is increased from acetyl to trimethylacetyl. The deacylation of acyl-α-chymotrypsins shows a marked compensation between the activation parameters with both ΔH^* and ΔS^* becoming more positive as steric bulk in the acyl group is increased.[10] The slope of the compensation plot for acyl groups that are not highly branched is 435°K.[10] Thus, again a pronounced difference can be observed between α-chymotrypsin deacylation and an imidazole, nucleophile-catalyzed reaction. Steric effects on the activation parameters of general base catalyzed hydrolysis reactions have yet to be determined.

Acknowledgment. This work was supported by a research grant from the National Institutes of Health. Support for J. B. Milstien was provided by the National Institutes of Health Research Training Grant GM-197.

The Properties of Thyroglobulin. XVI. Energy Transfer to Iodoamino Acids

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Abstract: The quenching of tryptophan fluorescence by iodoamino acids in native bovine thyroglobulin and in iodinated thyroglobulin and human serum albumin has been measured. Ionization of the iodoamino acids has a greater quenching effect in human serum albumin than in thyroglobulin. From the change in tryptophan fluorescence with iodoamino acid ionization average distances have been computed between emitter and recipient residues. Critical energy transfer distances from tryptophan and tyrosine to the ionized and un-ionized forms of mono- and diiodotyrosine and thyroxine have been calculated by the Forster equation.

The fluorescent properties of proteins have been the subject of considerable recent interest both with respect to their fundamental nature and as a means of detecting structural modifications. The emission spectra of most proteins which contain tryosine and tryptophan residues resemble that of tryptophan with only a minor contribution from the tyrosyl residues.[1b,2] The quantum yields of tryptophan fluorescence in proteins show wide variations which are determined by both local (environmental) and long-range interactions. The latter arise from radiationless energy transfer processes[3] and have received comparatively less attention than the former. A notable exception is the study by Weber and Teale[4] of the quenching of tryptophan fluorescence by heme in several heme-containing enzymes and proteins.

Few studies have been directed toward evaluating energy migration between different residues in nonconjugated proteins since the conditions for energy transfer, as shown by Förster, are satisfied only from tyrosine to tryptophan and tryptophan to ionized tyrosine. Because of the difficulty in resolving tyrosine emission, only the transfer from tryptophan to ionized tyrosine has received confirmation and evaluation.[5,6] Due to the spectral shift that occurs with iodination of tyrosine, energy transfer is possible between tryptophan and iodinated tyrosyl derivatives.[7] Iodotyrosyl residues occur in native thyroglobulin. They can also be introduced into proteins without significant structural change by careful iodination.[8,9] Their influence on tryptophan emission has been assessed in native and iodinated thyroglobulin.

Native bovine thyroglobulin is a very large protein of 670,000 molecular weight and contains approximately 75 tryptophanyl, 110 tyrosyl, and 30 iodoamino acid residues (~1% iodine).[8,10] Due to the large number of tryptophans and iodoamino acids the possibilities of energy transfer are extensive. A second protein, human serum albumin (HSA), containing only a single tryptophanyl residue, was therefore selected for comparison. The molecular properties of this protein are well known from numerous investigations.[11] A recent study showed that the synthesis of several moles of monoiodotyrosine (MIT) and diiodotyrosine (DIT) by iodination did not affect its molecular properties.[12]

Materials

HSA, four times recrystallized, was obtained from Nutritional Biochemicals. This preparation gave a single symmetrical peak by velocity sedimentation and a weight-average molecular weight of 72,400 by sedimentation equilibrium by the meniscus depletion method ($\bar{v} = 0.733$).

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Thyroglobulin was prepared from calf thyroid gland by a differential centrifugation procedure which gave a solution containing about 95% 19S and 5% 27S molecules,[13] followed by gel filtration through granulated agar which eliminated the 27S component.[14] Solutions were stored at −20°.

All chemicals were reagent grade. Glass-distilled water was used throughout.

Methods

A solution of 0.040 *M* I_2 in 0.16 *M* KI was added at a rate of 10 μl/min from an Agla syringe to either a 2% solution of HSA at pH 9.1 or a 1% solution of thyroglobulin at pH 9.35 in 0.05 *M* glycine–0.01 *M* KCl in a pH-Stat at 25° with magnetic stirring. KI was added to the iodinated solutions to bring them to the same final concentration of iodide, 0.03 *M*. At the levels of iodination employed, the protein solutions remained colorless. Further details of the iodination procedure have been described elsewhere.[8]

The concentrations of tyrosine, monoiodotyrosine (MIT), diiodotyrosine (DIT), and thyroxine (T_4) were determined spectrophotometrically on the intact proteins by a modification[15] of a procedure described elsewhere.[8] The tyrosyl and iodoamino acid composition of iodinated HSA and thyroglobulin is reported in Table I.

