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Synthesis and Characterization of a Metal-Binding Peptide Fragment of Human Carbonic Anhydrase B[1]

REBECCA L. CHAN, NGET HONG TAN, AND E. T. KAISER[2]

Department of Chemistry, University of Chicago, Chicago, Illinois 60637

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A 36-amino acid residue peptide containing the presumed metal-binding ligands at the active site of human erythrocyte carbonic anhydrase B was synthesized by the standard solid phase method. The synthetic peptide was purified by ion-exchange chromatography and was homogeneous as judged by cellulose acetate gel electrophoresis. Amino acid analysis, dansylation, C-terminal determination, and four cycles of Edman degradation all gave results consistent with the anticipated sequence. The peptide binds Co(II) with an apparent dissociation constant of about 7×10^{-5} *M* (uncorrected) but has little, if any, of the catalytic activity of carbonic anhydrase. Possible explanations for the weak binding of the metal ion are discussed along with prospects and strategies for designing polypeptide models of enzymatic catalysts.

INTRODUCTION

Solid phase peptide synthesis, first launched and developed by Merrifield (*1*), has been successfully applied to the synthesis of a growing number of biologically interesting polypeptides (*1*) and has been proven to be particularly useful in studies of the structure–function relationships of polypeptides of small to moderate molecular weight.

Our laboratory has undertaken studies on solid phase peptide synthesis with a view toward using this method in the design of polypeptide model enzymatic catalysts. In this paper, we present our results for the solid phase synthesis of a medium-sized peptide containing the presumed metal-binding ligands at the active site of human erythrocyte carbonic anhydrase B (HCAB). This enzyme has been selected for our studies because of its unusual properties. In particular, the enzyme consists of a single polypeptide chain (*2*) without any disulfide linkages, a feature relatively rare among enzymes. It is a metalloenzyme, with one very firmly bound zinc ion per molecule. The stability constant of the Zn(II)–protein complex is 10^{12} *M* at pH 7.5 (*3*). Furthermore, in its catalysis of the reversible hydration of CO_2, the enzyme exhibits a turnover number (*4*) of about 10^4 to 10^5 sec^{-1}, which is among the highest known for any enzyme. The complete amino acid sequence of HCAB (*5*) as well as its X-ray structure (*6*) have been established. The three-dimensional structure of its isozyme, human erythrocyte carbonic anhydrase C (HCAC), had been determined previously (*7*), and there were strong indications that the two isozymes were very similar (*5*). The X-ray work shows that three histidine residues

[1] This paper is dedicated with admiration to Professor William S. Johnson on the occasion of his 65th birthday.

[2] To whom correspondence should be addressed.

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Phe–Gln–Phe–His–Phe(95)–His–Trp–Gly–Ser–Thr(100)–Asn–Glu–His–Gly–Ser(105)–Glu–His–Thr–Val–Asp(110)–Gly–Val–Lys–Tyr–Ser(115)Ala–Glu–Leu–His–Val(120)–Ala–His–Trp–Asn–Ser–Ala

FIG. 1. Linear sequence of amino acids in the synthetic peptide. The numbers in parentheses give the positions of the corresponding amino acid residues in native HCAB.

in HCAC bind to the zinc ion. For HCAB, the corresponding histidine residues occur at positions 94, 96, and 119, spanning only a very short section of the entire sequence (see Fig. 1). As part of our program of synthetic studies on carbonic anhydrase, we felt it would be of interest to synthesize this portion of the enzyme. We wished to determine whether this peptide, containing the necessary metal-binding ligands, would form a strong complex with zinc or other metal ions and, if it did, whether it would accelerate reactions normally catalyzed by carbonic anhydrase. An advantage in studying the possible catalytic activity of fragments of carbonic anhydrase is that, even if they are far less active than the enzyme itself, activity might still be detectable, whereas for a less active enzyme this might not be true. The peptide we chose to synthesize constitutes residues numbered 91–126 in the sequence of HCAB (Fig. 1). Preliminary experiments showed that this peptide precipitated upon the addition of Zn(II). We therefore confined our studies to Co(II) since it has been shown that Co(II)–carbonic anhydrase has a specific activity comparable to that of the native enzyme–Zn(II) (*3*).

EXPERIMENTAL

General

Specific rotations at the sodium D line were measured with a Perkin–Elmer Model 141 electronic polarimeter which reads to 0.001°. Ultraviolet and visible spectra were measured with a Cary 15 or with a Beckman Model DU spectrophotometer modified with Gilford Model 2400 attachments. Circular dichroism spectra were measured on a Cary 60 spectropolarimeter. All pH measurements were carried out on a Beckman Research pH meter or a Radiometer Model PHM 4c instrument with combination glass–calomel electrodes (Thomas No. 4094 L60). The meters were standardized against Fisher certified standard buffers prior to use. Deionized water was obtained by passing distilled water through a Continental demineralizer. Amino acid analyses were performed on a Beckman Spinco Model 121 amino acid analyzer. Resin–peptide samples (about 10 mg) were hydrolyzed either at 130°C for 6–8 hr with 0.5 ml of 1 : 1 (v/v) concentrated hydrochloric acid: propionic acid or at 110°C for 20–24 hr with 0.3 ml of 1 : 1 (v/v) concentrated hydrochloric acid : dioxane in vacuum-sealed tubes. Free peptide samples were hydrolyzed at 110°C for 20–24 hr with 0.3 ml of 6 *N* hydrochloric acid containing 0.1% (w/v) phenol in vacuum-sealed tubes.

Synthesis of the Peptide Fragment of Human Erythrocyte Carbonic Anhydrase B

Reagents and solvents used in this synthesis were the same as described in our previous synthesis of pancreatic trypsin inhibitor (Kunitz) (*8*). Boc-L-alanine resin ester [2% cross-linked, chloromethylated styrene–divinylbenzene (DVB) copolymer] was purchased from Schwarz/Mann. The tert-butyloxycarbonyl amino acid derivatives used

were the following: Ala, Asp (β-benzyl), Asn-*p*-nitrophenyl ester, Glu (γ-benzyl), Gln-*p*-nitrophenyl ester, Gly, His (N^{im}-tosyl), Leu, Lys (ε-2-chlorobenzyloxycarbonyl), Phe, Thr (benzyl), Ser (benzyl), Trp, Tyr (2,6-dichlorobenzyl), and Val. These were all purchased from Bachem, Inc., Marina del Rey, California. The purity of each amino acid derivative was checked by thin-layer chromatography on silical gel chromatogram sheets (Eastman) before use (*9*).

A glass reaction vessel (4.8 × 15 cm) similar to that described by Merrifield (*1*) was used in conjunction with a Burrell wrist-action shaker. Pyridine hydrochloride titration for the determination of resin-bound amino groups (*10*) was performed either manually by the modified Volhard method of chloride determination (*10*) or potentiometrically with a Radiometer Titrator II in conjunction with a Radiometer SBR 2c titrigraph and a Model 25 pH meter. A combined silver–mercurous sulfate electrode (Type PK499), Radiometer was used.

