Optical Activity of Disulfide Bonds in Proteins. 1. Studies on Plasmin Modified Human Somatotropin†

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ABSTRACT: The reduction and alkylation of the two disulfide bonds in a preparation of human pituitary growth hormone which had been previously modified by limited proteolysis with the enzyme plasmin have been studied. Quantitative and selective reduction of the carboxyl-terminal disulfide, as well as total reduction of both disulfides, has been achieved in the absence of denaturants. Circular dichroism spectra of the various reduced and reduced-alkylated derivatives have provided sufficient information to allow an estimation of the individual contributions of each disulfide bond to the total optical activity of the protein. These contributions were found to represent a significant portion of the total optical activity between 290 and 250 nm. The carboxyl-terminal bond exhibits negative dichroism with an apparent center near 258 nm ($[\theta]_{M,258nm} = -2100$ deg cm^2 dmol^{-1}). By comparison, the contribution of the remaining disulfide is red-shifted to 273 nm, is also negative in sign, and somewhat more intense ($[\theta]_{M,273nm} = -3200$ deg cm^2 dmol^{-1}). Circular dichroism measurements have also been used to approximate the rate of reduction of the protein.

Since Holzwarth's (1964) initial publication of the use of circular dichroism (CD)[1] for studying the conformation of proteins in solution, much experimental effort has been expended by many investigators with the purpose of resolving the CD spectra of proteins into their constituent bands and assigning each optically active band to a specific chromophore or chromophore type. Considerable success has been achieved in resolving that portion of the spectrum dominated by amide bond absorption, providing reasonably reliable estimates of the secondary structural contents of the polypeptide backbone (see, for example: Holzwarth and Doty, 1965; Cassim and Yang, 1967; Greenfield and Fasman, 1969; Chen et al., 1972, 1974). Simultaneously, a much improved understanding of the near-UV optical properties of the side-chain chromophores, in particular the aromatic groups, has also been obtained (Beychok, 1966; Horwitz et al., 1969, 1970; Strickland et al., 1969). More recently, the near-UV optical activity associated with chiral disulfide bonds has been successfully measured in small organic compounds (Carmack and Neubert, 1967; Dodson and Nelson, 1968), the amino acid cystine (Beychok, 1965; Coleman and Blout, 1968; Ito and Takagi, 1970; Casey and Martin, 1972; Yamashiro et al., 1975), oxidized glutathione (Beychok, 1965), small cyclic polypeptides (Beychok and Breslow, 1968; Casey and Martin, 1972; Yamashiro et al., 1975), and antibiotics (Nagarajan et al., 1968; Ludescher and Schwyzer, 1971; Nagarajan and Woody, 1973).

However, at present, little is known about the contribution of disulfide bonds to the total optical activity of the larger globular proteins. The relatively low content of these chromophores and their characteristically broad CD bands, devoid of vibrational fine structure, make their recognition and direct measurement in such materials very difficult. In many instances it has been assumed that their contribution is negligible, although in others where the disulfide content is unusually high and/or the aromatic chromophore content is low, this is known not to be the case (Horwitz et al., 1970; Breslow, 1970; Bewley et al., 1972, 1974; Puett, 1972; Menendez-Botet and Breslow, 1975; Holladay and Puett, 1975).

Human pituitary growth hormone (HGH) is a single chain polypeptide containing 191 amino acid residues (mol wt 22 100) with a single tryptophan, 8 tyrosines, 13 phenylalanines, and two disulfide bonds linking ½-cystines 53 to 165 and 182 to 189 (Li, 1972). In 1968, Bewley et al. described the reduction and carbamidomethylation of the two disulfide bonds in HGH in the absence of denaturants. The derivative was found to retain full biological potency. Physicochemical evaluation suggested that the conformation of the modified protein was essentially indistinguishable from that of the native hormone (Bewley et al., 1969). It was further suggested (Bewley and Li, 1970) that the small difference between the CD spectra of the native and the fully reduced protein represented the total contribution of the two disulfide bonds to the optical activity of the native molecule. However, the individual contributions of each disulfide could not be determined. Adjunct to a study of a modified form of HGH produced by removal of the hexapeptide comprising residues 135 through 140, brought about by digestion with the enzyme plasmin (Li and Graf, 1974), procedures were developed which allowed the quantitative and selective reduction and carbamidomethylation of only one disulfide bond (Li and Bewley, 1976a). Both the plasmin digested derivative (PL-HGH) and the fully reduced-carbamidomethylated PL-HGH were found to retain full biological activity (Li and Bewley, 1976a). Additional investigation of these modified proteins has now provided information which may be used to estimate the individual con-

