Conformational Studies on the β Subunits of Human Hemoglobin and Their Arginyl-COOH Peptides†

Clara Fronticelli Bucci* and Enrico Bucci

ABSTRACT: The β subunits of hemoglobin upon alkylation of the cysteinyl residues with iodoacetamide showed a sedimentation velocity with an $s_{20,w}$ near 1.8 as for monomeric subunits. They reacted with α chains to give a tetrameric hemoglobin with a sedimentation constant near 4.4. Their CD spectrum was indistinguishable from that of untreated β chains below 270 nm, otherwise they showed some deviation that became pronounced in the Soret region, where the optical activity of the alkylated subunits was definitely lower than that of the native subunits. Upon removal of the heme the apo-β subunits showed a decreased optical activity in the far-uv region of the spectrum indicating a substantial loss of helical content. Their sedimentation behavior was consistent with the presence of large aggregates, which dissociates into monomers upon reconstitution with cyanoheme. The apo-β subunits could be renatured from 6 *M* guanidine hydrochloride. They showed a stoichiometric reaction with the heme in the molar ratio 1:1. Upon reconstitution with the heme their optical activity became similar to that of the native β chains in the far-uv region of the spectrum, but remained lower in the near-uv and Soret regions. After acylation of the lysyl residues with citraconic anhydride the apo-β subunits were digested with trypsin and the arginyl-COOH peptides β(1–30), β(31–40), β(41–104), and β(105–146) were separated by gel chromatography. With the exception of the peptide β(105–146), which was insoluble at neutral pH, the sedimentation behavior of the other peptides showed the presence of small polymers. The sedimentation behavior of the peptide β(31–40) was not tested. The percentage of α helix, β conformation, and of random coil (or unordered structure) of the various proteins and peptides was measured fitting their CD spectra in the far-uv region with the parameter published by Y. H. Chen et al. ((1974), *Biochemistry 13,* 3350) and by N. Greenfield and G. D. Fasman ((1969), *Biochemistry 8,* 4108). In this way the helical content of the native and reconstituted alkylated β subunits appeared to be near 76%, a value very near to that present in the same subunits in the hemoglobin crystal. The helical content of the apo-β subunits in 0.04 *M* borate buffer at pH 9.6 decreased to a value near 45%. The helical content of the isolated peptides in electrolyte solutions was in any case near 10% indicating an almost complete loss of the structure that they have in the hemoglobin crystal. Cyanoheme reacted with the peptide β(41–104), however, the reaction was not stoichiometric indicating a low affinity of the heme for the peptide. With the exception of the peptide β(31–104), all of the other peptides recovered some of their helical structure when dissolved in 50% methanol. Notably also the apo-β subunits did so suggesting that the loss of structure upon the removal of the heme could be in part due to the exposure of the heme pocket to water.

The study of the physicochemical, functional, and immunological properties of large fragments of proteins has been used by several authors in order to obtain clues to the nature of the forces that transform a sequence of amino acids into a biologically active protein (Atassi and Singhal, 1970; Brown and Klee, 1971; Crumpton, 1968; Epand and Scheraga, 1968; Hermans and Puett, 1971; Scheraga, 1971; Taniuchi and Anfinsen, 1969, 1971; Toniolo et al., 1974). Similar studies conducted on the apohemoglobin subunits and on fragments of it might also contribute to our understanding of this problem. A detailed knowledge of the structure and function of hemoglobin is already available. Apohemoglobin is a well-characterized protein and its interaction with heme has been extensively investigated (see Antonini and Brunori, 1971). Also, spontaneous mutations are available which offer the possibility of testing the effect of a single amino acid substitution on the structure of the entire protein and of the "mutant" fragments. Quaternary structure is present in the system, and the interactions among the subunits affect both the structure and function of hemoglobin.

Yip et al. (1972) have recently conducted studies on the conformation and interaction of the isolated subunits of hemoglobin with and without heme. It is our intention to continue that line of investigation and extend it to large fragments of those subunits.

In this report the physicochemical properties of the β subunits of hemoglobin, after alkylation of the SH groups with iodoacetamide, have been investigated. The properties of the apoprotein and of the arginyl-COOH peptides which can be obtained from the βIAA[1] chains were also studied. Alkylation of the SH groups was necessary in order to prevent the formation of intermolecular bonds.

Materials and Methods

Human hemoglobin was prepared from fresh washed red cells hemolyzed with toluene (Drabkin, 1946).

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[1] Abbreviations used are: apo-β chains, native apoprotein obtained from β subunits; apo-βIAA, apoprotein obtained from the βIAA subunits; βIAA, β subunits of hemoglobin alkylated with iodoacetamide; Gdn-HCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetate; Mec, exponential average molecular weight; MRW, mean residue weight; PMB, *p*-chloromercuribenzoate.

Hemoglobin subunits were prepared in the carboxy form, by the method of Bucci and Fronticelli (1965). Regeneration of the sulfhydryl groups in the β chains was accomplished by the procedure of Waks et al. (1973). For the α chains the SH groups were restored by shaking the solution with *N*-dodenecathiol as described by De Renzo et al. (1967). Titration with *p*-chloromercuribenzoate (Boyer, 1954) indicated that 95% or more of the SH groups was restored.

Alkylation of the β subunits was performed in 0.1 *M* sodium phosphate buffer (pH 7.0). The iodoacetamide concentration was 50 molar excess over that of cysteine. After allowing the reaction to proceed for 90 min in the dark at room temperature, it was stopped by pouring the solution into acid acetone as described for the heme removal. Titration with PMB (Boyer, 1954) demonstrates that at least 98% of the SH groups was alkylated. Amino acid analysis showed that only cysteinyl residues were reacted with iodoacetamide. A complete alkylation was not apparent, probably due to the cyclization of the alkylated residues during the analytical procedure (Bradbury and Smyth, 1973).

Removal of the heme was carried out at room temperature, by slowly pouring the protein into a solution of 2% HCl in acetone (Clegg et al., 1966). The protein was collected and dialyzed extensively against water and stored in the freezer.

Addition of heme to apo-βIAA in 0.04 *M* borate (pH 9.3) and to the peptide β(41–104) in 0.1 *M* phosphate (pH 8.0) was done as described by Rossi Fanelli et al. (1958).