A 9.0-g batch of Boc-L-alanine resin ester (0.37 mmol of alanine per g of resin) was placed in the reaction vessel. The synthesis of the peptide was carried out as described previously, except that it was performed manually. After the incorporation of tryptophan, mercaptoethanol was added to the deprotection mixture (4%, v/v) (*11*). Completeness of the coupling reactions was monitored by the ninhydrin test (*12*). Repeated coupling was carried out when the ninhydrin test yielded positive results. At the earlier stages of the synthesis acetylation was used to terminate the unreacted chains if the resin still gave positive ninhydrin color after recoupling. Acetylation was performed by reacting the resin with a mixture of 4 ml of acetic anhydride (J. T. Baker Chemical Co., distilled) and 2 ml of *N*-methylmorpholine (Aldrich) in 50 ml of dimethyl formamide (DMF) (*13*) for 30 min.

At the end of the synthesis, the resin was washed with absolute ethanol, dried to constant weight, and subjected to liquid HF cleavage (*14*) as described previously (*8*). To obtain the free peptide, the resin beads were placed on a 30-ml sintered-glass filter funnel, washed first with 200 ml of water and then with 100 ml of 50% acetic acid. The water and acetic acid extracts were pooled and lyophilized.

Purification of the Synthetic Peptide

Sodium acetate and sodium formate, tris(hydroxymethyl)aminoethane, and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were used without purification. Reagent grade urea (*15*) was recrystallized from aqueous ethanol prior to use. Sephadex SEC- or SPC-25 was obtained from Pharmacia Fine Chemicals.

The crude peptide obtained after HF cleavage was subjected to the following purification steps. In the initial purification, three buffers were used: (A) pH 3.0, 0.1 *M* sodium formate–formic acid, containing 4 *M* urea and 0.05 *M* Tris; (B) pH 5.0, 0.1 *M* sodium acetate–acetic acid, containing 4 *M* urea and 0.05 *M* Tris; and (C) pH 8.0, 0.15 *M* Tris–HCl, containing 4 *M* urea.

A sample of the crude peptide (60–100 mg) in 5 ml of buffer A was applied onto a column (2.8 × 25 cm) of the cation-exchange resin Sephadex SEC-25 (or SPC-25) preequilibrated with buffer A. The column was eluted in succession with 400 ml each of buffers A and B followed by 300 ml of buffer C. The flow rate was about 40 ml/hr. The elution pattern observed is shown in Fig. 2. The fractions eluted under the major peak

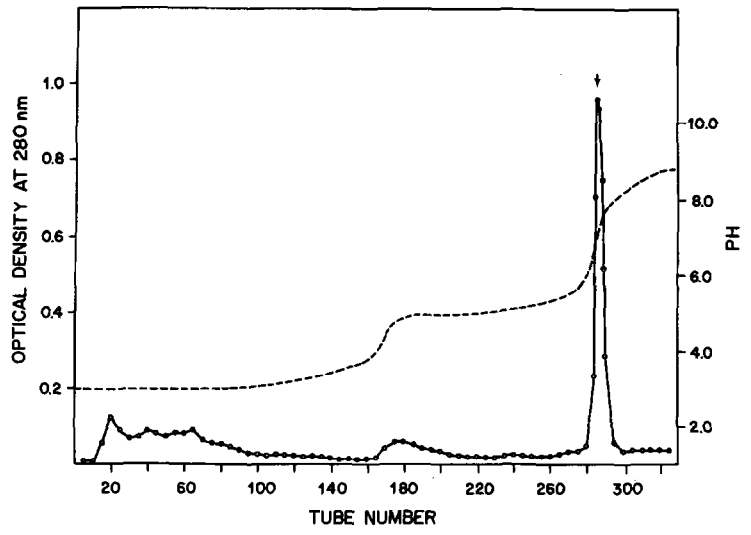


FIG. 2. Chromatography of the crude synthetic peptide on a Sephadex SEC-25 column: sample, 64 mg; column size, 2.8 x 25 cm; fraction size, 4 ml; yield, tubes 284–295 (from the peak marked with an arrow), 30 mg. The pH values of the fractions collected are indicated by the broken line.

absorbing at 280 nm were pooled, concentrated by lyophilization, and desalted on a 4 × 48-cm Sephadex G-50 column. The resulting peptide was lyophilized.

The partially purified peptide eluted with buffer C was rechromatographed on the same cation exchanger. A series of MES buffers (0.1 *M*, containing 0.05 *M* Tris and 4

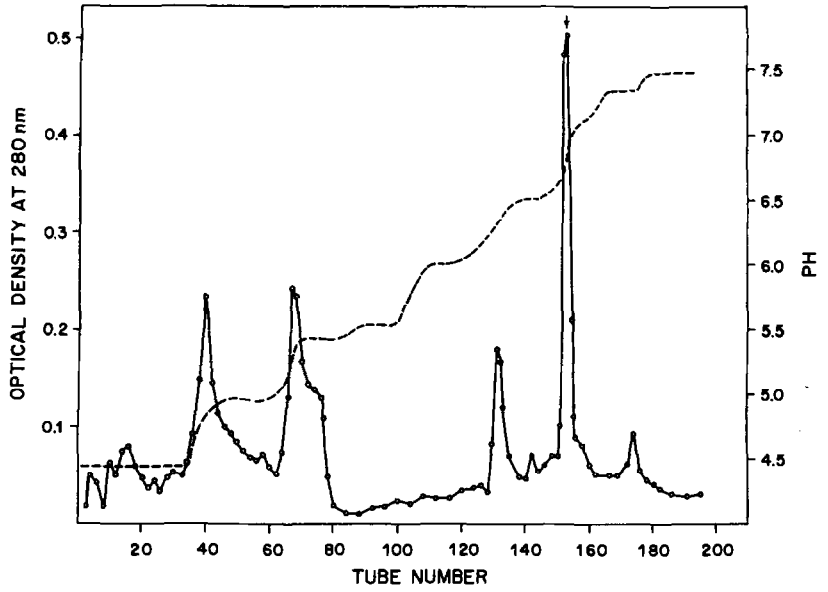


FIG. 3. Rechromatography of partially purified peptide on Sephadex SEC-25 column: sample, 10 mg; column size, 1.4 x 15 cm; fraction size, 3 ml; yield, tubes 150–160 (from the peak marked with an arrow), 0.95 mg. The broken line indicates the pH values of the fractions collected.

TABLE 1

PERCENTAGE YIELDS IN THE SYNTHESIS AND PURIFICATION OF THE SYNTHETIC PEPTIDE FRAGMENT OF HUMAN CARBONIC ANHYDRASE B

(1) Crude product, calculated from the initial amount of alanine in the starting material, Boc-L-alanine resin; 28%.[a]
(2) Product obtained after first ion-exchange chromatography, based on the amount of material eluted with buffer C: 48%.
(3) Product obtained from second ion-exchange chromatography, based on the amount of material eluted between pH 6.5 and pH 7.0: 11.2%
(4) Overall yield: 1.5%.

[a] If corrections are estimated for the amounts of material removed during the course of the synthesis for analytical purposes and lost during the manipulation involved in the HF cleavage process, then the crude yield may actually be about 40%.

