Conformational Properties of Cyanogen Bromide-cleaved Glucagon*

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SUMMARY

The circular dichroism of the cyanogen bromide peptide of glucagon, measured in dilute aqueous solution, in chloroethanol, and in urea is similar to that of glucagon. However, in more concentrated aqueous solutions the cyanogen bromide peptide does not rapidly aggregate to structures of higher helical content as does glucagon. Thus this property of glucagon is dependent on the presence of the two COOH-terminal amino acids.

Intramolecular quenching of tryptophan fluorescence by tyrosinate is observed in the cyanogen bromide peptide. From this quenching the approximate distance between aromatic residues is calculated and it is concluded that the peptide is in a compact conformation. The lack of quenching in unmodified glucagon is interpreted as due to the fixed angle between the aromatic residues.

Nuclear magnetic resonance studies at 250 MHz reveal small differences in the chemical shift of several of the protons between the cyanogen bromide peptide in water and the random coil spectrum. These differences suggest that the conformational change between a randomly coiled peptide and the glucagon monomer in water involves several sections of the molecule.

The frictional coefficient calculated from sedimentation velocity results along with the fluorescence and nuclear magnetic resonance data suggests that the cyanogen bromide peptide, like glucagon, folds into a compact, globular structure in dilute aqueous solution.

EXPERIMENTAL PROCEDURE

Materials—The amino acid analysis of crystalline glucagon (Sigma, Lot 80C-2900) agreed to within 5% of the reported value (10, 11) for each amino acid. Urea (Matheson, Coleman and Bell, reagent grade) was purified by passing an aqueous solution over a mixed bed deionizing resin (Bio-Rad resin AG501-X8(D)) followed by crystallization in the cold. Tris buffer was made from the free base (Sigma, Trizma grade) and HCl. 2-Chloroethanol (Eastman) was distilled over anhydrous potassium carbonate and the fraction distilling at 127-128° was collected and stored in the dark at 4°. Aqueous solutions of chloroethanol were made up immediately before use. Urea-d₄ was purchased from Merck, Sharp and Dohme. Ultra pure grade (Schwarz-Mann) guanidine hydrochloride was used.

Amino Acid Analyses—The analyses were determined with a Beckman 120 C analyzer. Samples were hydrolyzed at 110° for 22 hours in sealed, evacuated ampoules containing 6 N HCl. The solution was then taken to dryness under vacuum on a rotary evaporator; the residue dissolved in water and a portion taken for determination of homoserine. The pH of this portion was adjusted to 9 with a dilute solution of sodium hydroxide and after 15 min at room temperature again evaporated to dryness. Homoserine, produced from homoserine lactone, was eluted between serine and glutamic acid and the area of the peak was compared with that of a standard (Sigma, n-n-homoserine, Lot 10813-2250). Amino acid analyses are calculated to give the expected total number of amino acids. Ammonia was determined from analysis of the fraction not treated with base. The ammonia peak overlapped with that of homoserine lactone. For the estimation of asparagine and glutamine in the carboxypeptidase-hydrolyzed material, the amino acid analyzer was operated at 35° instead of the usual 55°.

Circular Dichroism Measurements—These measurements were performed with a Cary, model 61, spectropolarimeter calibrated according to the values given by Cassim and Yang (12). The temperature of the sample was maintained at 25° by means of a thermostable cell holder attached to a circulating constant temperature bath. Cells of 0.01 to 2.5 cm path lengths were used.

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Ultraviolet Spectra—The spectra were measured in a Cary 14 spectrophotometer at ambient temperature.

Infrared Spectra—These spectra were recorded on a Beckman IR 12 spectrophotometer using cells of thallium bromide iodide.