Citraconylation of the apo-β subunits was performed in 0.1 *M* phosphate buffer at pH 8.1. To the magnetically stirred protein solution, several aliquots of citraconic anhydride were added up to 150 molar excess over the lysyl residues. The pH of the solution was kept constant by addition of 5 *M* NaOH, using a Radiometer automatic titrator. At the end of the reaction (about 3–4 hr) the protein was dialyzed extensively against 0.5% ammonium bicarbonate at pH 9.8.

Tryptic digestion of the citraconylated protein was carried out at 38° in 0.5% ammonium bicarbonate at pH 9.8 for a period of 4 hr. Trypsin was added in two equal portions 1 hr apart. The final protein to trypsin ratio was 50:1 in weight. The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma Chemical Co. type 1-S) in a weight ratio 2:1 to the trypsin. Since all the lysyl residues were acylated, digestion occurred only at the peptide bond formed by the arginyl residues in positions β30, β40, and β104.

Removal of the masking groups from citraconyl peptides was carried out in 0.1 *N* HCl at room temperature over a 24-hr period. The deacylation was tested using: the reaction with tetranitrobenzenesulfonate (Habeeb, 1966) for lysyl residues; the reaction with hydroxylamine (Riordon and Vallee, 1964) for tyrosyl residues; and the reaction with $FeCl_3$ (Habeeb and Atassi, 1969) for seryl and threonyl residues. In all cases the deacylation appeared complete.

Protein concentration was measured spectrophotometrically for the various hemoproteins on a heme basis using the following extinction coefficient: 1.4×10^4 at 540 nm and 1.95×10^5 at 420 nm for the carboxy derivatives, 1.1×10^4 at 540 nm for the cyano derivatives. For hemin a value $\epsilon = 5 \times 10^4$ at 385 nm was used (Gibson and Antonini, 1963). The concentrations of apo-βIAA and of the peptides were measured by quantitative amino acid analysis on the basis of the yield of acidic and neutral amino acids.

Spectrophotometric measurements were performed with a Cary Model 14 spectrophotometer.

Circular dichroism (CD) measurements were performed with a Cary Model 60 spectropolarimeter with a 6001 CD attachment. All samples were filtered through a Millipore 0.45-μ filter before scanning. Ellipticity values are presented on a mean residue weight basis.

Measurements of Sedimentation Velocity and Equilibrium. These experiments were performed with a Beckman Model E ultracentrifuge. For the sedimentation velocity schlieren optics were used, while for the equilibrium experiments the interference system was adopted.

In equilibrium experiments the fringe displacement with the radial distance was fit with an exponential that allowed the estimation of the exponential average molecular weight "Mec"[2] over the concentration span of the solute in the liquid column.

The fractions of α helix, β conformation, and random coil present in the various polypeptides were calculated following the procedure outlined by Chen et al. (1974). At any wavelength

$$[\theta]^{MRW} = f_h X_h + f_\beta X_\beta + f_r X_r$$

in which $[\theta]^{MRW}$ is the overall mean residue ellipticity, X_h is the $[\theta]^{MRW}$ of a peptide 100% in α-helical conformation, and in similar way X_β and X_r are $[\theta]^{MRW}$ of the 100% β conformation and random coil, respectively; finally f_h, f_β, and f_r are the fractions of α helix, β conformation, and random coil of the polypeptide investigated. The X values were those published by Chen et al. (1974) or by Greenfield and Fasman (1969). The set of simultaneous equations was solved using the Marquardt algorithm (1963).

Urea and iodoacetamide were obtained from Sigma Chemical Co. Urea was purified by passage through a mixed bed resin column. Iodoacetamide was purified by recrystallization from hot water. Gdn·HCl used for CD measurements was from Schwarz/Mann Chem. Co. Citraconic anhydride obtained from Baker was purified by distillation under reduced pressure. All other reagents were analytical grade or better and used without further purification.

Results

Preparation of the Arginyl Peptides of the Alkylated β Chains. The following procedure was carried out on 100–300 mg of βIAA chains. The heme was removed as described, and the apoprotein was extensively dialyzed against water. The protein was carefully collected from the dialysis bag, and resuspended in 0.1 *M* phosphate (pH 8.1). Citraconylation was performed as described, and during this treatment the protein redissolved completely. After extensive dialysis against 0.5% ammonium bicarbonate (pH 9.8), the tryptic digestion was performed, as described. The peptide mixture so obtained was lyophilized, redissolved in 2–5 ml of 0.5% ammonium bicarbonate at pH 9.3, and filtered through a Sephadex G-50 column.

Figure 1 shows the elution profile obtained monitoring the optical density of the effluent as described in Figure 1.

In order to restore the acylated residues and resolve the peptides contained in the third peak, this fraction was lyophilized twice, dissolved in 2–5 ml of 0.1 *M* HCl, and let

[2] Mec is a quantity close to the classic average Mwc = $(c_a - c_b)/AC^0(r_a^2 - r_b^2)$ where a and b represent the meniscus and bottom of the cell, respectively, and C^0 is the initial concentration of the protein. Also, $A = \omega^2(1 - \bar{V}\rho)/2RT$ (E. Bucci, in preparation).

