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Optical Activity of Disulfide Bonds in Proteins: Studies on Human Choriomammotropin and Bovine Pituitary Somatotropin†

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ABSTRACT: The contributions of the homologous carboxyl-terminal disulfide bonds in human choriomammotropin (human chorionic somatomammotropin, human placental lactogen) and bovine somatotropin (pituitary growth hormone), to the near ultraviolet circular dichroism spectra of these two proteins have been evaluated. The disulfide bond in the human placental protein displays a broad, negative band centered near 260 nm ($[\theta]_{M,\,260nm} = -2100 \pm 160$ deg cm^2 dmol^{-1}) which is equivalent, within experimental error, to the band previously assigned to the identical disulfide in plasmin modified human somatotropin. The homologous disulfide in the bovine hormone also exhibits a negative band, very similar in intensity ($[\theta]_{M,\,254nm} = -2200 \pm 210$ deg cm^2 dmol^{-1}), with an estimated band center blue-shifted relative to the human proteins to 252-255 nm. Reoxidation of either partially reduced protein results in complete repair of the circular dichroism spectrum to that of the native protein. No definite contributions could be assigned below 240 nm to the optical activity of these disulfide bonds. Circular dichroism measurements have also been used to approximate the rates of reduction of the two proteins.

In a previous publication (Bewley, 1977) the individual contributions of each of the two disulfide bonds in plasmin modified somatotropin (PL-HGH)[1] to the total optical activity of the protein were estimated from circular dichroism spectra of a series of partially and completely reduced, as well as partially and completely reduced-carbamidomethylated derivatives of the hormone. It was found that the disulfide bond forming the smaller eight-membered loop near the carboxyl terminus of the molecule could be selectively and quantitatively reduced with DTT in the absence of denaturants without loss of biological or immunological activities (Li and Bewley, 1976). Within experimental error, no differences were found in the CD spectra of the unmodified and modified forms of the protein in the region of amide bond absorption. In the region of side-chain absorption, the reduced and reduced-carbamidomethylated derivatives appeared to fully retain the CD bands previously assigned to tryptophan, tyrosine, and phenylalanine chromaphores in PL-HGH and HGH. However, the CD spectra of the derivatives lacking the carboxyl-terminal disulfide bond were slightly shifted toward less negative ellipticity values between 285 and 245 nm, relative to PL-HGH. The maximum shift appeared around 258 nm. Subtraction of the CD spectrum of the partially reduced or partially reduced-carbamidomethylated derivative from that of PL-HGH resulted in a weak, broad, negative CD band centered near 258 nm ($[\theta]_{M,\,258nm} = -2100$ deg cm^2 dmol^{-1}) which was assigned to the optical activity of the disulfide bond linking half-cystine residues 182 and 189. Reoxidation of the partially reduced protein produced a complete repair of the side-chain CD spectrum. In a similar manner the second disulfide bond, linking half-cystine residues 53 and 165, could also be reduced and carbamidomethylated without loss of biological activity or change in the far-UV CD spectrum (Li and Bewley, 1976; Bewley, 1977). In the region of side-chain absorption the CD of the completely reduced-carbamidomethylated derivative again appeared to largely retain the bands originating from the aromatic chromophores in PL-HGH. Subtraction of this spectrum from that of the partially reduced-carbamidomethylated derivative produced a second broad, negative CD band, somewhat more intense than the first with its center red-shifted to around 273 nm ($[\theta]_{M,273nm} = -3200$ deg cm^2

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[1] Abbreviations used are: PL-HGH, human pituitary growth hormone modified by limited digestion with human plasmin; HGH, human pituitary growth hormone; HCS, human choriomammotropin (human chorionic somatomammotropin, human placental lactogen); BGH, bovine pituitary growth hormone; o-PR, ovine pituitary prolactin; DTT, dithiothreitol; CD, circular dichroism; UV, ultraviolet; SE, standard error.

dmol^{-1}). This band was assigned to the optical activity of the disulfide bond linking half-cystine residues 53 and 165 in PL-HGH. Reoxidation of the fully reduced protein again resulted in the complete repair of all CD changes brought about by reduction (Bewley, 1977).

HCS is a human protein of placental origin which shows a remarkable degree of sequence homology to HGH (Bewley et al., 1972). Both proteins contain 191 amino acid residues with two disulfide bonds (Li, 1972; Li et al., 1973). A total of 167 residues appear in identical sequence positions in the two proteins including all of the tryptophan and tyrosine residues, 11 of the 13 phenylalanine residues and the two disulfide bonds. Both proteins also contain an identical eight-membered disulfide loop very near the carboxyl terminus. In addition, the two hormones exhibit overlapping biological and immunological properties (Josimovich and MacLaren, 1962; Li, 1970). It has been previously reported that one of the disulfide bonds in HCS can be selectively reduced in the absence of denaturants (Li, 1970; Neri et al., 1973; Breuer, 1969; Aubert et al., 1973), although identification of the reactive bond has not been published. Reduction of the second bond requires the presence of denaturants (Li, 1970; Breuer, 1969; Neri et al., 1973; Aubert et al., 1973).

