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Intramolecular Energy Transfer in Adrenocorticotropin*

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ABSTRACT: The efficiencies for singlet energy transfer for the Tyr-2 and Tyr-23 residues to the Trp-9 residue were measured by excitation spectroscopy. Using Förster's theory for long-range energy transfer the intramolecular distance between residues 2 and 9 is estimated to be 10 Å and that between residues 9 and 23 is estimated to be 19 Å or more, the experimental uncertainties being about 15%. The emission spectrum of adrenocorticotropin shows that Trp-9 is well hydrated. The experimental methods are applicable to several peptide hormones and small proteins.

There exist several peptide hormones, as well as a few proteins, which contain a sufficiently small number of aromatic amino acids that the efficiency of singlet energy transfer between pairs of the aromatic residues may be measured unambiguously. With the aid of the Förster theory (Förster, 1948, 1966) which has been tested successfully under various conditions (Ermolaev and Sveshnikova, 1963; Bennett, 1964; Bennett *et al.*, 1964; Kellogg, 1964; Stryer and Haugland, 1967; Birks and Georghiou, 1967; Latt *et al.*, 1965; Conrad and Brand, 1968), it is then possible to determine r, the distance between the donor and acceptor chromophores. This method is often remarkably accurate in spite of uncertainties in some of the experimental parameters which enter into the calculation, as will be seen below. The separation r is not only a sensitive probe for changes in the polypeptide conformation but may usually be determined as an absolute distance. The fluorescence of larger proteins containing many aromatic amino acids (Konev, 1967; Weber, 1961; Teale, 1960) is generally too complex to permit such an analysis although the use of bound fluorescent labels (which may however alter the normal conformation of the protein) leads to information of a similar type (Weber, 1952; Stryer, 1960, 1968).

In the present paper we shall describe how r may be determined by fluorescence excitation spectroscopy and will illustrate the method by giving results for adrenocorticotropin, drawing additional conclusions about the structure of this hormone from its emission spectrum.

Experimental Section

The fluorescence spectra were obtained with an instrument which has been described previously (Eisinger, 1969b). For excitation spectroscopy this fluorimeter was modified by using a 45° Suprasil plate to deflect a small fraction of the excitation light into a quantum counter consisting of a cuvet containing a solution of 1-dimethylaminonaphthalene-5-sulfonate, a 350-nm cut-off filter, and 1P28 photomultiplier. In this way the excitation and emission intensities could be recorded simultaneously. The samples were in quartz tubes with an outside diameter of 2 mm, and their image at the emission monochromator entrance slit was smaller than the slit width. As a result the observed fluorescence intensity is proportional to the fluorescence quantum yield as long as the sample is optically thick (absorbance per cm greater than 10) at the excitation wavelength. Quantum yields were obtained by comparison with *p*-terphenyl in cyclohexane (Berlman, 1965) ($\Phi_f = 0.87$) as the standard after applying suitable corrections to the spectra (Eisinger, 1969b).

Synthetic adrenocorticotropin β^{1-24} was kindly put at our disposal by Dr. Gaunt of CIBA, Summit, N. J. The samples used for emission and absorption spectroscopy had concentrations of about 30 mg of hormone/ml and were at pH 6.4. The synthetic hormone analog adrenocorticotropin β-[$(Gly)_{1,2,3}$,4–24] and adrenocorticotropin β-[1–16-NH_2] which has an amide terminus were the generous gift of Dr. W. Rittel of CIBA, Basel. They will be referred to as ACTH (4–24) and ACTH (1–16), respectively. β-Melanotropin (β-MSH) was kindly given to us by Dr. S. Lande.

Theory

Let $\epsilon_{Trp}(\lambda)$, $\epsilon_{Tyr}(\lambda)$, and $\epsilon_{Phe}(\lambda)$ be the wavelength-dependent molar extinction coefficients of Trp, Tyr, and Phe, respectively. If the polypeptide under consideration contains n_{Trp}, n_{Tyr}, and n_{Phe} of these amino acids the fraction of light absorbed by Trp at any wavelength λ is given by

$$f_{Trp}(\lambda) = \frac{n_{Trp}\epsilon_{Trp}(\lambda)}{n_{Trp}\epsilon_{Trp}(\lambda) + n_{Tyr}\epsilon_{Tyr}(\lambda) + n_{Phe}\epsilon_{Phe}(\lambda)} \quad (1)$$

with corresponding expressions for the fractions of light absorbed by Tyr and Phe, $f_{Tyr}(\lambda)$ and $f_{Phe}(\lambda)$, respectively. Curves showing the wavelengths dependence of these three parameters for the hormones under consideration ($n_{Trp} = 1$; $n_{Tyr} = 1, 2$; and $n_{Phe} = 1$) are given in Figure 1.

