long time periods suggests that it is a preliminary step in the salt-induced dissociation process.

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Titration of Ribonuclease T_1*

Shozo Iida and Tatsuo Ooi

ABSTRACT: Potentiometric and spectrophotometric titrations have been carried out to investigate the structure and properties of ribonuclease T_1. The results of these experiments suggest the following conclusions: nine out of the twelve side-chain carboxyl groups have an abnormally low pK_0 of 3.85, and the other three have a high one of 5.0; the three imidazole groups can be classified into two sets, two groups with pK_0 = 6.6, and one with pK_0 = 7.4; the single lysine has a strikingly low pK_0 of 8.6; the terminal carboxyl and amino appear normal, with pK_0 = 3.85 and 7.4, respectively; only two of the nine phenolic groups titrate normally. The existence of such an unusually large number of abnormal groups presumably reflects the tertiary structure of the ribonuclease T_1 molecule. Titrations in the presence of an inhibitor, 2′-guanosine monophosphate, show that over the pH region 5–9 fewer groups ionize in the complex than in the free protein. The difference titration curve for ribonuclease T_1 and its 2′-guanosine monophosphate complex has a maximal value of *ca.* 1 mole of hydrogen ion/mole of protein at pH 6.3, which can be interpreted as reflecting a shift in the pK of one group from 4.9 to 7.8, and therefore as suggesting that one carboxyl group, presumably Glu-58, is masked by binding of the inhibitor. The pH at which abnormal tyrosines become exposed to the solvent through unfolding of the molecule shifts toward higher pH in the presence of inhibitor; binding of 2′-guanosine monophosphate apparently stabilizes the native conformation of ribonuclease T_1. One or both of the normal tyrosines may interact with 2′-guanosine monophosphate.

Ribonuclease T_1 (RNase T_1) is a globular enzyme that specifically hydrolyzes guanosine 2′,3′-cyclic phosphate and the 3′-phosphodiester linkages of guanosine nucleotides in RNA (Egami *et al.*, 1964). Takahashi (1965) has determined the sequence of the single chain of 104 amino acid residues and the location of the two disulfide bridges. It should be of interest to compare the properties of this protein with those of the ribonuclease of bovine pancreas, which exhibits different substrate specificity (Carter and Cohn, 1950; Schmidt *et al.*, 1951) and is probably not homologous.

The following hydrogen ion titration experiments explore the environments of the ionizable groups of RNase T_1. An improved hydrogen electrode, which does not require bubbling of the titration solution (Iida and Imai, 1969), was preferred

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because of the greater accuracy and reproducibility that could be obtained over the more generally used glass electrode. Some data that bear upon the titration properties of RNase T_1 have been reported. Egami *et al.* (1964) have shown that the molecule has a net negative charge at neutral pH (in contrast, RNase A is positively charged: Tanford and Hauenstein, 1956). The γ-carboxyl group of Glu-58 recently has been located at or near the active center (Takahashi *et al.*, 1967), suggesting that this ionizable group may have a role in the enzymic function.

Experimental Section

Materials. RNase T_1, isolated from Taka-diastase and purified by column chromatography on DEAE-Sephadex, was a gift from Sankyo Co., Ltd. In order to remove bound ammonium ion, the protein was deionized using a mixed-bed ion-exchange column. Protein concentration was determined by absorbance at 278 mμ, using $\epsilon_{278}^{1\%}$ 19.1 (Egami *et al.*, 1964). The concentrations used for potentiometric titrations were *ca.* 1–4 mg/ml, and those for spectrophotometric titration *ca.* 0.5–1.5 mg/ml. An inhibitor, 2′-GMP, was isolated from commercial 2′,3′-GMP (C. F. Boehringer and GmbH, Mannheim) by chromatography on Dowex-1 equilibrated with formic acid (Cohn and Volkin, 1951). Carbonate-free NaOH solutions for titration measurements were prepared freshly from saturated solutions of NaOH. HCl solutions were standardized using Na_2CO_3 (obtained from Asahi Glass; heated at 500° for 50 min) as primary standard. NaOH solutions were standardized by titration against the HCl. Glass-redistilled water was used.