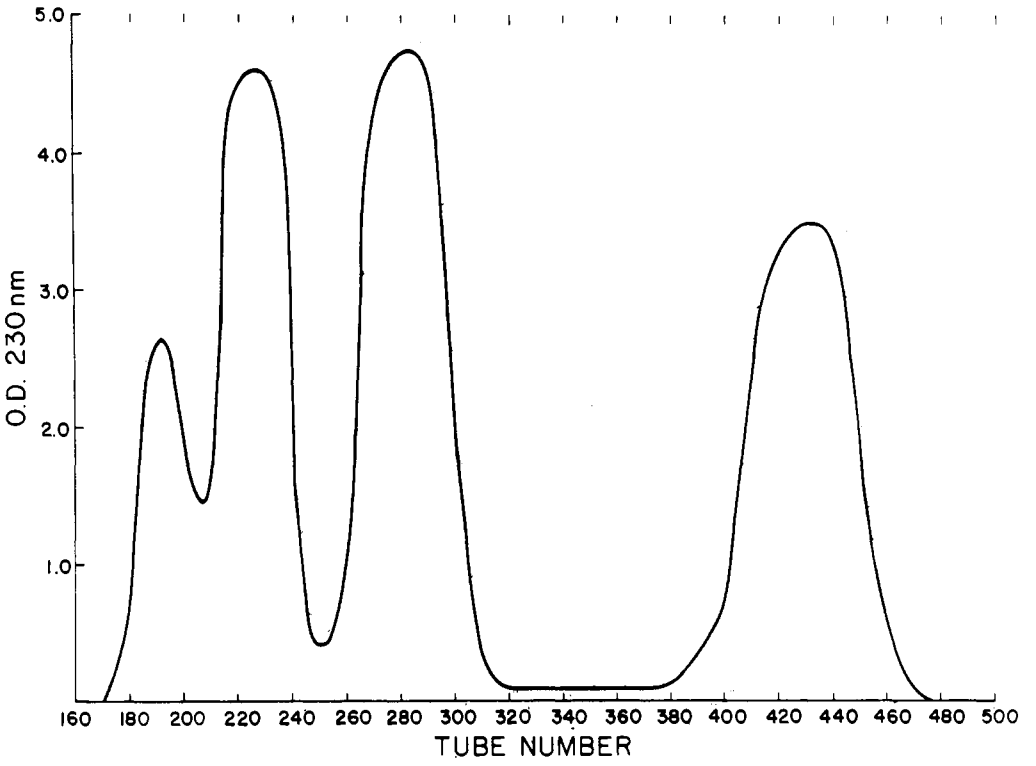

FIGURE 1: Chromatographic resolution at 4° of the arginyl-COOH peptides on Sephadex G-50 fine, eluted with 0.5% ammonium bicarbonate at pH 9.3. Column dimensions 5 × 100 cm, elution rate 20 ml/hr, fractions of 3 ml each. The optical density at 230 nm of the various fractions is plotted against the tube number. From the left, the first peak contained various complexes of trypsin, trypsin inhibitor, and partially digested β chains, the second peak contained the peptide β(41–104), the third peak contained a mixture of the peptides β(1–30) and β(105–146), the fourth peak contained the peptide β(31–40).

Table I: Amino Acid Composition of the β Arginyl-COOH Peptides.

	1–30		31–40		41–104		105–146	
	Theory	Found	Theory	Found	Theory	Found	Theory	Found
CmCys[b]	0	0.00	0	0.00	1	0.786	1	0.77
Asp	2	2.06	0	0.03	9	8.98	2	2.01
Thr	2	1.83	1	0.93	3	2.82	1	0.806
Ser	1	0.878	0	0.02	4	3.71	0	0.03
Glu	4	4.14	1	1.14	3	3.16	3	3.15
Pro	1	0.93	1	*a*	3	2.80	2	1.86
Gly	4	4.09	0	0.03	6	5.97	3	2.98
Ala	3	3.02	0	0.01	5	4.89	7	6.79
Val	5	4.54	2	1.96	4	3.85	7	6.16
Met	0	0.00	0	0.00	1	0.97	0	0.00
Leu	3	3.23	2	1.99	8	8.15	5	4.94
Tyr	0	0.00	1	0.94	0	0.00	2	2.01
Phe	0	0.00	0	0.02	6	5.87	2	1.94
Lys	1	1.02	0	0.02	2	2.08	3	2.98
His	1	0.60	0	0.01	1	0.90	4	4.02
Arg	1	1.02	1	*a*	1	1.04	1	0.98
Trp	1	*a*	1	*a*	0	*a*	0	*a*

[a] Not calculated. [b] Carboxymethylcysteine.

stand for 24 hr. The sample was then filtered on a Bio-Gel P10 column as described in Figure 2. To further purify the peptide β(41–104) and restore its acylated residues, it was twice lyophilized, then dissolved in 0.1 *M* HCl, let stand for 24 hr, and chromatographed on a column, 2.5 × 30 cm, of Sephadex G-25 fine, equilibrated with 0.01 *M* acetic acid. The eluted fractions were analyzed spectrophotometrically for the presence of tryptophan in order to eliminate residual β chains present in small amounts in the initial fractions of the peptide.

The purity of the peptides was tested by amino acid analysis. Table I shows the amino acid composition of each of the peptides.

Optical Activity of the βIAA Chains and of Their Apoprotein. In the far-uv region of the spectrum, shown in Figure 3, the βIAA chain had the same optical activity as the native subunits. Upon removal of the heme the optical activity decreased and was partially restored by addition of heme at alkaline pH. When this material was dialyzed against neutral pH some protein precipitated and the super-

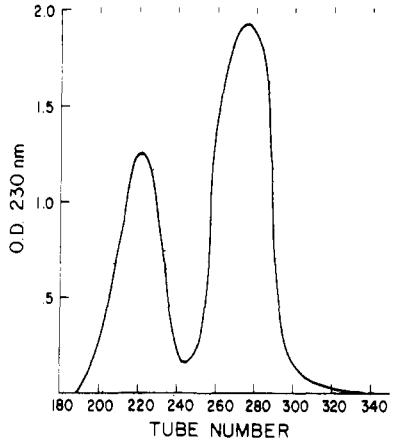

FIGURE 2: Chromatographic resolution at 4° of the peptides β(1-30) and β(105-146) on Bio-Gel P10, 100-200 mesh, eluted with 0.1 *M* acetic acid. Column dimensions 5 × 100 cm, elution rate 10 ml/hr, fractions of 3 ml each. The optical density at 230 nm of the various fractions is plotted against the tube number. From the left, the first peak contained the peptide β(105-146) and the second one the peptide β(1-30).