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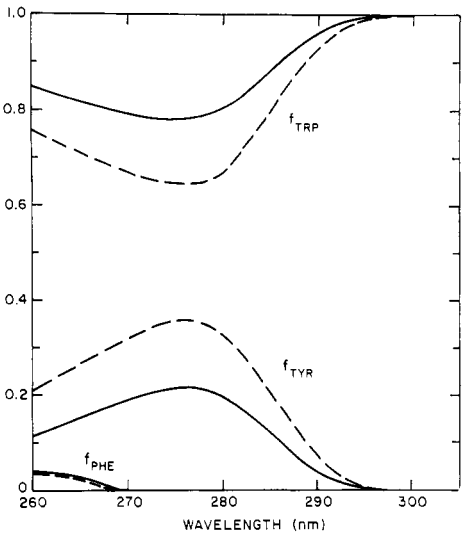

FIGURE 1: The solid curves labeled f_{Tyr}, f_{Trp}, and f_{Phe} represent the fractions of light absorbed by Tyr, Trp, and Phe, respectively, and an equimolar mixture of these three amino acids. The dashed curves represent the corresponding fractions in a mixture in which Tyr, Trp, and Phe are present in the ratios 2:1:1. These curves apply to ACTH (1–24), while the solid ones apply to ACTH (1–16) and ACTH (4–24).

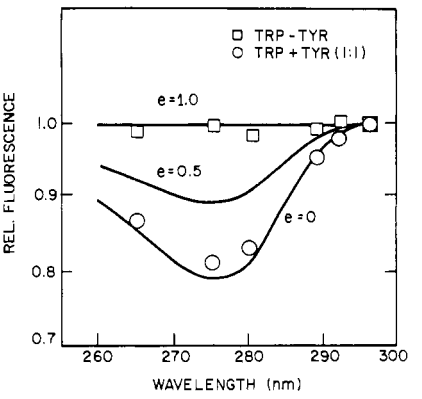

FIGURE 2: The fluorescence excitation spectra of the dipeptide Trp-Tyr and of an equimolar mixture of Trp and Tyr compared with theoretical curves calculated for different efficiencies for the Tyr → Trp energy transfer. The experimental points and theoretical curves are normalized at λ 292.5 nm. The excitation monochromator slit width was 3.2 nm and the fluorescence intensity was monitored at 355 nm.

If, as is often the case, Trp can be identified as the only fluorescent moiety, the quantum yield of the polypeptides depends upon the excitation wavelength, λ, and is given by[1]

$$\Phi_{PP}(\lambda) = \Phi_{Trp}[f_{Trp}(\lambda) + e_{Tyr}f_{Tyr}(\lambda) + e_{Phe}f_{Phe}(\lambda)] \quad (2)$$

where e_{Tyr} and e_{Phe} are the wavelength-independent efficiencies with which excitation energy is transferred to Trp from Tyr and Phe, respectively (transfer in the opposite directions is negligible) (Eisinger *et al.*, 1969). Φ_{Trp} is the quantum yield of Trp when incorporated in the polypeptide and in the absence of energy transfer and is, like the quantum yield of Trp in water, wavelength independent. The function given by eq 2 is then simply the appropriately normalized excitation spectrum of fluorescence monitored at a wavelength at which only Trp emits. It can be seen from Figure 1 that when λ exceeds 290 nm, $f_{Phe}(\lambda)$ and $f_{Tyr}(\lambda)$ are negligible and $\Phi_{PP}(\lambda)$ approaches Φ_{Trp}. When λ is 275 nm on the other hand some 35% of the light exciting adrenocorticotropin β^{1-24} is absorbed by the two Tyr residues and in the absence of energy transfer $\Phi_{PP}(275)$ would be $0.65\Phi_{PP}(297)$. If the energy transfer from Tyr to Trp is 100% efficient, $\Phi_{PP}(\lambda)$ is wavelength independent. This is seen to be the case for the dipeptide tryptophanyltyrosine, whose excitation spectrum is shown in Figure 2. A small amount of energy transfer is observed even in the equimolar mixture of Trp and Tyr, probably because the average distance between these two monomers (about 70 Å) was not sufficiently great to preclude intermolecular energy transfer.

Since f_{Trp}, f_{Tyr}, and f_{Phe} are known from the absorption spectra of the aromatic amino acids, e_{Tyr} and e_{Phe} may be determined in principle from eq 2. In practice Phe absorbs so weakly and only at wavelengths below 270 nm that special care must be used to find e_{Phe} by this method.