M urea) with pH values ranging from 4.5 to 7.5 in 0.5 pH unit steps was prepared. A 1.5 × 15-cm column of Sephadex SEC-25 was equilibrated with the first buffer (pH 4.5) in the series. Ten to twenty milligrams of the partially purified material (in 1 ml of the pH 4.5 MES buffer) was added to the column and a stepwise elution with about 75 ml of each of the buffers followed. A typical separation is shown in Fig. 3. The eluents giving uv absorption peaks at 280 nm were separately desalted and lyophilized. The peptide material obtained from elution with the pH 7.0 buffer was used in all subsequent experiments. The yields in the syntheses and purifications are shown in Table 1. In our experience there is, in general, a 10–20% loss in the HF cleavage step due to manipulations. Furthermore, in Table 1 we have not corrected for losses due to removal of material for analytical purposes during the course of the synthesis. If the crude yield in Table 1 were corrected for losses in the HF cleavage process and for the other losses, we estimate that it would be approximately 40%.

Carboxyl-Terminal Determination

About 100 nmol (0.3–0.4 mg) of the purified peptide in 300 μl of 0.05 *M* pH 7.8 phosphate buffer was digested with 10 μl of a carboxypeptidase A stock solution (Worthington) (concentration 0.6×10^{-4} *M* in 0.02 *M* pH 7.5 MES buffer containing 0.5 *M* NaCl) at room temperature. Samples (25 μl) were removed at timed intervals and added to 800 μl of pH 2.2 citrate buffer to terminate the digestion. The solution was directly applied to the amino acid analyzer without further treatment (*16*).

Edman Degradation

The following reagents were all of Sequenal grade purchased from Pierce Chemical Co: *N,N*-dimethyl-*N*-allylamine, phenylisothiocyanate, trifluoroacetic acid, *n*-propanol, ethyl acetate, and benzene. All reagents were stored under nitrogen.

About 100 nmol of the purified peptide was subjected to four cycles of Edman degradation (*17*). The procedure employed was essentially that of Peterson *et al.* (*18*) with minor modifications.

Following the conversion of the 2-anilino-5-thiazolinone derivative to the 3-phenyl-2-thiohydantoin (PTH) derivative by 1 *N* HCl, the aqueous solution was saturated with 1 *M* Na_2HPO_4 before extraction with ethyl acetate (4 × 0.5 ml). After evaporation to

dryness under a stream of nitrogen, the ethyl acetate extract was hydrolyzed with 100 μl of hydrogen iodide (58.2%, Fisher Scientific Co.) at 130°C for 20 hr in vacuum-sealed tubes. The resulting hydrolyzate was evaporated to dryness and the residue was analyzed on the amino acid analyzer.

Dansylation

Dansylation and identification of dansylated amino acid residues were carried out as described previously (*8*).

Cellulose Acetate Gel Electrophoresis

A microzone electrophoresis cell (Beckman Model R-101) was used together with a Beckman Duostat dc power supply. The buffer used was pH 5.0 bis-Tris–HCl [bis(2-hydroxyethyl)-imino-tris(hydroxyethyl)methane hydrochloride, General Biochemicals] containing 4 *M* urea. Cellulose acetate sheets (Gallard-Schlesinger Chemical Manufacturing Corp.) were stored in methanol at 4°C. They were soaked in the buffer for 2 hr prior to use.

The sheet was sponged dry right before use and was mounted on the electrophoresis cell. Samples of the purified peptides (1–5 μg) were spotted onto the sheet with a microapplicator. After 30 min at 300 V, the sheet was carefully removed from the electrophoresis cell and dipped into a 10% solution of trichloroacetic acid (2 × 15 min) to fix the peptide. This was followed by a 45-min treatment at 50°C with a 1% (w/v) solution of Coomassie brilliant blue R (Sigma Chemical Co.) in MeOH:H_2O:HOAc (5:4:1, v/v/v). The gel was destained first by a brief treatment (about 5 min) with 10% aqueous HOAc, followed by several changes of a destaining solution: MeOH:H_2O:HOAc (5:4:1, v/v/v). Destaining was complete within 30 min.

Co(II) Binding Studies

(*a*) *Apparatus and materials.* Reagent grade 1,10-phenanthroline was obtained from Aldrich Chemical Co. and was used without further purification. Dithizone (diphenylthiocarbazone) was purchased from J. T. Baker Chemical Co. and was recrystallized according to the method of Sandell (*19*). Tetramethylammonium chloride (Aldrich Chemical Co.) was recrystallized from absolute ethanol.

Dialysis tubings were viscose cellulose casings (width: 1 in.; average wall thickness: 0.0008 in.; molecular weight cutoff: 12 000) obtained from Arthur H. Thomas Co.

Two types of equilibrium dialysis cells were used. Plexiglas cells with 1-ml capacity were purchased from Lapine Scientific Co. Microequilibrium dialysis cells containing twin sets of dialysis chambers were an adaptation of the design of Englund *et al.* (*20*). These were constructed from cast Lucite rods (1-in. diameter). The volume of each chamber was about 250 μl.

The cobalt concentrations were determined on a Varian AA120 atomic absorption spectrophotometer with a Varian Techtron Model 63 carbon rod atomizer. A Varian multielement (Fe/Co/Cu/Cr/Ni/Mn) hollow cathode lamp was used.

Standard solutions of cobalt were prepared by dissolving known weights of cobalt powder (99.9%, K and K Laboratories) in hydrochloric acid (Ultra Pure 33%, Alfa Products), removing the excess HCl by evaporation *in vacuo*, and making up the solution to the desired volume with the buffer solution (see below).

(*b*) *Treatment of apparatus.* All of the glassware utilized in the metal-binding studies was soaked in 2 *N* nitric acid for at least 6 hr, followed by rinsing with deionized water. It was then washed with a concentrated solution of the disodium salt of ethylene diaminotetracetic acid (EDTA, from Aldrich Chemical Co.) and rinsed extensively with deionized water. All solutions were stored in polyethylene containers that had been treated with EDTA and rinsed thoroughly with deionized water. Equilibrium dialysis cells were soaked in concentrated EDTA solutions for 2 hr and rinsed with deionized water prior to use.

(*c*) *Preparation of buffer solution.* The buffer solution was prepared as follows: A solution containing 0.025 *M* Tris (Schwarz/Mann, Ultra Pure grade) and 0.4 *M* tetramethylammonium chloride was made up. The pH was brought to about 6.5 with concentrated HCl. This solution (1 liter) was extracted three times with 100-ml portions of a 0.01% (w/v) solution of dithizone in carbon tetrachloride. After repeated extractions with carbon tetrachloride to remove all dithizone, the pH of the aqueous solution was adjusted to 7.2 with solid Tris. The final concentration of Tris was about 0.027 *M*.

(*d*) *Acetylation of dialysis membrane.* The molecular weight cutoff of the commercially available dialysis tubing used was 12 000. These membranes had to be acetylated so as to decrease the pore size. The following method of acetylation is a modification of the procedure of Craig (*21*).

