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Dissociation Kinetics of Tetra-*S*-carbamidomethylated Plasmin-Modified Human Somatotropin†

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ABSTRACT: The dissociation of tetra-*S*-carbamidomethylated, plasmin-modified human somatotropin at 25 ± 2 °C has been found to be a slow, exergonic equilibrium reaction; an equilibrium constant of 1.77 mol/L and a standard free energy of dissociation of −340 cal/mol have been calculated. The major products of the dissociation have been shown to consist of a monomeric form of the 51-residue carboxyl-terminal fragment and a trimeric form of the 134-residue amino-terminal fragment. The carboxyl-terminal fragment appears to be a random coil, while the amino-terminal fragment retains a considerable degree of secondary and tertiary structures.

It has been reported that human somatotropin (HGH)[1] can be subjected to limited digestion with human plasmin resulting in the loss of a hexapeptide comprising residues 135 to 140 (see Figure 1) without loss in biological or immunological activities (Clarke et al., 1974; Li and Gráf, 1974; Mills et al., 1973; Yadley and Chrambach, 1973). In the absence of denaturants, reduction and carbamidomethylation of the two disulfide bonds in the plasmin-modified hormone (PL-HGH) remove the remaining covalent link (the disulfide bond linking half-cystine-53 to half-cystine-165) between the amino terminal 134-residue fragment and the 51-residue carboxyl-terminal fragment (Li and Gráf, 1974). Preliminary studies (Li and Bewley, 1976) showed that the tetra-*S*-carbamidomethyl-PL-HGH does not immediately dissociate but remains intact

† From the Hormone Research Laboratory, University of California, San Francisco, California 94143. *Received March 21, 1978.* Human somatotropin series paper no. 57. This work was supported in part by Grant NIH-AM 18677 from the National Institutes of Health and the Hormone Research Foundation.

[1] Abbreviations used: HGH, human somatotropin (growth hormone); PL-HGH, the product produced by limited proteolysis of HGH with human plasmin; DTT, dithiothreitol; CD, circular dichroism; UV, ultraviolet; V_e/V_0, the ratio of elution to void volume on exclusion chromatography.

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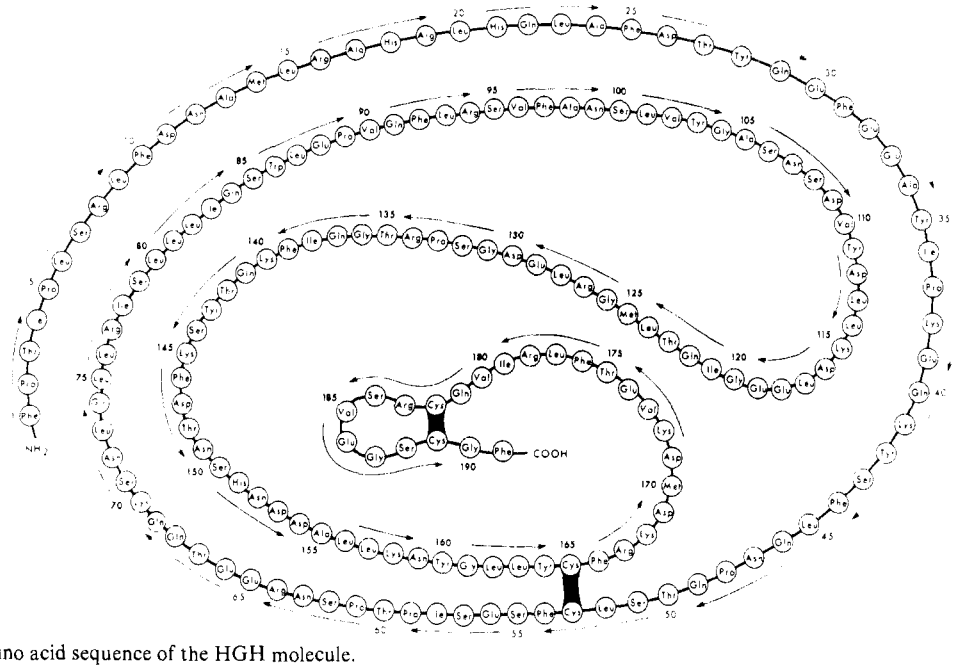