The so-called Förster distance, R_0, for which the energy-transfer rate, k_t, is equal to the spontaneous deactivation rate, k, of the donor is given by (Förster, 1948, 1966)

$$R_0^6 = 8.8 \times 10^{-25}\kappa^2\Phi_D n^{-4}J_{AD}' \quad (3)$$

with

$$J_{AD}' = \int_0^\infty F_D(\nu)\epsilon_A(\nu)\nu^{-4}d\nu \quad (4)$$

where κ^2 is an orientation factor which may vary between 0 and 4 and whose value for intramolecular energy transfer is discussed below, Φ_D is the donor fluorescence quantum yield, and n is the index of refraction of the intervening medium. J_{AD}' is the overlap integral between the molar decadic absorption coefficient of the acceptor (ϵ_A) and the spectral distribution of the fluorescence of the donor normalized to unit (F_D) modified by the wave-number factor ν^{-4}. Values for J_{AD}' and R_0 for pairs of aromatic amino acids in environments which obtain in polypeptides have recently been calculated (Eisinger *et al.*, 1969) and differ somewhat from earlier estimates (Karreman *et al.*, 1958).

Since the efficiency of transfer, $e = k_t/(k_t + k)$, between a donor and acceptor separated by a distance r becomes one-half when r is R_0, it can readily be shown that r may be obtained from R_0 and e by use of the following expression

$$r = (e^{-1} - 1)^{1/6}R_0 \quad (5)$$

By differentiating eq 5, the fractional error made in determin-

[1] If Tyr emission contributes to the fluorescence intensity at the wavelength used to monitor $\Phi_{PP}(\lambda)$, the emission spectrum will show additional intensity peaked at 310 nm at excitation wavelengths where Tyr absorbs (*i.e.*, for $\lambda > 290$ nm). Figure 5 shows small Tyr contributions in the spectra of ACTH (1–24) and adrenocorticotropin (4–24). To allow for this Tyr contribution, the left side of eq 2 must be divided by a correction factor $1 + [I_{Tyr}(\lambda)/I_{Trp}(\lambda)]$, where $I_{Tyr}(\lambda)$ and $I_{Trp}(\lambda)$ are the contributions to the fluorescence at the monitored emission wavelength arising from Tyr and Trp emission, respectively, when the excitation wavelength is λ. In the present case, the correction factor was always less than 1.02 at an emission wavelength of 355 nm.

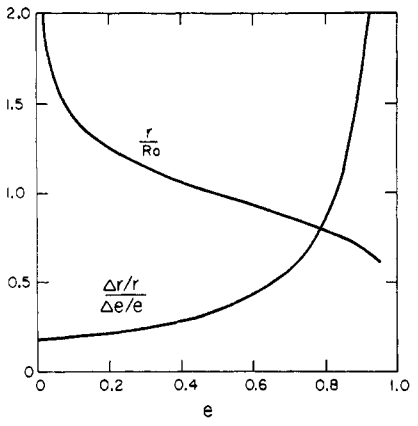

FIGURE 3: The distance between donor and acceptor, r, in terms of the Förster distance, R_0, as a function of the efficiency of energy transfer, e. The figure also shows the fractional error in r, $\Delta r/r$, in terms of the fractional uncertainty in e ($\Delta e/e$) as a function of e.

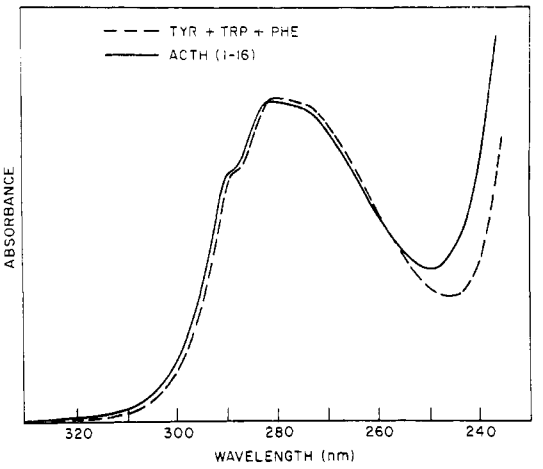

FIGURE 4: The absorption spectrum of ACTH (1–16) compared with that of the corresponding "dummy" solution containing an equimolar mixture of Tyr, Trp, and Phe.

ing r is seen to depend upon the fractional uncertainty of e in the following way

$$\frac{\Delta r}{r} = \frac{1}{6}\left(\frac{1}{1-e}\right)\left(\frac{\Delta e}{e}\right) \tag{6}$$

Figure 3 gives plots of eq 5 and 6 and shows the comparative insensitivity of r to an error in the measured transfer efficiency as long as it does not exceed 0.8 approximately.

The other uncertainties which enter into the calculation of r are those in Φ_D and κ^2. Since both of these quantities enter as sixth roots into the expression for R_0 (see eq 3), even approximate values will suffice.

When the donor is Tyr, Φ_D may be estimated from typical values which are observed for oligopeptides containing tyrosine (Konev, 1967; Cowgill, 1963) and for proteins containing tyrosine but no tryptophan, such as ribonuclease (Konev, 1967; Teale, 1960; Eisinger, 1969a). Such values range from 0.02 to 0.07 compared with 0.14 for tyrosine in water.[2] In some cases, Φ_{Tyr} may be estimated directly by measuring the quantum yield of the remaining emission of Tyr, $(1 - e_{Tyr})\Phi_{Tyr}$. This method is illustrated in the the next section.

