe displacements in microns as a function of the radius of rotation squared. The weights given are calculated from a consecutive series of points taken at positions across the cell between the indicated arrows. Buffer conditions are given in the Experimental Section. Equilibrium was reached at a rotor speed of 21,000 rpm after 24 hr: (a) pH 4.39; (b) pH 2.26; (c) pH 7.70 reassociated after dissociation at pH 2.26.

of enzyme of molecular weight 94,000. Zinc was removed by dialysis against *o*-phenanthroline, and the chelator subsequently removed by dialysis against metal-free buffer (details given in the Experimental Section). The resulting apoenzyme

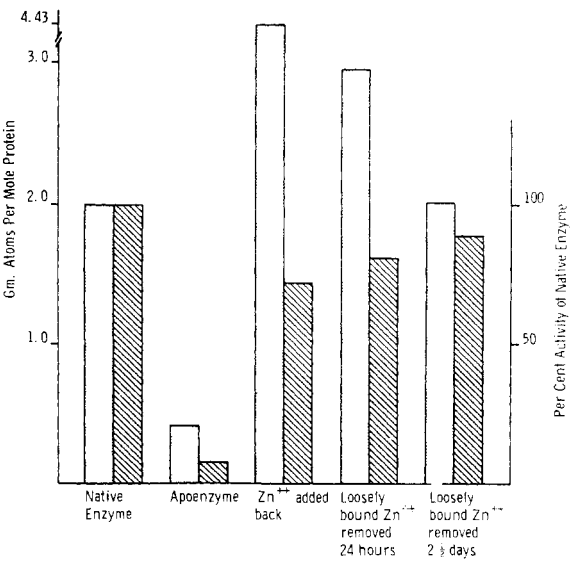

FIGURE 3: Reconstitution of the apoenzyme of renal dipeptidase by dialysis. Dotted bars indicate zinc content in gram atoms zinc per mole of protein (mol wt 94,000). Lined bars indicate per cent activity of the native enzyme. Zinc was restored to the apoenzyme by dialysis for 24 hr against 10^{-4} M $ZnSO_4$ in 2×10^{-3} M Tris-HCl buffer at pH 7.60. Loosely bound zinc was removed by dialysis against 2×10^{-3} M Tris-HCl buffer at pH 7.60 which had been prepared metal free.

contained 20% of the original zinc content and 7% of the original activity of the enzyme. Attempts to further reduce the zinc content of the peptidase by this method led to irreversible denaturation of the peptidase. When apoenzyme was prepared by the second method involving dialysis against *o*-phenanthroline at 25° and removal of chelator by Sephadex gel filtration, the final product contained 12% of the original zinc content and less than 1% of the original activity of the enzyme. The apoenzyme prepared by the second method was employed in the subsequent studies presented in Figures 4 and 5. In Figure 4, zinc was added back directly to the apoenzyme solution in increments up to 3 g-atoms of zinc per mole of protein. Following the addition of the carefully measured increments, the enzyme was assayed for zinc and specific activity. In Figure 5, cobalt was added back directly to the apoenzyme solution (4 mg/ml) which was then analyzed for zinc and cobalt contents and peptidase activity.

Spectral and Kinetic Analyses of Co^{2+} Renal Dipeptidase. The visible absorption spectra of cobalt renal dipeptidase measured over the wavelength range 440–700 mμ is presented in Figure 6. The absorption maxima detected at this enzyme concentration occur at 530 and 600 mμ. The rates of dipeptide hydrolysis catalyzed by zinc and cobalt renal dipeptidases were measured as a function of substrate concentration over the range 1.50×10^{-3}–8.00×10^{-5} M glycyldehydrophenylalanine. The data were analyzed by standard reciprocal plots (Lineweaver and Burk, 1934). The K_m estimated for the zinc enzyme and for the cobalt enzyme from these data was 0.9×10^{-3} M. The V_{max} for the zinc enzyme was 80 μmol min^{-1} mg^{-1} and for the cobalt enzyme was 47 μmol min^{-1} mg^{-1}.