FIGURE 1: The amino acid sequence of the HGH molecule.

for 24–36 h and exhibits full biopotency. Slightly alkaline solutions of tetra-*S*-carbamidomethyl-PL-HGH are completely resistant to dissociation when either frozen or kept at 2 °C for periods up to 3 months. In contrast, if incubated at 25 ± 2 °C, the derivative begins a slow dissociation after 24–36 h. The present investigation extends these preliminary observations (Li and Bewley, 1976) on the kinetics of the dissociation reaction as well as the nature and properties of the products.

Materials and Methods

HGH was isolated from fresh pituitary glands (Li et al., 1962). Digestion of the hormone by human plasmin was carried out as previously described (Li and Gráf, 1974). A monomeric form of PL-HGH was obtained by gel filtration on Sephadex G-100 in 0.1 M Tris-Cl buffer of pH 8.2 containing 0.02% NaN_3 and used directly without lyophilization. DTT and α-iodoacetamide were obtained from Calbiochem. α-Iodo[1-^{14}C]acetamide was purchased from New England Nuclear. All other chemicals were of reagent grade and used without further purification.

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 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). A mean residue weight of 115 was used for PL-HGH and tetra-*S*-carbamidomethyl-PL-HGH. Mean residue weights of 114 and 120 were used for the purified 134-residue amino-terminal and 51-residue carboxyl-terminal fragments, respectively. 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).

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 Beavan and Holiday (1952). The absorptivities of PL-HGH and tetra-*S*-carbamidomethyl-PL-HGH were assumed to be equivalent to that previously reported for native HGH (Bewley et al., 1969).

Fluorescence emission spectra were obtained on a Hitachi-Perkin-Elmer spectrofluorimeter, Model MPF-2A, according to procedures described elsewhere (Bewley and Li, 1975). Unless stated otherwise, the excitation monochromator was set at 294 nm in order to selectively detect the indole emission. Relative fluorescence emission at 340 nm was read against a sealed sample of native HGH in the Tris-Cl buffer.

Stepwise reduction–carbamidomethylation of the two disulfide bonds (providing a tetra-*S*-carbamidomethyl-PL-HGH in quantitative yield, in which half-cystine residues 182 and 189 are selectively labeled with a [1-^{14}C]carbamidomethyl group, while half-cystines-53 and -165 contain a nonradioactive carbamidomethyl group) was performed as previously described (Bewley, 1977). An undissociated, monomeric derivative was obtained by gel filtration on Sephadex G-100 immediately following the second stage reduction–carbamidomethylation reaction. This product was obtained in 90% yield and exhibited a specific radioactivity of 2.90 μCi/μmol. The purified, intact derivative was concentrated to 0.95 mg/mL by ultrafiltration (Amicon PM-10 membrane); one aliquot of the concentrate was kept within a sealed CD cell, another within a sealed rectangular cell used subsequently for both UV absorption and fluorescence spectra. A third aliquot was sealed in a test tube with parafilm. Small aliquots of the latter sample were chromatographed on Sephadex G-100 at regular intervals. All three sealed aliquots were maintained at 25 ± 2 °C throughout the dissociation experiment. CD, UV absorption, and fluorescence spectra were taken at regular intervals.