The choice of an appropriate value for κ^2 depends upon the donor lifetime and the rigidity of structure in which the donor (D) and acceptor (A) molecules are embedded. One might consider two extreme cases. In the first, the relative orientation of A and D does not change in a time which is comparable with the donor lifetime. This case is expected to obtain in globular proteins with well-developed tertiary structure and without a detailed knowledge of the protein structure it is impossible to know what value of κ^2 is appropriate. In the second case, which in its limit approaches the case of intermolecular transfer in solution, there exists sufficient rotational freedom in the polypeptide linking A and D that the relative orientation of A and D is averaged over all directions in a time which is short compared with the donor lifetime. It is not unreasonable to expect polypeptides to correspond to the second case since the $C_\alpha C_\beta$ and $C_\beta C_1$ bonds generally provide two rotatable bonds for both A and D even when the polypeptide between them is quite rigid (*e.g.*, α helical). If the orientational averaging is complete, $\langle\kappa^2\rangle_{av}$ is $^2/_3$ (Förster, 1948, 1966) if it occurs in a time which is short compared with the donor lifetime and $\langle\kappa^2\rangle_{av}$ is 0.475 (Maksimov and Rozman, 1968; Steinberg, 1968) if the relative motion of A and D is slow compared with the donor lifetime.

A more complete discussion of this problem has recently been presented by Eisinger *et al.* (1969). These authors also consider several other aspects of the problem of intramolecular energy transfer, including the effect of translational diffusion during the donor lifetime, and they estimated that in a random coil polypeptide the value obtained for r by the method outlined here will be low by about 1 Å if the donor lifetime is 0.5×10^{-9} sec.

Results

The anterior pituitary gland hormone adrenocorticotropin is a polypeptide consisting of 39 amino acids but the fragment consisting of the first 24 of these (adrenocorticotropin β^{1-24}) has been shown to have virtually the same activity as adrenocorticotropin (Schwyzer and Kappeler, 1963). Its primary sequence is known (Li, 1956) and it has been synthesized, but little is known about its secondary structure.

Adrenocorticotropin β^{1-24} or simply ACTH (1–24) contains tyrosines in positions 2 and 23 (Tyr-2 and Tyr-23), a phenylalanine in position 7 (Phe-7) and a tryptophan in position 9 (Trp-9).

Figure 4 shows the absorption spectrum of ACTH (1–16) as well as that of a dummy solution containing the same proportions of aromatic amino acids. The near coincidence of these two spectra supports an assumption, implicit in the analysis used here, that the absorption properties of the chromophores in the polypeptide are the same as those in the isolated solvated aromatic amino acids.

Figure 5 shows the fluorescence spectrum of the hormone

[2] The published values for the fluorescence quantum yields, Φ_F (Konev, 1967; Cowgill, 1963), have been lowered by us by one-third since they were calculated with a calibration based on $\Phi_F = 0.21$ for tyrosine and 0.20 for tryptophan (Teale and Weber, 1957). Several recent redeterminations (Eisinger, 1969b; Børresen, 1967; Chen, 1967) seem to point to a lower value for Φ_F of Tyr and Trp, which has been adopted in the present work.

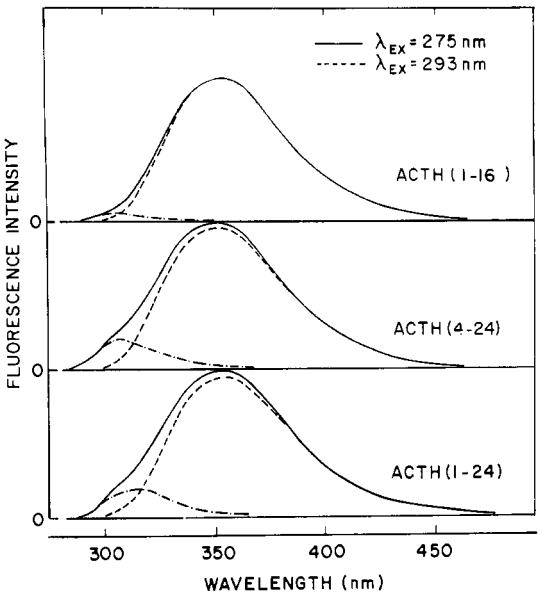

FIGURE 5: The fluorescence spectra of ACTH and two analogs excited at 275 nm, where some of the light is absorbed by the tyrosine residues and also excited at 293 nm, where tryptophan is the only absorbing chromophore. The difference spectra obtained in this way have the shape characteristic of tyrosine emission and their areas are used to estimate Φ_{Tyr}.