A lesser degree of sequence homology (60–65%) exists between either of the two human proteins and BGH (Gráf and Li, 1974), although the latter also contains 191 amino acid residues with two disulfide bonds. The carboxyl-terminal disulfide bond in BGH forms a nine-membered loop displaying fair homology with the eight-membered loops in HGH and HCS. Reduction of BGH with DTT in the absence of denaturants is reported to selectively and quantitatively modify only this carboxyl-terminal disulfide (Gráf et al., 1975).

In this paper the partial reductions of HCS and BGH have been studied by means of CD spectra. The reducible disulfide in HCS has been identified as the carboxyl-terminal bond and the contributions of the carboxyl-terminal disulfides to the total optical activity of both proteins have been evaluated and compared with that previously reported for PL-HGH as well as that previously reported for a synthetic cyclic undecapeptide containing the carboxyl-terminal disulfide bond of another closely related molecule, ovine pituitary prolactin (Yamashiro et al., 1975). Circular dichroism measurements have also been used to estimate the rates of reduction of the two hormones.

Materials and Methods

The isolation and purification of HCS (Li, 1970) and BGH (Li, 1954) have been previously described. The monomeric form of HCS and dimeric form of BGH were prepared by exclusion chromatography on Sephadex G-100 in 0.1 M Tris-Cl buffer (pH 8.2) as described elsewhere (Bewley and Li, 1971; Bewley and Li, 1972). The appropriate protein fractions were used directly without lyophilization. In certain instances aliquots of the protein solutions were concentrated by ultrafiltration (Amicon ultrafiltration apparatus, membrane type PM-10). Protein concentrations were determined spectrophotometrically as previously described (Bewley and Li, 1971; Bewley and Li, 1972). DTT was obtained from Calbiochem and [1-^{14}C]-α-iodoacetamide from New England Nuclear. Cyanogen bromide was obtained from Eastman Organic Chemicals. All other chemicals were of reagent grade and were used without further purification.

Circular Dichroism. Circular dichroism spectra were taken on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment. All spectra, including baselines, were scanned two–five times. With the exception of instances below 205 nm, all data reported herein were obtained at dynode voltages below 400 V. Protein concentrations were varied from 0.5 to 2.5 mg/mL in separate experiments with appropriate changes in path length allowing optimization of the signal-to-noise ratio. Each individual experiment was carried out at a constant protein concentration. A mean residue weight of 115 was used in all cases. Both mean residue ($[\theta]_{MRW}$) and molar ($[\theta]_M$) ellipticities are shown.

Analytical Procedures. Amino acid analyses were carried out on 22-h hydrolysates using a Beckman Model 120-C analyzer according to the procedures of Spackman et al. (1958). The radioactivity of appropriate samples was determined in a Packard TriCarb liquid scintillation counter, Model 3320.

Reduction of the Proteins. Reductions of HCS and BGH were carried out in 0.1 M Tris-Cl buffer (pH 8.2) at various protein concentrations, directly within the CD cells. After establishing the baseline and CD spectrum of the native protein, the appropriate amount of DTT was added in a volume of the Tris buffer which was less than 1% of the protein sample volume. No corrections were made for this small dilution factor. When the DTT had been thoroughly mixed with the protein solution, the ellipticity at 265 nm was followed as a function of time until the reaction appeared to have reached a stable state as evidenced by no further changes in ellipticity. At this point, the entire CD spectrum was repeatedly rescanned two–five times to test for additional time-dependent changes at other wavelengths. Having established the CD of the reduced protein, some samples were set aside to autoxidize. Autoxidation and purification of the reoxidized proteins were carried out as described elsewhere (Bewley, 1977). Several partially reduced samples of HCS were reacted with α-iodoacetamide or α-iodoacetic acid and the modified proteins purified as previously described (Bewley, 1977). In one instance [1-^{14}C]-α-iodododoacetamide was used to label the cysteine residues produced by partial reduction of HCS with a radioactive group. Cleavage of this product with cyanogen bromide and purification of the carboxyl-terminal peptides was carried out as previously described (Li et al., 1973).