Results

In three separate dissociation experiments, the optical density of the mixture was followed. In all three cases, there were no significant changes in the wavelength maximum or the maximum optical density. This observation is of some impor-

tance, as it can be used to estimate the individual absorptivities of the two fragments. Native HGH is reported to exhibit an experimental molar extinction coefficient of 2×10^4 (Bewley et al., 1969). This may be compared to a value of 1.6×10^4, calculated for HGH as described by Beavan and Holiday (1952). It has been assumed that the additional absorptivity of HGH, over the calculated value, is due to conformational effects on the aromatic chromophores (Bewley and Li, 1975). There is no evidence for a change in either conformation or extinction coefficient upon limited plasmin digestion or reduction–carbamidomethylation of the two disulfide bonds in HGH (Bewley and Li, 1975; Bewley, 1977). Apparently, during dissociation of tetra-*S*-carbamidomethyl-PL-HGH there is again no net change in the conformational effects. This is not equivalent to saying that no conformational changes occur, only that there is no net effect on the total optical density of the mixture. As will be shown below, the smaller, carboxyl-terminal fragment is obtained in an essentially random coil, monomer form which should be devoid of any conformational effects on its seven chromophores (tyrosine-143, -160, and -164 plus phenylalanine-146, -166, -176, and -191). Accordingly, the *calculated* extinction coefficient for this fragment is probably valid, and we have used this value (ϵ_m 4020 and $\epsilon_{276nm}^{0.1\%,1cm} = 0.675$) in estimating the concentrations of this fragment. In contrast, as also shown below, the larger, amino-terminal fragment retains a remarkable degree of both secondary and tertiary structures. It is very probable that conformational effects do still exist in this fragment or that new ones have come into play and a calculated extinction coefficient would undoubtedly be in error. This proposition is supported by the observation of a conservation of the entire UV-absorption spectrum during dissociation. We assume that the conformational effects characteristic of native HGH are either retained or equaled in the free form of the amino-terminal fragment. Therefore, the molar extinction coefficient of this fragment can be estimated at ϵ_m 15 980 ($\epsilon_{277nm}^{0.1\%,1cm} = 1.04$), by simply subtracting the calculated value for the random-coil, carboxyl-terminal fragment from that of native HGH.

Exclusion Chromatography of Dissociating Mixtures. The extent of dissociation at any time may be evaluated by exclusion chromatography of a small aliquot of the mixture on Sephadex G-100 as shown in Figure 2. The elution positions of the intact molecule (V_e/V_0 = 2.12), the amino-terminal fragment (V_e/V_0 = 1.75), and the carboxyl-terminal fragment (V_e/V_0 = 3.00) have been determined previously on this same column with authentic samples of each (Li and Bewley, 1976). The carboxyl-terminal fragment is detected by its radioactivity, the amino-terminal fragment by indole fluorescence, and the intact form by the superposition of both radioactivity and fluorescence. Since the fluorescence quantum yield and emission maximum are constantly changing during dissociation, quantitative estimation of the degree of dissociation is computed only on the basis of radioactivity and the conservation of mass. As can be seen in Figure 2A, the elution pattern of the entire sample (2 mL at ~1 mg/mL), chromatographed 2 h after the final carbamidomethylation step, exhibits no radioactivity at the position of the free carboxyl-terminal fragment, indicating that no perceptible dissociation has occurred. The slight shoulder in both radioactivity and fluorescence appearing at $V_e/V_0 \simeq 1.70$ is due to a small amount of dimer of the intact protein produced during the reduction–carbamidomethylation reactions. As can be seen, this dimer of the intact form also ultimately dissociates into the two fragments. The chromatograms at 22 to 92 h demonstrate increased amounts of dissociation, as evidenced by the free carboxyl-terminal fragment. However, the free form of the amino-terminal fragment becomes evident only after 140 h. It will be shown below that this free form is trimeric. This demonstrates that, under these conditions, trimerization of the amino-terminal fragment occurs more slowly than the dissociation. Figures 2B and 2C also demonstrate that monomeric or dimeric forms of the amino-terminal fragment cannot be resolved from the intact form in this chromatographic system.