Ultracentrifugal Studies—These studies were performed at 90° with a Spinco model E ultracentrifuge using sapphire windows. A double sector, capillary-type, synthetic boundary cell was used for the sedimentation velocity runs with the schlieren optical system and a speed of 60,000 rpm. The peak positions were calculated by the method of second moments. The sedimentation equilibrium runs were performed with a 12-mm cell at a calculated by the method of second moments. The sedimentation equilibrium runs were performed with a 12-mm cell at a speed of 39,500 rpm, and a peptide concentration of 0.2% making use of the combined Rayleigh interference and schlieren optical systems (13). Results calculated solely from the schlieren system by the method of Lamou (14) gave very good agreement with the method using the combined optics. These procedures circumvent the use of a synthetic boundary run with the undialyzable peptide. The partial specific volume of the cyanogen bromide peptide was estimated to be 0.71 ml per g from the amino acid composition by the method of Cohn and Edsall (18), treating homoserine as threonine. Attainment of equilibrium was verified by comparison of photographs taken at intervals of several hours.

Fluorescence Emission Spectra—These spectra were measured on Mark I spectrofluorometer (Farrand Optical Co.) at an ambient temperature of 20° in 0.1 M KCl, the pH being adjusted with KOH. The sample was excited at 270 nm and the emission spectra were recorded. The excitation wave length increases the tyrosyl to tryptophenyl emission ratio and is about at the isobestic point for the ionization of N-acetyltyrosinamide so that the fraction of light absorbed by tryptophan does not change with pH (16). Emission spectra were measured with 1-cm2 cells for solutions having optical densities of less than 0.05 at 280 nm and are proportional to concentration.

Proton Magnetic Resonance Spectra—These spectra were obtained with a modified Varian high resolution spectrometer which operates at a frequency of 250 MHz. The proton signal of the D2O solvent was used as the internal lock. In separate experiments the position of this solvent line was measured with respect to 2,2-dimethyl-2-silapentane-5-sulfonate and spectra are reported with respect to this latter reference. All spectra were taken with a single scan and repeated at least once.

Concentration of Protein Solutions—The concentration of protein solutions was determined from amino acid analysis using an internal standard of norleucine.

Reaction of Glucagon with Cyanogen Bromide—This reaction was done by two methods. In the first method a 0.9% solution of glucagon was reacted with a 35 molar excess of cyanogen bromide in 0.1 N HCl containing 9% dioxane for 24 hours at room temperature. Dioxane inhibits aggregation in acidic solution (17). At the end of the reaction the material containing some precipitate was lyophilized.

The reaction was also carried out in 70% formic acid (18), using 0.1% glucagon with a 200 molar excess of cyanogen bromide. The reaction was allowed to proceed for 24 hours at room temperature at the end of which time the clear solution was diluted with 3 volumes of cold water and lyophilized.

Separation of Cyanogen Bromide Peptides—Separation of the cyanogen bromide peptides was done on a column (1.5 x 100 cm) packed with Sephadex G-10 (Lot 3411) which had been equilibrated with an alcohol-water solvent containing 2 volumes of ethanol per volume of water. Before application of the peptide several void volumes of solvent were passed through the column until the eluant was transparent in the ultraviolet. The hydrostatic pressure was adjusted to give a flow rate of 10 ml per hour. Glucagon is eluted in the void volume at about 60 ml. Because of its moderate solubility in the water-alcohol mixture this is a convenient procedure to separate glucagon from low molecular weight substances.

The lyophilized products of the cyanogen bromide reaction were partially dissolved in the water-alcohol mixture. A few drops of 0.1 N NaOH were added to increase solubility of the 70% formic acid product and then immediately placed on the Sephadex column. In both cases only one peak absorbing at 278 nm was eluted at the void volume. The cyanogen bromide peptide was lyophilized after dilution with 10 volumes of water. Ninhydrin analysis revealed a second peak at about twice the void volume whose amino acid analysis showed only aspartic acid and threonine in equimolar amounts.

Carboxypeptidase Hydrolysis—One milliliter of a suspension containing 4 mg of disopropyl phosphorofluoridate-treated carboxypeptidase A (Nutritional Biochemicals) was centrifuged and the precipitate washed twice with 1 ml of water; 0.1 ml of 2 M NH4HCO3 was added, and the resulting solution was clarified by centrifugation. Ten microliters of this solution were added to 0.1 ml of a 0.5% solution of peptide in 4 M urea, 10-2 M Tris buffer, pH 8. The solution stood for 3 hours at room temperature after which time the reaction was stopped by acidification, and the material was immediately used for amino acid analysis.