Results

Partial Reduction of HCS. The CD spectra of native and partially reduced HCS, in the region of side-chain absorption, are shown in Figure 1A. At least six negative optically active bands may be seen in the spectrum of the native protein. The spectrum of the partially reduced protein also shows the same six bands. However, in the latter spectrum there is an overall trend toward increasingly less negative ellipticity values beginning at about 287 nm and extending to around 260 nm. Below 260 nm the two CD spectra appear to converge again, being identical within experimental error below 240 nm as further shown in Figure 1C. Unfortunately, spectra between 235 and 250 nm, accurately depicting the reconvergence, are extremely difficult to obtain due to the combination of a highly unfavorable signal-to-noise ratio and the rapidly increasing negative dichroism of the $n\rightarrow\pi^*$ transition of the α-helical amides. An actual instrument tracing of a portion of the side-chain spectra of the native and partially reduced forms, including baseline, is shown in Figure 2. Although the differences are small, it is evident that they can be readily measured under these conditions. It is of special interest to note that the CD spectra of either a carboxymethylated or carbamidomethylated derivative of the partially reduced protein are essentially identical with that of the reduced form, while the spectrum following reoxidation back to the disulfide form (90–95% yield of monomer) is identical with that of the native protein (Figure 1A). Below 240 nm, spectra of the partially reduced, carbamidomethylated, carboxymethylated, and

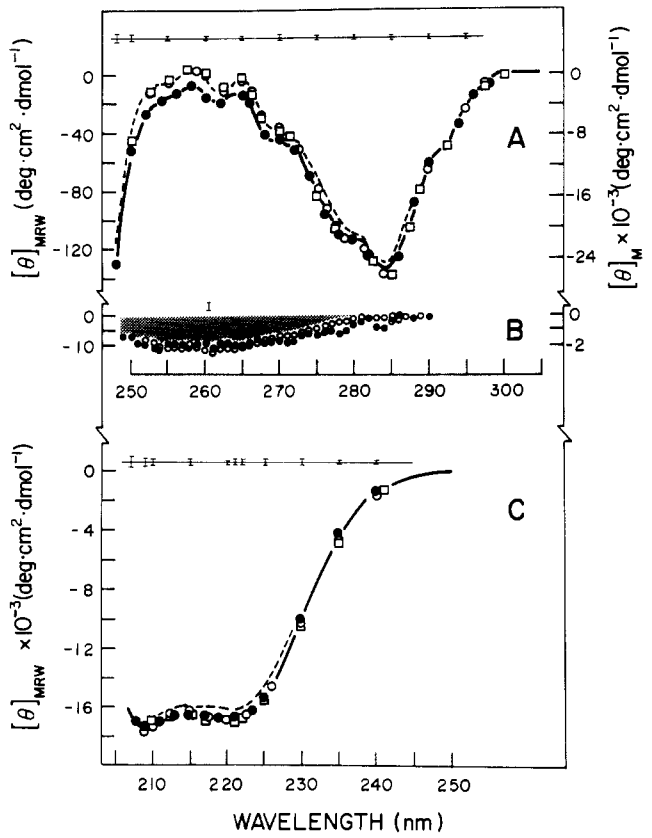

FIGURE 1: CD spectra of native HCS (—), partially reduced HCS (- - -), partially reduced-carbamidomethylated HCS (□), partially reduced-carboxymethylated HCS (○), and reoxidized HCS (●), in the region of side-chain absorption (A) and amide bond absorption (C). For the native protein, the mean of seven spectra is shown while the mean of four spectra appears for the partially reduced protein. The standard errors of the mean for the partially reduced protein are shown at selected wavelengths on separate abscissas above the appropriate portion of the spectrum. The standard errors of the mean for the native hormone are not shown, but are equal to or slightly smaller than those shown for the partially reduced form, at all wavelengths. The points depicting the derivatives are taken from individual spectra and are not mean values. In B the difference spectrum (●) obtained by subtracting the spectrum of the partially reduced protein from that of the native hormone is coplotted with a similar difference spectrum for PL-HGH (○) taken from Bewley (1977). The standard error at the negative maximum of the difference spectrum in B, calculated as described in Table III, appears above the shaded region as a vertical bar at 260.5 nm. Note the change of scale in the ordinates for A and B.

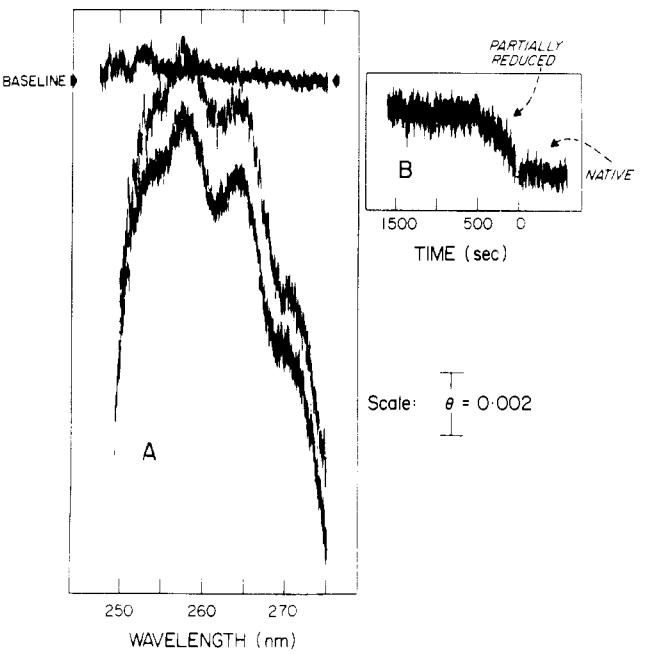

FIGURE 2: Photograph of an instrument tracing of the CD spectra of native (lowest spectrum) and partially reduced HCS (middle spectrum) with baseline. The protein concentration was 0.97 mg/mL and the path-length was 2.0 cm. In A the spectra from 275 to 250 nm are shown, while in B the change in ellipticity at 265 nm is presented as a function of time. There is no baseline trace in B.