Discussion

The introduction of preparative polyacrylamide gel electrophoresis into the purification procedure for renal dipeptidase eliminates the necessity of performing carboxymethylcellulose chromatography and Sephadex gel filtration to achieve adequate purity for the physical-chemical

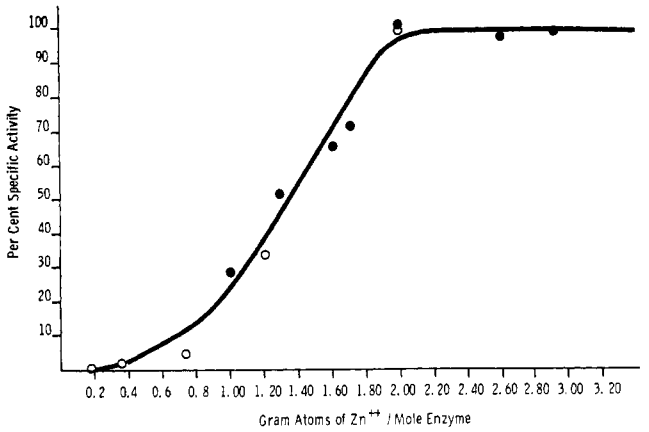

FIGURE 4: Titration of the apoenzyme of renal dipeptidase with zinc. The apoenzyme was titrated with increments of standard zinc solution (spectroscopically pure $ZnSO_4 \cdot 7H_2O$ in 2×10^{-3} M Tris-HCl buffer at pH 7.60). After each increment the solution was assayed for zinc content and peptidase activity. The peptidase assays were performed within 3 min after addition of the standard zinc solution; (○) and (●) indicate the results of two independent experiments carried out under the same conditions. Plotted on the ordinate is the per cent specific activity of the native zinc enzyme.

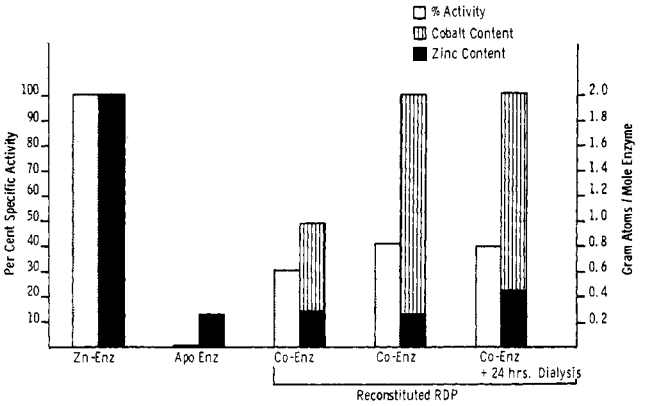

FIGURE 5: Preparation of cobalt renal dipeptidase. The cobalt dipeptidase was prepared by the addition of spectroscopically pure $Co(NO_3)_2 \cdot 6H_2O$ dissolved in metal-free 2×10^{-3} M Tris-HCl buffer at pH 7.60. Peptidase activity and cobalt content were assayed as described in the Experimental Section. Zinc analyses of the cobalt peptidase preparations were performed to ensure that restored activity was not due to zinc contamination. The cobalt enzyme was dialyzed against metal-free 2×10^{-3} M Tris-HCl buffer at pH 7.60 for 24 hr.

characterization of the enzyme. The electrophoretic technique permits the processing of relatively large quantities of enzyme (100–200 mg) in one step.

The isoelectric point of 4.90 for purified renal dipeptidase is consistent with the value that might be expected from the amino acid content of the enzyme reported by René and Campbell (1969). It is not surprising that little activity was found associated with the protein following isoelectric focusing. Ampholines are strong metal chelators, and little zinc would be expected to be bound to the enzyme in the presence of ampholines at low pH. Also prolonged exposure of the peptidase to low pH has been shown to irreversibly inactivate the enzyme (Campbell *et al.*, 1966). Since the diffusion coefficient was determined at only one protein concentration (7.15 mg/ml), the value of 5.74×10^{-7} cm²/sec must be regarded as an approximation to be refined when sufficient data are available to extrapolate to zero concentration. When the sedimentation coefficient was measured as a function of protein concentration there appeared little concentration dependence for $s_{20,w}$. This relatively small concentration dependence of the sedimentation coefficient suggests that renal dipeptidase is a compact globular macromolecule (Schachman, 1960). The molecular weight calculated from the Svedberg equation using the apparent diffusion coefficient (5.72×10^{-7} cm²/sec) and the value of 5.42 S for the sedimentation coefficient is 83,300. A comparison of the apparent sedimentation coefficient of the apoenzyme of renal dipeptidase (5.33 S) with that of the native enzyme (5.42 S) suggests that zinc can be removed from the native enzyme without producing the dissociation that occurs when the enzyme is subjected to low pH (Figure 2). Since only one sedimenting component was observed in the sedimentation velocity pattern of the apoenzyme, and since the value of that component was approximately that of the native enzyme, it seems likely that bonds other than metal–protein bonds are involved in maintaining the quaternary structure of the macromolecule.