The dialysis tubing (width: 1 in.) was cut open into rectangular strips (approximately 2 × 12 cm) and immersed in a mixture of pyridine and acetic anhydride (1 : 5, v/v). The container was sealed with Parafilm and covered with aluminum foil. It was left in a well-ventilated hood at room temperature for 24–48 hr. At the end of this period, the pyridine : acetic anhydride mixture was carefully poured off. The membranes were thoroughly rinsed first with deionized water and then with 0.01 *M* acetic acid. They were next cut up into squares measuring 2 × 2 cm. These were boiled for 1 hr in water containing 0.05 *M* sodium bicarbonate and 0.05% EDTA. The boiling was repeated once. The membranes were then washed with deionized water and boiled for 1 hr in water. Finally, they were refluxed for 20 min in 70% aqueous ethanol followed by thorough rinsing with deionized water to ensure complete removal of excess acetic anhydride: pyridine mixture and were stored in deionized water at 4°C. The membranes were washed with deionized water and sponged dry right before use.

Part of the dialysis tubing was acetylated without being cut open. This was used for preparing peptide stock solutions. The acetylation procedure was the same as that described above except that a longer time (3–4 days) of acetylation was required.

The permeability of each preparation of acetylated dialysis membranes toward the synthetic peptide and Co(II) ions was checked before use. For this purpose, using equilibrium dialysis cells of 1-ml capacity, one compartment was filled with a peptide solution [or a Co(II) solution, containing approximately 100 μg of Co(II)/ml] in the buffer and the other compartment was filled with the buffer only. The cells were mechanically shaken at room temperature. The approach to equilibrium was monitored at regular intervals by withdrawing 100 μl of solution from both compartments of the Co(II)-containing cells. The Co(II) concentration was measured colorimetrically using nitroso-R salt (1-nitroso-2-naphthol-3,5-disulfonic acid disodium salt hydrate, Aldrich Chemical Co.) according to Sandell (*19*). When the result showed that equilibrium was

reached, the absorbances at 280 nm of the solutions in both compartments of the peptide containing cells were measured to determine the peptide concentrations. The membranes were considered suitable for use in the equilibrium dialysis experiments if equilibrium [for Co(II) ions] could be reached within 24 hr with no more than 5% of peptide diffusing through it during this period. Acetylated dialysis tubing was similarly tested by immersing the dialysis sacs containing the peptide solution or Co(II) solution in a beaker containing the buffer and checking the concentration of the peptide [or Co(II)] in solutions both inside and outside the dialysis sacs.

(*e*) *Preparation of stock solutions of the purified peptide.* Approximately 2 to 3 mg of the purified peptide was dissolved in about 2 ml of the pH 7.2 buffer containing 2×10^{-3} *M* 1,10-phenanthroline. This solution was placed in an acetylated dialysis tubing and dialyzed at room temperature against 250 ml of the same buffer in a plastic beaker. After 24 hr, the buffer in the beaker was replaced by one not containing the chelating agent. Dialysis was continued with four or five changes of buffer at 12-hr intervals. The concentration of 1,10-phenanthroline in the buffer, as determined by uv measurements ($\varepsilon_{264} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$), generally dropped to a negligibly low level after three changes of buffer.

After the procedure involving extraction with the chelating agent, the peptide solution was passed through a Millipore filter (0.45-μm pore size). The concentration of the filtrate was measured by uv absorption at 280 nm. The solution which had a pH of 7.2 $\pm$ 0.05 was stored in a plastic vial kept in the freezer.

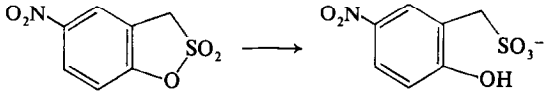
(*f*) *Equilibrium dialysis.* One compartment of the microequilibrium dialysis cell (with acetylated dialysis membrane) was filled with 150 μl of the peptide solution obtained by suitable dilution of the stock solution with the pH 7.2 buffer. The other compartment was filled with an equal volume of a solution of cobalt chloride in the buffer. The concentration of the peptide varied from about 1×10^{-5} to 3×10^{-5} *M*. The concentration of Co(II) was 1.5- to 2.5-fold that of the peptide. One-milliliter plastic syringes graduated to 0.01 ml and fitted with fine Teflon tubings were used in filling or emptying the equilibrium dialysis cells. The cells were left on a wrist-action shaker at room temperature for 20–24 hr. The contents were then withdrawn, suitably diluted, and stored in small polyethylene silylation tubes (Beckman) at 4°C before analysis of the cobalt content.

The following settings on the atomic absorption spectrometer were used: wavelength, 2407 Å; slit height, 6.5 μm; slit width 200 μm. The atomic absorption peak heights were not linear with concentration and the instrument had to be calibrated before use with a series of standard Co(II) solutions in the buffer (0.5–1.5 ppm). Five microliters of sample solution was used for each reading. Absorption peaks were checked for matrix effects, i.e., nonatomic absorption in flameless atomization due to molecular absorption by matrix components and/or light scattering by carbon particles in the absorption cell. This was done employing the Varian H_2 continuum light source. The absorption measurements obtained with the continuum source were subtracted from those obtained with the hollow cathode line source to yield the sample's true atomic absorption (*22*). In our case, the matrix effects were generally negligible. At least three consistent readings were taken for each sample. These were averaged and the cobalt contents determined from the calibration curve were converted into concentration in moles per liter.

Activity Measurements

The two reactions that carbonic anhydrase catalyzes with greatest efficiency are the reversible hydration of carbon dioxide [Eq. (1)] and the hydrolysis of 5-nitro-2-hydroxy-α-toluenesulfonic acid sultone (*23*) [Eq. (2)]. Kinetic measurements on the effects of synthetic peptide–Co(II) mixtures on these reactions were carried out as described below.

$$H_2O + CO_2 \rightleftharpoons HCO_3^- + H^+ \quad (1)$$



(2)

(*a*) *Hydration of CO_2.* CO_2 stock solution was prepared by bubbling the pure gas (J. T. Baker Chemical Co., Bone Dry grade) into freshly deionized water for 45 at room temperature. This gives a concentration of about 3.5×10^{-2} *M* (*24*). The buffer used was a 0.025 *M* Tris solution containing 0.05 *M* sodium sulfate adjusted to pH 7.6 with concentrated sulfuric acid. Bovine carbonic anhydrase from Worthington (lot CA OFA) was a mixture of the electrophoretic components A and B and was used without purification. A stock solution of the enzyme (1.87×10^{-5} *M*) in the above buffer was prepared. Stock solutions of the purified peptides were prepared by dissolving about 3 mg of the peptide in 3 ml of buffer and passing the solution through a 0.45-μm Millipore filter. The peptide concentration was determined from uv absorption at 280 nm. A 2.5% solution of phenol red (sodium salt, Allied Chemical Co.) in the buffer was used as the indicator in the CO_2 hydration experiments. A solution of $CoCl_2$ (1.696×10^{-2} *M*) in the above buffer was prepared from cobalt powder as described in the section on Co(II)-binding studies.