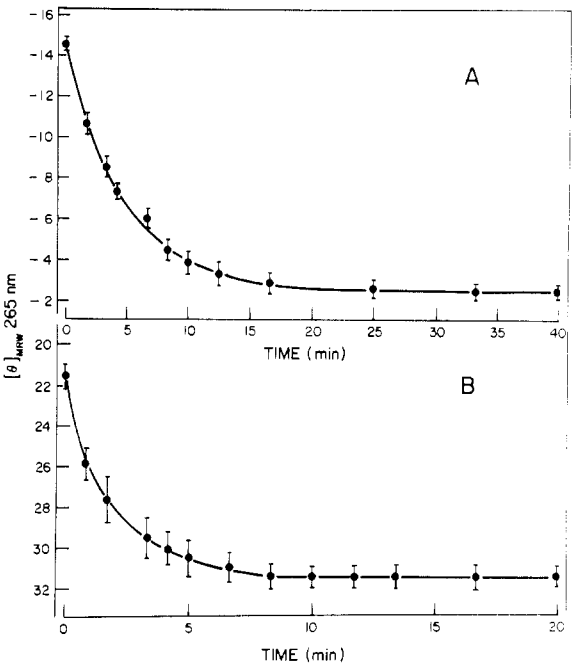

FIGURE 3: The rate of change in ellipticity at 265 nm for the partial reduction of BGH with a 5 mol excess of DTT (B). Both curves represent the means of four experiments at protein concentrations near 1 mg/mL. Standard errors of the means are shown as vertical bars.

reoxidized forms are all essentially equivalent within experimental error to that of the native hormone (Figure 1C). Subtraction of the side-chain spectrum of the partially reduced protein from that of the native hormone produces the "difference" spectrum shown in Figure 1B. This difference appears as a broad negative band beginning near 285 nm and extending below 250 nm. This band is very similar to the previously reported band generated by subtracting the spectrum of partially reduced PL-HGH from that of "native" PL-HGH (Bewley, 1977). However, unlike the PL-HGH difference spectrum, the HCS difference spectrum also exhibits three additional very weak negative maxima which may be seen at approximately 284, 278, and 272 nm.

The rate of change in ellipticity at 265 nm upon addition of a 10 mol excess of DTT over the total disulfide content of the protein is shown in Figure 3A. The reaction appears to be 50% complete in about 3.5 min and reaches a stable state in 25 to 30 min. No further changes occur at this wavelength or any other as shown by repeated complete scans taken over an additional period of several hours. This stable state is retained even if the DTT concentration is increased from a 10 mol to a 100 mol excess. Carbamidomethylation, carboxymethylation, or reoxidation of the partially reduced protein was begun 60 to 90 min after adding the DTT. Amino acid analyses confirm that only a single disulfide bond can be reduced under these conditions.

Figure 4 shows the elution pattern of radioactive peptides produced by cyanogen bromide cleavage of partially re-

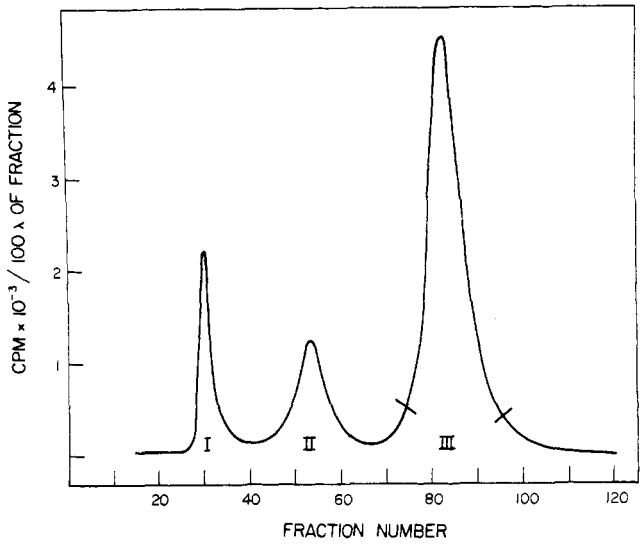

FIGURE 4: The elution pattern of radioactive fragments produced from cyanogen bromide cleavage of partially reduced-[1-^{14}C]-carbamidomethylated HCS prepared as described in the text. Chromatography on Sephadex G-25 (2.5 × 65 cm) was carried out in 0.1 N acetic acid as described by Li et al. (1973). Fraction volumes were 1.9 mL with a 75-mL forerun. Peak III was pooled as shown and submitted to amino acid analysis.

