Structural Studies on Polypeptide Hormones

I. FLUORESCENCE

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SUMMARY

The structures of three polypeptide hormones (glucagon, a 25-residue analogue of adrenocorticotropin, and parathyroid hormone) which contain enough residues to permit organization have been evaluated in aqueous solution by fluorescence and circular dichroism. The effects of pH, temperature, and guanidine on both tyrosyl and tryptophanyl emission have been measured. None of the fluorescence parameters could be interpreted as revealing a structural modification.

Glucagon and parathyroid hormone show weak circular dichroic activity near 222 nm, the wave length region of the \( n \rightarrow \pi \) transition of the peptide bond in the \( \alpha \) helix. However, much stronger dichroic activity occurs in all three hormones near 200 nm, the region where the random form of the peptide bond is optically active.

Although glucagon appears to be almost completely unorganized in aqueous solution, it is \( \alpha \)-helical when crystalline. The structure of this polypeptide of 29 amino acid residues appears to undergo a helix to coil transition between its crystalline and aqueous states.

This paper is an evaluation of the potentialities of fluorometry in analyzing for tertiary structure in polypeptide hormones. Fluorometry therefore complements the methods of optical rotatory dispersion and circular dichroism, which measure helical or secondary structure in polypeptides. The analysis of tertiary structure in polypeptide hormones near 200 nm, the wave length region of the \( n \rightarrow \pi \) transition of the peptide bond, is a important characteristic of fluorometry that much less material is needed for observations than with most methods of studying macromolecules in solution. The feature has been particularly important in the study of parathyroid hormone, of which only very small quantities were available. Concentrations were determined from absorption measurements at 280 nm. Molar extinction coefficients were calculated from the tryptophan and tyrosine contents of the hormones.

The CD measurements were made with a Cary spectropolarim-
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eter, model 60, equipped with a Pockels cell. The optical density of all solutions for the experimental path length was close to 2 at ~200 nm. A spectral curve was obtained on the solvent either before or after each solution was measured. The data are reported as the ellipticities (θ) recorded on the instrument in order to indicate the observed values. The molecular ellipticity, in units of deg cm² per decimole, is

\[ \theta = \frac{100 \theta}{l (\text{cm}) \text{m} (\text{molarity})} \]

A residue weight of 115 was used in calculating the molarity.

Materials—Parathyroid hormone was a highly purified product prepared by Dr. G. D. Aurbach, National Institutes of Health, from bovine parathyroid glands (12). Bovine PTH is a single chain of 83 amino acid residues, including 1 of tryptophan and 1 of tyrosine. A 25-residue analogue of ACTH, p-Ser-Neu-(Val-NH₂)-β-corticotropin (1-25) was a gift from Dr. R. S. Boissonnas (9). This material is a synthetic product with greater biological activity than the natural hormone. It has tyrosyl residues at positions 2 and 23, and a tryptophanyl residue at position 9. The natural porcine hormone of 39 residues, ACTH, was obtained from Sigma. Glucagon (Lilly and Sigma), a crystalline product (13, 14), was prepared from a mixture of bovine and porcine pancreatic glands. This preparation will be referred to as glucagon. There are 29 amino acid residues in porcine glucagon, with tyrosyl residues at positions 10 and 13 and a tryptophanyl residue at position 23 (14). Crystalline bovine glucagon (referred to as bovine glucagon), which has the same amino acid composition as porcine glucagon, was a gift from Dr. W. W. Bromer. Trp-Gly₄-Tyr and N-acetylglycylamide, prepared by Dr. M. Wilchek (Weizmann Institute, Rehovoth, Israel), were chromatographically pure. N-Acetyl-L-tryptophanamide, purchased from Cyclo Chemical Corporation, was recrystallized first from acetone and then from ethanol. No change was observed in its near ultraviolet absorption spectrum with recrystallization.

Guanidine hydrochloride, purchased from Aldridge Chemical Company, Washington, D. C. showed very little absorption or fluorescence in 5 M solution. Other chemicals were reagent grade. Glass distilled water was used.

RESULTS

Fluorescence Parameters

Emission Spectra—The emission spectra of Trp-Gly₄-Tyr and PTH are almost superimposable from 250 to 400 nm, as seen in Fig. 1. The tryptophanyl peak occurs at 347 nm, and the emission due to tyrosine is evident as a prominent shoulder at ~300 nm. The spectra of ACTHₙ and glucagon (Fig. 1) are displaced slightly from that of PTH to longer wave lengths, with maxima at 350 nm, and also show shoulders near 300 nm. The wave lengths of the emission peaks of the three hormones are comparable to those observed with tryptophan and simple tryptophan peptides in aqueous solution (15).