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[1] Abbreviations used: CD, circular dichroism; HGH, human pituitary growth hormone; PL-HGH, HGH modified by limited digestion with human plasmin; Cys(SH)^{182,189}-PL-HGH, the product formed by reduction of one disulfide bond in PL-HGH; Cys(Cam)^{182,189}-PL-HGH, the product formed by alkylation of Cys(SH)^{182,189}-PL-HGH with α-iodoacetamide; Cys(SH)^{53,165,182,189}-PL-HGH, the product formed by reduction of both disulfide bonds in PL-HGH; Cys(Cam)^{53,165,182,189}-PL-HGH, the product formed by alkylation of Cys(SH)^{53,165,182,189}-PL-HGH with α-iodoacetamide; Cys(Cam)^{53}-HGH-(1-134), the 134-residue N-terminal fragment prepared from Cys(Cam)^{53,165,182,189}-PL-HGH; Cys(Cam)^{165,182,189}-HGH-(141-191), the 51-residue C-terminal fragment prepared from Cys(Cam)^{53,165,182,189}-PL-HGH; REOX-PL-HGH, reduced and autooxidized PL-HGH; DTT, dithiothreitol; DTNB, 5,5′dithiobis(2-nitrobenzoic acid); SEM, standard error of the mean; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane.

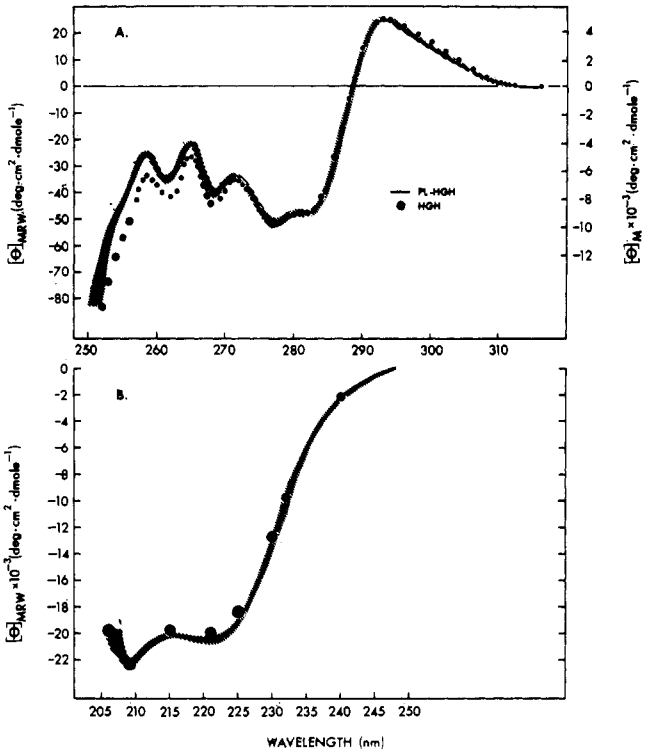

FIGURE 1: Circular dichroism spectra in the region of side-chain absorption (A) and the region dominated by amide bond absorption (B) of PL-HGH (—) and HGH (●) in 0.1 M Tris-Cl buffer, pH 8.2. In A the spectrum of PL-HGH is the mean of ten separate spectra using three preparations of the protein. The shaded area represents the SEM for these spectra as a function of wavelength. Similarly, the diameter of the circles represents the SEM for seven spectra of HGH. In B, three spectra were used for PL-HGH and four for HGH.

tributions of each disulfide bond to the total optical activity of the plasmin modified hormone. Results of these studies are reported herein.

Materials and Methods

HGH was prepared as described by Li et al. (1962). The plasmin modification was carried out as previously reported by Li and Gråf (1974). A monomeric form of PL-HGH was obtained by exclusion chromatography on Sephadex G-100 in 0.1 M Tris-Cl buffer (pH 8.2). This monomer was used directly without lyophilization. DTT and α-iodoacetamide were obtained from Calbiochem. [1-^{14}C]-α-Iodoacetamide was obtained from New England Nuclear. All other chemicals were of reagent grade and were used without further purification.

Exclusion Chromatography. All exclusion chromatography was carried out in Tris-Cl buffer (pH 8.2) on Sephadex G-100. The sample was applied to the bottom of a column (1.5 × 58.5 cm) and eluted upward at a flow rate of 4.9 ml/h with an LKB peristaltic pump (Model 10200). The column was repeatedly calibrated with Blue Dextran 2000 (Pharmacia), myoglobin, bovine serum albumin (Miles Research Products), and native HGH.

Circular Dichroism. Circular dichroism spectra were taken on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment according to procedures outlined previously (Bewley et al., 1972). No data were used at dynode voltages greater than 420 V. A mean residue weight of 115 was used in all cases. All spectra, including baselines, were scanned from three to five times each. Protein concentrations were varied from 0.8 to 1.9 mg/ml. The content of α helix was estimated as previously described (Bewley et al., 1969).

Fluorescence. Fluorescence emission spectra were obtained on a Hitachi Perkin-Elmer spectrofluorimeter, Model MPF-2A, according to procedures described elsewhere (Bewley and Li, 1975). The excitation monochromator was set at 294 nm in all cases.

Protein Concentration. Protein concentrations were determined from absorption spectra taken in the Tris-Cl buffer on a Beckman DK-2A spectrophotometer from 360 to 250 nm. All spectra were corrected for light scattering as described by Beaven and Holiday (1952). The absorptivity of PL-HGH and the modified forms were assumed to be equivalent to that previously reported (Bewley et al., 1969) for native HGH.

