Glucagon Conformation: Use of Optically Detected Magnetic Resonance and Phosphorescence of Tryptophan to Evaluate Critical Requirements for Folding of the Polypeptide Chain†

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ABSTRACT: The triplet state of Trp-25 in native glucagon, *des*-Met[27], Asn[28], Thr[29]-glucagon (glucagon-(1-26)), and smaller peptide fragments was examined by optical detection of magnetic resonance (ODMR) and phosphorescence emission spectra. Glucagon(1-26) has ODMR and phosphorescence spectra similar to the five-residue glucagon peptide (22-26) and to shorter peptides also lacking Met-27, Asn-28, and Thr-29. However, the phosphorescence emission of glucagon-(1-29) is red-shifted by about 3 nm. In addition the ODMR zero-field splittings (zfs) show about a 40-MHz decrease in $|D - E|$ and a 95-MHz increase in $|2E|$ when compared with glucagon-(1-26). The tryptophan triplet state parameters of the 18-29 and 19-29 fragments are similar to those of glucagon-(1-29), while those of the 22-26 and shorter peptides are similar to those of glucagon-(1-26). The ODMR line widths of the glucagon-(1-29) zfs transitions are significantly narrower than those of glucagon-(1-26) and are close to those of the 18-29 and 19-29 fragments. *Otherwise the line width generally decreases with increasing chain length.* Comparing the triplet state data with circular dichroism studies of glucagon in the literature, and with the theoretical predictions for α-helical structure in glucagon made by Chou, P. Y., and Fasman, G. D. ((1975), *Biochemistry 14*, 2536) indicates that the 15-20% α-helical structure is located in the region of Trp-25. Acquisition of secondary structure in glucagon takes place in peptides of eight residues or longer, and/or in those which include the sequence Met[27]-Asn[28]-Thr[29].

An understanding of the dilute solution conformation of polypeptide hormones is limited by the number of physical methods available for attacking the problem. One of the most useful tools has been circular dichroism (CD),[1] although signal resolution is a problem at very low concentrations, and interpretation of the spectrum itself can be controversial (Fasman et al., 1970; Strauss et al., 1969; Chen et al., 1974). Nevertheless, generalized conformational changes are detectable by this approach, and the fractional number of residues in α-helical or β conformations has been estimated in this manner for a variety of proteins and polypeptides.

This paper demonstrates the use of optically detected magnetic resonance (ODMR) (Sharnoff, 1967; Kwiram, 1967, 1972), and its use with phosphorescence spectra to complement the conformational information obtained by CD. We present here detailed evidence for the localization of specific structure in glucagon, a 29-residue (Bromer et al., 1957) adenylate cyclase stimulating hormone (Makman and Sutherland, 1964; Murad and Vaughn, 1964). The primary amino acid sequence is shown in Figure 1. Our approach utilizes the triplet state properties of tryptophan as an endogenous probe for studying folding of the polypeptide chain. By comparing shifts in the triplet state zero-field splittings (zfs) and phosphorescence emission of the single tryptophan (Trp-25) in glucagon with those of selected polypeptide fragments, we find a pattern consistent with the published CD results (Srere and Brooks, 1969; Contaxis and Epand, 1974; Panijpan and Gratzer, 1974) as well as with the theoretical predictions for α-helical structure in the 18-29 region of the hormone (Chou and Fasman, 1975). The x-ray crystal structure of glucagon by Blundell and coworkers (Sasaki et al., 1975) shows that near neutral pH the polypeptide chain is largely helical, whereas CD studies in dilute solution indicate 15-20% α helix.

Our data suggest that in dilute solid solution des-Met[27], Asn[28], Thr[29]-glucagon and fragments of five or fewer residues lacking these three carboxyl-terminal amino acids are not folded in a specific conformation. On the other hand, the 19-29 and 18-29 peptides appear to adopt an ordered structure similar to the segment in native glucagon containing these same residues. Preliminary results have been presented elsewhere (Ross et al., 1976).