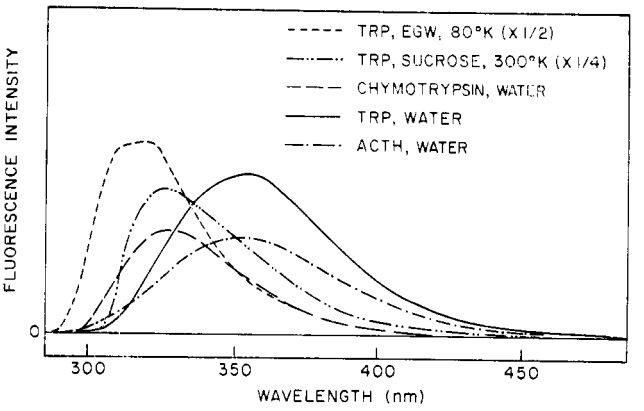

FIGURE 6: The fluorescence spectra of Trp in different environments. Note that the emission spectrum of ACTH resembles that of Trp in water.

and two analogs excited at 275 and 293 nm. It is clear that while most of the emission has the spectral distribution typical of tryptophan in water (Konev, 1967; Eisinger and Navon, 1969) (λ_{max} 355 nm), there is a small contribution from tyrosine which appears only at the shorter excitation wavelength. This contribution is peaked at 310 nm which is typical of Tyr fluorescence whose spectral distribution is fairly insensitive to its environment (Teale, 1960; Longworth, 1968).

The sensitivity of the Trp fluorescence spectrum to the polarity and viscosity of the solvent shell surrounding the chromophore is illustrated in Figure 6. While Trp in water is seen to have an emission spectrum peaked at 355 nm, it fluoresces at 320 nm in nonpolar solvents (Konev, 1967), at about 330 nm in ribonuclease T_1, where Trp is in a nonplanar environment (Longworth, 1968). A polar solvent does not, on the other hand, assure that a red-shifted fluorescence will appear, an additional requirement for this being the ability of the solvent molecules to reorient themselves during the lifetime of the excited singlet state (Eisinger and Navon, 1969). Thus, Trp in a polar glass at 80°K or in a sucrose glass at room temperature emits at 320 and 330 nm, respectively (see Figure 6). The observed spectrum of adrenocorticotropin indicates, therefore, that Trp-9 is surrounded by several water molecules whose rotation is virtually unhindered, and it is safe to say that the indole ring is likely to stick out into the water and is surely not buried in a hydrophobic pocket of the polypeptide. In many proteins the Trp emission has a peak between 320 and 355 nm (see Figure 6).

The quantum yield of adrenocorticotropin at 281 nm is $\Phi_F = 0.08 \pm 0.01$. At 297 nm, on the other hand, where Trp is the only amino acid capable of absorbing the exciting light, Φ_F is 0.10 ± 0.01. This is only slightly less than Φ_F for Trp in water (0.14), another indication that Trp-9 in adrenocorticotropin is not greatly perturbed by nearly amino acids.

The quantum yields of Tyr-2 in ACTH (1–16) and of Tyr-23 in ACTH (4–24) may be estimated from the difference fluorescence spectra show in Figure 5. If A_{Tyr} and A_{Trp} are the areas under the Tyr and Trp spectra excited at 275 nm, it is clear that

$$\frac{A_{Tyr}}{A_{Trp}} = \frac{f_{Tyr}(275)(1 - e_{Tyr})\Phi_{Tyr}}{[f_{Trp}(275) + e_{Tyr}f_{Tyr}(275)]\Phi_{Trp}} \tag{7}$$

As will be seen below (Figure 7), approximate values for $e_{Tyr\text{-}2}$ and $e_{Tyr\text{-}23}$ are 0.5 and 0.15, respectively. With $\Phi_{Trp} = 0.10$ obtained above and these two values one obtains the following estimates for the quantum yields of the Tyr residues from eq 8.

$$\text{ACTH (1–16)}: \Phi_{Tyr\text{-}2} = 0.02 \tag{8}$$

$$\text{ACTH (4–24)}: \Phi_{Tyr\text{-}23} = 0.05 \tag{9}$$

In Figure 7 are shown the experimental excitation spectra of the hormone fluorescence yield monitored at 355 nm along with the theoretical curves calculated according to eq 2. The experimental points (shown dashed) corresponding to λ 296.7 nm fall somewhat below the theoretical curves presumably because at that wavelength the samples were not quite optically thick. The excitation wavelengths used are those of prominent mercury lines and the light passed through a double monochromator with slit widths corresponding to 3.2 nm. While the fit between theory and experiment is not perfect, the following values for the Tyr transfer efficiencies in the three hormone analogs appear reasonable.

$$\text{ACTH (1–16)}: e_{Tyr\text{-}2} = 0.5 \pm 0.15 \tag{10}$$

$$\text{ACTH (4–24)}: e_{Tyr\text{-}23} = 0.15 \pm 0.1 \tag{11}$$

$$\text{ACTH (1–24)}: \bar{e}_{Tyr} = 0.33 \pm 0.18 \tag{12}$$

We feel that the scatter of the data at λ 265.5 nm does not permit one to draw a conclusion about the efficiency of Phe → Trp transfer. $\bar{e}_{Tyr}$ is the efficiency of transfer from both Tyr residues to Trp.