Effect of Acid—The pH dependence of the fluorescence intensities of the tryptophanyl and tyrosyl residues of the three hormones between neutrality and pH 2 is shown in Figs. 2 to 4. The changes in emission of both chromophores are small, and cannot be attributed to structural modifications. The intensity changes are presumably related to the influence of the charged state of the carboxyl groups which ionize in this pH region. Similar decreases in tryptophanyl emission have been observed in three proteins (pepsin, ovalbumin, and lysozyme) which are configurationally stable in acid (16).

Effect of Guanidine Hydrochloride—The peaks of tryptophanyl emission in a large group of proteins are shifted from ~330 to 340 nm in water to 350 nm in 8 M urea (17). Guanidine, however, should be a more useful reagent than urea in normalizing

FIG. 1. A comparison of the emission spectra of ○, glucagon, pH 5.0, 0.02 M KCl; ▲, ACTHₙ, pH 6.0, 0.08 M KCl-0.02 M lysine; O, Trp-Gly₄-Tyr, pH 5.0, 0.02 M acetate; and △, PTH, pH 6.0, 0.08 M KCl-0.02 M lysine.

FIG. 2. The pH dependence of tryptophanyl and tyrosyl fluorescence of PTH in alkali (0.09 M KCl-0.02 M lysine) and in acid (0.02 M Tris and 0.02 M KCl). In Figs. 2, 3, and 4, the fluorescence intensities in acid are observed values; those in alkali have been normalized to equal values at neutral pH.
the emission characteristics, since it appears that there is relatively little or no organized structure left in proteins in concentrated guanidine solutions (18). In addition, guanidine has only a minor effect on tryptophan and tyrosine emission intensity, as seen in Fig. 5, whereas urea increased the intensity of tryptophan emission significantly (19).

The effect of 5 M guanidine (pH 5.5) on the emission spectrum of Trp-Gly Tyr is seen in Fig. 6. Only relatively minor changes are observed in guanidine as compared with water. The fluorescence intensity of the tryptophanyl peak decreased by 15%, and that at the tyrosyl peak (300 nm) increased by about 25% in 5 M guanidine. The changes in emission intensities of ACTH, PTH, and glucagon (and of bovine glucagon) at pH 5 to 6 were in the same directions but smaller in magnitude. The wave lengths of the tryptophanyl peaks were unchanged in 5 M guanidine, except for PTH, where there was a small (~2 nm) red shift. It should be mentioned that misleading results were obtained when the pH of the aqueous stock solutions of glucagon (~0.06%) which were used to make the 5 M guanidine and aqueous solutions (~0.006%) was not adjusted either to below ~4 or to above 9. Glucagon is not completely soluble between pH ~4 and 9 at concentrations as low as 0.06%.

Effect of Temperature—There is a monotonic decline in the quantum yields of tryptophan and tyrosine with increasing temperature, the former changing more rapidly than the latter (20). Consequently, any variation in emission intensity of either chromophore which does not fall uniformly with temperature is abnormal and probably implies a structural alteration. Inflections in the temperature-intensity profiles of tryptophanyl emission have revealed structural transitions in pepsin and pepsinogen (21), chymotrypsinogen (16), and Bence-Jones protein (20). The curves of tryptophanyl emission in Trp-Gly Tyr and ACTH are indistinguishable (Fig. 7). The data for glucagon and PTH fall on a single curve, with temperature coefficient only slightly greater than that of ACTH and Trp-Gly Tyr. The temperature dependence of tryptophanyl fluorescence of the three hormones declines continuously and at a rate only somewhat smaller than that of tryptophan.

The thermal dependence of tyrosyl emission of Trp-Gly Tyr and the three hormones is very similar, and there is an almost
linear decline between 20° and 60° (Fig. 8). All show greater temperature coefficients than tyrosine. This result is unexpected, since the quantum yields of ACTH and Trp-Gly-Tyr are only about 20% and 30%, respectively, of that of tyrosine (22). If the lifetimes were proportional to the quantum yields, a greater rate of thermal quenching would be anticipated for tyrosine because of its higher quantum yield.