TABLE I: Amino Acid Composition of the Major Radioactive Fraction (III) Containing Cyanogen Bromide Fragments CB-5 and CB-6.[a]

Amino acid	^{14}C-labeled[a] fraction III	CB-6[b] (residues 180–191)	CB-5[b] (residues 173–179)
Lys	1.1		1
Arg	1.5	1	1
CM-Cys[c]	2.0	2.0[d]	
Asp	1.1		1
Thr	0.5		1
Ser	1.7	2	
Glu	3.0	2	1
Gly	2.1	2	1
Val	2.1	2	1
Leu	0.9		1
Phe	1.7	1	1

[a] Molar ratios assuming glutamic acid equal to 3.0. Plus traces of histidine, alanine, isoleucine, and tyrosine (<0.2 mol). [b] Composition taken from Li et al. (1973). [c] *S*-Carboxymethylcysteine. [d] Assuming reduction and carbamidomethylation of carboxyl-terminal disulfide.

duced-carbamidomethylated HCS in which the sulfhydryl groups formed during reduction were blocked with [1-^{14}C]-α-iodoacetamide. Seventy percent of the radioactivity elutes in a single peak near the position previously reported (Li et al., 1973) for the carboxyl-terminal peptides CB-5 and CB-6, comprising residues 171–179 and 180–191, respectively. Peptide CB-6 contains the two half-cystines at positions 182 and 189 which form the carboxyl-terminal disulfide bond. Amino acid analysis of the major radioactive fraction (Table I) indicates it to be an equimolar mixture of peptides CB-5 and CB-6. Two minor radioactive peaks occur at elution positions which indicate them to be larger peptides or possibly aggregates of smaller peptides. No attempt was made to purify and identify these minor components.

Partial Reduction of BGH. The CD spectra of native and partially reduced BGH, in the region of side-chain absorption,

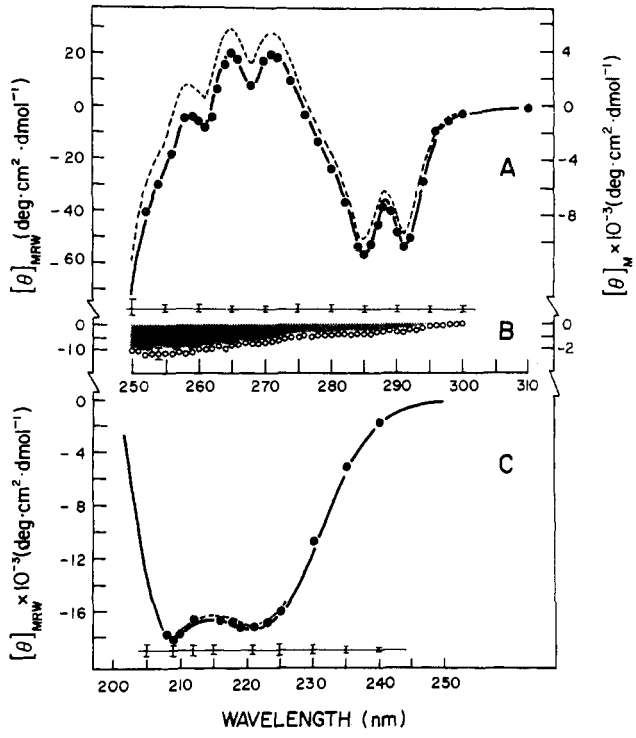

FIGURE 5: CD spectra of native BGH (—), partially reduced BGH (- - -), and reoxidation BGH (●), in the region of side-chain absorption (A) and amide bond absorption (C). For both the native and partially reduced protein the mean of three experiments is shown. The standard errors of the mean for the partially reduced hormone are presented at selected wavelengths on separate abscissas below the appropriate portion of the spectrum. The standard errors for the native protein are essentially as shown for the partially reduced form. The points representing the reoxidized protein are from a single spectrum and do not constitute mean values. In B, the difference spectrum (○) obtained by subtracting the spectrum of the partially reduced protein from that of the native hormone is depicted. The standard error at the negative maximum in B was calculated as described in Table III and appears as a vertical bar at 254 nm.

are presented in Figure 5A. Two major negative bands appear at 291 and 285 nm with a very weak negative band above 300 nm. A distinct change in curvature appears at 279–281 nm. Two relatively intense negative bands occur at 268 and 261 nm superimposed on a stronger positive envelope. Finally, a weak, negative band may be seen as a change in curvature between 256 and 253 nm. As seen in the experiments with native and partially reduced HCS and PL-HGH, the native and partially reduced BGH samples appear to contain identical band systems, with the difference between the two spectra increasing smoothly from about 295 nm, and reaching a maximum near 252–255 nm. The two spectra appear to reconverge in the region below 250 nm although again, because of an unfavorable signal-to-noise ratio, the reconvergence is very difficult to demonstrate precisely. Subtraction of the spectrum of the partially reduced protein from that of the native hormone generates the broad, negative band seen in Figure 5B. The rate of change in the ellipticity at 265 nm is presented in Figure 3B. Using a 5 mol excess of DTT, the reaction appears to be 50% complete in just over 1 min, with a stable state being achieved in about 10 min. During the next hour,[2] repeated rescanning of the entire spectrum of the partially reduced protein showed