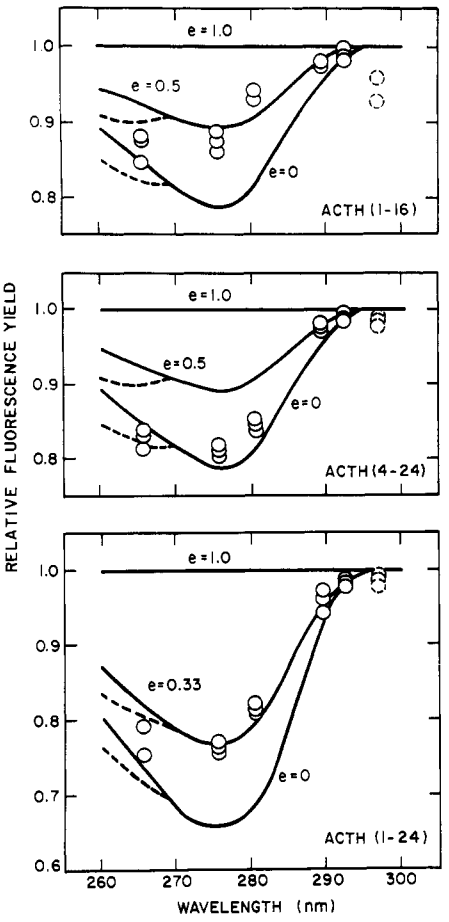

FIGURE 7: The excitation spectra ACTH (1–24) and two of its analogs compared with theoretical curves which represent different efficiencies of Tyr → Trp energy transfer. The solid curves were calculated assuming 100% efficient energy transfer from Phe-7 to Trp-9, while the dashed curves correspond to $e_{\text{Phe-7}} = 0$. The excitation monochromator slit width was 3.2 nm and the emission was monitored at 355 nm.

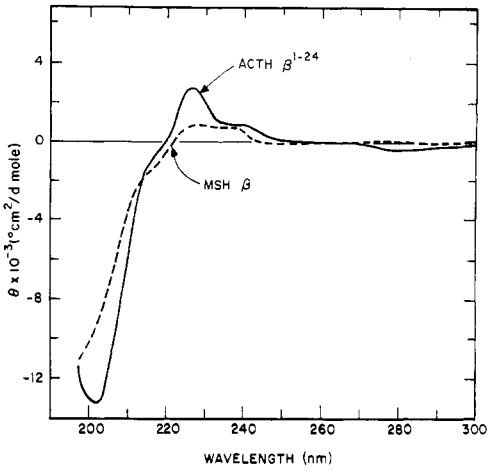

FIGURE 8: The circular dichroism spectra of ACTH (1–24) and β-MSH at neutral pH.

We will now analyze these results to derive values for $r_{2,9}$ and $r_{9,23}$, the distances between Trp-9 and Tyr-2 and Tyr-23, respectively. To do this, we make the assumption that $r_{2,9}$ is the same in ACTH (1–16) and in ACTH (1–24) and that $r_{9,23}$ is the same in ACTH (4–24) and in ACTH (1–24). Since $\bar{e}_{\text{Tyr}}$ for ACTH (1–24) in which both Tyr-2 and Tyr-23 may transfer their energy to Trp-9 is equal to the average of $e_{\text{Tyr-2}}$ measured in ACTH (1–16) and of $e_{\text{Tyr-23}}$ measured in ACTH (4–24), this model is consistent with the experimental results. Using the curves in Figure 3 and the values for $e_{\text{Tyr-2}}$ and $e_{\text{Tyr-23}}$ given above, we obtain $r_{2,9} = (1.0 \pm 0.1)R_0$, and $r_{9,23} \geq (1.7 \pm 0.13)R_0$. The inequality sign is included in the latter expression since the possibility of *intermolecular* energy transfer cannot be excluded (see Figure 2).

Since the experimental results for ACTH (1–24) appear to have less uncertainty associated with them than those for its analogs and because they refer in any case to the hormone which is of primary interest here, it may be preferable to use only $\bar{e}_{\text{Tyr}}$ obtained for this polypeptide which represents the average energy transfer efficiency for the two tyrosine moieties. We make the assumption that Tyr-23 → Trp-9 transfer is negligible compared to Tyr-2 → Trp-9 transfer (this being approximately true in the two analogs) and in this way obtain a value for $e_{\text{Tyr-2}}$ of 0.66 ± 0.2. This leads to a value of $r_{2,9}$ only slightly different than that given above, namely, $r_{2,9} = (0.9 \pm 0.1)R_0$.