Experimental Section

Materials. Crystalline glucagon was purchased from Elanco Products, and purified by ion-exchange chromatography (Ross, 1976). Glucagon peptides were gifts from Dr. W. W. Bromer of Lilly Research Laboratories, Indianapolis, Ind.; Dr. H. Edelhoch of the National Institutes of Health, Bethesda, Md.; Dr. A. Fontana of the Institute for Organic Chemistry, Padova, Italy; and Professor K. Wüthrich of the Swiss Federal Institute of Technology, Zürich, Switzerland.

Sample Preparation. Polypeptides were dissolved at a final concentration of about 10^{-5} M in 50% (v/v) aqueous ethylene glycol (Chromatoquality; Matheson, Coleman and Bell) buffered at pH 7.4 with 0.1 M K_xPO_4. This solvent gives a good clear glass for low temperature spectroscopy.

Luminescence Measurements. A conventional right-angle optical configuration was used for measuring phosphorescence spectra. Exciting light was provided by either a 150-W high-pressure xenon arc (Canrad-Hanovia, Newark, N.J.) or a 100-W high-pressure mercury arc (Illumination Industries

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[1] Abbreviations used: CD, circular dichroism; ODMR, optically detected magnetic resonance; zfs, zero-field splitting; ELDOR, electron double resonance; OD/ELDOR, optically detected ELDOR.

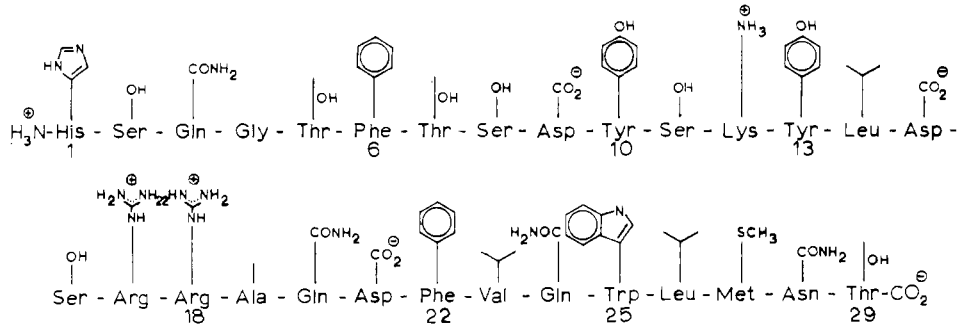
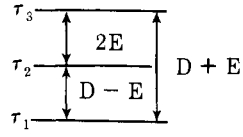


FIGURE 1: Amino acid sequence of glucagon (after Bromer et al., 1957) showing the residue side chains. The numbered residues 1, 6, 10, 13, 22, and 25 identify the aromatic amino acids. Residue 18 is the amino-terminal residue of the longest peptide fragment which includes Met-27, Asn-28, and Thr-29.

Inc., Sunnyvale, Calif.). The excitation wavelength was 297 nm, selected by a 0.25-m Jarrell-Ash monochromator (Fisher Scientific Co., Waltham, Mass.) set for either a 1.5- or 3-nm bandpass, depending upon the intensity required for optimum excitation of tryptophan while excluding significant excitation of tyrosine. Phosphorescence was detected through a second 0.25-m Jarrell-Ash monochromator using a 3-nm bandpass, by an EMI 9558 phototube (EMI Gencom Division, Plainview, N.Y.) coupled to an SSR 1105 photon counter (Princeton Applied Research Corp., Princeton, N.J.). The contributions of scattered exciting light and fluorescence were eliminated by a rotating can chopper. The samples were contained in 3-mm quartz tubes and positioned inside a microwave helix. The helix, terminating a rigid 50-ohm co-axial sample holder, was suspended inside a liquid helium Dewar. The Dewar was equipped with a quartz tail to allow near-ultraviolet excitation of the sample at temperatures ranging from 1.3 to 77 K. Acquisition of the phosphorescence spectrum over the range 350 to 564.5 nm was carried out using 600-s scans distributed over 1024 channels on a PDP 8/E computer (Digital Equipment Corp., Maynard, Mass).