Amino Acid Analysis. Amino acid analyses of the alkylated proteins were carried out on 22-h acid hydrolysates using a Beckman Model 120-C analyzer according to the procedures of Spackman et al. (1958).

Reduction of the Protein. Reduction of the protein was carried out in the Tris-Cl buffer at protein concentrations between 0.8 and 1.9 mg/ml within the CD cell. Before beginning the reduction, the CD of the sample was taken from 320 to 200 nm. The reducing agent (dissolved in the Tris-Cl buffer) was added in either a 2.5 or 25 mol excess over the total protein disulfide content. The added volume of the reducing agent was less than 1% of the sample volume in all cases. Following addition of the reducing agent, the ellipticity at 265 nm was recorded as a function of time until the reaction appeared to reach a stable state. The entire CD spectrum was then rescanned. In some cases the reaction was carried out on a single sample in two steps: first reducing with a 2.5 mol excess, rescanning the spectrum after 2×10^3 s, and then raising the excess to 25 mol to complete the reduction, monitoring as before at 265 nm with a final set of rescans after 2×10^4 s.

Alkylation of the Reduced Protein. Following the final CD scan, the pH of the reduction mixture was adjusted from 8.2 to 8.5 (Radiometer pH meter, Model 26) and a 20 mol excess of α-iodoacetamide (dissolved in the Tris-Cl buffer) was added with vigorous (but smooth) stirring. The 20 mol excess was calculated on the basis of the moles of thiol contained in the DTT used for reduction. The alkylation was allowed to proceed for 10 min after which the reaction mixture was desalted on Sephadex G-25. The alkylated protein was concentrated by ultrafiltration (Amicon Diaflo apparatus, PM-10 membrane) and submitted to exclusion chromatography on Sephadex G-100. One sample of PL-HGH was alkylated with a 20 mol excess of [1-^{14}C]-α-iodoacetamide, 2×10^3 s after adding a 2.5 mol excess of DTT. The radioactive protein (2.9 μCi/μmol) was desalted on Sephadex G-25 and a monomer obtained from Sephadex G-100. This monomer was reduced a second time using a 25 mol excess of DTT. After 2×10^4 s, the reduced protein was again alkylated, but this time with a 20 mol excess of "cold" α-iodoacetamide. After desalting on Sephadex G-25, the fully reduced-carbamidomethylated protein was purified on Sephadex G-100. The monomeric form was allowed to spontaneously dissociate into its two fragments comprising residues 1-134 and 141-191 by standing at room temperature for 800 h as described elsewhere (Li and Bewley, 1976a). The dissociated protein was then submitted to exclusion chromatography on Sephadex G-100. Fractions were assayed for both tryptophan fluorescence and radioactivity (Packard TriCarb liquid scintillation counter, Model 3320).

Reoxidation of the Reduced Protein. Two samples of PL-HGH were reduced for 2×10^4 s with a 25 mol excess of

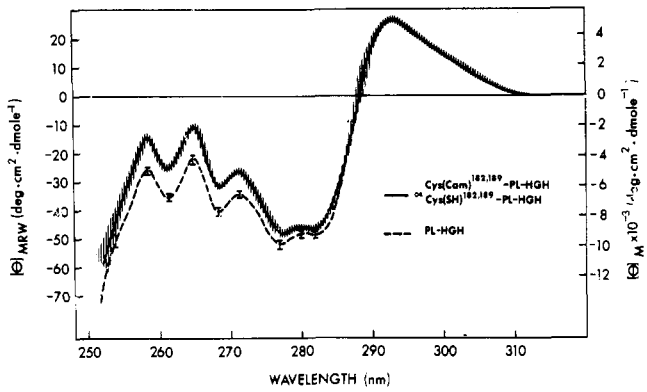

FIGURE 2: Circular dichroism spectra in the region of side-chain absorption of Cys(SH)^{182,189}-PL-HGH (—) and PL-HGH (- - -). Spectra of the reduced form were taken after 2×10^3 s in the presence of a 2.5 mol excess of DTT; the shaded area represents the SEM for four preparations. The SEM for the PL-HGH spectrum is the same as in Figure 1A, but shown here as vertical bars. The spectrum of Cys(Cam)^{182,189}-PL-HGH is identical with that of Cys(SH)^{182,189}-PL-HGH.

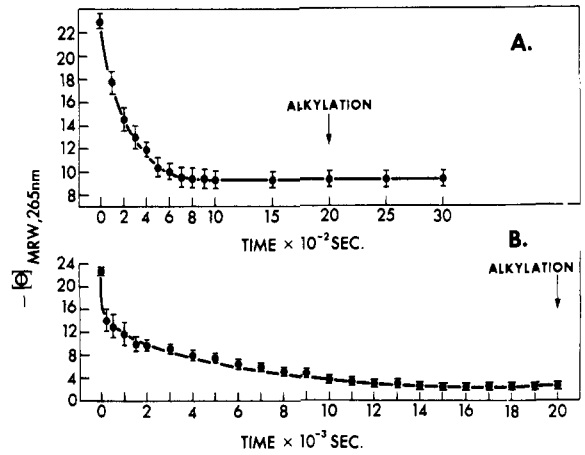

FIGURE 3: The rate of change in the ellipticity at 265 nm for PL-HGH treated with (A) a 2.5 mol excess of DTT and (B) a 25 mol excess of DTT. In both curves, the filled circles represent the mean values for four experiments; SEM values are shown as vertical bars.