Energy Transfer from Tryptophanyl to Ionized Tyrosyl—The quenching of tryptophanyl emission by radiationless energy transfer to ionized tyrosine has been shown to be an important source of quenching in proteins in the alkaline pH range (16, 23). In a study on synthetic peptides containing terminal tryptophanyl and tyrosyl residues, quenching of tryptophanyl emission was almost complete in Trp-Tyr but fell to about 40% in Trp-Gly-Tyr (24). If a molecule has only one tyrosyl residue, its phenolic ionization can be followed fluorometrically, since ionized tyrosine does not fluoresce at 300 nm. If two or more tyrosyl residues are present, the fluorescence at 300 nm may be quenched by energy transfer from un-ionized to ionized phenolic groups (25).

The effect of tyrosyl ionization on tryptophanyl emission of PTH is shown in Fig. 2, where both emissions are normalized to the same value at neutral pH. It should be noted that above pH 11.5 to 12.0, tryptophan emission is also quenched by hydroxyl ions (24). In the pH zone of tyrosyl ionization there is a constant proportionality of 30% between tryptophanyl quenching and tyrosyl quenching. This result strongly suggests that the quenching of tryptophanyl fluorescence is due solely to energy transfer to the ionized tyrosyl residue.

A much smaller degree of tryptophanyl quenching (~10%) of PTH by ionized tyrosyl was found in 5 M guanidine solution. This result does not necessarily imply that a new structure is formed in this solvent, but may simply reflect a greater extension of the molecule in guanidine since the latter is a much better solvent than water for peptides (26). In fact, energy transfer experiments offer a method of evaluating the effects of different solvents on polymer dimensions (27).

There are tyrosyl residues in glucagon at positions 10 and 13. The ionization of either one will strongly quench the other (if the phenolic groups possess rotational freedom), as has been observed with Tyr-Gly-Tyr (25). There is almost no quenching of tryptophanyl fluorescence in glucagon (or bovine glucagon) in
the pH region of tyrosyl ionization (Fig. 4). The independence of tryptophanyl emission from tyrosyl ionization is expected if glucagon has a random coil structure in this pH region, since the tryptophanyl residue is at position 25. If glucagon were globular it would almost certainly show quenching in alkali by energy transfer.

In ACTH$_{1-24}$, tryptophanyl fluorescence is quenched 15% by ionized tyrosyl residues (Fig. 3). The two tyrosyl residues are far apart (positions 2 and 23), while the tryptophanyl residue is at position 9. The poor quenching efficiency is in keeping with the distance between the chromophores, provided, of course, that the chain is structureless and the indole and phenol groups have adequate rotational freedom.

Circular Dichroism

The CD spectra of ACTH$_{1-24}$ and ACTH are illustrated in Fig. 9. There does not appear to be any optical activity associated with a chromophore with a CD peak above 200 nm in either hormone. In particular, there is no dichroic activity at 222 nm, the peak of the $n \rightarrow \pi$ transition of the peptide bond in the $\alpha$ helix (10). A CD band, which is due to the random coil form of the peptide chromophore, is centered close to 200 nm, with $[\theta] = -14$ and $-10.5 \times 10^3$, respectively, for ACTH$_{1-24}$ and ACTH. When the CD spectra are compared with those of poly-L-glutamic acid or poly-$L$-lysine in their helical forms (10), it is apparent there is no $\alpha$-helical structure in either the synthetic or the natural form of ACTH.

In glucagon, in addition to a CD band with a peak near 200 nm, there is a weak band near 220 to 225 nm, with a molecular ellipticity of $-3000$ at 222 nm (Fig. 10). Similar CD spectra were obtained at pH 3.5 and 9.5. The molecular ellipticities, $[\theta]$, at the well resolved minima ($\sim 200$ nm) are $-14.5$ and $-11.0 \times 10^3$ at the two pH values, respectively. The origin of the weaker band (or bands) cannot be assigned unequivocally, since its structure is not adequately resolved owing to overlap with the much stronger neighboring band. If it is due to helical peptide bonds, its intensity indicates that about 10% of the peptide bonds contribute. Thus only a few of the 28 peptide bonds of glucagon contribute to helical structure, apparently not enough to form a helix with a single turn (10). It is not unlikely that the CD band arises from other sources, i.e. $\beta$ structures, or from the aromatic chromophores which have strong absorption bands in this wave length region (28).