Optical Detection of Magnetic Resonance. The optical configuration was similar to that used for the luminescence measurements. Rather than utilizing the chopper, however, scattered exciting light and fluorescence were removed by 0-51 Corning filters inserted between the sample and the detector during steady-state excitation (steady-state ODMR). Alternatively, a millisecond-resolution shutter in front of the emission monochromator was opened several seconds after closing a similar shutter on the excitation side of the sample (delayed ODMR; Moore and Kwiram, 1974). The PDP 8/E computer was used to operate the shutters, to control the Hewlett-Packard (Palo Alto, Calif) Model 8690B and Alfred (Singer, Palo Alto, Calif.) Models 621 BK, 622 BK, and 623 BK microwave generators, and to accumulate and average the signal. Microwave frequencies were monitored by a frequency counter (EIP, Inc., Santa Clara, Calif.).

At 1.3 K all the tryptophan zfs of the various glucagon peptides could be detected by a combination of steady-state ODMR, delayed ODMR, and optically detected ELDOR (Hyde et al., 1968; Kuan et al., 1970), a microwave double resonance experiment (OD/ELDOR) which can be described as follows: Since in tryptophan the $|D - E|$ and $|2E|$ transitions have the principal radiative level τ_2 in common (Zuclich et al., 1974; Rousslang and Kwiram, 1976) as shown in the diagram, both could be readily observed by steady-state or



delayed ODMR. The levels τ_1 and τ_3 are not as well coupled as τ_2 to the ground state; hence the $|D + E|$ transition is of very low intensity. By saturating either the $|D - E|$ or $|2E|$ transitions—thus equilizing the spin populations of their respective sublevels—and then irradiating with microwaves corresponding to the energy splitting of τ_1 and τ_3, the $|D + E|$ transition was readily detected. We obtained the best signal to noise ratio with delayed OD/ELDOR (for example, saturating the $|2E|$ transition of glucagon-(19-29), the signal:noise was 30:1 with 12 scans at 90 MHz/s). To obtain a flat baseline, a scan made in the absence of microwaves was subtracted from a scan made in the presence of microwaves.

During steady-state ODMR we observe a shift in the ODMR line position depending on whether we scan toward higher or toward lower frequencies. With scan rates as slow as 6 MHz/s, the spectra for up and down scans cannot be superimposed. Slower scan rates reduce but do not eliminate the observed differences. The change in the ODMR line position and shape depends upon the scan rate and the dynamic parameters of the triplet state. In our system the conditions for slow passage are not satisfied. The term "slow passage" used in zero-field ODMR is taken from high-field magnetic resonance, and refers to the steady-state solution to the Bloch equations. The analogue of these solutions at zero-field has been discussed by Rousslang (1976). It follows from the latter that the degree to which steady-state is maintained throughout resonance will depend upon how quickly the equilibrium spin populations are regained relative to the sweep rate. Ideally during microwave slow passage with steady near-ultraviolet excitation, a new quasisteady state is continuously established, and the line shape of the magnetic resonance response is unaltered by the lifetime of the sublevels.

For delayed ODMR the exciting light is extinguished prior to a slow microwave sweep. Thus the conditions for steady state obviously cannot be satisfied.

Since the line positions (hence the center frequencies) are sensitive to the experimental conditions, we report the average of the peak position of scans made toward increasing and decreasing frequency (see Kwiram, 1971). This provides an internal correction for observations taken at different times with varying scan rates. In the region where we monitor the phosphorescence (432 nm), changing the emission wavelength by a few nm in either direction has no significant effect on the zfs parameters.