Kinetic experiments were performed on an Aminco-Morrow stopped-flow spectrophotometer. All solutions were kept in a 25 ± 0.1°C thermostated water bath for at least 1 hr before use.

The CO_2 solution obtained by diluting the stock solution 10-fold with deionized water was placed in one syringe, and a solution containing the peptide ($1.4–1.8 \times 10^{-5}$ *M*), Co(II) (1.7×10^{-4} *M*), and phenol red (100 μl of stock/100 ml of solution) in the pH 7.6 buffer was placed in the other syringe. The CO_2 hydration reaction was followed at 450 nm. The total change in transmittance did not exceed 5%, and the oscilloscope trace readings were used directly for making first-order plots to calculate the hydration rate constants. The pH of the solution after mixing usually fell to a value between 7.45 and 7.54.

Control experiments for the metal–peptide complex-catalyzed reaction were also carried out in the absence of Co(II) or in the absence of peptide. The concentration of the substrate in these controls was adjusted to be the same as that in the above runs by adding a suitable volume of the buffer. The spontaneous and enzymatic (enzyme concentration = 0.94×10^{-7} *M*) rates of hydration of CO_2 were also determined.

(*b*) *Hydrolysis of 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone.* The sultone was prepared and recrystallized from ethanol, mp 148–149°C [lit. mp 148.5–149.5°C (*23*)]. A stock solution of this compound (1.04×10^{-3} *M*) in distilled acetone was kept

tightly stoppered and refrigerated. Buffer, enzyme stock solution, and peptide stock solution were prepared as described in the previous section on hydration of CO_2.

The kinetic runs were performed on a Cary 15 recording spectrophotometer or a Beckman DU spectrophotometer equipped with a Gilford Model 242 recorder. The cell compartments were thermostated at 25 ± 0.1°C.

Hydrolysis experiments in the presence of the peptides were carried out using 1-cm path-length, 1-ml capacity quartz cuvettes. In a typical run, 650 μl of the pH 7.6 buffer was introduced to the cuvettes, followed by 150 μl of the peptide and 50 μl of the Co(II) stock solutions. The resulting concentration of peptide was 0.6–0.8 × 10^{-4} *M* and that of Co(II) was 1 × 10^{-3} *M*. The reaction was initiated by adding a small aliquot (5 μl) of the sultone stock solution to the cuvette and mixing. The reactions were followed at 407 nm (λ_{max} of the reaction product) for at least three half-lives, and the infinity absorbance was taken after 7–10 half-lives. At the end of the run, the pH of the reaction mixture was measured. The reading generally changed by no more than 0.03 unit. The pseudo-first-order hydrolysis rate constant (k_{obs}) was calculated from a first-order plot of the absorbance trace.

Controls similar to those in the hydration of CO_2 experiments were performed. The spontaneous and enzymatic hydrolysis rate constants at pH 7.6 were also determined as described by Kaiser and Lo (*23*) using 4-ml capacity, 1-cm path length cuvettes.

RESULTS

The Synthesis and Purification of the Carbonic Anhydrase Peptide Fragment

Although the yield of crude product based on the starting Boc-L-alanine content is given in Table 1 as only 28%, this value is not corrected for the removal of material for analysis and for the loss of product from manipulations in the HF cleavage step.

Recoupling was found necessary for the following amino acid residues: Ala-116, Tyr-114, Trp-97 (recoupled twice), His-96 (recoupled twice), Phe-95, His-94, Phe-93, Gln-92, and Phe-91 (recoupled twice). Following the incorporation of Ser-115, Lys-113, and Glu-106, the resin was acetylated. The amounts of resin-bound amino groups were determined after the incorporation, subsequent acetylation step, and deprotection of Ser-115 and Glu-106. The results are shown in Table 2.

The resin–peptide mixture obtained after HF cleavage was extracted by water and 50% acetic acid and then lyophilized. It was first fractionated on a cation-exchange

TABLE 2

PYRIDINE HYDROCHLORIDE TITRATION RESULTS IN THE SYNTHESIS OF PEPTIDE FRAGMENT OF HCAB

N-Terminal amino acid residue	Total amount of free amino groups (mmol)
Ala-126	3.32 ± 0.10
Ser-115	3.04 ± 0.03
Glu-106	3.04 ± 0.05

TABLE 3

AMINO ACID COMPOSITION OF PURIFIED SYNTHETIC PEPTIDE

Amino acid	Number of residues expected	Number of residues found
Lys	1	0.96
His	6	6.06
Asp	3	3.00
Thr	2	1.81
Ser	4	3.38
Glu	4	4.45
Gly	3	3.22
Ala	3	2.89
Val	3	3.10
Leu	1	0.93
Tyr[a]	1	0.52
Phe	3	2.96

[a] The low value of Tyr found is believed to be due to oxidation of its side chain during acid hydrolysis.

column of Sephadex SEC-25. The peptide eluted by the pH 8.0 Tris buffer was collected and lyophilized (Fig. 2). The partially purified peptide was rechromatographed on the same cation exchanger and eluted by MES buffers of increasing pH values. The elution pattern is shown in Fig. 3. A solution of the peptide eluted by pH 7 buffer was collected, desalted, and lyophilized. This material was used in all subsequent experiments.

As indicated in Table 1, the overall yield of the target peptide is 1.51%, uncorrected for the removal of analytical samples, etc.

Characterization of the Synthetic Peptide

The purified peptide had the expected overall amino acid composition (Table 3), and carboxypeptidase A digestion indicated that alanine was the C-terminal residue (Table 4) as anticipated. Four cycles of Edman degradation gave results consistent with the

TABLE 4

RESULTS ON CARBOXYL-TERMINAL DETERMINATION ON THE PURIFIED SYNTHETIC PEPTIDE

Amino acid	Amount (nmol)			
	0 hr	0.5 hr	1 hr	3 hr
Lys	0.09	0.03	0.05	0.02
His	0.16	0.14	0.17	0.22
Ser	0.31	0.53	1.24	2.04
Ala	1.21	1.62	2.12	2.75
Val	0.14	0.21	0.09	0.14
Leu	0.22	0.17	0.08	0.15

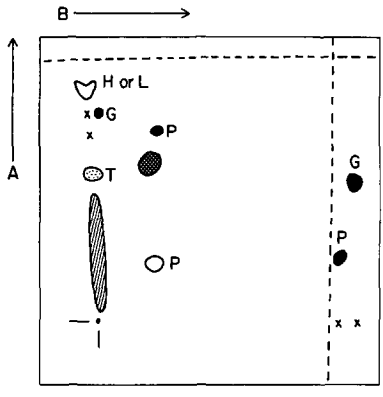


FIG. 4. Bidimensional thin-layer chromatography on a polyamide sheet of the dansyl derivative obtained from the purified peptide. Standard dansyl amino acids (●) used were: N^{α}-His (H); N^{ϵ}-Lys (L); Glu (G); and Phe (P). (◍) blue (dansyl-OH); (◎) pink (*O*-dansyl tyrosine); all other spots are green. (◉) Dansyl-NH_2. Solvent; (A) H_2O:88% formic acid (100:1.5, v/v); (B) benzene:acetic acid (9:1, v/v). The sample was applied at the lower left corner.