Under these conditions all of the amino acids up to aspartate (residue 21), a poor substrate for carboxypeptidase (19), are completely released (Table II). Blank runs, containing only the carboxypeptidase, released only traces of amino acids.

RESULTS AND DISCUSSION

Treatment of glucagon with cyanogen bromide will convert the methionine residue at position 27 to homoserine and liberate the COOH-terminal dipeptide, asparaginylthreonine. We have separated the two products of the cyanogen bromide cleavage by gel filtration. This procedure would not be expected to separate the 37 amino acid cyanogen bromide peptide from unreacted glucagon. We conclude that the peptides produced by cleavage in 0.1 N HCl contain only a few percent of unreacted glucagon and that the reaction in 70% formic acid is virtually quantitative because of the loss of the sole methionine residue, the recovery of 1 residue of homoserine, the loss of approximately 1 residue of aspartic acid and threonine (Table I), the recovery of these two amino acids in another fraction of the column eluent and the enhanced solubility of the final product. The product of the reaction in 70% formic acid was further analyzed by digestion of the COOH-terminal amino acids with carboxypeptidase (Table II). The amino acids released from glucagon correspond to the 8 COOH-terminal amino acids and show that the digestion proceeded up to the slowly hydrolyzed amino acid residue 21, aspartic acid. The remaining peptide containing residues 1 to 21 was isolated by Sephadex chromatography and its identity confirmed by amino acid analysis. The amino acids released from the cyanogen bromide peptide also correspond to digestion up to residue 21 with the liberation of 6 amino acids; homoserine replacing methionine. The absence of unreacted glucagon in the cyanogen bromide peptide is most clearly demonstrated by the release of only 0.02 residue of threonine, the COOH-
Table I

Amino acid analysis of glucagon and its cyanogen bromide peptide

| Amino acid | Glucagon | CNBr peptide |
|------------|----------|--------------|
|            | Experimental | Calculated | Experimental | Calculated |
| Lysine     | 0.97      | 1           | 0.85        | 0.88       | 1           |
| Histidine  | 1.02      | 1           | 0.78        | 0.75       | 1           |
| Arginine   | 4.05      | 4           | 5.23        | 0.08       | 3           |
| Arginine   | 1.56      | 2           | 1.89        | 2.02       | 2           |
| Aspartic acid | 4.10    | 4           | 3.39        | 3.34       | 3           |
| Threonine  | 2.94      | 3           | 2.24        | 2.13       | 2           |
| Serine     | 3.81      | 4           | 3.77        | 4.05       | 4           |
| Glutamic acid | 3.16  | 3           | 3.04        | 2.80       | 3           |
| Glycine    | 1.00      | 1           | 0.99        | 1.05       | 1           |
| Alanine    | 1.00      | 1           | 0.99        | 1.08       | 1           |
| Valine     | 1.00      | 1           | 0.83        | 1.05       | 1           |
| Methionine | 0.98      | 1           | 0.07        | 0          | 0           |
| Leucine    | 1.00      | 2           | 2.01        | 2.14       | 2           |
| Tyrosine   | 2.03      | 2           | 2.11        | 2.13       | 2           |
| Phenylalanine | 2.04 | 2           | 2.08        | 2.09       | 2           |
| Tryptophan | 1.00      | 1           | 1.00        | 0.9        | 0.9         |
| Valine     | 1.00      | 1           | 1.00        | 1.0        | 1.0         |
| Homoserine | 0.02      | 0           | 0.93        | 0.95       | 1           |

a Using 0.1 N HCl as solvent.
b Using 70% formic acid as solvent.
c c, not determined.