Results

Luminescence Spectra. A comparison of the tryptophan phosphorescence of glucagon-(1-29) and des-Met[27], Asn[28], Thr[29]-glucagon-(1-26) is shown in Figure 2. It is readily apparent that the emission of the intact hormone is red-shifted

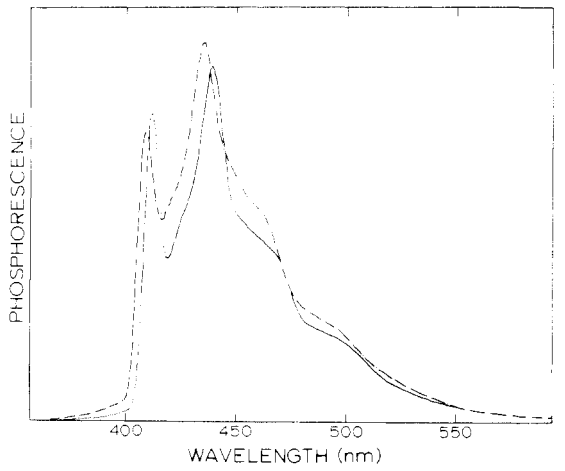


FIGURE 2: The phosphorescence emission spectra of native glucagon (—) and glucagon-1-26 (— · —) at a concentration of about 10^{-5} M in 0.1 M K_xPO_4 (pH 7.4) buffered aqueous ethylene glycol (50% v/v), at 77 K.

with respect to that of the 1-26 peptide in the same solvent. The phosphorescence emission maxima of Trp-25 in glucagon and glucagon fragments are listed in Table I. Peptides of eight or more residues and including Met-27, Asn-28, and Thr-29 have a phosphorescence red-shifted from that of glucagon-(1-26) toward that of glucagon-(1-29). The observed shifts are independent of temperature in the range 1.3-77 K.

Optical Detection of Magnetic Resonance. An example of the ODMR spectra from one of the peptides is shown in Figure 3. As reported in an earlier communication (Ross et al., 1976), we observed a change in both the $|D - E|$ and $|2E|$ triplet state transitions of Trp-25 in glucagon as a function of chain length.[2] The zfs of the various peptides are summarized in Table II. In general, as the peptide chain increases in length, the values of $|D - E|$ decrease and those of $|2E|$ increase. However, glucagon-(1-26) is an exception since the zfs of this peptide are quite similar to those of the five-residue and shorter peptides terminated by Leu-26.

In addition to the change in the zfs, we observed a decrease in the $|D - E|$ and $|2E|$ transition linewidths of Trp-25 in the longer peptides, again except for glucagon-(1-26). For example, as shown in Table II, the $|D - E|$ line width of native glucagon is about 100 MHz, whereas that of the 1-26 peptide is about 140 MHz. Their respective $|2E|$ line widths are about 205 and 295 MHz.

The $|D + E|$ transition of the various fragments, detected by delayed OD/ELDOR, was within error the sum of $|D - E|$ and $|2E|$. Either $|D - E|$ or $|2E|$ could be saturated to measure $|D + E|$, although the best signal-to-noise ratio was observed by saturating $|2E|$.

Discussion

The major changes in the zfs and phosphorescence of Trp-25 in glucagon and glucagon peptides are dependent upon the presence of the three carboxyl-terminal amino acids, Met-27, Asn-28, and Thr-29.

We have previously considered the possibility that the perturbation to the zfs arises from an interaction between the indole ring of Trp-25 and the heavy sulfur atom of Met-27 (Ross et al., 1976). However, second-order perturbation theory predicts that the effect due to increased spin-orbit coupling would give at most a 0.03 MHz contribution to the triplet *energies*.[3] According to first-order perturbation theory, such a contribution from spin-orbit coupling would result in a decrease in the phosphorescence *lifetime* of at least two orders of magnitude. This is not observed experimentally. In addition, the zfs of the control model dipeptides Gly-Trp and Met-Trp do not differ by more than a few MHz from each other or from Trp-Leu-amide (a model peptide for the 22-26 segment in glucagon) in either $|D - E|$ or in $|2E|$; nor do the phosphorescence lifetimes of these dipeptides differ from each other to any discernible extent. Thus it is clear that the changes in the zfs of Trp-25 in native glucagon cannot be attributed to an interaction between the side groups of Trp-25 and Met-27.