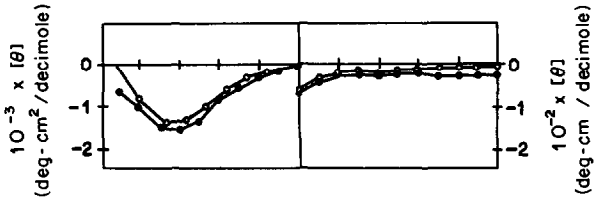


FIG. 5. Circular dichroism spectra of the purified synthetic peptide (●) and of the synthetic peptide incubated with Co(II) (○). The solvent was a pH 7.6 buffer containing 0.025 *M* Tris and 0.05 *M* Na_2SO_4. θ is the molecular ellipticity.

sequence. The bidimensional thin-layer chromatography of dansyl derivatives of the purified peptide is shown in Fig. 4. In agreement with the Edman degradation results, it is seen that the N-terminal residue for the purified peptide is phenylalanine. The pattern seen on cellulose acetate gel electrophoresis showed a single spot, indicating that the purified peptide is homogeneous.

Figure 5 shows the circular dichroism (CD) spectra of the synthetic peptide in the absence and presence of Co(II). The trough near 217 nm is an indication of the presence of β structure. The CD spectrum of the synthetic peptide does not change appreciably after incubation with Co(II).

Co(II) Binding Studies

The difference in concentration of Co(II) in the two compartments of the equilibrium dialysis cell gives the concentration of the Co(II)–peptide complex. From the known initial concentration of the peptide, the apparent dissociation constant (K_{app}) of the Co(II)–peptide complex was calculated according to Eq. (3), where $\Delta[\text{Co(II)}] = [\text{Co(II)}]_{\text{side 1}} - [\text{Co(II)}]_{\text{side 2}}$.

$$K_{app} = \frac{[\text{Co(II)}]_{\text{side 2}} \times ([\text{Peptide}]_o - \Delta[\text{Co(II)}])}{\Delta[\text{Co(II)}]} \tag{3}$$

TABLE 5

APPARENT DISSOCIATION CONSTANT OF THE Co(II)–PEPTIDE COMPLEX

Peptide concentration ($\times 10^5$) (M)	Co(II) concentration ($\times 10^5$) (M)			K_{app}[b] ($\times 10^5$) (M)
	Initial Co(II)	Side 1[a,b]	Side 2[b,c]	
3.1	4.25	2.05 (2.47)	1.48 (1.78)	6.5 (6.2)
2.5	3.40	1.36 (1.91)	1.06 (1.49)	7.6 (7.4)

[a] Peptide compartment.

[b] Values in parentheses were corrected for the drifting in atomic absorption peak intensities.

[c] Co(II) compartment.

The results of the binding experiments are summarized in Table 5. The atomic absorption peak intensities were found to drift with time so that addition of the concentrations of Co(II) on either side of the equilibrium dialysis cell (as read from a calibration curve determined before the measurements) did not give good correspondence to the values of the initial Co(II) concentrations given in column 3 of the Table. The values corrected for this drift are shown in parentheses. It can be seen that the values of K_{app} (ca. 7×10^{-5} M) are not much affected by the drift in the atomic absorption peak intensities.

The Donnan effects in our equilibrium dialysis experiments can be assumed to be negligible [cf. Ref. (*25*)]. The atomic absorption peak intensities of Co(II) were not affected by the presence of peptides.

Activity Measurements

The results on the sultone hydrolysis and CO_2 hydration experiments are shown in Tables 6 and 7, respectively. The synthetic peptide in the presence or absence of Co(II)

TABLE 6

HYDROLYSIS OF 5-NITRO-2-HYDROXY-α-TOLUENESULFONIC ACID SULTONE

Reaction conditions[a]	$k_{obs} \times 10^4$ (sec^{-1})
Spontaneous[b]	7.29 ± 0.04
Co(II)[c]	7.80 ± 0.11
Peptide[d]	7.75 ± 0.09
Peptide + Co(II)	7.80 ± 0.07

[a] Kinetics measured in 0.025 M Tris containing 0.05 M Na_2SO_4, pH 7.6.

[b] $k_{enz} = (2.33 \pm 0.10) \times 10^{-2}$ sec^{-1} at an enzyme concentration of 0.93×10^{-7} M

[c] Co(II) $= 1 \times 10^{-3}$ M.

[d] Peptide $= 0.8 \times 10^{-4}$ M.

TABLE 7

HYDRATION OF CARBON DIOXIDE

Reaction conditions[a]	$k_{obs} \times 10^2$ (sec^{-1})
Spontaneous[b]	4.85 ± 0.30
Co(II)[c]	4.66 ± 0.04
Peptide[d]	4.92 ± 0.04
Peptide + Co(II)	4.79 ± 0.12

[a] Kinetics measured in 0.025 *M* Tris containing 0.05 *M* Na_2SO_4, pH 7.6.
[b] k_{enz} = (3.36 ± 0.03) sec^{-1} at an enzyme concentration of 0.47×10^{-7} *M*.
[c] Co(II) = 0.85×10^{-4} *M*.
[d] Peptide = 0.9×10^{-5} *M*.

possesses little, if any, catalytic activity. As will be discussed later, a maximum activity of only about 0.0002% of that of the native enzyme could be seen for the peptide. This value falls within the experimental error.

DISCUSSION

Solid Phase Synthesis of the Carbonic Anhydrase Peptide Fragment

At the present stage of development of solid phase peptide synthesis, complete reaction throughout all cycles in a synthesis cannot generally be achieved (*1*). Repeated coupling using carbodiimide to ensure more complete reaction was first suggested by Weygand *et al.* (*26*) and was employed at several stages of our work. In our preparation of a carbonic anhydrase fragment, considerable difficulty was encountered toward the end of the synthesis where steric hindrance became especially important due to the presence of three bulky phenylalanine residues (phenylalanines 91, 93, and 95 in the HCAB sequence) and two histidine residues with bulky imidazole-protecting tosyl groups (histidines 94 and 96) in close proximity to each other.

Purification and Yield in the Synthesis

Purification of crude products obtained by solid phase peptide synthesis is indispensable. Merrifield (*27*) has pointed out that, "even with ideal conditions and very nearly quantitative yields at every step, it is too much to expect and has never been claimed, that pure products will be obtained directly." Initially, we tried to purify our crude products by isoelectric focusing (*28*). This proved to be unsatisfactory due to precipitation of the peptide at the isoelectric point. Therefore, we resorted to the relatively simple but often used technique of ion-exchange chromatography for purification.