Table II

Amino acids released by carboxypeptidase digestion

| Amino acid | Calculated from glucagon sequence | Determined for glucagon | Determined for CNBr peptide |
|------------|----------------------------------|-------------------------|-----------------------------|
| Threonine  | 1                                | 1.0                     | 0.02                        |
| Asparagine and glutamineb | 2 | 2.0 | 1.0 |
| Methionine | 1                                | 1.0                     | 0                           |
| Homoserine | 0                                | 0                       | 1.0                         |
| Leucine    | 1                                | 1.1                     | 1.1                         |
| Tryptophan | 1                                | 1.0                     | 1.0                         |
| Valine     | 1                                | 1.0                     | 1.0                         |
| Phenylalanine | 1 | 1.0 | 1.0 |

Assuming hydrolysis proceeds up to the slowly hydrolyzed aspartate at residue 21.
b Asparagine and glutamine cochromatograph.

terminal amino acid of glucagon, which along with asparagine is absent in the cyanogen bromide peptide. The small amount of threonine released could have originated from the dipeptide asparaginylthreonine which was not completely separated on the Sephadex column, from autolysis of carboxypeptidase A and from a maximum of about 2% unreacted glucagon. Unless otherwise stated, the cyanogen bromide peptide prepared in 70% formic acid was used for further study.

The circular dichroism of the cyanogen bromide peptide shows a small negative ellipticity in the spectral region of the amide chromophore which is diminished in the presence of urea and enhanced by chloroethanol (Fig. 1). This is very similar to the behavior of glucagon (4, 7). However, the enhanced negative ellipticity exhibited by more concentrated aqueous solutions of glucagon does not occur with the cyanogen bromide peptide, the ellipticity of which is independent of concentration (Fig. 1, Curve B).

Glucagon also exhibits cotton effects with magnitudes of about 40 degree cm² per decimole in the spectral region of the aromatic amino acid chromophores which are enhanced in more concentrated solutions (7). These cotton effects were not detected for the cyanogen bromide peptide whose mean molar residue ellipticity was found to be 0 ± 15 degree cm² per decimole at peptide concentrations of 0.03 and 0.05% in Tris buffer. This suggests that the aromatic residues of glucagon have a fixed orientation, with respect to the asymmetric centers, which is lost in the cyanogen bromide peptide.

The lowered ability of the cyanogen bromide peptide to form aggregates of higher helical content may also be correlated with the loss in orientation of the aromatic chromophores. The importance of the aromatic side chains to the aggregation process in glucagon was shown by difference spectra (2) and by circular dichroism (7). The aromatic residues have not been chemically altered in the production of the cyanogen bromide peptide since its ultraviolet absorption spectra (from 215 to 330 nm at 0.03% peptide in Tris buffer at pH 8) and fluorescence emission (from 280 to 400 nm at 5 PM peptide in water) spectra are coincident with that of glucagon.

The cyanogen bromide peptide has a higher solubility at pH 8 than does glucagon. This can be correlated with its lowered tendency to aggregate and to form structures of higher helix content. We cannot rule out the possibility that some hydrolysis of the side chain amide bonds occurred during preparation of the cyanogen bromide peptide. However, if it were an important factor we should expect to observe more hydrolysis in the

Fig. 1. Circular dichroism of the cyanogen bromide peptide of glucagon at 25°. Curve A, 8 m urea, pH 8, 10⁻² m Tris buffer; Curve B, pH 8, 10⁻² m Tris buffer; Curve C, 90% 2-chloroethanol prepared by diluting a solution of the peptide in Tris buffer to 10 times its volume with 2-chloroethanol. Protein concentration is 0.1 mg per ml. Concentrations up to 5 mg per ml for Curve B give spectra which are superimposable on the one shown.
preparation made in 70% formic acid. This preparation as well as the one from 0.1 N HCl exhibited enhanced solubility in neutral aqueous solutions and had identical circular dichroic properties.

In addition to rapid formation of aggregates of higher helical content, glucagon has also been shown to slowly form \( \beta \) structures at high peptide concentrations, both in acidic (3, 17) and in basic (20) aqueous solutions. A 0.5% solution of the cyanogen bromide peptide in Tris buffer, pH 8, on standing overnight at room temperature forms a gel-like substance. The gel has been identified as a \( \beta \) structure on the basis of its infrared spectra when suspended in D\( _2 \)O. The observed peak of the amide I band at 1618 cm\(^{-1}\) in D\( _2 \)O is typical of a \( \beta \) structure and lies between 1613 cm\(^{-1}\) and 1626 cm\(^{-1}\), the absorption maximum found for the gel formed from acidic (5) and basic (20) D\( _2 \)O solutions.