DTT. Protein concentrations were between 1 and 1.5 mg/ml. Following completion of the reduction period, the reaction mixture was transferred to a test tube and covered with gauze and the entire mixture allowed to autooxidize at room temperature. After 72 h, no detectable thiol could be found using DTNB as described by Ellman (1959). The oxidized reaction mixture was submitted to chromatography on Sephadex G-100.

Preparation of Oxidized DTT. Oxidized DTT, dissolved in the Tris-Cl buffer, was prepared from the reduced form by three different procedures: air oxidation for 96 h, oxidation with a 5 mol excess of H_2O_2, and oxidation with a 1.5 mol excess of bovine pituitary growth hormone (Li, 1954). In the latter experiment the oxidized DTT was separated from the reduced protein on Sephadex G-25. The absence of reduced DTT was verified with DTNB. The concentration of oxidized DTT was determined spectrophotometrically as described by Cleland (1964).

Results

Circular Dichroism of Reduced and Oxidized DTT. The CD spectra of both reduced and oxidized DTT samples were measured at concentrations 10–100 times the maximum present in any of the spectra reported below, and in the same path length cells as used in the protein spectra. No significant dichroism was detected in any of the oxidized or reduced DTT preparations between 320 and 220 nm.

Circular Dichroism of PL-HGH. Figure 1A shows the near-UV CD spectrum of PL-HGH compared with the spectrum of native HGH. Except for very small differences above 294 nm and below 266 nm, it can be seen that the two spectra are essentially identical. The positive, asymmetric band above 289 nm has been previously assigned to transitions arising from the tryptophan residue at position 86 in HGH (Bewley et al., 1972; Bewley and Li, 1972a; Aloj and Edelhoch, 1972; Holladay et al., 1974). The negative bands at 282 and 277 nm have been assigned predominantly to tyrosine residues, with the two negative bands at 268–269 and 261–262 nm being assigned to phenylalanine (Bewley and Li, 1972a; Aloj and Edelhoch, 1972; Holladay et al., 1974). The same assignments may be applied to the bands in PL-HGH. From Figure 1B, it can be seen that, in the region dominated by amide bond absorption, PL-HGH and HGH again display essentially the same CD spectrum. The content of secondary structure in PL-HGH is estimated at 55 ± 5% excess right-hand α helix as previously reported for native HGH (Bewley et al., 1969).

Reduction and Alkylation of PL-HGH with a 2.5 Mol Excess of DTT. The side-chain CD spectrum of PL-HGH after 2×10^3 s in the presence of a 2.5 mol excess of DTT is shown in Figure 2 along with the spectrum of PL-HGH for comparison. It is clear that the difference between the two spectra far exceeds the standard error in either spectrum alone and is therefore well beyond experimental error. The positive indole band is unchanged but there is an increasing loss of negative dichroism in the reduced protein from 286 to about 260 nm. Below 260 nm, the two spectra converge again with no significant differences appearing between them at wavelengths below 240 nm. Figure 3A shows the rate of loss of negative dichroism at 265 nm. The reaction is more than 50% complete after the first 5 min with an essentially stable state being reached in about 15 min. This state remains unchanged for the next several hours. Repeated scanning of the spectrum after 2×10^3 s disclosed no further time dependence at any wavelength between 320 and 200 nm. Alkylation of the reduced product was performed after the final CD scan had been completed. A monomeric form of the alkylated product was obtained from Sephadex G-100 in 95% yield. Amino acid analysis of the product (Table I) indicated that only one of the two disulfide bonds had been modified. Within experimental error, the entire CD spectrum of the carbamidomethylated product, Cys(Cam)^{182,189}-PL-HGH, was indistinguishable from that of the reduced form, Cys(SH)^{182,189}-PL-HGH. This includes both the near- and far-ultraviolet regions (see also Table II).[2] Moreover, the far-ultraviolet CD of both these derivatives are indistinguishable from either PL-HGH or native HGH (Table II).

Reduction and Alkylation of PL-HGH with a 25 Mol Excess of DTT. Figure 4 shows the side-chain CD spectrum of PL-HGH after 2×10^4 s in the presence of a 25 mol excess of DTT. Again, the spectrum of the starting material is included for comparison. A small but significant loss of positive dichroism above 285 nm is seen, with a somewhat greater loss of negative dichroism below 285 nm than found in the case of

[2] In the interest of brevity, the far-UV CD spectra are indicated by the ellipticities at the two negative maxima at 221 and 209 nm. No significant differences were found at any other wavelengths below ≈240 nm.

TABLE I: The Extent of Modification of Partially and Fully Reduced PL-HGH.