The synthetic peptide contains one lysine, one aspartic acid, three glutamic acids, six histidine residues, together with a free amino and a free carboxyl group. All other amino acid residues in the peptide have neutral side chains. Based on the acid dissociation

constants of the above-mentioned amino acid residues (*29*) and applying the Henderson–Hasselbach equation [Eq. (4)], where (B)/(A) is the ratio of the concentrations of the conjugate base B and conjugate acid A of the ionizable amino acid side chains (or of the free amino or carboxyl groups) at a given pH, we have estimated that the isoelectric point, p*I*, of the carbonic anhydrase fragment we have prepared lies between 6 and 7. This forms the basis of the purification procedure we employed. Columns containing the cation exchangers Sephadex SEC-25 or SPC-25 were used. At the isoelectric point, the peptides, having a net charge of zero, should be displaced from the ion-exchange column by the eluting solvent. The material eluting at pH 7 (peaks marked with an arrow in the elution patterns shown in Figs. 2 and 3) should therefore possess the correct isoelectric point and seems likely to correspond to the desired peptide in the purified form. We used 4 *M* urea in all eluting buffers to ensure solubility of the peptide, especially at its isoelectric point.

$$\mathrm{pH} = \mathrm{p}K_a + \log (\mathrm{B})/(\mathrm{A}) \tag{4}$$

The overall yield of the crude product we obtained is 28%. This low yield, which is not unusual for solid phase peptide synthesis, can be explained by the following points: (i) repeated removal of samples for ninhydrin color tests; (ii) manipulations in HF cleavage resulting in a 5–15% loss in general; (iii) introduction of an acetylation step after a few unsuccessful recouplings; and (iv) acidolytic cleavage of the benzyl ester linkage joining peptide chain and the polymeric backbone during repeated treatments with 50% TFA in deprotection steps. The last possibility is likely to account for the major loss during solid phase synthesis, as has been reported by many authors [e.g., Ref. (*31*)]. If we correct for the losses due to the first two causes, we estimate that the overall yield of the crude polymer-bound product was actually about 40%. The synthetic peptide had the expected overall amino acid composition and was homogeneous as judged by cellulose acetate gel electrophoresis. Dansylation, Edman degradation, and C-terminal determination all gave the expected results.

With substantial additional effort, improvements in the synthesis of our carbonic anhydrase fragment could undoubtedly be made and preparative results of a quality comparable to those achieved with bovine pancreatic trypsin inhibitor (Kunitz) (*8*, *31*) might be obtained. However, the objective of the present work was to prepare a sufficient amount of the peptide corresponding to residues 91–126 of the HCAB sequence to test its metal-binding and catalytic properties, and this goal was attained.

Sensitivity of the Co(II)-Binding Experiments and the Activity Measurements

The Co(II)-binding experiments utilizing atomic absorption measurements gave only a rough estimate of the apparent dissociation or stability constants. The error, however, is not likely to exceed 50% (estimated from variations in atomic absorption peak heights, various dilution processes, a possible 5% diffusion of peptide across the acetylated membrane, and the accuracy of the calibration curves). The results in Table 5 were uncorrected for binding of Co(II) by anionic species (Tris and Cl^-) present in the buffer. These anions competed with the peptide for Co(II), thereby decreasing the effective concentration of free metal ions in solution. In an analogous determination (*32*) of the stability constants of various metal ion–carboxypeptidase A (CPA) complexes in a buffer containing 0.05 *M* Tris and 1.0 *M* NaCl, a correction for the

binding of anionic buffer species with metal ions raised the stability constant of the CPA–Co(II) complex from $10^{5.8}$ (apparent stability constant) to 10^7. At these concentrations of Tris and NaCl the correction in the case of Co(II) thus amounts to approximately an order of magnitude. In our case, we used a buffer containing only 0.027 *M* Tris and 0.4 *M* Cl^-. The corrected value of the Co(II)–peptide complex should thus be equal to or less than 10^5 (the uncorrected value is approximately $10^{4.2}$).

The reason for our choice of the carbonic anhydrase fragment prepared and studied in the present investigation has been briefly touched upon in the Introduction. Here we

TABLE 8

LIMIT OF SENSITIVITY IN THE ACTIVITY MEASUREMENTS FOR THE SYNTHETIC PEPTIDE

	Hydrolysis of sultone	CO_2 hydration
(Enzyme)	0.93×10^{-7} *M*	0.47×10^{-7} *M*
(Peptide)	0.8×10^{-4} *M*	0.8×10^{-5} *M*
k_{enz}	2.3×10^{-2} sec^{-1}	3.3 sec^{-1}
$k_{spont.}$	7.3×10^{-4} sec^{-1}	4.9×10^{-2} sec^{-1}
$k_{enz\ (corrected)}$	20 sec^{-1}[a]	5.7×10^{-2} sec^{-1}[b]
Sensitivity limit	0.0006%	0.0013%
max k_{obs} for Co(II)–peptide complex	7.8×10^{-4} sec^{-1}	4.79×10^{-2} sec^{-1}
Enzymatic activity observed (%)[c, d]	0.00025%	0.0002%

[a] Corrected to an enzyme concentration of 0.8×10^{-4} *M*.

[b] Corrected to an enzyme concentration of 0.8×10^{-5} *M*.

[c] Calculated from the maximum k_{obs} for Co(II)–peptide complex and $k_{enz\ (corrected)}$.

[d] The catalytic activities of bovine carbonic anhydrase and the human carbonic anhydrases do not differ sufficiently to affect significantly the conclusions drawn (*23*).

wish to comment on the limit of sensitivity in the measurement of the catalytic activity of the synthetic peptide and its complex with Co(II). From the data in Tables 6 and 7, an activity of about 15% above the spontaneous reaction rates (i.e., a value of k_{obs} at or greater than 9.1×10^{-4} sec^{-1} in sultone hydrolysis or 5.6×10^{-2} sec^{-1} in CO_2 hydration) would fall outside the range of experimental error and therefore could be confidently taken as an indication of activity for the synthetic peptide–Co(II) complexes. The sensitivity limits (relative to k_{enz}, corrected to an enzyme concentration equal to that of the peptide) are shown in Table 8. We see from this table that in the case of carbonic anhydrase the activity tests are very sensitive and as little as 0.0006% activity relative to the enzyme could have been detected for the peptide and its Co(II) complex. In the cases of many other enzymes, comparable sensitivity in assaying the activity of models cannot be achieved.

Table 8 also shows that a maximum activity of only about 0.0002% could be seen at a peptide concentration of 0.8×10^{-4} *M* in the hydrolysis of the sultone and of $0.8 \times$

10^{-5} *M* in the CO_2 hydration experiments. This value, however, falls within the range of experimental error. We can, therefore, conclude that the synthetic peptide and its Co(II) complex possess little, if any, significant carbonic anhydrase activity.

The Significance of the Results on Co(II)-Binding Experiments

The apparent association constant of the Co(II)–peptide complex (10^5) is certainly not very impressive when compared to that of the Co(II)–carbonic anhydrase complex (approximately 10^9). [The apparent stability constant (*3*) is 10^7 at pH 5.5; at pH 7.2, the value is likely to be higher by about two orders of magnitude.] It is, however, more comparable to that of the Co(II) CPA (10^7 at pH 7) (*32*).