Sedimentation velocity studies confirm that the cyanogen bromide peptide has a diminished ability to rapidly aggregate in 0.1 m NaCl, 10\(-2\) m Tris buffer, pH 8, compared with that of glucagon (6). The observed \( s_{20,w} = 0.77 \) S is virtually independent of peptide concentration between 0.1 and 0.4%. The lowered ability of the cyanogen bromide peptide to aggregate is concomitant with a lowered tendency to form structures of higher helical content in water. These two phenomenon are linked equilibria (7).

Molecular weights calculated from the sedimentation equilibria runs in 0.1 m NaCl, 10\(-2\) m Tris buffer, pH 8, gave values which varied with distance from the center of the rotor. The average slope, calculated by least squares, gave a molecular weight of 4500 while near the meniscus and bottom of the cell the molecular weight is 3500 and 6600, respectively. These values are for the average molecular weight.

The centrifuge studies are done in the presence of 0.1 m NaCl to minimize charge effects. The addition of 0.1 m NaCl causes little change in the circular dichroism of the peptide, although its magnitude seems to decrease about 10%, only slightly more than the experimental error. The molecular weight calculated in the region of the cell near the meniscus (3500) is close to that expected on the basis of the amino acid composition of the monomer (3238). The cause for the apparent increase in molecular weight near the bottom of the centrifuge cell is not known but could arise from the formation of aggregates in the form of a \( \beta \) structure. We have shown that this type of aggregation occurs with the cyanogen bromide peptide in concentrated solutions over a period of many hours. These conditions are fulfilled during the sedimentation equilibrium run where 24 hours are needed for the apparent attainment of equilibrium and the concentration at the bottom of the cell is greater than the initial concentration. This effect does not occur in the sedimentation velocity runs where only an hour is required for its completion and the concentration at the solution side of the observed boundary is never greater than the initial concentration. It is possible that some of the poor reproducibility between runs in the sedimentation equilibrium studies of glucagon (6) can be explained by the formation of \( \beta \) structure aggregates. This could also explain why the association constants calculated by Swann and Hamnes (6) from this data are higher than those calculated by Gratzer and Beaven (7) from circular dichroism studies. This effect due to the formation of \( \beta \) structure is more important for the cyanogen bromide peptide than for glucagon since the lower solubility of glucagon causes some of it to precipitate before \( \beta \) structure is formed.

The observed sedimentation coefficient of the cyanogen bromide peptide reflects its hydrodynamic properties thus giving information about the shape of the particle in solution (21). From the observed sedimentation coefficient, \( s_{20,w} = 0.77 \) S, we calculated the ratio of the observed frictional coefficient to the minimum possible frictional coefficient for an unhydrated sphere to be 1.10. This low value is similar to those of other globular proteins and is indicative of a compact spherical molecule.

Long range radiationless transfer of singlet excitation energy has been used to measure the distance between the transition moments of two interacting chromophores. The efficiency of energy transfer is determined by the distance between the chromophores and also by the relative orientation of their transition dipoles (22). Singlet excitation transfer in proteins can be measured by the quenching of tryptophan fluorescence in alkaline pH due to radiationless transfer to tyrosinate. No such quenching was observed with glucagon (16) suggesting either that the single tryptophan was further than 16 A from either of the two tyrosines or that the relative orientations of the transition dipoles of donor and acceptor were such as to preclude energy transfer.