Measurement of pH. Measurements of pH were with an improved hydrogen electrode, made from gold plate with bright platinum (described in detail by Iida and Imai, 1969). Hydrogen gas is flowed continuously *over* the titration solution. Bubbling the gas over the submerged electrode is not necessary. The titration cell was immersed in a temperature-controlled bath (25 ± 0.05°). Before beginning an experiment the titration solution was stirred magnetically for several hours to saturate with hydrogen gas and remove CO_2. The reference electrode was saturated KCl calomel. The cell potential was measured with a Shimadzu type PD potentiometer and galvanometer. A laboratory-made amplifier connected to the potentiometer improved the sensitivity of the detector to 2×10^{-4} pH/mm of the galvanometer mirror scale. Reproducibility of the measurements was $\pm 1 \times 10^{-3}$ pH unit.

Hydrogen Ion Titration. NaOH or HCl was added to titration solutions with a microsyringe (Metrohm E 374) that had a total capacity of 0.5 ml and a smallest scale division of 1×10^{-4} ml. Titration data were calculated as the amounts of added NaOH (or HCl) to bring a protein solution to a given pH from a reference pH (usually the isoionic), after subtraction of solvent blanks that had been obtained by titration of a blank solution of the same salt concentration as the protein. The titration curves were analyzed by assuming they reflect the summed behavior of groups whose ionization could be described by the Linderstrøm–Lang equation

$$\mathrm{pH} = \mathrm{p}K_0^i - \log \frac{\alpha_i}{1 - \alpha_i} - 0.868wZ \qquad (1)$$

$$Z = \sum_i n_i \alpha_i \qquad (2)$$

α_i is the degree of ionization of the ith class of titrating groups, n_i is the number of groups in this class, $\mathrm{p}K_0^i$ is the corresponding intrinsic pK, Z is the net charge on the protein, and w is the electrostatic interaction factor

$$w = \frac{\epsilon^2}{2DRkT}\left(1 - \frac{\kappa R}{1 + \kappa a}\right) \qquad (3)$$

where ϵ is the unit charge, k is the Boltzmann constant, T is the absolute temperature, κ is the Debye–Hückel parameter, R is the radius of the protein, and a is the radial distance of closest approach, *i.e.*, that between the centers of the protein and salt ions.

Spectrophotometric Titration. Absorbance was measured with Zeiss PMQ II and Shimadzu MPS-50L spectrophotometers. The pH difference spectra of RNase T_1 were typical of phenolic group ionization, with maxima at 245 and 295 mμ. Because the changes at 245 mμ showed a contribution from other chromophores, especially 2′-GMP, the 295-mμ absorbance was used for tyrosyl titration. pH was measured with a Radiometer TTT1 meter equipped with a Radiometer G202B glass electrode. Since 2′-GMP has a pH-dependent absorption spectrum, tandem cells were used for difference measurements in the presence of 2′-GMP.

Results

Potentiometric titrations of RNase T_1 in 1, 0.1, and 0.01 M NaCl are shown in Figure 1. Reversibility was tested by back-titration from pH 10.5, 11.2, and 2.0. Reverse titration curves from pH 10.5 to lower pH and from pH 2.0 to higher pH coincided exactly with the forward, whereas those from pH 11.2 did not, suggesting that irreversible denaturation of the protein had occurred at pH above 10.5.

The titration data can be analyzed according to eq 1 and 2. The nature of the 28 ionizable groups of RNase T_1 is known from the primary structure (Takahashi, 1965). Values of pK_0 and w were determined with the accuracy of *ca.* 0.1 pH unit for pK_0 and 10% for w by best fit of calculated curves with the experimental, using, because of the irreversible denaturation above pH 10.5, only data obtained at pH below 10. By this procedure, the number of groups in each class and corresponding pK_0 could be obtained almost uniquely, *i.e.*, deviation of these value gave rise to an inferior fit with the experimental curve. The values of pK_0 and w and number of groups so obtained are summarized in Table I. The isoionic point (pH 3.8) is indicated by the arrow in Figure 1.