It is clear from either method of analysis that $r_{2,9}$ is about one Förster radius while $r_{9,23}$ is considerably greater.

To evaluate these distances absolutely J_{AD}' and R_0 must be evaluated. The fluorescence spectrum of ribonuclease A, an enzyme containing Tyr residues but no Trp residues, resembles the Tyr fluorescence (difference) spectra shown in Figure 5 and it was used for $F_{\text{D}}(\nu)$ to evaluate J_{AD}'. The difficulties in choosing the correct value for $\langle\kappa^2\rangle_{\text{av}}$ were outlined above. If we accept the view that a small polypeptide-like adrenocorticotropin is unlikely to have a rigid conformation (evidence for this view is given in the Discussion section), two-thirds appears to be reasonable value for $\langle\kappa^2\rangle_{\text{av}}$ and n was taken to be 1.5. Using the quantum yields for Tyr-2 and Tyr-23 given above this leads to $R_0 = 9.8$ Å for Tyr-2 → Trp-9 transfer and $R_0 = 11.0$ Å for Tyr-23 → Trp-9 transfer.

With these values, one finally obtains the following intramolecular distances: $r_{2,9} = (10 \pm 1.5)$ Å and $r_{9,23} \geq (19 \pm 2.5)$ Å, where the errors reflect the experimental uncertainties in e_{Tyr} given above and where the errors in Φ_{Tyr} were assumed to be $\pm 50\%$. Translational diffusion during the donor lifetime would increase these distances by less than 1 Å.

As far as the error introduced by the assumption of $\langle\kappa^2\rangle_{\text{av}}$ being equal to two-thirds is concerned it is clearly impossible to estimate its magnitude since it depends upon the details of the three-dimensional structure of the molecule. This remains a serious theoretical weakness of the method outlined here. In the case of short polypeptides with little evidence for rigid structure, as, for example, from optical rotation experiments, it is hoped that the polymer retains sufficient flexibility to make the assumptions for $\langle\kappa^2\rangle_{\text{av}}$ employed here a reasonable one.

Discussion

There exists at present very little information about the secondary structure, if any, of adrenocorticotropin which would help us evaluate these results. We shall therefore try to compare the experimental values given above with a few extreme

models ranging from a random coil to an α-helical conformation.

First of all it should be pointed out that the values for r given above represent values for $\langle r^{-6}\rangle_{av}^{-1/6}$. If one constructs an α-helix model for the adrenocorticotropin region from Tyr-2 to Trp-9 one obtains a minimum separation between these two chromophores of 12 Å and a maximum of 16.8 Å, corresponding to a linear average of $\langle r_{2,9}\rangle_{av}$ = 14.4 Å. The corresponding value for $\langle r_{2,9}{}^{-6}\rangle_{av}{}^{-1/6}$ is 13.9 Å. If we assume an error of 20% for the model which was constructed with a CPK kit our experimental value for $r_{2,9}$ is not inconsistent with an α-helical conformation. The region between Trp-9 and Tyr-23, on the other hand, contains two proline residues which make an α-helix model for $r_{9,23}$ very unlikely (Squire and Bewley, 1965).

The foregoing represents of course in no sense evidence for an α-helical structure. Moreover, the circular dichroism spectra of ACTH and β-MSH, a hormone which contains an amino acid sequence which is virtually identical with that between Tyr-2 and Trp-9 in adrenocorticotropin, indicate that the α-helical content, if any, is small (Squire and Bewley, 1965; Timasheff *et al.*, 1967; Gratzer *et al.*, 1968) (see Figure 8).

In order to compare the experimental intramolecular distances with estimated end-to-end distances, h, for polypeptides we turn to a model used by Brant *et al.* (1967). Because of the torsional rigidity of the amide bond a polypeptide may be considered to consist of a series of virtual bonds between successive C^α atoms 3.80 Å long and with two rotations (Ψ,Φ) about the $C^\alpha C$ and $C^\alpha N$ bonds determining the angles between successive virtual bonds and hence the conformation of the polypeptide. In order to estimate $\langle h^{-6}\rangle^{-1/6}$ for random coil heptapeptides which could then be compared with $r_{2,9}$ it is therefore necessary to calculate h distributions from the conformational energy maps (Brant *et al.*, 1967) which depict the steric and potential limitations of the Ψ and Φ rotations. While such a calculation has not been performed, one may use the method suggested by Brant and Flory (1965a) to estimate $\langle h^2\rangle$ for a heptapeptide random coil which makes use of the experimentally determined characteristic ratio $\langle h^2\rangle/nl$ for infinite random polymers. Here n is the number of links in the polymer, each link being of length, l. Using the value 8.8 for this ratio (Brant and Flory, 1965b) this method leads to an estimate of 20 Å for $\langle h^2\rangle^{1/2}$ of the random coil heptapeptide. By comparison the maximum extension of a heptapeptide is 27 Å while $r_{2,9}$ was seen to be 10 Å.