The fluorescence emission spectra for glucagon and for the cyanogen bromide peptide, at neutral pH, were identical. Tryptophan fluorescence was monitored at 350 nm while fluorescence due largely to tyrosine was recorded at 300 nm. Above pH 11.5 a small quenching by hydroxide occurs. The pH change does not cause a conformational change in glucagon as shown by the invariance of the peptide cotton effects between pH 2 and 11.3. Our results with glucagon are in very good agreement with those of Edelhoch and Lippoldt (16) and show very little, if any, quenching of the tryptophan fluorescence by the ionization of tyrosine (Fig. 2). However, the cyanogen bromide peptide shows a decrease of 20% in the tryptophan fluorescence at higher pH and this decrease is proportional to the decrease in tyrosine fluorescence (Fig. 3) demonstrating that it is caused by energy transfer to tyrosinate. The spectral red shift of the absorption spectrum of tyrosine upon ionization, to the region of tryptophan fluorescence, allows for intramolecular singlet excitation energy transfer between these two residues in basic solution. The NH\(^+\) group of lysine at residue 12 also ionizes near this pH region, but it would be expected to quench the fluorescence of the two tyrosines at residues 10 and 13 much more than the tryptophan at residue 25.

Eisinger et al. (23) have tabulated the Forster distance, \( R_0 \), for the singlet excitation transfer between pairs of aromatic amino acids. For energy transfer from tryptophan to tyrosinate this distance is about 10 A, assuming a typical quantum yield. The Forster distance is quite insensitive to the choice of quantum yield varying as the sixth root of this quantity. From the efficiency of energy transfer we can calculate the distance between the transition moments of the interacting dipoles in the cyanogen bromide peptide to be 12 A, assuming random orientation of the side chains (23). This distance is comparable to the calculated radius of 9.7 A for a spherical model of the cyanogen bromide peptide (molecular weight 3238; \( \bar{v} = 0.71 \) ml per g). The sole tryptophan residue in this peptide occurs at position 25 and the two tyrosines at positions 10 and 13. Using the method of Brant, Miller, and Flory (24) we can calculate that the distance between residues 13 and 25 in a randomly coiled polypeptide
The distance distribution function must also be considered for a polypeptide containing n virtual bonds of average lengths $l_i$. We have used their values of 5 for the ratio of the random coil in D$_2$O.

Although singlet excitation transfer is precluded for a polypeptide chain only if the distribution of end to end distances were broad. The absence of tyrosinate quenching of tryptophan fluorescence in the presence of guanidine hydrochloride (Fig. 4) shows that energy transfer is precluded when the peptide is unfolded. Guanidine hydrochloride causes only relatively minor changes in the emission spectra of tyrosine and tryptophan (18) and thus should not greatly affect the Forster distance, $R_0$. This experiment demonstrates the conformational dependence of the observed energy transfer. The distance between aromatic residues is too great in the random conformation to allow energy transfer, while the distance between the residues in water, 12 A, is comparable to the radius of the cyanogen bromide peptide as a compact sphere, 9.7 A.

The increased quenching at alkaline pH of the cyanogen bromide peptide compared with glucagon would result either from a folding of the molecular conformation of the two COOH-terminal amino acids or from a change in orientation of the transition dipoles of the aromatic amino acids. The former explanation seems unlikely and is contrary to the circular dichroism evidence. The latter explanation, however, is supported by the loss of the extrinsic Cotton effects on cleavage of glucagon and the increased quenching at alkaline pH of the cyanogen bromide peptide compared with glucagon.

The proton magnetic resonance spectra calculated for glucagon by the procedure of McDonald and Phillips (27) agree fairly well with that measured in 8 M urea (Figs. 5 and 6). The measured spectra shows somewhat greater resolution of peaks because of the small size of glucagon and the improved instrumentation at the slightly higher fields of 250 instead of 220 MHz. There are however some differences between the calculated and observed spectra (Table III). The methyl peaks of threonine, alanine, and glycine are however some differences between the calculated and observed spectra (Table III). The methyl peaks of threonine, alanine, and glycine are.