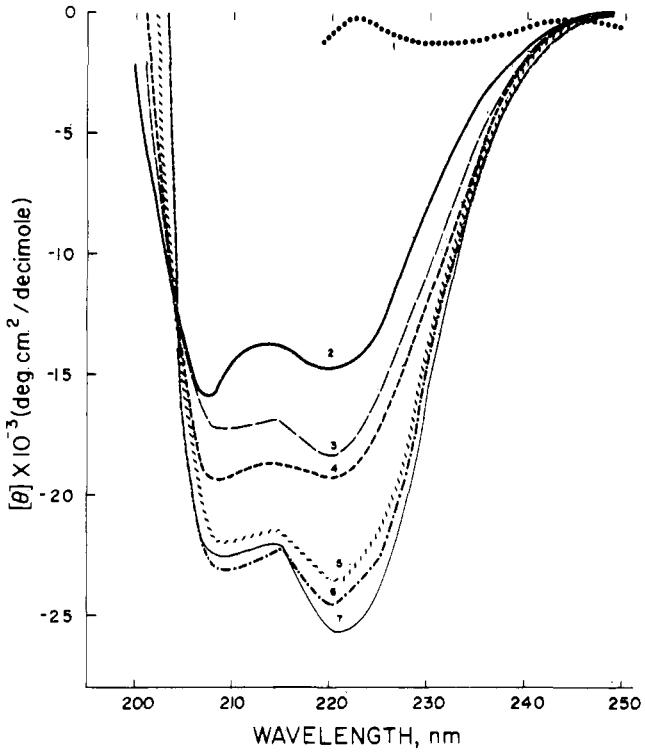

FIGURE 3: Mean residue weight ellipticity of the β system in the far-uv region of the spectrum. (1) Apo-βIAA chains in 6 *M* Gdn·HCl; (2) apo-βIAA chains in 0.04 *M* borate buffer at pH 9.5; (3) CN-ferric derivative of the reconstituted βIAA chains in 0.04 *M* borate buffer at pH 9.5; (4) apo-βIAA chains in 50% methanol; (5) CN-ferric derivative of the βIAA chains in 0.04 *M* borate buffer at pH 9.5; (6) CN-ferric derivative of the β chains in 0.04 *M* borate buffer at pH 9.5; (7) CN-ferric derivative of the reconstituted βIAA chains after dialysis against 0.01 *M* phosphate buffer at pH 7.0.

natant showed a spectrum very similar to that of the native protein. Similar results were obtained when the apo-βIAA chains were previously exposed to 6 *M* Gdn·HCl.

In the near-uv region of the CD spectrum, shown in Figure 4, it appears that the alkylation of the β chains decreased the ellipticity values between 280 and 310 mμ but did not affect the rest of the spectrum. Addition of heme to

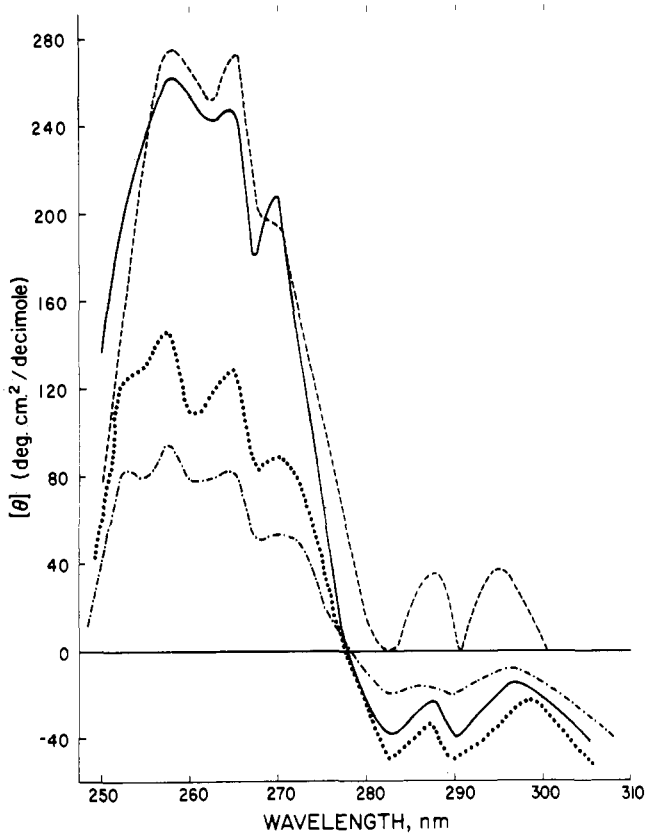

FIGURE 4: Mean residue weight ellipticity of the β chains system in the near-uv region of the spectrum. β chains in 0.04 *M* borate buffer at pH 9.5 (---), βIAA chains in 0.04 *M* borate buffer at pH 9.5 (—), reconstituted βIAA chains in 0.04 *M* borate buffer at pH 9.5 (- · -), and after dialysis against 0.01 *M* phosphate buffer at pH 7.0 (· · ·). All the hemoproteins were CN-ferric derivatives.

the apo-βIAA chains did not restore completely the optical activity of the subunits, even after dialysis at neutral pH.

In the Soret region of the spectrum, shown in Figure 5, it appears that the alkylation produced a decrease of the ellipticity in the βIAA chains. Also in this case, titration of the apo-βIAA subunits with cyanoheme did not completely restore the optical activity of the chains even after dialysis at neutral pH.

Sedimentation Behavior of the βIAA Chains and Their Apoprotein. All the experiments were performed in 0.04 *M* borate buffer at pH 9.5. The results are summarized in Tables II and III. The βIAA chains appeared to be monomeric and recombined with the α subunits to form tetramers. In both cases symmetrical single peaks were obtained in the schlieren diagrams. After removal of the heme the apo-βIAA subunits showed the presence in solution of several large polymeric species. Upon addition of cyanoheme to the apoprotein in a molar ratio 1:1 the reconstituted chains appeared homogeneous and monomeric.

Titration with Heme of the Apo-βIAA Chains. Titration of the protein with cyanoheme was followed 24 hr after the mixing with the heme either spectrophotometrically at 420 nm or polarimetrically at 222 nm. In both cases a stoichiometric recombination was present, with a break point near the molar heme-protein ratio 1:1 (Figure 6). The molar extinction coefficient at 420 nm of the reconstituted cyanoferric protein was 9.2×10^4. For the same derivative of the βIAA chains a value ϵ_{420} 1.10×10^3 was obtained in agreement with the value reported by Yip et al. (1972).