[2] Reductions of BGH carried out at pH values higher than 8.2 and/or at protein concentrations greater than 1.5 mg/mL invariably exhibited additional changes in both the near- and far-UV regions beyond those shown in Figure 5. These changes were always accompanied by a rapid increase in the turbidity of the sample with eventual (6–8 h) precipitation of the protein.

no additional changes at any wavelength. As shown in Figure 5C, there are no clearly significant differences in the spectra below 240 nm. Reoxidation of the partially reduced protein provided a dimeric product in 80% yield whose CD spectrum was identical with the native dimer in both the near and far-UV regions of the spectrum (Figures 5A and 5C).

Discussion

As pointed out in the initial publication in this series (Bewley, 1977), the disulfide bond in DTT, in either its reduced or oxidized form, does not display a measurable CD spectrum even at concentrations an order of magnitude higher than those used herein. However, the possibility that transient mixed disulfides, formed between the protein and the DTT, might produce a temporary CD contribution must be kept in mind, particularly when attempting to interpret the rates of change of ellipticity (Figure 3) in terms of reaction rates. As pointed out by Cleland (1964), in cases where the equilibrium constant for interchange between the protein disulfide and the DTT produces essentially quantitative reduction of the protein, the concentrations of mixed disulfides should rapidly become exceedingly small due to cyclization of the DTT to its fully oxidized form. Therefore, if the protein can be quantitatively reduced, the CD of the reduced protein will ultimately become free of any significant influences from the optical activity of mixed disulfides. Even though the rate of change in ellipticity does not necessarily reflect the precise rate of reduction, in the present cases involving essentially quantitative reduction, it seems justifiable to assume that the interchange between the DTT and the protein disulfide must be at equilibrium, and, therefore, as outlined above, free of any effects from mixed disulfides, at least by the time the CD reaches a stable state. Accordingly, it is proposed that the reduction reactions must have come to completion on or before the onset of the stable state. Determination of whether the bond in question has in fact been quantitatively reduced under the conditions employed, as well as the selectivity of the reduction are aspects which cannot be settled by CD measurements alone but require further evaluation by procedures such as carbamidomethylation of the reduced protein followed by amino acid analysis of the product, along with identification of the modified cysteine containing peptides following fragmentation of the modified protein.

Reduction and CD of HCS. Previous reports (Li, 1970; Neri et al., 1973; Breuer, 1969; Aubert et al., 1973) indicate that in the absence of denaturants only one of the two disulfide bonds in HCS can be reduced. From the results presented herein this observation can be extended to include reaction conditions containing as much as a 100 mol excess of DTT for as long as 24 h at 27 °C. Amino acid analysis of five preparations gave mean ± standard error of the mean values of 1.99 ± 0.22 mol of carboxymethylcysteine, and 1.93 ± 0.37 mol of half-cystine per mol of protein. The CD data (Figure 1 and 3A) indicate that, after a small initial change, a stable state is achieved. From these two results it may be concluded that the final set of complete CD spectra are free of any contributions arising from optically active mixed disulfides and therefore represent the CD of the partially reduced protein. This conclusion is further supported by the fact that CD spectra of the purified carbamidomethylated and carboxymethylated derivatives are essentially identical with that of the partially reduced protein. Analysis of fragments produced by cyanogen bromide cleavage of a partially reduced sample, which had been labeled with radioactive α-iodoacetamide, clearly demonstrates that the reducible disulfide is the carboxyl-terminal bond linking half-cystine residues 182 and 189. It is assumed that the two minor radioactive peaks in Figure 4 are due to larger peptides, containing the modified carboxyl-terminal disulfide, but which arise due to incomplete cleavage of methionine-179 as well as other methionine residues. The facts that increasing the molar excess of DTT from 10- to 100-fold produces no additional change in the CD spectrum and, following carbamidomethylation, amino acid analysis still shows only one bond being reduced strongly suggest that the second disulfide is completely unreactive, attesting to the selectivity of the reduction. The final problem is then to evaluate to what extent the spectral differences between the native and partially reduced protein represent only the optical activity of the carboxyl-terminal disulfide bond, or contain contributions from changes in the optical activities of other chromophores (Sears and Beychok, 1973; Strickland, 1974).