There are two possible explanations for the weak binding of our peptide to Co(II). (i) In our calculation of the apparent dissociation constant, we have assumed that the peptide in aqueous solution exists in only one conformation. From the CD spectra, we see that peptide in aqueous solution appears to possess some β-sheet structure. In fact, this peptide fragment itself constitutes a part of the twisted β-sheet structure in carbonic anhydrase. If, in aqueous solution, the peptide exists as an equilibrated mixture of the ordered (P_β, with β-sheet structure) and random species (P_r), and if only P_β binds well to Co(II), we have the situation represented by Eq. (5).

$$P_r \underset{}{\overset{K}{\rightleftharpoons}} P_\beta \underset{K'}{\overset{\text{Co(II)}}{\rightleftharpoons}} P_\beta - \text{Co(II)} \tag{5}$$

In Eq. (5), K' is the actual dissociation constant for the P_β form of the peptide complexed to Co(II) and K is the ratio P_β/P_r. At equilibrium Eq. (6) would hold where P_o = the total peptide concentration.

$$K' = \frac{[\text{Co(II)}]_{\text{side 2}} \times \{K([\text{Peptide}]_o - \Delta[\text{Co(II)}])\}}{\Delta[\text{Co(II)}]\,(1 + K)} \tag{6}$$

Therefore, if $K \ll 1$ (i.e., the peptide exists mainly in a random conformation), the value of K', the dissociation constant for the form of the peptide actually binding Co(II), would be significantly smaller than the value of K_{app} calculated from Eq. (3). We don't have a way of accurately measuring K for our peptide, but that fact that the CD spectrum of the peptide is almost the same in the absence and presence of Co(II) indicates that the equilibrium between P_r and P_β is not much perturbed by Co(II), which suggests that K cannot be very small. Thus, it seems that, even if we take the equilibrium between the random and ordered forms of our peptide into account, the stability constant of the peptide–Co(II) complex would at best be around 10^6, which is still about 10^3 times smaller than the value in the case of the native enzyme–Co(II) complex. This amounts to a difference of 4 kcal/mol in the free energy of binding.

(ii) The difference in free energy of binding can be explained in terms of the loss of entropy upon binding. We observe that, in carbonic anhydrase, the metal-binding ligands are rigidly fixed by a twisted β-sheet structure consisting of about 10 β fragments. The loss in entropy upon binding of the metal ion to the apoenzyme is undoubtedly much smaller than that with our synthetic peptide, which is unlikely to possess a very rigid structure. The flexibility of the synthetic peptide would be expected to result in an unfavorable increase in the entropy of binding.

Design of Polypeptide Model Enzymatic Catalysts

The synthesis of polypeptides as models for enzymatic catalysts could be a very powerful tool in the elucidation of the structure–function relationships of peptides and proteins and the nature of enzymatic catalysis. The development of classical peptide synthesis provided the necessary background for this approach. However, the classical methods of peptide synthesis have certain serious shortcomings, notably the large work load, the low yields, and the solubility problems encountered with large- or even medium-sized peptides. Not surprisingly, much of the earlier work employing peptide synthesis in the study of structure–function relationships has concentrated on small peptide hormones. In order to serve as a viable enzyme model, we believe that a polypeptide should contain at least 30 amino acid residues, as our model building suggests that this is probably the minimum size required to maintain stable conformations of the active and binding sites which are essential for effective enzymatic catalysis. The synthesis of polypeptides of this size by classical methods is a far from trivial task. Furthermore, at present, the state of our knowledge on the correlation of peptide primary structure and tertiary structure is still not developed enough for us to design with a high degree of confidence useful polypeptide models of enzymatic catalysts. Therefore, as far as we are aware, there have not been any truly serious attempts to synthesize polypeptide enzyme models by classical solution methods.

Merrifield's solid phase method (*1*), however, has made it much more reasonable to consider the synthesis of polypeptides as enzyme models. In favorable instances, solid phase peptide synthesis can proceed rapidly and can require much less tedious work than what is commonly required in solution phase synthesis. In several cases, reasonably pure polypeptides containing 30 to 60 amino acid residues have been synthesized with relative ease by this method (*8*, *31*, *33*, *35*). A very high standard of purity is demanded for synthetic polypeptides used for the studies of structure–function relationships of peptide hormones, as the truncated and failure sequences which are contaminants might be highly potent biologically, and this could obscure the interpretation of activity measurements. This is not likely to be a serious problem with polypeptides designed as enzyme models, as it is improbable that failure or truncated sequences produced in the preparation of the target molecules would be "superactive" catalytically or in binding. In view of this, solid phase peptide synthesis, in conjunction with currently available methods of predicting protein conformations (*35*, *36*), as well as comparative studies on protein structure and peptide fragment complementation/reconstitution may provide viable approaches to the development of effective polypeptide enzyme models.

Several alternative directions can be taken in the design of polypeptide model enzyme catalysts. One can base the model on the X-ray crystallographic structure of the intact protein molecule and "cut away" pieces that seem redundant, synthesizing a truncated polypeptide that consists of only the "essential" portions of the molecule.

Another approach is to start with the active core of the enzyme, extending the polypeptide chains to build a better model, possibly with the aid of theoretical predictions of conformations, the X-ray crystallographic structure of the target molecule, and of peptide complementation studies. Many different laboratories have reported the synthesis of active site fragments of various enzymes, but the results have

been very disappointing. For example, a pentadecapeptide active site fragment of rabbit muscle triosephosphate isomerase which was prepared by Channabasavaiah and Sivanandaiah (*37*) was devoid of catalytic activity. Syntheses of active site-containing fragments of lysozyme (*38*), HCAB (*39*) and serine proteinases (*40*, *41*) all gave rise to materials showing none of the catalytic activity of the corresponding native enzyme. Along somewhat different lines, a cyclic heptadecapeptide model of bovine pancreatic trypsin inhibitor has been synthesized in our laboratory (*31*). Although this model was found to bind strongly to trypsin, the association constant measured for its complex with trypsin is still 10^8 times smaller than that for the complex formed between the natural inhibitor and trypsin.

At this stage, the choice of the target enzyme will be very important in order to ensure success in the design of models of enzymatic catalysts. As indicated earlier, our current knowledge concerning the factors determining peptide and protein structure is still too primitive to permit us to do "sophisticated" designing. It seems likely that the first few models designed will have low enzymatic activity. This suggests that the target enzyme to be modeled should be one having a very sensitive and readily interpretable assay method, allowing the facile assessment of the effectiveness of the model. It would also be very helpful if the mechanism of action of the target enzyme were as simple as possible. In carbonic anhydrase, the various proposed mechanisms of action require only the presence of the metal-binding ligands, a binding cavity, and some means for proton transfer to be accomplished (*42*). It appears to us that the structural requirements in the active site of this very active enzyme may be less stringent than those in the active sites of many other enzymes which one might otherwise consider modeling.

Based on our results with the peptide fragment described in this paper, we believe that the next step in designing a carbonic anhydrase model should be preparation of a peptide capable of binding the active site metal ion more tightly. This probably can be achieved by synthesizing a sequence containing more β structure in order to increase the rigidity of the peptide. A possible candidate polypeptide is the fragment comprising residues 91–146 of HCAB. The synthesis of this peptide is currently underway in our laboratory.

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