It is clearly premature to propose any secondary structure of adrenocorticotropin, but the measured intramolecular distance appears to be in better agreement with some form of loop or helical structure than a random coil between residues 2 and 9. The rapid and complete deuterium exchange reported by Li (1962) suggests little or none of the hydrogen bonding which stabilizes an α helix, but some secondary structure may be maintained as a result of solvent interaction (Craig *et al.*, 1965) or weak interactions between residues which may be well separated in the primary sequence of the polypeptide. This possibility is currently being explored in a study of intramolecular distances in various synthetic modifications of adrenocorticotropin with different carboxyl terminations.

Acknowledgments

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Intramolecular Singlet Excitation Transfer. Applications to Polypeptides*

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ABSTRACT: A critical examination of the conditions under which the formalism of the Förster singlet energy-transfer theory may be used to determine the donor–acceptor separation from the experimental energy-transfer efficiency is presented. In particular, the importance of multipole transitions, exchange interaction, "before-relaxation" transfer, and translational and rotational diffusion and the useful range of distances are examined. The overlap integrals and Förster distances for transfer between aromatic amino acids are evaluated from experimental absorption and fluorescence data for various environments and temperatures.

The usefulness and limitations of energy-transfer experiments in the determination of intramolecular distances in polypeptides are discussed.

The long-range radiationless transfer of singlet excitation energy has in recent years been observed in many systems. Its theoretical basis is the dipolar interaction between the transition moments of two chromophores and has been developed and discussed in detail by Förster (1948, 1951, 1966). This theory has been tested experimentally under a variety of conditions (Ermoleav and Sveshnikova, 1963; Bennett, 1964; Bennett *et al.*, 1964; Kellogg, 1964; Stryer and Haugland, 1967; Birks and Georghiou, 1967; Latt *et al.*, 1965; Conrad and Brand, 1968) and the agreement between the calculated and observed transfer rates is excellent.

Many proteins contain several aromatic amino acids which fluoresce and exchange singlet excitation energy. It has been suggested that the measurement of energy-transfer rates among these chromophores offers in principle the possibility of determining a structure-sensitive parameter. While the application of this technique is complicated by the multiplicity of donors and acceptors in most proteins, it appears to be practical for peptide hormones (Eisinger, 1969b). In addition several experiments have been reported in which the transfer is to fluorescent labels bound to the protein (Edelman and McClure, 1968; Teale, 1960; Weber, 1952; Stryer, 1968). In the present paper we wish to evaluate the usefulness and limitations of studies of intramolecular energy transfer, particularly between the aromatic amino acids, as a means of studying molecular conformation, and to evaluate the spectral overlap integrals which are needed to translate experimental values of transfer efficiencies into intramolecular distances between the aromatic residues.

Förster Theory

Several reviews of Förster's theory for singlet energy transfer have appeared in the literature (Förster, 1967; Lamola, 1969). Here we wish to limit our discussion to those aspects of the theory which are our particular concern, *i.e.*, the transfer between Trp, Tyr, and Phe residues in polypeptide chains.

Transfer rates between pairs of aromatic amino acids have been previously estimated by Karreman *et al.* (1957, 1958). These authors made the following two approximations in calculating R_0, the Förster critical distance, which is defined below. (1) The fluorescence and absorption spectra of the donor are mirror images when plotted on a wave-number scale. This is justified only if the absorption band arises from a single transition and in the absence of solvent effects or geometrical changes in the excited state (see below). (2) The donor lifetime may be estimated from the absorption strength after correction for the emission quantum yield. This assumption is subject to the same limitations as the previous one.

Additional calculations of R_0 values for aromatic amino acids by Perlman *et al.* (1968) and Konev (1967) made use of the same approximations. Since it is now feasible to measure these energy-transfer rates to a reasonable precision it seemed worthwhile to reevaluate spectral overlap integrals and R_0 values using Förster's exact formulas (see below), so that these results might be used in the determination of intramolecular distances in proteins and peptide hormones. The results are, in general, subject to the following conditions. (1) The dipolar coupling between donor and acceptor is assumed to be small compared with the (unresolved) absorption band of the acceptor ("Very Weak Coupling" of Förster, 1951, 1966). (2) The point dipole approximation is assumed to be valid. This is the case if donor–acceptor separations are large compared with the dimensions of the chromophores. This condition also assures that higher multipoles need not be considered and that exchange interactions are negligible (see Appendix II). (3) Relaxation to the lowest vibronic level of the excited donor is fast compared with energy transfer. If this condition is not

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