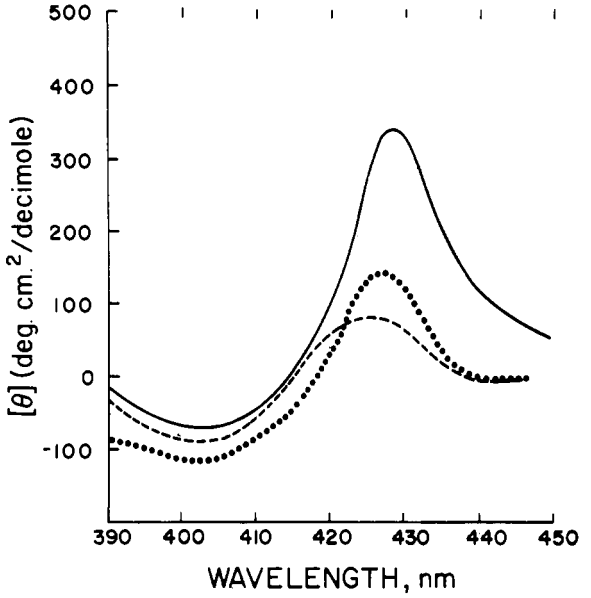

FIGURE 5: Mean residue weight ellipticity of the β chains system in the Soret region of the spectrum. β chains in 0.04 *M* borate buffer at pH 9.5 (—), βIAA chains in 0.04 *M* borate buffer at pH 9.5 (···), reconstituted βIAA chains in 0.01 *M* phosphate buffer at pH 7.0 (- - -). All the hemoproteins were CN-ferric derivatives.

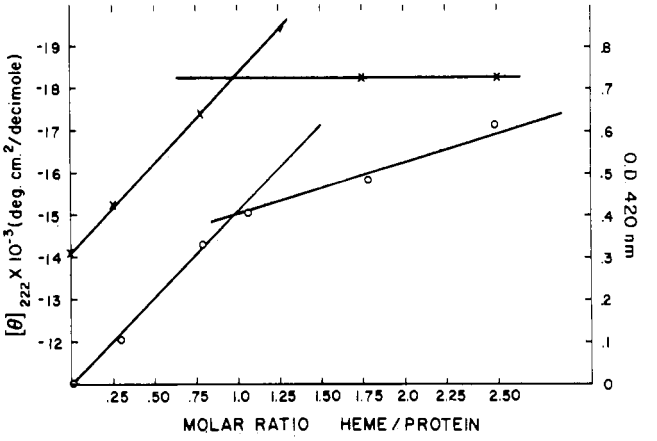

FIGURE 6: Titration with CN-heme of apo-β subunits in 0.04 *M* borate buffer at pH 9.5. The ellipticity at 222 nm or the optical density at 420 nm were monitored. The protein concentration was 4.53×10^{-6} *M* for the spectrophotometric measurements (○) and 6.68×10^{-6} *M* for the CD determinations (x). Ellipticity is calculated as mean residue weight.

Table II: Sedimentation Velocity of the βIAA System in 0.04 *M* Borate Buffer at pH 9.5.

Protein	$s_{20,w}$	Concn (mg/ml)
βIAA CO derivative	1.76	3.1
Apo-βIAA	1.69	2.5
+ CN heme	1.78	2.9
βIAA + α chains CN derivatives	4.22	4.4

Table III: Sedimentation Equilibrium of Apo-βIAA Chains and Their Arginyl-COOH Peptides.

Peptide or Protein	Solvent	Mec[a]	Mol wt from AA. Composition	Concn span (mg/ml)
Apo-βIAA	0.04 *M* borate buffer at pH 9.5	53,100	15,950	0.3–1.5
β(1–30)	0.1 *M* NaCl (pH 7.0)	4,200	3,150	0.7–2.2
β(41–104)	0.1 *M* NaCl (pH 7.0)	7,300	6,960	0.6–2.5
β(105–146)	0.033 *M* H_2SO_4	6,200	4,500	0.3–1.6

[a] See footnote 2.

Optical Activity of the Arginyl-COOH Peptides. Figures 7–9 show the CD spectrum in the far-uv region of the arginyl-COOH peptides β(1–30), β(41–104), and β(105–146) in different solvents. The absorption spectrum of the peptides is shown in the same figures. The peptide β(31–40) is not shown, because of the lack of secondary structure in all the solvents tested.

In all cases the CD spectrum of the peptides in electrolyte solutions indicated a low amount of helical structure. In 50% methanol a notable amount of helical structure was recovered by the peptides β(1–30) and β(105–146). The CD spectra in concentrated solutions of urea and guanidine hydrochloride suggested that the peptides were more unfolded.

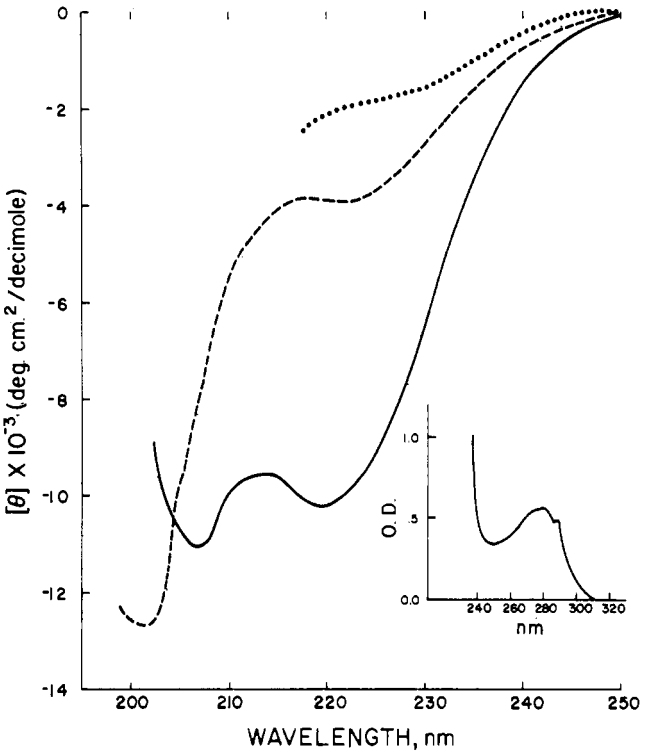

FIGURE 7: Mean residue weight ellipticity of the peptide β(1–30): in 0.033 *M* Na_2SO_4 (- - -), in 50% methanol (—), in 3 *M* urea (···). The inset shows the absorption spectrum in the uv region of the peptide in 0.01 *M* acetic acid at a concentration approximately 1×10^{-4} *M*.