![Fig. 2 (left). The pH dependence of the tryptophan and tyrosine fluorescence of glucagon in 0.10 M KCl. O---O, tyrosine fluorescence at 300 nm; •---•, tryptophan fluorescence at 350 nm.](image1)

![Fig. 3 (center). The pH dependence of the tryptophan and tyrosine fluorescence of the cyanogen bromide peptide of glucagon in 0.10 M KCl. O--O, tyrosine fluorescence at 300 nm; •--•, tryptophan fluorescence at 350 nm.](image2)

![Fig. 4 (right). The pH dependence of the tryptophan and tyrosine fluorescence of the cyanogen bromide peptide of glucagon in 6.0 M guanidine hydrochloride. O---O, tyrosine fluorescence at 300 nm; •---•, tryptophan fluorescence at 350 nm.](image3)

### Table III

| Peak | Identification | Number of protons | Chemical shifts$^a$ |
|------|----------------|------------------|---------------------|
|      |                | Calculated       | Measured            | Calculated for random coil $^a$ | 8 M urea | CNBr peptide in D$_2$O |
| 1    | Leu (2)$^e$ and Val (1) CH$_3$ | 18               | 4.0                  | 0.90                  | 0.69    | 0.74                  |
| 2    | Thr CH$_3$     | 9$^e$            | 8.6                  | 1.23                  | 1.14    | 1.80                  |
| 3    | Ala CH$_3$     | 3                | 2.8                  | 1.41                  | 1.40    | 1.37                  |
| 4    | Met CH$_3$     | 3                | 3.0                  | 2.06                  | 2.05    | 2.04                  |
| 5    | Val CH$_3$     | 1                | 2.3                  | 2.25                  | 2.23    | 2.14                  |
| 6    | Gln CH$_2$     | 4                | 4.3                  | 2.87                  | 2.86    | 2.31                  |
| 7    | Arg CH$_3$     | 4                | 6.0                  | 3.19                  | 3.14    | 3.00                  |
| A    | Tyr, ortho to OH | 4 $^e$          | 3.8                  | 6.82                  | 6.81    | 6.77                  |
| B    | Trp (1) C-5,6, Hist (1) C-4, Trp (2), meta | 7               | 6.4                  | 7.08                  | 7.07    | 7.04                  |
| C    | Trp (1) C-2, Phe (2), aromatic | 11               | 12.4                 | 7.25                  | 7.24    | 7.21                  |

$^a$ Parts per million from 2,2-dimethyl-2-silapentane-5-sulfonate.

$^b$ Measured for spectrum in 8 M urea.

$^c$ Calculated from data of McDonald and Phillips (27) for random coil in D$_2$O.

$^d$ Number in parentheses after amino acid indicates the number of residues per mole of glucagon when more than 1 residue is identified with a peak.

$^e$ Six for CNBr peptide.

$^f$ Methionine not present in CNBr peptide.

The measured spectra shows somewhat greater resolution of peaks because of the small size of glucagon and the improved instrumentation at the slightly higher fields of 250 instead of 220 MHz. There are however some differences between the calculated and observed spectra (Table III). The methyl peaks of threonine, alanine, and glycine are.

The observed energy transfer in the cyanogen bromide peptide could come from a randomly coiled chain only if the distribution of end to end distances were broad. The absence of tyrosinate quenching of tryptophan fluorescence in the presence of guanidine hydrochloride (Fig. 4) shows that energy transfer is precluded when the peptide is unfolded. Guanidine hydrochloride causes only relatively minor changes in the emission spectra of tyrosine and tryptophan (18) and thus should not greatly affect the Forster distance, $R_0$. This experiment demonstrates the conformational dependence of the observed energy transfer. The distance between aromatic residues is too great in the random conformation to allow energy transfer, while the distance between the residues in water, 12 A, is comparable to the radius of the cyanogen bromide peptide as a compact sphere, 9.7 A.