The negative shoulder at 291 nm has been assigned to the $[0 - 0]^1L_b$ transition of the tryptophan-86 residue (Bewley and Li, 1971). The negative maxima at 284.5 and 279 nm are largely due to the $[0 - 0]$ and $[0 + 800\ cm^{-1}]$ transitions of tyrosine residues (Bewley and Li, 1971), although both tryptophan and the disulfide bonds may also contribute in this region. Finally, the two negative maxima at 269 and 261.5 nm, as well as the very weak band near 254–255 nm, have been assigned to the $[0 - 0]$, $[0 + 930\ cm^{-1}]$, and $[0 + (2 \times 930)\ cm^{-1}]$ phenylalanine transitions respectively (Bewley and Li, 1971). There is no evidence that partial reduction produces any significant change in the $[0- 0]$ tryptophan transition at 291 nm and it may be presumed, therefore, that no significant changes have occurred in the higher vibronic modes of this transition appearing at wavelengths between 250 and 290 nm. The difference CD spectrum in Figure 1B does, however, exhibit a fair amount of scatter from the smooth curve which would be expected for an isolated disulfide CD band. In the region from 270 to 250 nm this scatter appears to be more or less random and may be largely attributed to experimental error rather than to changes in the optical activity of the phenylalanine CD bands. In contrast, between 285 and 270 nm, three fairly distinct negative maxima do appear in the difference spectrum. These bands can be seen more clearly if the difference CD spectrum obtained from the partial reduction of the identical carboxyl-terminal disulfide in PL-HGH (Bewley, 1977) is coplotted with the HCS difference spectrum. Although the PL-HGH spectrum also exhibits some statistical scatter, no definite bands can be discerned in it. With the somatotropin spectrum as a "baseline" for the HCS spectrum, the three bands may be seen centered near 284, 278, and 272 nm. Of course, the very low intensity of these bands, and this rather indirect method of "resolving" them, places considerable limitations on the accuracy of their description. This is particularly true of the band near 272 nm. Keeping these limitations in mind, we may nevertheless tentatively assign them, from their spacing on the wavelength axis, to small changes in the optical activity of the $[0 - 0]$, $[0 + 800\ cm^{-1}]$, and $[0 + (2 \times 800)\ cm^{-1}]$ transitions of one or more tyrosine residues. As noted above, similar perturbations of the tyrosyl bands do not appear when the identical disulfide bond in PL-HGH is reduced. Figure 1B suggests that the difference CD spectrum below 270 nm for the native and partially reduced HCS is equivalent, within experimental error, to that found in the previous PL-HGH study (Bewley, 1977). Accordingly, it is proposed that the entire CD contribution of the carboxyl-terminal disulfide in HCS is equivalent, within experimental error, to the previously published CD of the carboxyl-terminal disulfide in PL-HGH (Bewley, 1977); i.e., a broad negative band with a maximum near 258–262 nm ($[\theta]_{M,260nm} = -2100 \pm 160$ deg cm^2 dmol^{-1}). It is recognized that these two CD

TABLE II: Estimated Contribution of the Carboxyl-Terminal Disulfide Bonds in PL-HGH, HCS, BGH, and o-PR to the Total Optical Activity of the Parent Protein.

Protein	Disulfide linking ½-Cystines A and B		λ_{max} (nm)	$[\theta]_M$ (deg cm^2 dmol^{-1})	Δ^a (nm)
	A	B			
PL-HGH[b]	182	189	258–260	−2100 ± 120[c]	16–18
HCS	182	189	258–260	−2100 ± 160	16–18
BGH	182	189	252–255	−2200 ± 210	24–26
o-PR[d]	191	199	260–262	−1350	24–26

[a] Half-bandwidth in nm, measured at $1/e$ of the maximum ordinate. [b] Taken from Bewley (1977). [c] The uncertainty in these ellipticities has been calculated as the square root of the sum of squares of the standard errors taken from the CD spectra of the native and partially reduced hormones at λ_{max}. No estimate is available in the case of o-PR. [d] From Yamashiro et al. (1975).

bands could in fact be different by as much as 10–20% and still appear the same, since the experimental error is large in proportion to their intensity. The observation that the CD spectra of the carbamidomethylated and carboxymethylated derivatives are identical with that of the partially reduced HCS, while reoxidation returns the spectrum to that of the native hormone, provides further support to this assignment. Figure 1C demonstrates that, if this disulfide bond also contributes to the optical activity below 240 nm, that contribution is too weak to measure in the presence of the powerful CD bands generated by the α-helical amides. A similar situation has been noted in the case of both disulfide bonds in PL-HGH (Bewley, 1977).

Reduction and CD of BGH. It has been previously established that reduction of BGH under the conditions used herein results in both a selective and quantitative modification of the carboxyl-terminal bond (Gráf et al., 1975). Figures 3B and 5 demonstrate that the CD spectrum of the partially reduced protein reaches a stable state only slightly different from that of the native hormone. It may be noted that the CD of partially reduced BGH stabilizes more rapidly than in the case of HCS, even though the molar excess of reducing agent was twice as large for the latter protein. For the reasons described above, this difference in apparent rate cannot be clearly equated at present to actual differences in the rates of reduction of the two proteins. This point is under further investigation.