The CD spectrum of PTH, illustrated in Fig. 11, resembles that of glucagon. There is a weak, negative band centered near 225 nm ($[\theta]_{222} = -2,000$) and a stronger, well resolved minimum at 200 nm ($[\theta]_{200} = -12,000$). The latter dichroic band agrees very well with that of the peptide bond when in a random coil. The longer wavelength band is due to the peptide bond in an $\alpha$-helical form, the molecular ellipticity value suggests a helical content of about 10%. However, in polypeptides containing all the common amino acid residues, many other chromophore groups can contribute to the CD activity in the 210 to 240 nm wave length region (28).

Optical rotatory dispersion measurements on the same sample of PTH revealed a Cotton effect with a distinct minimum at 232 nm ($[\delta]_{232} = -2,500$). This value is not much more negative than that of random poly-$L$-lysine, and implies that very few helical bonds are present (29). It should also be noted that weak Cotton effects centered near 230 nm can originate from other types of interactions in polypeptides (30).
The fluorescence properties of three low molecular weight polypeptide hormones have been assessed. The molecular sizes of the hormones are in a range intermediate between those of proteins and simple polypeptides. It is of importance in understanding the biological properties of these hormones to know whether they are statistical coils or possess noncovalent interactions.

The degree of secondary structure in a polypeptide is readily evaluated by measurements of optical rotatory dispersion or circular dichroism. The tertiary interactions cannot be uniquely determined except by x-ray diffraction methods, since there exist almost unlimited possibilities. Nevertheless, we would suggest that an analysis of the behavior of the tryptophanyl and tyrosyl residues may suffice, in many cases, as an indicator of tertiary interactions. The basis of this proposal is the currently held concept that the interactions responsible for the three dimensional structure of native proteins are largely "hydrophobic" in nature (1-6). The thermodynamic basis for this point of view, as developed by Kauzmann (4), has been fully realized from the x-ray analysis of several proteins. The residues which contribute significantly in stabilizing the native structure of proteins should include nonpolar and aromatic groups, since the polar and charged groups tend to be situated on the surface of the protein (1-3). Consequently, if the fluorescence properties of the phenol and indole chromophores reveal them to be free of interactions other than with solvent and if the optical activity of the polypeptide hormone is a random backbone structure, then the polypeptide is probably neither globular nor fibrous. This deduction, of course, involves an extrapolation from the behavior of two aromatic residues to about ten non-or slightly polar residues. This assumption appears to be a reasonable one, since almost all proteins that have been studied carefully show abnormal tryptophanyl or tyrosyl emission spectra (17). The abnormalities observed in the absorption behavior of these two chromophores in most proteins (31, 32) should also be reflected in their fluorescence. In fact, the emission characteristics will usually be more affected than those of absorption (33, 34).

The emission maximum of the indole moiety is shifted from ~350 nm in water to lower wave lengths as the polarity of the solvent is reduced, and is near 325 nm in dioxane and 300 nm in hydrocarbons (34, 35). The blue shift observed in native proteins is assumed therefore to arise from the smaller polarity of the groups forming the atmosphere of the tryptophanyl residues. The 330 to 340 nm emission maxima of a large group of proteins are shifted to ~350 nm in 8 M urea (17). Since the emission maxima of the three hormones in water agree with that of proteins when denatured in urea or guanidine and of simple peptides in water, the tryptophanyl residue in the hormones appears to be extensively exposed to the water molecules of the solvent. It should be noted that the high wave length of the emission peak of the tryptophanyl residue in the three hormones (i.e. ~350 nm) permits resolution of the fluorescence of the tyrosyl residues by reducing the overlap between the two spectra.

The tryptophanyl emission of most native proteins is strongly quenched by resonance energy transfer in the pH region of tyrosyl ionization. The critical distance ($R_0$), where the probability of spontaneous deactivation is equal to that of transfer, has been calculated by the Förster equation (36) to be 11.2 Å if the indole and phenol groups are randomly oriented (37).

In all four hormones the predominant dichroic band is centered near 200 ± 2 nm, and agrees with the optical behavior of the peptide bond in the unorganized forms of model homologous polypeptides. However, the ellipticity of this band in all four molecules is between 10.5 and 14.5 × 10$^4$. These values are only about one-third to one-half of those observed in either random poly-L-glutamic acid or random poly-L-lysine (38). It seems unlikely that other dichroic bands of opposite sign are reducing their magnitude, since their symmetry and shape are quite similar to those of the model polypeptides. Since all the hormones have similar ellipticity values, the larger values found for the two homologous charged polypeptide models in their random form may not hold for polypeptides containing charged and uncharged side chain residues (39) (see also Reference 40).