Preparation	Residues/mol of Protein[a] SCM Cys	½-Cys
Cys(Cam)^{182,189}-PL-HGH	2.0 ± 0.2	1.6 ± 0.1
Cys(Cam)^{53,165,182,189}-PL-HGH	3.60 ± 0.1	<0.1[b]

[a] Mean ± SEM for three preparations of each derivative. [b] In two out of three preparations, no ½-Cys was detectable with less than 0.1 residue/mol appearing in the third.

TABLE II: Ellipticities[a] and α-Helix Contents[b] of Modified PL-HGH in the Region of Amide Bond Absorption.

Preparation	$[\theta]_{221nm}$[c]	$[\theta]_{209nm}$[c]	Estimated % α helix
HGH	−20 000	−22 400	55
PL-HGH	−20 600	−22 500	55
Cys(SH)^{182,189}-PL-HGH	−21 390	−23 390	55
Cys(Cam)^{182,189}-PL-HGH	−21 300	−23 400	55
Cys(SH)^{53,165,182,189}-PL-HGH	−20 400	−21 400	55
Cys(Cam)^{53,165,182,189}-PL-HGH	−21 500	−23 000	55
REOX-PL-HGH	−20 200	−23 700	55

[a] Mean residue weight ellipticities in deg cm^2 dmol^{-1}. All measurements were made in 0.1 M Tris-Cl (pH 8.2). [b] Estimated to the nearest 5%. [c] The SEM at these wavelengths is approximately ±500 deg cm^2 dmol^{-1} (see Figure 1B).

Cys(SH)^{182,189}-PL-HGH. Again the spectrum of the starting material and the reduced form converge below 260 nm, being identical within experimental error, below 240 nm (Table II). The rate of loss of ellipticity at 265 nm is shown in Figure 3B. The reaction is nearly 50% complete in the first 2 min. At this point a slow but progressive change in ellipticity continues to occur for the next 12–15 × 10^3 s, with a stable state being achieved by 2 × 10^4 s. Despite the greater difference in the side-chain CD, the far-ultraviolet CD of Cys(SH)^{53,165,182,189}-PL-HGH is virtually unchanged from that of the starting material (Table II).

Alkylation of the fully reduced protein was performed at 2 × 10^4 s and the alkylated protein immediately submitted to exclusion chromatography on Sephadex G-100. A monomeric product (mol wt ≈22 000) was obtained in approximately 90% yield. Amino acid analysis (Table I) indicated that both disulfide bonds had been quantitatively modified. Although the far-UV CD of this carbamidomethylated product was not markedly changed from that of PL-HGH (or Cys(SH)^{53,165,182,189}-PL-HGH), the side-chain CD showed a somewhat increased loss in positive dichroism above 289 nm, relative to that seen in the fully reduced form. The amount of this additional change depended on how much time had elapsed between the alkylation and the time the CD was taken. After chromatography, the monomeric Cys(Cam)^{53,165,182,189}-PL-HGH was immediately frozen in several aliquots at 1.0–1.5 mg/ml for future use. When thawed 3 months later, the side-chain CD of the "frozen" derivative surprisingly exhibited an indole dichroism identical in all respects with either PL-HGH or native HGH. The rest of the spectrum (below ≈289 nm) was changed from that of the freshly prepared Cys(Cam)^{53,165,182,189}-PL-HGH samples. The CD spectra of these

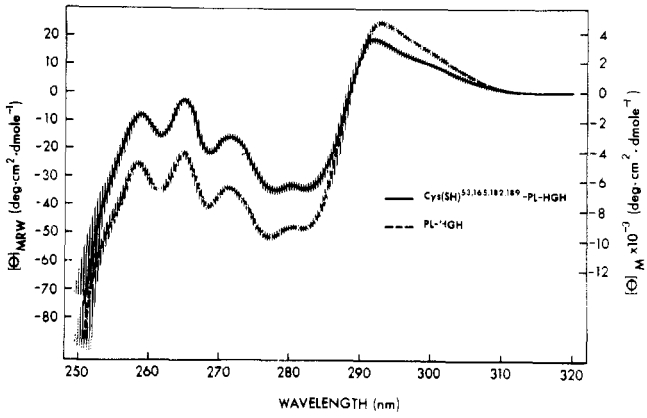

FIGURE 4: Circular dichroism spectra in the region of side-chain absorption of Cys(SH)^{53,165,182,189}-PL-HGH (- - -) and PL-HGH (—). The spectrum of the reduced form is the mean of eight measurements begun after 20 × 10^3 s in the presence of a 25 mol excess of DTT in the Tris-Cl buffer. The shaded area represents the SEM for these experiments. The spectrum of PL-HGH is the same as in Figure 1A.

thawed samples were quite stable at room temperature for periods of up to 24 h.

Reoxidation of Fully Reduced PL-HGH. Exclusion chromatography of two preparations of REOX-PL-HGH on Sephadex G-100 gave a 95% yield of monomeric material. Both the near- and far-UV CD spectra of the reoxidized protein were identical with PL-HGH.