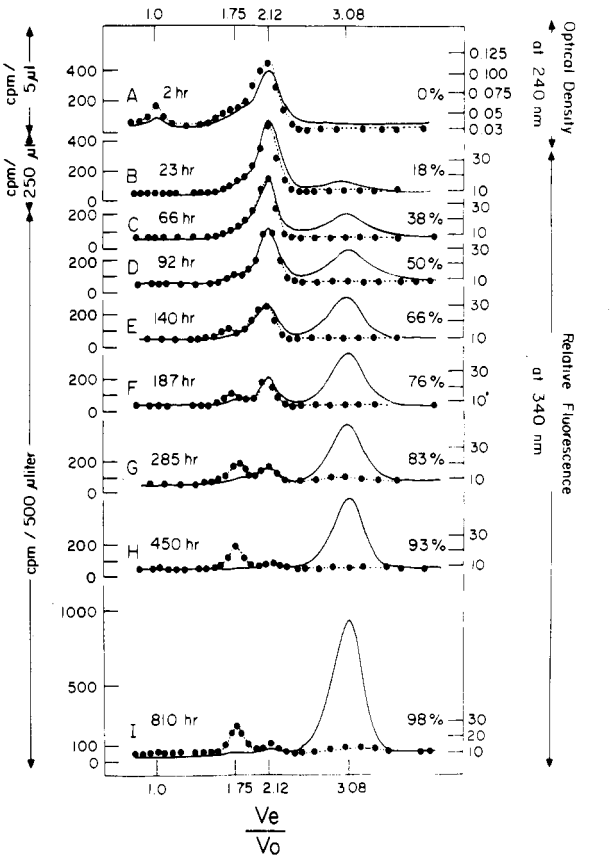

FIGURE 2: Exclusion chromatography of the dissociating mixture on Sephadex G-100. The times of incubation at 25 ± 2 °C are shown above each chromatograph at the position of V_0. The extent of dissociation, obtained from the integrated radioactivity profile, appears above each chromatogram at the far right. In curve A, 2 mg of protein was applied to the column; in curves B through H, approximately 50 µg of total protein was applied; in curve I, approximately 100 µg of total protein was applied. In all chromatograms, radioactivity is shown as a solid line; the filled circles represent optical density in A and relative fluorescence in B through H.

The dissociation proceeds smoothly, reaching an apparently stable state in about 500 h. Chromatograms run between 600 and 2000 h are all equivalent and show 96 to 98% dissociation. The small peak, representing 2–4% of the original intact molecules, remains regardless of how long the time is extended. It is of special interest to note that despite these very long incubations at 25 ± 2 °C no aggregated forms appear in the void volume, nor do small peptides containing indole fluorescence appear at positions with $V_e/V_0 > 2.12$. All components can be accounted for as either free fragments or intact protein. The kinetics of dissociation, as represented by the rate of appearance of the free carboxyl-terminal fragment, is shown in Figure 3A.

From its elution position on a calibrated G-100 column, a Stokes radius of 32.3 Å and an estimated molecular weight of 47 000 have been calculated for the amino-terminal fragment. From this Stokes radius and the previously reported (Li, 1975) sedimentation coefficient of 3.81 S, a molecular weight estimate of 51 000 is obtained. Both results are consistent with a largely trimeric product for the purified amino-terminal

TABLE I: Properties of HGH, PL-HGH, and Reduced-Carbamidomethylated Derivatives and Fragments of PL-HGH.[a]

preparation	V_e/V_0	S (Å)	$s_{20,w}$	fluor emiss max (nm)	$\epsilon_{277nm}^{0.1\%,1cm}$
HGH	2.12	23.8	2.50[c]	336	0.931
PL-HGH	2.13	23.6	2.41[c]	336-337	0.931
Cys(Cam)^{53,165,182,189}-PL-HGH	2.13	23.8		337-338	0.931
Cys(Cam)^{53}-HGH(1-134) (trimer)	1.75	32.3	3.81[c]	342-343	1.04
Cys(Cam)^{53}-HGH(1-134)(monomer or dimer)	~2.10	~25		338-339	
Cys(Cam)^{165,182,189}-HGH(141-191)	3.08			305-306[b]	0.657

[a] All measurements in 0.1 M Tris-Cl buffer (pH 8.2). [b] Excitation at 276 nm. [c] Taken from Li and Bewley (1976).