RNase T_1 has one α-, six β-, and six γ-carboxyl groups, for a total of thirteen. Ten of these have pK_0 = 3.85. This value is abnormally low for β- or γ-carboxyl groups. Nozaki and Tanford (1967) cite pK_0 = 4.0 for aspartic and 4.5 for glutamic acid side chains. Assuming the α-carboxyl is normal, nine β- and γ-carboxyl groups are of low pK_0. The other three carboxyls have pK_0 = 5.0, which is significantly higher than the expected values. The three imidazole groups are divided into two sets that contain, respectively, two groups of pK_0 = 6.4 and one group of pK_0 = 7.2. The terminal α-amino group has a nearly normal pK_0 of 7.4. The value 8.6 is tentatively assigned for the pK_0 of the single ε-amino group (of Lys-41). This is substantially lower than that normal for lysine side chains. Two tyrosines (see below) show a normal pK_0 of 9.7.

TABLE I: Intrinsic pK Values for Ionizable Groups of RNase T_1.

Ionic Strength		α-COOH	Side-Chain Carboxyl		Imidazole		α-Amino	ε-Amino	Phenolic	Guanido	w (Exptl)	w (Theory)
	No. from titration	1	9	3	2	1	1	1	2			
1		3.80	3.80	4.8	6.6	7.4	7.8	8.6			0.04	0.048
0.1		3.85	3.85	5.0	6.4	7.2	7.4	8.6	9.7		0.08	0.085
0.01		3.80	3.80	5.0	6.6	7.0	7.4	8.2			0.12	0.16
	No. from sequence	1	12[a]		3		1	1	9	1		
	Expected[b] intrinsic pK	3.6	4.0–4.6		6.4		7.8	10.4	9.6	12.5		

[a] Six aspartic and six glutamic. [b] From Tanford (1962), Steinhardt and Beychok (1964), and Nozaki and Tanford (1967).

The values of w calculated by eq 3, assuming R = 14.5 Å and a = 15.5 Å, are 0.16, 0.085, and 0.048, for 0.01, 0.1, and 1 M NaCl, respectively. These are in good agreement with those calculated from experiment (given in Table I).

When the pH is raised above pH 10.5, the titration curve shows a sharp increase, presumably due to the exposure and ionization of tyrosine residues buried within the molecule. The number of groups titrated between pH 9.5 and 11.5 is greater than the sum of the number of tyrosine and arginine residues by *ca.* 1 (see Discussion).

The results of spectrophotometric titrations of the phenolic groups are shown in Figure 2. The change in molar extinction coefficient per tyrosine was determined by dividing the total change in molar extinction at pH 13, where all tyrosines in the molecule were ionized, by the number of tyrosines (nine). The value determined was 2500 at 295 mμ, in agreement with that reported for other proteins (Beaven and Holiday, 1952). The 295-mμ absorbance increased only slowly with pH until pH 10.5, at which point it increased sharply, as did the hydrogen ion release described above. At pH higher than 10.5 the absorbance was time dependent; the data shown in Figure 2 were obtained after about 10-min reaction. The spectrophotometric titration was reversible below pH 10.5. Seven or eight phenolic groups are titrated at pH near 11.0; the remaining one or two in the alkali-denatured protein apparently ionize with pK = 11. The data show that most of the tyrosine residues in RNase T_1 are buried within the molecule. The num-