Identification of the More Readily Reduced Disulfide Bond. As described under Materials and Methods, one sample of PL-HGH was reduced and alkylated in two separate stages: first, using radioactive iodoacetamide to label the thiol groups from the more reducible bond and, second, using "cold" alkylating agent to block the thiols produced by reduction of the more resistant bond. This sample was allowed to spontaneously dissociate at room temperature and then an aliquot of the dissociation mixture was submitted to exclusion chromatography on Sephadex G-100. The elution profile as measured by indole fluorescence and radioactivity is shown in Figure 5. Fluorescence from the tryptophan-86 residue appears in a major peak at V_e/V_0 = 1.75 and a minor peak at V_e/V_0 = 2.12. These are precisely the elution positions previously reported (Li and Bewley, 1976a) for a trimer of the 134-residue amino-terminal fragment [Cys(Cam)^{53}-HGH-(1–134)] and intact Cys(Cam)^{53,165,183,189}-PL-HGH, respectively, on this column. A small amount of fluorescence also appears in the void volume of the column. Under these conditions, the amino-terminal fragment appears to exist predominantly in this trimeric form (Li and Bewley, 1976a). In contrast, 98% of the radioactivity is found in a single peak at V_e/V_0 = 3.08. This is the elution position previously reported (Li and Bewley, 1976a) for the 51-residue carboxyl-terminal fragment Cys(Cam)^{163,182,189}-HGH-(141–191).

Discussion

Comparison of PL-HGH with HGH. It has previously been reported that PL-HGH retains the growth promoting, crop-sac stimulating, and immunochemical activities of HGH (Lewis et al., 1971; Yadley et al., 1973; Li and Graf, 1974; Clarke et al., 1974; Li and Bewley, 1976a). The CD spectrum of PL-HGH demonstrates that the loss of the hexapeptide comprising residues 135 to 149 does not produce any pronounced alterations in either the secondary or tertiary structures of the molecule. Accordingly, the retention of full biological potency,

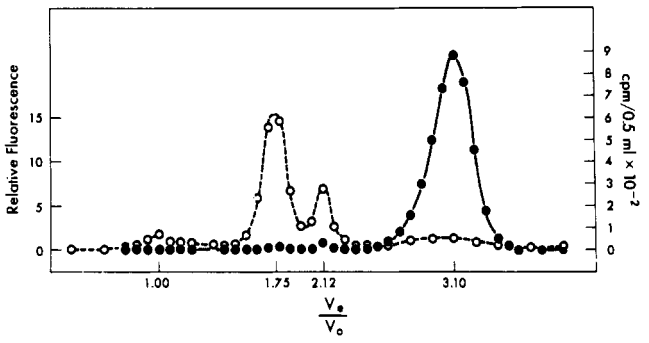

FIGURE 5: Exclusion chromatography of dissociated Cys-(Cam)^{53,165,182,189}-PL-HGH on Sephadex G-100. Fraction volumes were 2.5 ml. Reduction and radioactive labeling were carried out as described in the text. Relative fluorescence (- -o- -) was measured at 345 nm (excitation at 294 nm). Radioactivity is shown as filled circles (—●—).

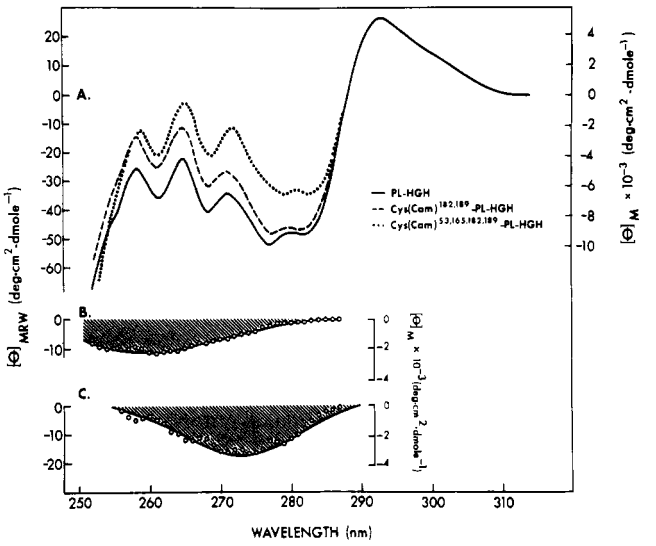

FIGURE 6: (A) Circular dichroism spectra in the region of side-chain absorption of PL-HGH (—), Cys(Cam)^{182,189}-PL-HGH (- - -), and Cys(Cam)^{53,165,182,189}-PL-HGH (●). (B) The CD band generated by subtracting the spectrum of Cys(Cam)^{182,189}-PL-HGH from that of PL-HGH. (C) The CD band generated by subtracting the spectrum of Cys(Cam)^{53,165,182,189}-PL-HGH from that of Cys(Cam)^{182,189}-PL-HGH.

by all criteria studied, may be presumed a further reflection of the near identity between the conformation of PL-HGH and that of the native hormone.