The Yphantis method of equilibrium sedimentation has provided a sensitive means for examining the homogeneity of a protein preparation. The linear plot obtained for renal dipeptidase revealed no significant deviation from homogeneity for renal dipeptidase under these conditions. The various molecular weight averages also confirm the homogeneity of the purified peptidase and indicate a molecular weight within the range from 94,000 to 96,000. However, evidence for dissociation of the peptidase into lower molecular weight species as the pH is decreased is provided by the data given in Figure 2. Lowering the pH to 4.39 resulted in a general lowering of the molecular weight averages to a range from 72,600 to 89,900. When the pH was decreased to 2.26 evidence of marked heterogeneity in the sample was observed. The Yphantis plot appears as a curve in Figure 2b, and no agreement was observed in the molecular weight averages ($\bar{M}_w$ and $\bar{M}_z$) calculated from these data. Increasing the pH from 2.26 to 7.70 seems to produce reassociation of the dissociated enzyme as evidenced in the data presented in Figure 2C. The reassociation does not result in a homogeneous preparation (range 50,000–116,000 $\bar{M}_w$) and appears to be nonspecific in that the catalytic activity of the enzyme was not restored upon reassociation. The dissociation of renal dipeptidase into smaller molecular weight components has been confirmed by recent preliminary observations using polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Armstrong, 1972).

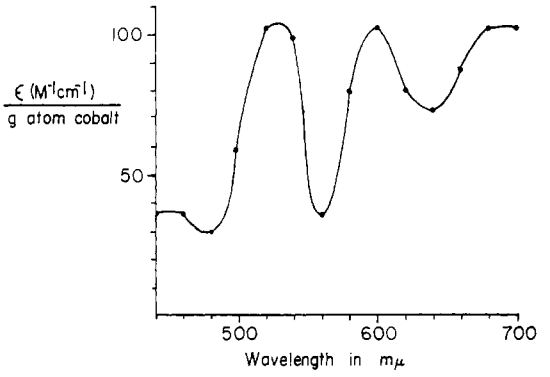

FIGURE 6: Visible absorption spectrum of cobalt renal dipeptidase. The cobalt peptidase was prepared as described in Figure 5. The spectra were measured using 5-cm path-length cells, and the protein concentration in each case was 4.0×10^{-5} M. The spectra were obtained by subtracting the absorptions due to $Co(NO_3)_2 \cdot 6H_2O$ and apoenzyme from the absorption due to the cobalt enzymes.

The results presented in Figure 3 demonstrate that zinc can be removed from the peptidase with subsequent loss in enzymic activity and that the activity can be completely restored when the enzyme is reconstituted by the addition of up to 2 g-atoms of zinc per mole of enzyme (mol wt 94,000). Addition of measured amounts of zinc to the apoenzyme as shown in Figure 4 did not demonstrate a linear relationship between the gram atoms of zinc present in the enzyme and the per cent of activity restored. The addition of the first gram-atom of zinc per mole of enzyme restored 24% of the original activity, and the addition of the second gram-atom restored the remaining 76% of the specific activity. Maximal activity was not obtained until a level of 2 g-atoms/mole of enzyme was achieved. Although inhibition of peptidase activity by excess zinc was observed when the metal ion was allowed to react with the apoenzyme over a period of 24 hr of dialysis against 10^{-4} M $ZnSO_4 \cdot 7H_2O$ (Figure 3), this inhibition was not observed when the metal ion was added directly and assayed immediately (Figure 4). This effect could have been caused by a time dependent inhibition of peptidase by excess zinc which was reversed upon dialysis against metal-free buffers over a period of 2.5 days. The data presented in Figure 5 indicate that the specific activity of the completely reconstituted cobalt enzyme is 42% of the specific activity of the native zinc enzyme. The addition of 0.67 g-atom of cobalt to the apoenzyme restored 84% of the specific activity which was reached when 2 g-atoms of cobalt/mol were added. These data do not permit discrimination between the possible catalytic and structural functions of the bound metal ions, but it is significant that in both cases 2 g-atoms of metal ion were required to completely restore the biological activity of the peptidases.

The visible spectrum of cobalt renal dipeptidase reported in Figure 6 exhibits maxima at wavelengths close to those reported for the visible spectra of cobalt carboxypeptidase (Latt and Vallee, 1969) and for cobalt alkaline phosphatase (Simpson and Vallee, 1968). The estimated extinction coefficients of approximately 102 for these maxima are also within the range of those reported for other reconstituted cobalt enzymes. Absorption maxima in the 510–515- and 605–615-mμ range have been reported to be characteristic of irregularly tetrahedral or five coordinate geometry in cobalt carbonic anhydrase and in cobalt alkaline phosphatase (Vallee and Williams, 1968).

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