Neither the temperature nor the pH profiles of the emission intensities of either chromophore suggest that ACTH$_{38}$ possesses structure. Its CD spectrum does not show any contribution from peptide bonds in an α-helical form. The fairly small extent of alkaline quenching is in accord with the large number of residues between the tryptophanyl and two tyrosyl residues in the chain if the structure is that of a random coil. The similarity of the emission spectra of ACTH$_{38}$ in water and in 5 M guanidine also precludes significant interactions between the fluorescent chromophores and other residues. Thus the CD and fluorescence spectral data are consistent in indicating the absence of secondary or tertiary interactions. ACTH$_{38}$ therefore appears to behave as a random chain polymer in water, devoid of important intramolecular interactions.

No significant effects on the emission properties of PTH which could be interpreted as involving a structural modification were observed with guanidine, temperature, or acid pH. The rapid oxidation of the methionine residues and the marked reactivity of the tryptophanyl residue afford chemical evidence of the accessibility of these two groups to the solvent (12). The circular dichroism and optical rotatory dispersion data also agree with a molecule which is largely disorganized but which may contain a small amount of structure involving the peptide or other chromophores (28, 30).

If PTH has a random coil structure, the distance between the tryptophanyl and tyrosyl residues can be estimated by comparing the extent of energy transfer in alkali with that of small peptide models. Present information on the amino acid sequence of PTH places the tyrosyl residue in an octapeptide that is either 4 or 10 residues removed from the tryptophanyl residue (41). The moderate degree of quenching fits much better with the shorter distance, thereby predicting the order of the two fragments in the sequence.

The negligible amount of tryptophanyl quenching in guacagon in alkali precludes a globular form. If guacagon were spherical its radius would be 10.0 Å (mol wt 3485, $\bar{v} = 0.72$). It seems very unlikely that both tyrosyl residues would be separated from the tryptophanyl residue by 1.44 × $R_0 = 16$ Å, the distance required for a transfer efficiency of 10%. It is possible, of course, that very inefficient transfer would result even if the residues were much closer to each other if they had fixed orientations, since the directions of the transition moments of the oscillators of emission and absorption also control the rate of transfer (36).

The weak CD band in guacagon observed in the region of the α-helical peptide bond suggests that a few residues may have a helical structure. The possibility of another assignment of this CD band should also be considered, since the molecular ellipticity...
is very small and the band lacks the structural features of the α-helical CD band. Cotton effects have been observed at 235 nm by optical rotatory dispersion by Gratzer et al., and have been interpreted as indicating that there may be a single helical turn in glucagon (42). Aside from the indications of minor elements of structure observed by optical rotatory dispersion by Gratzer et al. and by CD, both studies suggest that this hormone has relatively little secondary or tertiary structure in aqueous solution.

The three polypeptide hormones investigated appear to have very little, if any, organization in aqueous solution. This result, however, does not imply that they will have the same molecular form when bound to their receptor sites. If the water concentration at their binding sites is reduced significantly, as in lipid layers or cellular membranes (43), then the molecular form of the hormone may change. It has been reported by Gratzer et al. (42) that the helical content of glucagon increases continuously with 2-chloroethanol concentration and reaches about 90% in the absence of water. An X-ray study of glucagon also indicates that it has a helical structure in its crystalline form (13).

There has been considerable interest since the x-ray analysis of the structure of crystalline myoglobin in determining whether protein molecules, when isolated from their interactions with their neighbors in the crystal and exposed to 55 moles of water, retain the same form as they have in the crystal (44). The organization of several crystalline proteins (1, 45–47) appears to survive their solution, even if their secondary structure, as judged by analysis of their optical rotatory dispersion or CD spectra (10, 48–50), is lost. This may occur in structureless polypeptides when their environments can be expanded. It has been shown that polypeptide fragments, the S-peptide of ribonuclease (59), and a tetradecamer of 29 residues, have very little structure in solution but forms a helix when crystallized. It has been observed that polypeptide fragments, the S-peptide of ribonuclease (59), and a tetradecamer of 29 residues, have very little structure in solution but forms a helix when crystallized.

Evidently, important configurational changes can occur in structureless polypeptides when their environments are significantly modified.

REFERENCES

34. Koby, S. V., Excited electronic states of biopolymers, Nauka Toshioka, Minneapolis (1968).