Comparison of Reduced-Carbamidomethylated Derivatives with HGH and PL-HGH. The CD spectra of Cys-(Cam)^{182,189}-PL-HGH and Cys(Cam)^{53,165,182,189}-PH-HGH also demonstrate that further modification, through reduction and carbamidomethylation of either one or both of the disulfide bonds in PL-HGH, still does not produce any immediate, pronounced conformational changes. The changes in indole dichroism, brought about by reduction of the more refractory disulfide, are easily and completely reversed by incubation of the fully alkylated monomer in the cold. This conformational equivalence is also consistent with the demonstration of full biological potency in these two derivatives (Li and Bewley, 1976a). By contrast, when Cys(Cam)^{53,165,182,189}-PL-HGH is allowed to incubate at room temperature for extended periods of time (10^2 to 10^3 h), the molecule spontaneously dissociates into its two fragments. This dissociation is accompanied by dramatic conformational changes, as measured by CD, and substantial losses in biological and immunological activities (Li and Bewley, 1976a). The fact that dissociation could be effected only after the more refractory disulfide bond had been cleaved previously suggested that this bond was the covalent link between the two fragments, i.e., the bridge linking ½-Cys-53 to ½-Cys-165 (Li and Bewley, 1976a). This has now been concretely established by alkylation of the more readily reducible bond with [^{14}C]iodoacetamide. The elution profile shown in Figure 5 clearly demonstrates that virtually all the radioactivity resides in the smaller, carboxyl-terminal fragment, with the amino-terminal fragment being free of label. This chromatogram not only serves to identify the more readily reducible disulfide bond, but also attests to the selectivity of the partial reduction.

Before beginning to discuss the CD spectra of the reduced proteins, it is important to emphasize that neither reduced DTT nor the disulfide bond in oxidized DTT exhibits significant dichroism over the spectral range studied. This observation has been reported previously (Bewley and Li, 1970) and has been extended in this present study to include oxidation of the DTT by disulfide interchange with a chiral disulfide, i.e., the carboxyl-terminl disulfide bond in bovine pituitary growth hormone. Apparently, the chirality known to exist in the protein disulfide (Gráf et al., 1975) is not conserved in the oxidized DTT. Accordingly, the DTT present in the CD samples cannot be contributing to the final measured dichroism. The possibility that a transient mixed disulfide between the protein and the DTT may produce a temporary CD contribution will be discussed below.

From Figure 2 and Table II, it is evident that the entire CD spectra of PL-HGH and either Cys(SH)^{182,189}-PL-HGH or Cys(Cam)^{182,189}-PL-HGH contain only one area of significant difference. This difference begins at about 286 nm and increases to a maximum around 258-260 nm. Below ≈256 nm, the two spectra reconverge. Unfortunately, the reconvergence occurs in a region of the spectrum where an unfavorable signal-to-noise ratio makes accurate measurements very difficult and, in addition, presents the problem of measuring smaller and smaller differences between increasingly larger numbers. Nevertheless, simple subtraction of the spectrum of the modified protein from that of PL-HGH generates a reasonably smooth, broad negative band shown in Figure 6B. This band is very similar in shape and spectral position to the bands attributed to the disulfide dichroism in L-cystine (Beychok, 1965; Coleman and Blout, 1968; Ito and Takagi, 1970; Casey and Martin, 1972; Yamashiro et al., 1975), and also to a synthetic cyclic undecapeptide prepared according to the carboxyl-terminal sequence of ovine pituitary prolactin (Yamashiro et al., 1975). The band shown in Figure 6B is also nearly equivalent (though opposite in sign) to a band obtained by subtracting the CD spectrum of partially reduced and carbamidomethylated bovine pituitary growth hormone from that of the native protein (Gráf et al., 1975). In the bovine protein it has also been established that partial reduction affects only the carboxyl-terminal disulfide bond. It is noteworthy that the bands produced by these two subtractions are so similar, in view of the fact that the aromatic chromophores in the human and bovine hormones produce very different sidechain CD spectra (Bewley and Li, 1972b; Holladay et al., 1974). Accordingly, it is proposed that this "difference" CD band represents the optical activity of the disulfide bond linking ½-Cys-182 to ½-Cys-189 in PL-HGH and that all other chromophores are contributing essentially the same optical activity in both the reduced and oxidized forms of the protein. If the above hypothesis is true, then the rate of change in ellipticity at 265 nm (Figure 3A)

may closely represent the rate of reduction of the disulfide. However, it must be recognized that the rate of change of the CD might also be influenced by the transitory existence of optically active mixed disulfides formed between the reducing agent and the protein during the first stage of the interchange reaction. As pointed out by Cleland (1964) the concentration of such mixed disulfides should rapidly become exceedingly small due to cyclization of the DTT to its fully oxidized form. Therefore, the CD spectrum of the reduced protein should ultimately become free of any influence from mixed disulfides. The extent to which the rate of change of the CD spectrum accurately reflects the rate of reduction is presently under investigation. Nevertheless, it seems justifiable to assume that the bond in question must be fully reduced, and essentially free of mixed disulfides, by the time the CD reaches a stable state. The fact that there are no convincing differences between the CD spectra of these proteins in the region below ≈240 nm suggests that the far-UV contributions of this disulfide bond are too weak, relative to the powerful $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ amide bond transitions of the α helix, to be readily measured.