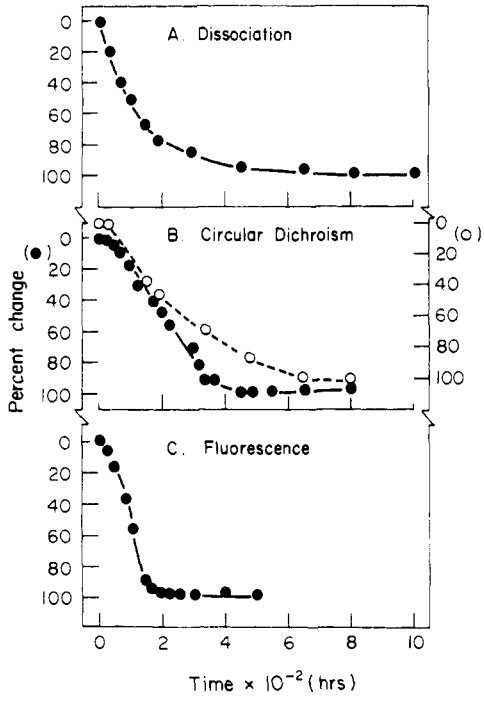

FIGURE 3: The rates of change of measured parameters as a function of time during dissociation. Each parameter is expressed as a percentage of the total change in that parameter. In A, the dissociation rate represents the rate of appearance of the radioactive carboxyl-terminal fragment at $V_e/V_0 = 3.08$. In B, the rates of change in the ellipticity at 298 (●) and 221 nm (○) are shown. In C, the rate of change in fluorescence emission at 340 nm (exitation at 295 nm) is presented.

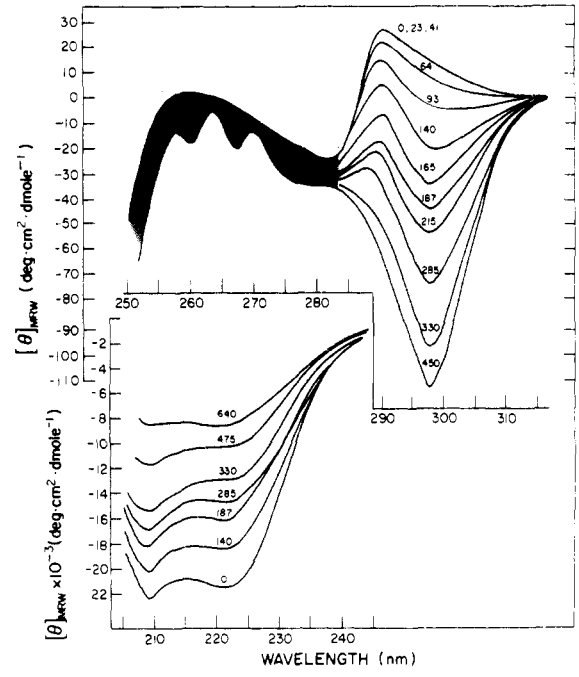

FIGURE 4: Circular dichroism spectra of the dissociating mixture at various times in the region of side-chain absorption (A) and the region dominated by amide bond absorption (B). The time of incubation is indicated above each spectrum.

fragment (monomer weight = 15 300).

Change in Fluorescence during Dissociation. Careful examination of Figure 2 demonstrates a continual loss of the total indole emission at 340 nm, summed over all components. At any excitation from 285 to 294 nm, native HGH and PL-HGH exhibit an emission maximum at 336 nm (Li and Bewley, 1976). In contrast, freshly prepared tetra-*S*-carbamidomethyl-PL-HGH shows an emission maximum shifted to 339-340 nm (Table I). The relative quantum yields of these are presently unknown. The purified amino-terminal trimer gives an additional shift in emission maximum to 342-343 nm (Table I). The loss of total indole fluorescence with accumulation of the trimer cannot be quantitatively explained as due only to this shift in emission maximum. Separate experiments demonstrate that the area under the emission spectrum for the intact molecule is more than twice as great as the area under the spectrum of the trimer, per OD unit. Since the trimer has lost much less than half of its optical density per mole (through loss of the carboxyl-terminal fragment), it is clear that the molar quantum yield of the indole groups in the trimer is only a fraction of the molar quantum yield of the same fluorophores in the intact molecule. The rate of loss in total fluorescence intensity at 340 nm is shown in Figure 3C. Obviously, the curve contains unresolved components produced by both the shift in emission maximum and the loss of total quantum yield.