We can think of two other possible explanations for our

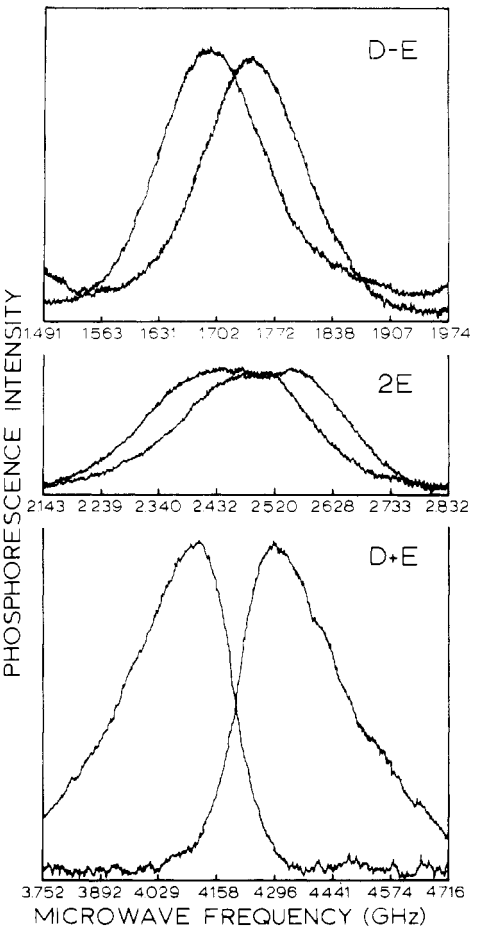


FIGURE 3: Optical detection of the triplet state zero-field splittings of Trp-25 in glucagon-(1-26). The $|D - E|$ and $|2E|$ transitions were measured by steady-state ODMR, and the $|D + E|$ transition was measured by delayed OD/ELDOR as described in the text. The two spectral lines in each frequency range result from scan accumulations made toward increasing frequency (right shifted), and toward decreasing frequency (left shifted).

[2] We previously observed that the 22-29 peptide had zfs similar to those of the 22-26 peptide (Ross et al., 1976). Unfortunately the single direction scan rates made were faster for 22-29 than for the other fragments which caused the relative zfs assignments to be in error. In fact we now find by using the method of averaged "up" and "down" scans somewhat better precision in estimating the relative zfs, and that the $|2E|$ and $|D - E|$ values of the 22-29 peptide fall between those of 18-29 and 22-26 glucagon.

[3] This calculation is based on the relationship $\tau_F/\tau_p\,(E^\circ_S - E^\circ_T) = E'_T - E^\circ_T$ where $E^\circ_S - E^\circ_T$ is the zero-order approximate energy gap between the first excited singlet and triplet states (10^4 cm^{-1}), τ_p is the phosphorescence lifetime assumed to be 10 s, and τ_F is the fluorescence lifetime assumed to be on the order of a nanosecond. $E'_T - E^\circ_T$ is the second-order spin-orbit coupling effect on E°_T. The equation is due to McClure, D. S. ((1952), *J. Chem. Phys. 20,* 682). See also Boorstein, S. A., and Gouterman, M. (1963), *J. Chem. Phys. 39,* 2443.

TABLE I: Phosphorescence Maxima (λ_1 and λ_2 in nm) of Glucagon and Glucagon Peptides.[a]

	Peptides ending in Thr-29						Peptides ending in Leu-26			
	1-29	18-29	19-29	20-29	21-29	22-29	1-26	22-26	23-26	25-26
λ_1:	411.6	411.2	411.7	410.4	410.3	410.4	409.2	409.5	408.6	408.1
λ_2:	438.9	437.2	438.6	436.4	436.4	436.7	435.0	435.6	435.2	434.0

[a] Spectra are measured at 1.3-77 K with excitation at 297 nm. The solvent is pH 7.4 (0.1 M K_xPO_4) 50% (v/v) ethylene glycol/water. Estimated precision of the data is ±0.5 nm.