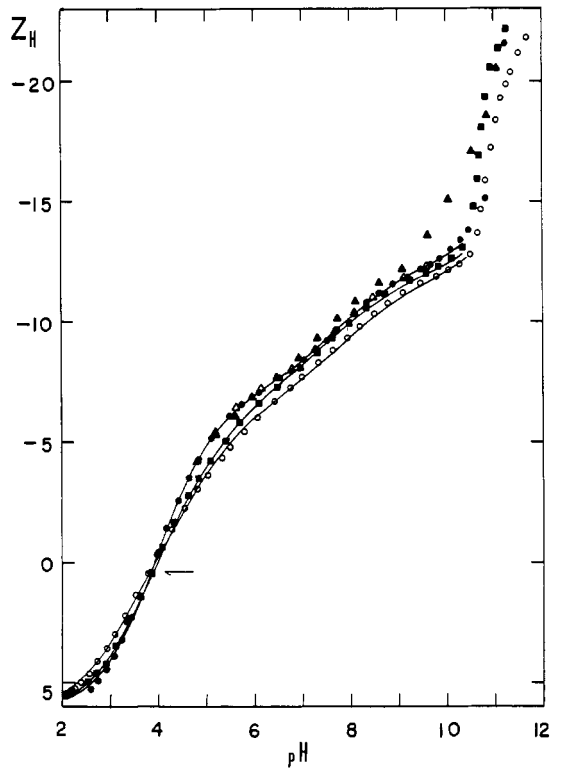

FIGURE 1: Potentiometric titrations of ribonuclease T_1 in 1 M (●), 0.1 M (■), and 0.01 M (○) NaCl at 25°. Back-titrations from pH 11.2 and 10.5 are shown by (▲) and (△), respectively. Solid lines are theoretical curves calculated according to eq 1. The isoionic point is indicated by the arrow in the figure.

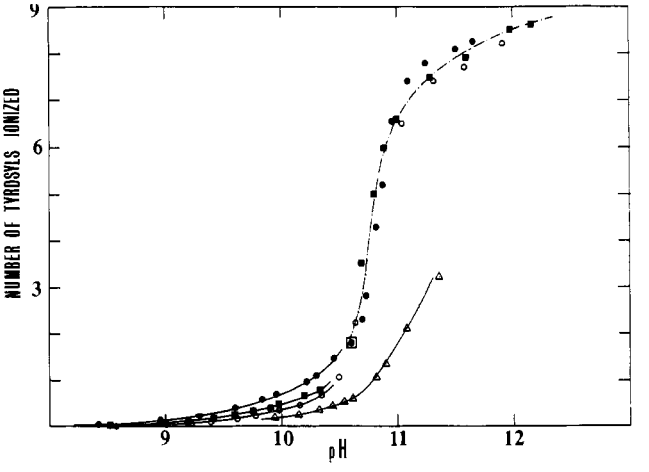

FIGURE 2: Spectrophotometric titrations of ribonuclease T_1 in 1 M (●), 0.1 M (■), and 0.01 M (○) NaCl at 25° and in the presence of 2′-GMP, in 0.1 M carbonate buffer (△).

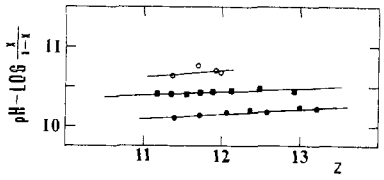

FIGURE 3: Logarithmic plots of the data in Figure 2, calculated assuming that only two tyrosines ionize at pH below 10.5.

ber of abnormal tyrosines was determined by examining according to eq 1 the region of reversible ionization (*i.e.*, that below pH 10.5), where the abnormal tyrosines did *not* ionize. As shown in Figure 3, the assumption of two normal tyrosines allowed the data to be fit well, indicating that seven tyrosines are abnormal. Because the region of analysis was narrow and far from the isoionic pH of 3.8, this number should be considered an estimate. It is in fact possible that all the tyrosines are abnormal, and that the reversible region represents partial ionization of many or all of these residues.

When a competitive inhibitor such as 2′-GMP binds to RNase T_1, some ionizable groups on the molecule may be masked, giving rise to shifts in titration behavior. Ionizable groups involved in the interactions between RNase T_1 and 2′-GMP can in principle be defined by comparison of titrations of RNase T_1 done in the presence of 2′-GMP with titrations of the free protein and the free ligand. The results of such measurements are given in Figure 4. Because of the ionizable phosphate and amino protons of 2′-GMP, more groups were titrated in the presence than in the absence of inhibitor. However, after correction for the ionization of 2′-GMP, in the pH region 5–9 fewer groups titrated in the complex than in free RNase T_1. The effect of 2′-GMP is shown in Figure 5, as the difference in number of groups titrated as a function of pH, *i.e.*, the difference titration curve corrected for the contribution of 2′-GMP. The same difference curve was obtained at molar ratios (RNase T_1 to 2′-GMP) of 1:1.38 and 1:2.1, suggesting that the differences are real, and also that the association constant for 2′-GMP is large. The curve has a maximum at pH 6.3, where the difference in protons bound (ΔZ) is 1. The results indicate that ionizable groups are involved in binding of substrate. It is of interest that the pH dependence of ΔZ is similar to the pH dependence of the enzymic activity (Shiobara *et al.*, 1962).