Change in Circular Dichroism during Dissociation. Figure 4 presents the changes in the circular dichroism spectra occurring during dissociation. It is evident from Figure 4A that the positive, asymmetric band above 290 nm, which has been previously assigned to the single tryptophan-86 residue (Bewley et al., 1969, 1972), is lost, being replaced by a much stronger negative band at 298 nm. From model compound studies (Strickland et al., 1969), this band may also be assigned to the indole ring of tryptophan-86. It should be noted that no changes can be seen in the CD spectra until after ~50 h, although Figure 2 demonstrates that approximately 30% of the molecules dissociate in this time period. Very little change can be seen at any time in the tyrosine and phenylalanine bands between 250 and 289 nm, although there is a general tendency for these bands to weaken in intensity toward the end of the incubation period. In contrast, a very significant change occurs below 250 nm in the region dominated by amide bond absorption. The two negative bands at 221 and 209 nm, characteristic of the 55 ± 5% α helix in native HGH or intact tetra-*S*-carbamidomethyl-PL-HGH (Li and Bewley, 1976), are

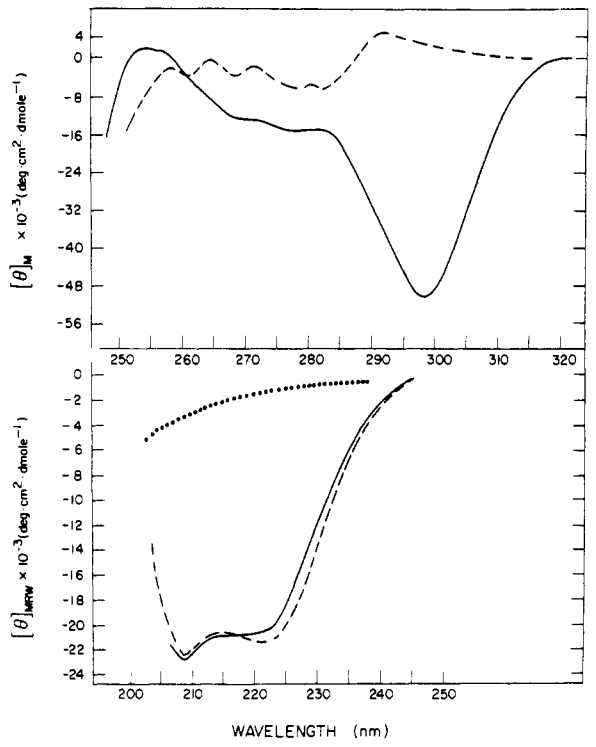

FIGURE 5: Circular dichroism spectra of the purified amino-terminal fragment (—) and the purified carboxyl-terminal fragment (· · ·) in the region of side-chain absorption (A) and the region dominated by amide-bond absorption (B). The CD spectrum of undissociated tetra-*S*-carbamidomethyl-PL-HGH (- - -) is added for comparison.