The reduction of the second disulfide in PL-HGH requires a much larger excess of reducing agent in order to effect modification within a reasonable period of time. Even with a 25 mol excess the reduction appears to take 4–5 h to come to completion.[3] This again assumes that the rate of reaction may be approximated by the rate of change in ellipticity. If the loss in dichroism at 265 nm is largely a direct measurement of the disappearance of the disulfide optical activity, then the rate of reduction of PL-HGH is considerably slower than the rate for native HGH under similar conditions (Bewley et al., 1969; Bewley and Li, 1970). Clearly, this change in rate is a result of some alteration in the rate of reduction of the second bond. This suggests the existence of a very subtle conformational difference between HGH and PL-HGH. The reduction of the second disulfide bond in PL-HGH also produces greater subsequent effects than the total reduction of HGH. Even before the reduction is complete, noticeable changes have begun to occur in the indole dichroism. No such changes occur on total reduction of HGH (Bewley et al., 1969; Bewley and Li, 1970). However, these changes in the spectrum of reduced PL-HGH are reversible. If the monomeric form of Cys-(Cam)^{53,165,182,189}-PL-HGH is transferred to the cold, the indole CD will repair to what it was before the reduction began. When thawed,[4] the samples appear quite stable at room temperature, with the tryptophanyl CD being equivalent to that of PL-HGH or HGH for a period of approximately 24–36 h. Only after this lag period do the changes in the indole dichroism slowly reappear, to be followed ultimately by dissociation into fragments.

There is no evidence for similar changes in the tyrosine, phenylalanine, or the far-UV bands, either during total reduction or as a result of freezing and thawing. Very recently it has been shown (Li and Bewley, 1976b) that, when the two carbamidomethylated fragments prepared by dissociation of Cys(Cam)^{53,165,182,189}-PL-HGH are recombined and incubated at 4 °C, they slowly recombine by complementation to produce intact Cys(Cam)^{53,165,182,189}-PL-HGH in about 25% yield. This product is indistinguishable from the fully reduced-alkylated starting material, displaying full biological and immunological potencies and exhibiting a CD spectrum that is identical in all respects with that of the "frozen" Cys-(Cam)^{53,165,182,189}-PL-HGH shown in Figure 6A.

As in the case of Cys(Cam)^{182,189}-PL-HGH, the difference between the CD of "frozen" Cys(Cam)^{53,165,182,189}-PL-HGH and PL-HGH can be attributed largely to the loss of two optically active disulfide bonds, rather than to conformational changes in other chromophores occurring as a result of the reduction. This is further supported by the fact that reoxidation of the fully reduced protein completely reverses *all* CD changes produced by reduction. The difference between the spectrum of Cys(Cam)^{182,189}-PL-HGH and "frozen" Cys-(Cam)^{53,165,182,189}-PL-HGH (Figure 6C) has been assigned to the optical activity of the disulfide bond linking ½-Cys-53 to ½-Cys-165. The band generated by this subtraction is also broad with no clear-cut fine structure. The center of this band appears to be red-shifted to 272–274 nm relative to the carboxyl-terminal disulfide. It is generally accepted that low-energy disulfide bond transitions may occur at any wavelength between 250 and 340 nm, depending on the dihedral angle and immediate environment of the chromophore (Bergson, 1958, 1962; Linderberg and Michl, 1970; Woody, 1973; Nagarajan and Woody, 1973). The two disulfide bonds in PL-HGH, therefore, appear to be in different local conformations, most probably with differing dihedral angles. It is tempting to assign an absolute sense of chirality to both disulfides. According to empirical rules (Carmack and Neubert, 1967; Dodson and Nelson, 1968) which have been confirmed by crystal studies (Ito and Takagi, 1970), a negative long-wavelength CD band is associated with a left-handed screw sense of the disulfide. However, according to the quadrant rule governing disulfide bond optical activity (Linderberg and Michl, 1970; Ludescher and Schwyzer, 1971; Woody, 1973), it would first be necessary to know whether the dihedral angles of the two disulfides in HGH were smaller or larger than 90°. No such information is available at present. Moreover, as pointed out by Woody (1973), in the case of disulfides occurring in proteins, coupling between the disulfide transitions and other more powerful centers of optical activity in their immediate environment may obviate routine application of the quadrant rule. Similar coupling phenomena may also be affecting the rotational strength of these bands (Woody, 1973). Nevertheless, it is important to note that the two proposed bands shown in Figure 6B and 6C contribute a significant portion to the total optical activity of the protein in this region of the spectrum.

In contrast, the reduction of both disulfide bonds still does not produce a significant change in the CD spectrum below ≈240 nm. Presumably this again reflects the relative rotational strengths of the far-UV transitions of the disulfides and the α-helical amides.

The CD band shown in Figure 6C is not as smooth as the one shown in Figure 6B. Whether this is simply statistical "scatter" produced by the subtraction of two rapidly changing patterns, or contains contributions from small changes in the CD bands associated with tyrosine residues, cannot be clearly evaluated at present. Nevertheless, it is proposed that the major contributor to this band is the disulfide bond linking ½-Cys-53 to ½-Cys-165.

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[3] A 12.5 mol excess requires 15–20 h to effect the same change in the CD spectrum.

[4] The kinetics of this repair is presently under investigation.

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