Sedimentation Behavior of the Arginyl-COOH Peptides. Table III summarizes the results obtained in sedimentation equilibrium experiments. The peptide β(105–146) showed a marked tendency to aggregate that resulted in poor solubility at neutral pH, and for this reason the experiment was performed in acid. The other peptides tested, β(1–30) and β(41–104), showed some tendency to polymerize. Their average molecular weight indicated the presence of at least dimers. Presumably at the protein concentration used in the

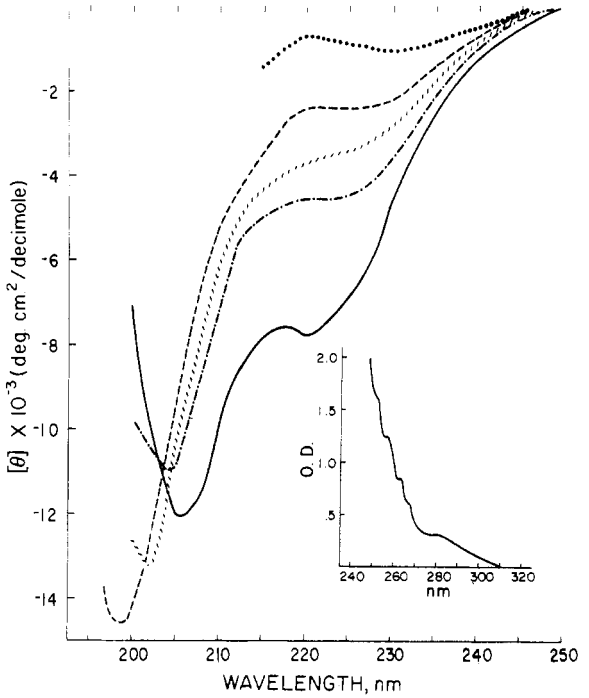

FIGURE 8: Mean residue weight ellipticity of the peptide β(41-104): in 0.033 M Na_2SO_4 (- - -), in 50% methanol (/ / / /); after addition of 1 mol of CN-heme per mol of peptide in 0.1 M phosphate buffer at pH 8.0 (- · - ·); after addition of an equal volume of methanol to the mixture of CN-heme and peptide above described (—) in 6 M Gdn·HCl (···). The inset shows the absorption spectrum in the uv region of the peptide in 0.01 M acetic acid at a concentration approximately 1×10^{-4} M.

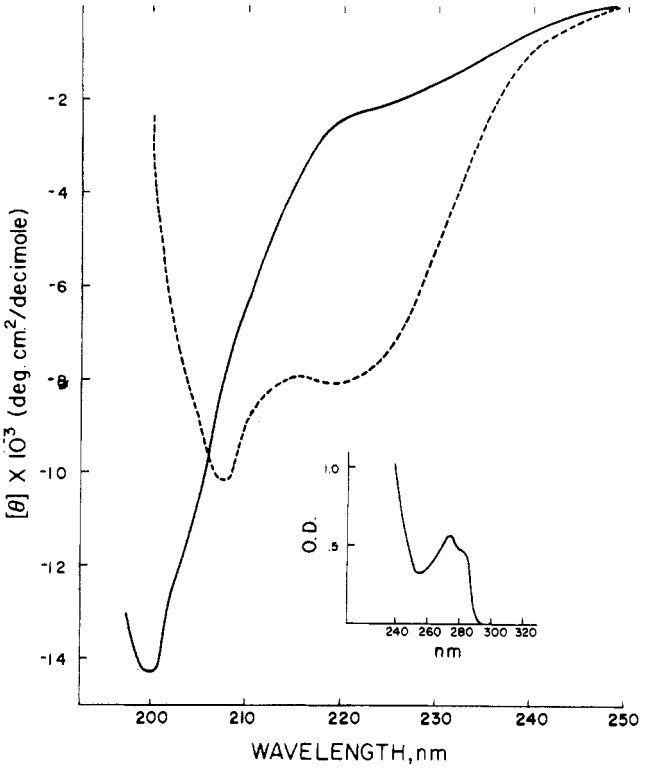

FIGURE 9: Mean residue weight ellipticity of the peptide β(105-146): in 0.033 M H_2SO_4 (—); in 50% methanol (- - -). The inset shows the absorption spectrum in the uv region of the peptide in 0.01 M acetic acid at a concentration approximately 1×10^{-4} M.

CD experiments (0.1-0.2 mg/ml) the peptides β(1-30) and β(41-104) were mostly in monomeric form.

Titration with Cyanoheme of the Peptide β(41-104). Addition of cyanoheme to the peptide β(41-104) in the molar ratio 1:1 increased the amount of helical structure of the peptide. Addition of an equal volume of methanol to these solutions further increased the helical content of the peptide. However, when the titration was followed spectrophotometrically at 420 nm, or polarimetrically at 222 mμ, it failed to show a stoichiometric reaction indicating a low specificity of the peptide for cyanoheme.

Fractions of α Helix, β Conformation, and Random Coil in the Various Polypeptides. The numerical fittings obtained with the parameters of Chen et al. (1974) are illustrated in Figure 10 and the fractions of the various conformations so calculated are listed in Table IV. The fittings were good in general, and in the case of the βIAA chains, the calculated amount of helical conformation corresponded to that of the β chains in the hemoglobin crystal.

Discussion

βIAA System. Upon alkylation of the SH groups with iodoacetamide, the β chains became monomeric. This phenomenon was observed by Neer (1970) after iodoacetate alkylation of the β112 cysteine. She suggested that it could be explained either by a conformational change of the protein or by the The modification of the optical activity of the protein in the near-uv and Soret region of the spectrum indicates the presence of a conformational change and suggests that this was the reason for the formation of monomers.