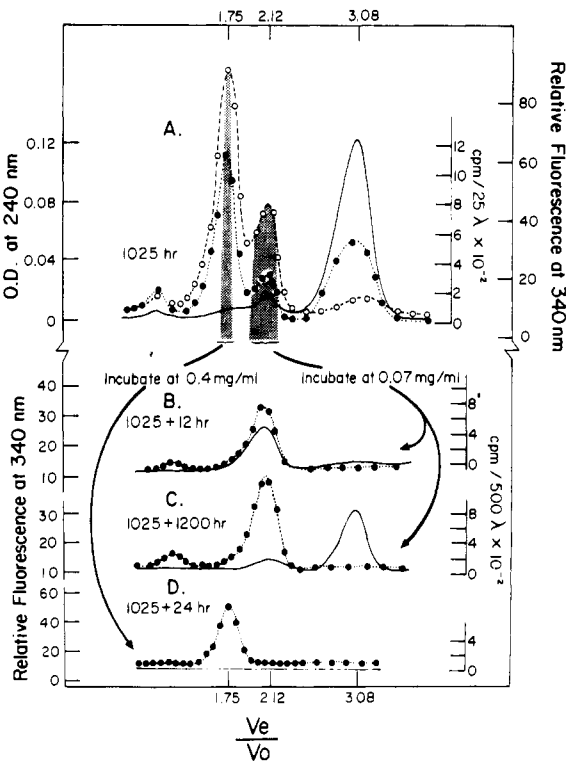

FIGURE 6: Exclusion chromatography on Sephadex G-100. In A, the final chromatogram after 1025-h incubation is shown. Approximately 1 mg of total protein was applied to the column. The peak representing intact protein (V_e/V_0 = 2.12) was concentrated by ultrafiltration to 0.07 mg/mL total protein. One aliquot of this concentrate was rechromatographed after an additional 12 h (B), while a second aliquot was allowed to incubate for an additional 1200 h before rechromatography (C). In D, the peak tube of the amino-terminal fragment (V_e/V_0 = 1.75, concn = 0.4 mg/mL) was directly rechromatographed after an additional 24 h of incubation. All incubations were carried out at 25 ± 2 °C. In all chromatograms radioactivity is shown as a solid line, optical density as open circles, and relative fluorescence as filled circles.

progressively lost with increasing time. A stable state is reached after ~500 h, corresponding to an average α-helical content of 30–35% for the entire mixture (when calculated as though still intact). The rates of change in the ellipticities at 298 and 221 nm are shown in Figure 3B. The negative band at 298 nm increases in intensity more slowly than either the rate of dissociation (Figure 3A) or the change in indole fluorescence (Figure 3C). The rate of attenuation of the α-helical band at 221 nm is even slower than the changing indole CD at 298 nm.

Circular Dichroism of Purified Fragments. The CD spectra of the amino-terminal trimer and the carboxyl-terminal monomer which have been purified by rechromatography on Sephadex G-100 are presented in Figure 5. The carboxyl-terminal fragment exhibits no discernible bands in the region of side-chain absorption and no evidence of any ordered form of secondary structure in the region dominated by amide bond absorption. In sharp contrast, the trimeric amino-terminal fragment shows a very intense negative band at 298 nm which has been assigned to the Trp-86 residue. In addition, this fragment exhibits very intense negative bands at 221 and 209 nm, characteristic of the α helix. This spectrum (Figure 5B) is very similar to that of native HGH or intact tetra-*S*-carbamidomethyl-PL-HGH, representing approximately 55 ± 5% α helix in the trimer form of the fragment.

Chromatography of Dissociated Mixture. Figure 6A shows the elution pattern of the dissociated mixture (1.9 mg of total protein in 2.0 mL) after 1025 h at 25 ± 2 °C. Careful examination of the optical density and fluorescence patterns demonstrates that the fluorescence emission at 340 nm is much greater per OD unit for the intact form (V_e/V_0 = 2.12) than for the amino-terminal trimer (V_e/V_0 = 1.75). When the peak tube of the trimer form is incubated for 24 h at 25 ± 2 °C (concentration 0.4 mg/mL) and then rechromatographed, a purified trimer is recovered in good yield (Figure 6D).

When the intact form (V_e/V_0 = 2.12) from Figure 6A is pooled and concentrated approximately twofold to 0.070 mg/mL by ultrafiltration, essentially no radioactivity can be detected in the filtrate from a PM-10 membrane (Amicon Inc.). Since this membrane is highly permeable to free carboxyl-terminal fragment, it would appear that this material is indeed an undissociated form. Immediate rechromatography of this pooled material also demonstrates it to be intact (Figure 6B). However, after incubation at this relatively low concentration (0.07 mg/mL) for extended periods, the intact protein further dissociates to an extent of 90–95% (Figure 6C). It should be noted that, in contrast to the incubations carried out at higher concentrations, the amino-terminal fragment now elutes at a position (V_e/V_0 = 2.10–2.12) indicating a monomer or possibly dimer form, with no evidence of trimer formation. Any attempt to concentrate it by lyophilization or ultrafiltration results only in trimer and more highly aggregated forms. At present, the only property we have been able to demonstrate for this form is a fluorescence emission maximum at 338–339 nm (Table I).