TABLE II: Zero-Field Splittings of Glucagon and Glucagon Peptides.[a]

		Peptides ending in Thr-29					Peptides ending in Leu-26				
		1-29	18-29	19-29	20-29	21-29	22-29	1- 26	22-26	23-26	25-26
$\lvert D-E\rvert$:	Frequency (GHz)	1.682	1.679	1.674	1.692	1.684	1.694	1.721	1.716	1.720	1.744
	Line width (MHz)[b]	102	116	116	135	144	144	139	135	140	175
$\lvert 2E\rvert$:	Frequency (GHz)	2.592	2.590	2.586	2.574	2.578	2.576	2.498	2.515	2.506	2.470
	Line width (MHz)[b]	206	217	231	220	246	279	293	282	263	283

[a] Average maximum position of magnetic resonance spectra of peptides dissolved in pH 7.4 (0.1 M K_xPO_4) 50% (v/v) ethylene glycol/water at 1.3 K, monitored at 432 nm with excitation at 297 nm. Estimated precision of the data is ±8 MHz. [b] The $|D-E|$ and $|2E|$ ODMR line widths (full-width at half-height) are reported for scans of 12 MHz/s and 28 MHz/s, respectively, and are accurate to about ±7% for the specified scan rates.

observations: the perturbation of the triplet state of Trp-25 could be (1) a consequence of intermolecular association, or (2) a result of intrinsic secondary structure in certain of the polypeptide chains.

The first alternative does not seem likely. We used dilute concentrations of peptides (ca. 10^{-5} M) for these experiments, and ultracentrifuge measurements at 20 °C in pH 7.4, 0.1 M phosphate buffer indicate that this is below the limit where oligomers are detectable in aqueous solution (Ross, J. B. A., and Deranleau, D. A., unpublished results). Furthermore, the self-association of glucagon is considerably less in 50% (v/v) ethylene glycol (Contaxis and Epand, 1974). When the glucagon concentration exceeds 10^{-4} M, the steady-state ODMR line widths begin to broaden and change shape. This indicates an increase in the total number of tryptophan environments, *and in agreement with the ultracentrifuge data, the ODMR line broadening in the more concentrated samples must be due to concentration dependent aggregation.*

We previously presented the hypothesis that the zfs of Trp-25 could be perturbed by local interactions resulting from the presence of secondary structure in glucagon and in the 18-29 peptide fragment (Ross et al., 1976). We supposed that, in keeping with current views on protein structure, a critical number of residues of a given sequence having a high potential for folding are required to obtain a stable secondary structure. Two questions follow: (1) What is the critical amino acid sequence for folding in the region of Trp-25; and (2) how is the perturbation reflected in the zero-field parameters $|D|$ and $|E|$?

Ignoring for the moment the contributions of specific amino acid residues, we show in Figure 4 the zero-field parameters $|D|$ and $|E|$, each as a function of chain length. The parameter $|D|$ varies somewhat for the different fragments, but there is no obvious trend. The major change is in $|E|$, and it correlates with the shift in the luminescence data given in Table I. All of the measured parameters $|E|$, λ_1, and λ_2 indicate that the perturbation begins to manifest itself when the chain length of the region including Trp-25 is greater than five residues, and *includes* residues 27-29.

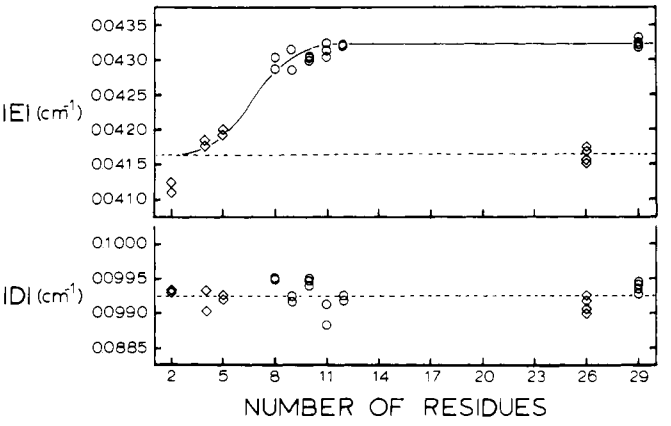


FIGURE 4: Plot of $|D|$ and $|E|$ as a function of chain length for peptides terminated by Thr-29 (○) and by Leu-26 (◇).