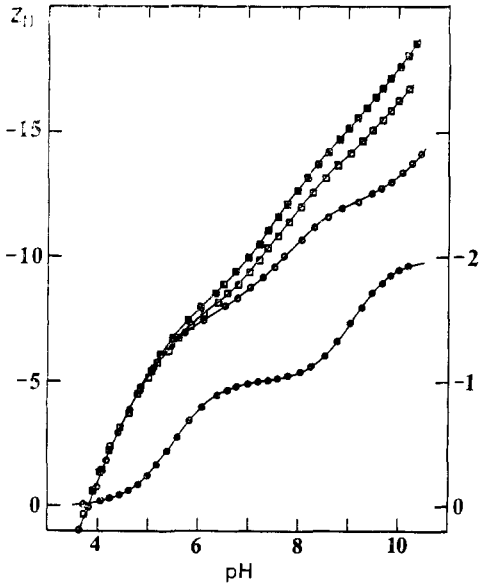

FIGURE 4: Potentiometric titrations of ribonuclease T_1 in the presence of 2′-GMP, in 1 M NaCl. Molar ratios of RNase T_1 to inhibitor were 1:1.38 (□) and 1:2.1 (■). (○) Titration of ribonuclease T_1 alone. (●) Titration of 2′-GMP (right ordinate scale).

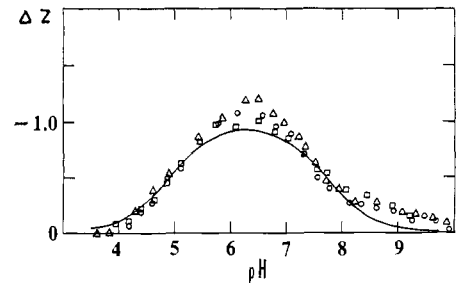

FIGURE 5: The difference of hydrogen ion bound per mole of protein, Z_h, in the presence and absence of 2′-GMP, as a function of pH. Molar ratios of RNase T_1 to inhibitor were 1:1.38 (△) and 1:2.1 (○, □).

The effect of 2′-GMP on the spectrophotometric titration of tyrosine residues is shown in Figure 2. The curve in the presence of 2′-GMP shows with increased pH only slightly increased absorbance up to pH 10.8, above which an appreciable rise in optical density occurs; in the absence of 2′-GMP the break was observed at pH 10.5, *i.e.*, 0.3 pH unit lower. The absorbance in the presence of 2′-GMP was time dependent at pH above 10.8. The extent of reversible tyrosine titration is reduced in the complex. Binding of the inhibitor perhaps increases by one the number of buried tyrosines, as well as stabilizing the structure of the molecule. The activity of RNase T_1 is very low at the pH of tyrosyl titration but binding still occurs.

Discussion

Potentiometric titrations of proteins have usually been carried out with the glass electrode. This has a time-dependent asymmetry potential, and unless a large amount of protein is available, use of the glass electrode leads to considerable error, especially at extremes of pH where the blank is large. The hydrogen electrode is stable and reliable, provided for biological macromolecules there is no bubbling of gas that might denature them, and it is particularly recommended for the titration of proteins available only in small amounts.

Below pH 10.5 titration was reversible, and the number of titrated groups was consistent with that determined from the primary structure (Figure 1 and Table I). However, the number of groups titrated above pH 10.5 was larger by *ca.* 1 than that predicted from the primary structure, and the back-titration from pH above 10.5 did not coincide with the forward. Exposure to high alkaline pH in the presence of hydrogen gas presumably results in irreversible unfolding of the molecule and perhaps chemical modification of some amino acid residues.

The spectrophotometric titration experiments showed ap-

proximately two normal tyrosines. Solvent perturbation measurements (Herskovits and Laskowski, 1962) can give the same sort of information. However, because of the small fraction of normal residues (2 out of 9), the observed changes in absorbance produced by several perturbants tried were too small to obtain an accurate number; the data were consistent, however, with two exposed (*i.e.*, normal) tyrosines. Chemical modification experiments have indicated that two tyrosines are highly accessible (H. Kasai and T. Ando, personal communication), and we presume that these residues correspond to the normal tyrosines observed in the present measurements.