The alkylation of the SH groups allowed the renaturation of the apo-βIAA chains after exposure to 6 M Gdn·HCl. The unsuccessful attempt of Yip et al. (1972) to renature their apo-β subunits was probably due to the presence of free SH groups which upon denaturation formed intermolecular bonds and stabilized the unfolded form of the protein. The renatured apo-βIAA chains were still not soluble at neutral pH and showed a lower helical content and a higher polymerization than the native apo-β chains obtained by Yip et al. (1972) indicating a different conformation of the two proteins. After recombination with the heme and dialysis at neutral pH most of the protein that was not properly folded was eliminated by precipitation, and the resulting material showed in the far-uv a CD spectrum very similar to that of the native chains. However, the optical activity in the near-uv and Soret region of the spectrum remained lower than that of the native protein. Also, the extinction coefficient of the reconstituted protein was lower than that of the native one. In these regions of the spectrum the rotatory power of the hemoproteins is determined by the interaction of the heme with the amino acid residues present in the heme pocket (Hsu and Woody, 1971, Beychok et al., 1967). Therefore the incomplete recovery of optical activity would suggest that the recombination with the heme occurred either in a different way or was heterogeneous in the sense that it occurred in more than one way. This heterogeneity was not apparent in the titration of the apo-βIAA chains with the heme (Figure 6).

Evaluation of the Fractions of α Helix, β Conformation, and Random Coil. For these calculations we used the ellipticity values reported by Greenfield and Fasman (1969) and by Chen et al. (1970). The assumptions underlying these calculations are: (1) that the optical activity of the polypeptides investigated was due only to the contributions of the α helix, β conformation, and random coil and (2) that the nonsystematic ("random") structure of proteins has the optical activity of the random coil of synthetic polypeptides.

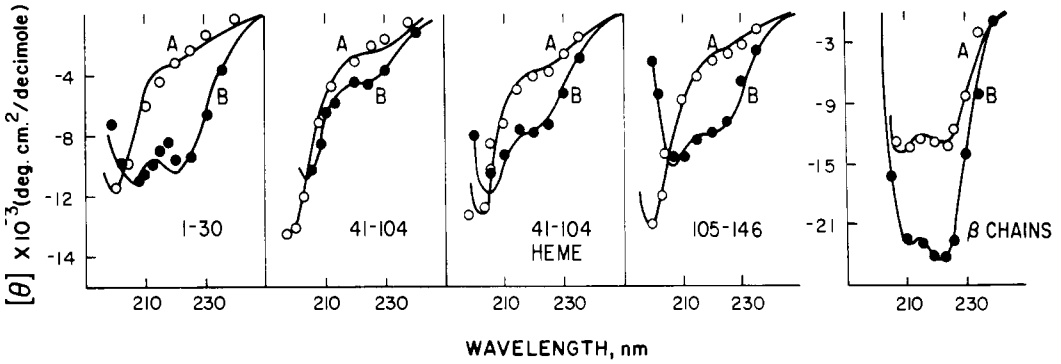

FIGURE 10: Simulation of the circular dichroic spectra, of the β chains based on the data of Chen et al. (1974). In the frame marked "β chains" A is the CD spectrum of the apo-βIAA subunits and B is that of the βIAA subunits, both in 0.04 *M* borate buffer at pH 9.5. In the other frames A refers to the peptides in 0.033 *M* Na_2SO_4 (0.033 *M* H_2SO_4 for the β(105-146)) and B to the peptides in 50% methanol. The mixtures of CN-heme and peptide β(41-104) in electrolyte and methanol solutions were obtained as described for Figure 9. (—) Experimental measurements; (○,●) simulated data.

Table IV: Secondary Structure of βIAA Subunits and Their Arginyl-COOH Peptides Obtained Using the Parameter of Chen et al. (1974).

Protein or Peptide	α Helix (%)	β Conformation (%)	Random Coil (%)
βIAA 0.04 *M* borate (pH 9.4)	76	0	24
Apo-βIAA 0.04 *M* borate (pH 9.4)	45	21	34
Apo-βIAA 50% methanol	67	0	33
β(1–30) Na_2SO_4, 0.033 *M*	10	21	69
β(1–30) 50% methanol	35	0	65
β(41–104) Na_2SO_4, 0.033 *M*	11	15	74
β(41–104) 50% methanol	18	3	79
β(41–104) + CN heme 0.1 *M* phospate (pH 8)	16	6	78
β(41–104) + CN heme; 0.05 *M* phospate (pH 8) and 50% methanol	28	5	67
β(105–146) H_2SO_4, 0.033 *M*	11	11	77
β(105–146) methanol, 50%	27	14	59

Table V: Helical Structure of βIAA Subunits and Their Arginyl-COOH Peptides in Electrolyte Solutions.

Peptide or Protein	% of α Helix in Hemoglobin Crystal	% of α Helix Expected Minimum Value[a]	% of α Helix Expected Maximum Value[b]	% of α Helix Measured from CD Spectrum
βIAA[c]	79		81	76
Apo-βIAA[c]			77	45
β(1–30)[d]	77	40	67	11
β(41–104)[d]	67	34	66	11
β(105–146)[e]	86	47	83	11

[a] Calculated on the basis of the effect of the heme removal and protein fragmentation. [b] Maximum computable value obtained by the procedure of Kabat and Wu (1973). [c] In 0.04 *M* borate at pH 9.5. [d] In 0.033 *M* Na_2SO_4. [e] In 0.033 *M* H_2SO_4.

These assumptions are not necessarily correct. When the nonsystematic structure of proteins forms sharp corners, it can show an optical rotatory dispersion similar to that of the β conformation (Woody, 1974). The solvent iself can also produce optical activity when it interacts with polypeptides (Pysh, 1974). However the quantities involved in these errors appear to be small and recently a matrix rank analysis performed by Bannister and Bannister (1974) has shown that the optical activity of the "five proteins" used by Chen et al. (1974) in their investigation can be explained in terms of three components only.

Only the calculations based on the parameters of Chen et al. (1974) are reported in Tables IV and V, and Figure 10. Those based on the parameters by Greenfield and Fasman (1969) gave similar results and showed systematically a lower amount of helical structure. The parameters obtained by Greenfield and Fasman measuring the optical activity of synthetic polypeptides refer to helical segments of "infinite" length, while those reported by Chen et al. (1974) refer to helical segments with an average length of eight residues. This average corresponds to that present for the helical segments of the β subunits in the hemoglobin crystal. It is worth noting that addition of urea or Gdn·HCl decreased the ellipticity at 222 nm of the peptides where the calculations showed the presence of only 10% α helix, suggesting a good accuracy of the determinations.

The Secondary Structure of the βIAA and Apo-βIAA Chains. In the βIAA subunits the amount of α helix calculated with the parameters of Chen et al. (1974) corresponded very well with that found in the hemoglobin crystal.