The negative maximum at 291 nm has been assigned previously to the $[0 - 0]$,^1L_b transition of the tryptophan-86 residue (Bewley and Li, 1972; Holladay et al., 1974), while the band at 285 nm is regarded as a composite of a $[0 - 0]$ tyrosine transition plus a $[0 + 850\ cm^{-1}]^1L_b$ indole transition (Bewley and Li, 1972; Holladay et al., 1974). The weak shoulder above 292 nm has been assigned to two ^1L_a indole transitions (Holladay et al., 1974) and the change in curvature near 278–280 nm has been assigned to the $[0 + 800\ cm^{-1}]$ tyrosine transition (Holladay et al., 1974). The three negative bands at 268, 261, and 254–255 nm have been assigned to the $[0 - 0]$, $[0 + 930\ cm^{-1}]$, and $[0 + (2 \times 930)\ cm^{-1}]$ transitions of phenylalanine, respectively (Bewley and Li, 1972; Holladay et al., 1974).

The difference CD spectrum (Figure 5B), obtained by subtracting the spectrum of the partially reduced protein from that of the native hormone, is a broad, negative band apparently centered around 252–255 nm ($[\theta]_{M,254nm} = -2200 \pm 210$ deg cm^2 dmol^{-1}). Unlike the case of HCS, this difference CD appears relatively smooth, the scatter of points mostly reflecting experimental error rather than significant perturbations of the optical activities of chromophores other than the carboxyl-terminal disulfide bond. Reoxidation of the disulfide produces a complete repair of the CD back to that of the native hormone. It is therefore proposed that the CD band in Figure 5B represents the contribution of the carboxyl-terminal disulfide bond to the total optical activity of BGH. As shown in Figure 5C, any CD bands below 240 nm, associated with this disulfide bond, are again too weak to detect with surety in the presence of the α-helical amides.

The amino acid sequences around the carboxyl-terminal disulfide bonds in HGH and PL-HGH (Li, 1972), HCS (Li et al., 1973), BGH (Li et al., 1972), and o-PR (Li et al., 1970; Li, 1976) are shown below.

HGH, PL-HGH and HCS:

-Gln-Cys-Arg-Ser-Val-( )-Glu-Gly-Ser-Cys-Gly-Phe$_{191}$-COOH

BGH:

-Lys-Cys-Arg-Arg-Phe-Gly-Glu-Ala-Ser-Cys-Ala-Phe$_{191}$-COOH

o-PR:

-Asn-Cys-Arg-Ile-Ile-Tyr-Asn-Asn-Asn-Cys$_{199}$-COOH

It is evident that there is either identity or a fair degree of homology between these structures. The present estimates of the contributions which these four disulfide bonds make to the total optical activities of their respective parent hormones are listed in Table II. Given the fact that the experimental error in these measurements is high in proportion to the optical activity of these bonds, further work will be required to establish whether the small differences in the intensities, apparent half-band widths, and band centers shown in Table II are real or not. Nevertheless, these data clearly demonstrate that these four chromophoric groups exhibit very similar optical activities. This may be in keeping with the close structural relationships noted in the sequences around these chromophores, although even the two identical species (PL-HGH and HCS) need not necessarily exhibit the same properties depending on the local environments contributed by the remainder of the two parent proteins. The o-PR data were obtained from studies of a synthetic undecapeptide rather than from experiments on the natural hormone (Yamashiro et al., 1975). As pointed out by Woody (1973), coupling between the disulfide bonds and stronger centers of asymmetry within their local environments may sharply affect the intensity and even the sign of the near-ultraviolet disulfide CD bands. There is a greater probability for such coupling phenomena in the intact proteins than in a small peptide, which may in part explain the more significant difference between the intensities of the three disulfides measured in the intact proteins and the disulfide in the undecapeptide. The partial reduction and CD of native o-PR are now under investigation. It will be of great interest to see if the properties of this disulfide in the intact hormone are significantly different from those in the synthetic peptide.

It would be tempting to assign an absolute sense of chirality

to these disulfide bonds from the sign of their CD bands, or at least to state that all four must have the same screw sense. However, until the problem of potential coupling mentioned above can be solved and more detailed information about the dihedral angles of the bonds can be obtained, such assignments cannot be unequivocally made (Woody, 1973). Nevertheless, the data do indicate that the dihedral angles of all four disulfides must be fairly close to 90°, probably within 10° of that value (Woody, 1973).

Finally, it should be noted that the optical activities assigned to these disulfides represent significant contributions to the total optical activities of their parent hormones in the region of side-chain absorption. Accordingly, whenever chemical modifications involving destruction of the disulfide bonds in these hormones are employed, great care must be exercised in interpreting small changes in the CD spectra. As evidenced above, such changes may merely reflect the loss, through chemical modification, of centers of intrinsic optical activity rather than conformational changes in the protein. This precaution can probably be generalized to include many disulfide bond-containing proteins. Each situation should be carefully evaluated before firm interpretations are made.

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