The assignment of pK_0 values to groups and classes of groups is a somewhat arbitrary procedure. A large number of different microscopic constants contribute to the over-all titration properties of a protein, and in principle there should be a different pK_0 and interaction constant, w, for each group. This much information cannot be unambiguously extracted from experiment, however; the best that can be done is to make the simplest assignments of pK_0 that fit the data within experimental error, and that are consistent with the amino acid composition, with pK_0 values for side chains in model compounds and other proteins, and with other chemical studies of the protein in question. Clearly, it is possible, for example, that one of the groups of $pK_0 = 6.4$ could be an abnormal carboxyl and one of these of $pK_0 = 5.0$ could be an imidazole of abnormally low pK. Also, the split of the 13 carboxyl ionizations into only two classes is likely an artifice.

In spite of the difficulties just described, several significant conclusions can be drawn. At least several carboxyl groups must be of pK_0 about 0.5 unit higher than normal, and several others of pK_0 about 0.5 unit lower than normal. The value of 8.6 found for the single lysyl side chain is almost two units below that expected (10.4). Any other assignment would make it yet lower. At least seven of the nine tyrosyl side chains are not accessible to protons in the native structure. This is remarkable and represents to our knowledge a larger proportion of "buried" tyrosyl side chains than reported for other small proteins. Indeed, if we consider the carboxyl ionizations as well as the tyrosyl and lysyl, 10 and probably more of the 28 ionizing groups of RNase T_1 are significantly perturbed. This is an unusually high fraction (10% of the ionizations are perturbed for most proteins, a conclusion drawn from the reviews of Tanford (1962) and Steinhardt and Beychok (1964)). Crystallographic studies have shown that, with only rare exceptions, ionizable groups are on the surface of a protein and exposed to solvent. Either this must not be true for an unusually large number of such groups of RNase T_1, or there must be an unusually large number of strong side-chain interactions between surface charged groups. Further speculation on the explanation seems pointless without crystallographic information.

The difference titration curve of Figure 5 shows that binding of the inhibitor 2′-GMP alters the ionization of one or more groups. There are several ways to interpret data of this kind. As must done for the full titration curve, it is necessary to specify at least the number of the groups affected, their pK, and in addition for each the extent of the pK shift. We should expect several plausible choices for these parameters. In addition, the effect of the electrostatic factors, w, has little effect on the difference titration curve at such a high salt concentration as the present experiment, 1 M (Beychok and Steinhardt, 1959). In the absence of other information, it seems appropriate to consider only the simplest—which is that only one ionizable group is involved. In this case the pK in the free protein and that in the inhibitor–protein complex are defined by the best fit of a calculated difference curve to the data. As shown by the curve in Figure 5, $pK = 4.9$ for the free protein and 7.8 for the complex reproduce the data reasonably well, with a maximum discrepancy of 0.2 mole of H^+/mole of protein. Since the difference data were obtained by subtraction of direct titration curves, errors of the order of 0.1 are likely. It is tempting to conclude that one carboxyl group of $pK = 4.9$ is masked, perhaps by hydrogen bonding to some element of the inhibitor. In accord with this analysis Sato and Egami (1965) have shown that reaction of RNase T_1 with bromoacetate is inhibited by 2′-GMP. Takahashi *et al.* (1967) have identified Glu-58 as the residue carboxymethylated.

Finally, the data of Figure 2 show that at least one tyrosine is perturbed by the binding of 2′-GMP. In this case there is no doubt about the nature of the group affected, but detailed interpretation of the data is difficult. As already noted, specification of the pK of the tyrosine titrating reversibly is uncertain for even the free protein. Also, there may be reversible conformational changes that occur at pH below 10.5 (at which point irreversible changes take place), and if this is true, binding of 2′-GMP might only stabilize a low pH conformation. In this connection, 2′-GMP shifts to higher pH the onset of irreversible changes. Thus, we can only conclude that one or two tyrosines might be at the active site, and that further experiments that examine the tyrosines would be of particular interest.

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Intramolecular Energy Transfer in Adrenocorticotropin*

Josef Eisinger

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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