The increased quenching at alkaline pH of the cyanogen bromide peptide compared with glucagon would result either from a folding of the molecular conformation of the two COOH-terminal amino acids or from a change in orientation of the transition dipoles of the aromatic amino acids. The former explanation seems unlikely and is contrary to the circular dichroism evidence which shows identical intrinsic Cotton effects for the two peptides and to hydrodynamic evidence indicating compact structures for the peptides. On the other hand, a change in orientation of the aromatic residues is supported by the loss of the extrinsic Cotton effects on cleavage of glucagon with cyanogen bromide. It thus appears that monomeric glucagon is in the form of a compact molecule and that no energy transfer occurs between tryptophan and tyrosine because of the fixed orientation of their transition dipoles. Glucagon thus possesses at least two properties in common with larger globular proteins, its compact structure and the fixed position of its aromatic side chains.
leucine, and valine and the $\delta$CH$_2$ peak of arginine are shifted to higher field in the observed spectra. Peak 1 is especially shifted and is also seen in the spectra of Patel (8). Most of the peaks of the glucagon spectra in 8 M urea, however, do agree very well with the calculated value and their assignments are confirmed by measurement of the peak areas. Because of the overlap of Peaks 8 and 9 the measured total number of protons for these two peaks agrees well with the calculated value while each individual peak does not. In addition the measured area of Peak 11 is somewhat high because of overlap with other resonance lines.

There are several discernible differences between the spectra of glucagon in 8 M urea and that of the cyanogen bromide peptide in water. The peaks of the cyanogen bromide peptide are slightly broader and more complex having several overlapping peaks, indicating protein folding; especially for Peak B in Fig. 6.

In addition, there is clear indication of a change in chemical shift for Peaks 3, 8, and 11 between glucagon in 8 M urea and the cyanogen bromide peptide in water. This shift is less for other peaks and Peak 9 and part of Peak 3 do not shift at all indicating that the shift is not caused by an unspecified solvent effect. The two remaining threonine residues in the cyanogen bromide peptide produce resonance lines at 1.06 and 1.13 ppm. The resonance at 1.13 ppm occurs at the same position as Peak 3 in 8 M urea while that at 1.06 ppm occurs in a region where no signal is found in the 8 M urea spectrum. This suggests that one of the threonine residues is still exposed to the solvent while the other is in a folded region of the peptide. The amino acids which are implicated as being in the structured regions of the peptide are a threonine residue at position 5 or 7 and the valine and arginine residues at positions 23, 17, and 18. Thus the conformational
difference between the cyanogen bromide peptide in water and that of a random coil involves several regions of the polypeptide chain and is not due to a local ordering.

Glucagon has been shown to adapt several conformational forms (2-5, 28, 29). The ability of the peptide to form aggregates of higher helical content in concentrated aqueous solutions appears to be dependent on the presence of the two COOH-terminal amino acids, as we have found that the cyanogen bromide peptide does not exhibit this property. In contrast, the ability of glucagon to fold into a compact molecule in dilute aqueous solution is retained in the cyanogen bromide peptide. The globular nature of the cyanogen bromide peptide is suggested by the quenching of the tryptophan fluorescence by tyrosinate, the shift of several peaks in the proton magnetic resonance from their position in the random coil and from the frictional coefficient obtained from the sedimentation velocity studies. Since glucagon and its cyanogen bromide peptide exhibit similar cotton effects in the region of the peptide chromophore, in dilute aqueous solution, the two peptides have similar conformations in the monomeric form. The results of this work thus strengthen the conclusion, based on viscosity measurements (20), that the glucagon monomer is in the form of a compact structure.

The cyanogen bromide peptide has been found to be active in stimulating adenylate cyclase activity in the liver plasma membrane (30) and in activating lipolysis in isolated fat cells (31). In addition, the removal of approximately the last 7 amino acids by carboxypeptidase digestion did not result in loss of biological activity (32). The immuno reactivity of glucagon does not depend on the COOH-terminal amino acid residues either. A synthetic peptide containing residues 9 to 23 (33) and a tryptic peptide of residues 1 to 17 (34) were shown to have the ability to aggregate to structures of higher helical content (32). The immuno reactivity of glucagon does not depend on the COOH-terminal amino acid residues either. A synthetic peptide containing residues 9 to 23 (33) and a tryptic peptide of residues 1 to 17 (34) were shown to have the ability of combining with antibodies to glucagon. Our results on the conformational properties of the cyanogen bromide peptide of glucagon indicate that its ability to aggregate to structures of higher helical content is not essential for its biological activity.

Note Added in Proof—Our preparation of the cyanogen bromide peptide of glucagon activates the membrane-bound adenylate cyclase of rat liver, although at somewhat higher concentrations than that of glucagon.

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