Discussion

Under the conditions described herein, the dissociation of reduced-carbamidomethylated-PL-HGH is a slow, spontaneous reaction. The clear separation of radioactivity from the fluorescent peaks attests to the selectivity of the first-stage reduction and ^{14}C-carbamidomethylation of the carboxyl-terminal disulfide bond, as well as quantitative cleavages

during plasmin digestion and reduction–carbamidomethylation of the second disulfide. These two points are further demonstrated by the additional spontaneous dissociation of the 2–4% intact form as shown in Figure 6C.

As demonstrated by exclusion chromatography, the products of the reaction appear to consist of a monomeric form of the carboxyl-terminal fragment [HGH(141–191)], devoid of secondary or tertiary structure, and a trimer form of the amino-terminal fragment [HGH(1–134)], which contains (or retains) a considerable degree of both secondary and tertiary structures. In addition, 2–4% of the intact form also appears in the chromatogram. At present, we cannot detect the presence of any monomeric or dimeric form of the amino-terminal fragment, but small amounts of these forms may be present near or under the peak at $V_e/V_0 = 2.12$. This point will require further experimentation.

The small amount of intact hormone which always remains at $V_e/V_0 = 2.12$ could have arisen for several reasons: because of incomplete cleavage of the peptide backbone during the plasmin digestion, incomplete reduction, or alkylation of the second disulfide bond, or both. In contrast to these mechanisms, the presence of this form might represent an equilibrium state between the intact and the dissociated molecules. Figure 6A,C clearly demonstrates that this is indeed the case, since the intact sample will itself spontaneously dissociate further when separated from the fragments, reestablishing an equilibrium state. If we assume the overall reaction to be an equilibrium between intact, monomeric, and trimeric forms:

$$3[\text{Cys(Cam)}^{53,165,182,189}\text{-PL-HGH} \rightleftharpoons [\text{Cys(Cam)}^{53}\text{-HGH(1-134)}]_3 \text{ (trimer)} + 3[\text{Cys(Cam)}^{165,182,189}\text{-HGH(141-191)}] \text{ (monomer)}$$

an equilibrium constant at 25 ± 2 °C may be calculated from the molar concentrations of these three components. These concentrations may be computed from the known starting concentration of the intact form and the degree of dissociation at equilibrium taken from the elution pattern of radioactivity (Figures 2 and 3). Thus, the equilibrium constant is found to be 1.77 mol/L, corresponding to a standard free energy of dissociation of −340 cal/mol at 25 ± 2 °C.

It is apparent from Figure 2 that dissociation is accompanied by a relatively rapid quenching of the indole fluorescence. This alteration in fluorescence properties includes a shift in the indole emission maximum (Table I). Figure 4 demonstrates that there are also simultaneous changes in the CD spectrum. Specifically, these changes involve a loss of α-helix content for the total sample and distinct alterations in the local environment of the tryptophan-86 side chain.

Purified samples of the amino-terminal trimer contain approximately 55% α helix, corresponding to an average of 75 residues. Native HGH (Bewley et al., 1969) or intact tetra-*S*-carbamidomethyl-PL-HGH (Li and Bewley, 1976) is also reported to contain 55% α helix or about 105 residues. Since the rate of loss of α helix corresponds kinetically with the formation of amino-terminal trimer rather than with the liberation of carboxyl-terminal fragment, it may be tentatively concluded that nearly all of the α helix in the intact tetra-*S*-carbamidomethylated-PL-HGH, and probably in native HGH itself, must be contained within the first 134 residues.

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