A substantial loss of helical structure was produced by the removal of the heme. The notable amount of β conformation calculated in this case is consistent with the loss of helical content. As expected also, the amount of nonordered structure was increased upon the removal of the heme.

The Secondary Structure of the Arginyl-COOH Peptides. In order to appreciate the extent of the conformational change produced in these fragments by exposure to the solvent of regions normally buried inside the protein molecule, we tried to estimate what their secondary structure would be if no other changes occurred beside those produced by the breaking of the peptide bonds and by the removal of the heme.

Making reference to the hemoglobin crystal, it might be expected that, whenever the tryptic digestion broke a peptide bond inside a helical segment, three residues above and three below the breaking point would lose their helical structure. The removal of the heme also produced a loss of helical structure that in the absence of more detailed information was averaged through the entire molecule. The results of these considerations are numerically expressed in the first column of "expected" values of helical content listed in Table V for the various peptides. In so doing an overestimation of the structural loss is likely to occur. In fact the same loss can be counted twice if the tryptic digestion

occurred in a region where the helical structure had been already lost because of the removal of the heme. In other words these values should be considered minimum values.

Another way to approach the problem is to calculate the helical content of the peptide by the empirical procedure of Kabat and Wu (1973). These values are also listed in Table V and were calculated trying to maximize the helical content in order to represent maximum possible values.

In Table V it clearly appears that the amount of helical structure present in electrolyte solutions of the arginyl-COOH peptides is far below the minimum expected values. This indicates a substantial change in the peptide conformation when they are isolated from the rest of the protein.

One function of the polypeptide length is to define the minimum energy of conformation in which the polypeptide prefers to exist. The removal of part of the sequence would give energetic relevance to preexisting or newly formed "local" minima, thereby giving to the peptides a large degree of mobility and conformational freedom. This situation would result in an extensive heterogeneity in the conformation of the peptides.

It has also been shown that binding of water molecules to polypeptides inhibits the formation of helical structure, as it occurs in synthetic polylysine (Barteri and Bispisa, 1973). In fact it is a common observation that when the water activity was reduced by addition of organic solvent, more helical structure was formed in synthetic polypeptides and protein fragments (Epand and Scheraga, 1968; Hermans and Puett, 1971; Atassi and Singhal, 1970; Toniolo et al., 1974).

In our case it might be noted that not only in the arginyl peptides but also in the entire apo-βIAA chains, addition of the methanol increased the amount of helical structure of the protein. This might suggest that after the removal of the heme, part of the structural loss is produced by the exposure of the heme pocket to water.

Studies are now in progress on the behavior of the arginyl-COOH and other peptides in different solvents.

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References

Antonini, E., and Brunori, M. (1971), *Front. Biol. 21,* 1.

Atassi, M. Z., and Singhal, R. P. (1970), *J. Biol. Chem. 245,* 5122.

Bannister, W. N., and Bannister, J. V. (1974), *Int. J. Biochem. 5,* 123.

Barteri, M., and Bispisa, B. (1973), *Biopolymers 12,* 2309.

Beychok, S., Tyuma, I., Benesh, R. E., and Benesh, R. (1967), *J. Biol. Chem. 242,* 2460.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76,* 4331.

Bradbury, A. F., and Smyth, D. G. (1973), *Biochem. J. 131,* 637.

Brown, J. E., and Klee, W. A. (1971), *Biochemistry 10,* 470.

Bucci, E., and Fronticelli, C. (1965), *J. Biol. Chem. 240,* PC551.

Chen, Y. H., Yang, J. T., and Chau, K. H. (1974), *Biochemistry 13,* 3350.

Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 211.

Clegg, J. B., Naughton, H. A., and Weatherall, D. J. (1966), *J. Mol. Biol. 19,* 91.

Crumpton, N. J. (1968), *Biochem. J. 108,* 18.

DeRenzo, E. C., Ioppolo, C., Amiconi, G., Antonini, E., and Wyman, J. (1967), *J. Biol. Chem. 242,* 4850.

Drabkin, D. L., (1946), *J. Biol. Chem. 164,* 703.

Epand, R. M., and Scheraga, H. A. (1968), *Biochemistry 7,* 2864.

Gibson, Q., and Antonini, E. (1963), *J. Biol. Chem. 238,* 1384.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8,* 4108.

Habeeb, A. F. S. (1966), *Anal. Biochem. 14,* 328.

Habeeb, A. F. S., and Atassi, M. Z., (1969), *Immunochemistry 6,* 555.

Hermans, J., Jr., and Puett, D. (1971), *Biopolymers 10,* 895.

Hsu, M. C., and Woody, R. W. (1971), *J. Am. Chem. Soc., 93,* 3515.

Jevaherian, K., and Beychok, S. (1968), *J. Mol. Biol. 37,* 1.

Kabat, E. A., and Wu, T. T. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1473.

Marquardt, D. W. (1963), *SIAM Rev. 11,* 431.

Neer, E. J. (1970), *J. Biol. Chem. 245,* 566.

Pysh, E. S. (1974), *Biopolymers 13,* 1575.

Riordon, J. F., and Vallee, B. L. (1964), *Biochemistry 3,* 1768.

Rossi Fanelli, A., Antonini, E., and Caputo, A. (1958), *Biochim. Biophys. Acta 30,* 605.

Scheraga, H. A. (1971), *Chem. Rev. 71,* 195.

Singhal, R. P., and Atassi, M. S. (1970), *Biochemistry 9,* 4252.

Taniuchi, H., and Anfinsen, C. B. (1969), *J. Biol. Chem. 244,* 3864.

Taniuchi, H., and Anfinsen, C. B. (1971), *J. Biol. Chem. 246,* 2291.

Toniolo, C., Fontana, A., and Scoffone, E. (1974), *J. Pept. Protein Res. 6,* 371.

Waks, H., Yip, Y. K., and Beychok, S. (1973), *J. Biol. Chem. 248,* 6462.

Woody, R. W. (1974), Rehovot Symposium on Polyamino Acids, Polypeptides and Proteins and their Biological Implication, Israel.

Yip, Y. K., Waks, M., and Beychok, S. (1972), *J. Biol. Chem. 247,* 7237.