An indication that this perturbation results from acquisition of ordered structure in the peptide chain can be found in the observed difference in the line widths of $|D-E|$ and $|2E|$ in, for example, glucagon-(1-26) and the native hormone. The inhomogeneous line width of the indole side chain is sensitive to the multiplicity of interactions which arise as a consequence of its immediate surroundings, and should decrease in a more ordered environment (Rousslang et al., 1977). Fully solvent available models such as free tryptophan or *N*-acetyltryptophanamide have considerably larger line widths than tryptophan in proteins like lysozyme (von Schütz et al., 1974; Rousslang et al., 1977) or chymotrypsin (Maki and Co, 1976).

Our spectroscopic observations fit nicely with the theoretical predictions for α-helical structure in glucagon made by Chou and Fasman (1975). Briefly, their theory states: When clusters of four helix formers out of six residues occur in sequence in any native protein segment, the nucleation of α-helical secondary structure begins and propagates in both directions until terminated by tetrapeptide breakers (peptides containing 50%

or more helix breaking or indifferent residues). They conclude from their analysis that the peptide segment 19–27 fulfills the necessary requirements for helical folding. By their criteria Asn-28 is a helix breaker, Thr-29 is indifferent, and the tetrapeptide 15–18 is a helix terminator. Therefore we expect that glucagon-(1–26), lacking the strong helix former Met-27, would not fold in the region of Trp-25. This is consistent with the observed phosphorescence and zfs of glucagon-(1–26).

Our hypothesis that the low temperature triplet state perturbation of Trp-25 is due to the presence of α helix in the 18–29 and 19–29 segments has an experimental basis in the CD studies of glucagon which indicate the presence of between one and two helical turns in dilute aqueous solution (Srere and Brooks, 1969; Contaxis and Epand, 1974; Panijpan and Gratzer, 1974). By contrast Bromer (1976) finds that glucagon-(1–26) is essentially a random coil and suggests that the three carboxyl-terminal residues are necessary for formation of an α helix in glucagon. The fact that both the phosphorescence spectrum and zfs of glucagon-(1–26) are closely similar to those of the five residue and smaller fragments is completely consistent with Bromer's findings.

Independent evidence that glucagon in dilute solution exhibits stabilized intramolecular interactions is the finding by McBride-Warren and Epand (1972) that at pH 3 and 0 °C approximately 8 (±2) amide hydrogens exchange more slowly than in randomly coiled polypeptides. This finding is consistent with the presence of a stable helix of roughly two turns (see also Panijpan and Gratzer, 1974). Although the experiments were carried out at acid pH, it seems likely that a similar situation is present at neutral pH since Srere and Brooks (1969) find that the CD is essentially constant from pH 2 to 10.

We conclude from our data that (1) the helical structure is localized in the region of Trp-25, and that (2) at least the 18–29 and 19–29 peptides provide essentially the same tryptophan environment as native glucagon. Also, it is evident that ODMR, complemented by other spectroscopic methods, is a site-specific technique for interrogating conformation in polypeptide chains. This is reflected not only in the zfs shifts, but also by the change in the intrinsic ODMR line widths (Rousslang et al., 1977). The magnetic resonance perturbations observed in the microwave domain—within the triplet state itself—are roughly correlated with the electronic perturbations observed in the optical domain; that is, a general relationship exists between the changes in the zfs and the shifts in the phosphorescence emission of the different fragments. However, more detailed information is available from ODMR; this should be potentially useful for understanding the way in which proteins fold, and the relationship of structure to function.

Acknowledgments

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