Table I. Tyrosine and Iodoamino Acid Composition of Iodinated HSA and Thyroglobulin[a]

No.	I_2	Tyr	MIT	DIT	T_4
		Human Serum Albumin			
1	0	17.6	0	0	0
2	3	15.6	0.9	0.3	
3	6	14.4	1.7	0.8	
4	12	12.4	2.3	2.0	
5	18	10.9	2.4	3.2	0.2
6	24	8.9	2.6	4.9	0.2
		Thyroglobulin			
1	0	109	12.2	11.7	5.8
2	10	104	14.7	15.4	4.9
3	20	100	14.9	18.8	5.2
4	40	88.6	16.6	26.7	4.9
5	60	82	17.0	33.5	5.2
6	80	75.6	15.6	38.5	6.3
7	100	69.5	16.0	44.0	5.8
8	130	60.1	16.0	48.2	6.4

[a] All the amino acid residue concentrations are in moles per mole of protein. The iodine concentrations are the number of moles of iodine added per mole of protein.

Ultraviolet Fluorescence. The fluorescence of 0.01% solutions of HSA and thyroglobulin was measured in an Aminco-Bowman spectrofluorometer. Titrations were performed by the addition of small amounts of HCl and KOH. The temperature was maintained at 25°; 275 mμ was selected for excitation since the absorption of tyrosine and the iodotyrosines does not change appreciably with ionization at this wavelength. The isosbestic wavelength of the iodotyrosyl ionization is at 290 mμ in HSA and at 295 mμ in thryroglobulin. These wavelengths could not be used for excitation since the absorption of tyrosyl residues increases significantly with ionization. The fluorescence due to tryptophan was measured at 350 mμ.

Calculation of Energy Transfer Distances. Calculations of the critical distances for transfer of electronic excitation energy from tyrosine and tryptophan to several acceptor amino acids were made according to the Förster equation[3,16]

$$R_0 = \sqrt[6]{\frac{1.69 \times 10^{-33}}{n^2} \frac{\tau J\bar{\nu}}{\bar{\nu}_0^2}}$$

where R_0 is the critical transfer distance, at which the probability of energy transfer equals the probability of spontaneous deactivation of the excited state. The refractive index, n, was assumed to be 1.5. τ, the actual lifetime of the excited state in the absence of energy transfer, was taken as 3×10^{-9} sec for both tryptophan and tryosine.[17] $\bar{\nu}_0$, the wave number of the 0–0 transition of the energy donor, was approximated as the mean of the absorption and emission maxima; for tryptophan, this was 32.8×10^3 cm^{-1} (corresponding to absorption and emission maxima of 279 and 335 mμ, respectively), and for tyrosine 34.7×10^3 cm^{-1} (absorption and emission maxima of 275 and 302 mμ). $J\bar{\nu}$, the overlap integral, was measured graphically from the absorption spectra of the various acceptors and the fluorescence emission spectra of tryptophan and tyrosine in water, normalized by setting the maximum fluorescence intensity equal to the molar extinction coefficient at the absorption maximum. The absorption spectra of MIT and DIT in 0.1 *M* HCl and 0.1 *M* KOH were kindly given to us by Professor S. Lissitsky. All the other spectral data used in these calculations were obtained in our laboratory. It should be noted that the correction of the Förster equation suggested by Latt, *et al.*,[18] does not apply to the equation as used here, in which the experimental fluorescence spectra are treated as mirror images of the absorption spectra. These calculations assume the random orientation of the donor and acceptor transition moments for emission and absorption, respectively, *i.e.*, $K^2 = {}^2/_3$.[19] It should be noted that these values are only approximate since the actual lifetimes vary[17] and the tryptophan emission peak can vary from 350 mμ in aqueous to about 300 mμ in nonpolar solvents. The absorption spectra of the chromophores are changed relatively trivially by changing the solvent.

Results

Fluorescence of Iodinated HSA. The relative fluorescence intensity of native and several samples of iodinated HSA as a function of pH is presented in Figure 1. Native HSA has a complicated pH–fluorescence curve, with strong quenching below pH 4[20] (associated with an important conformational change)[11] and above

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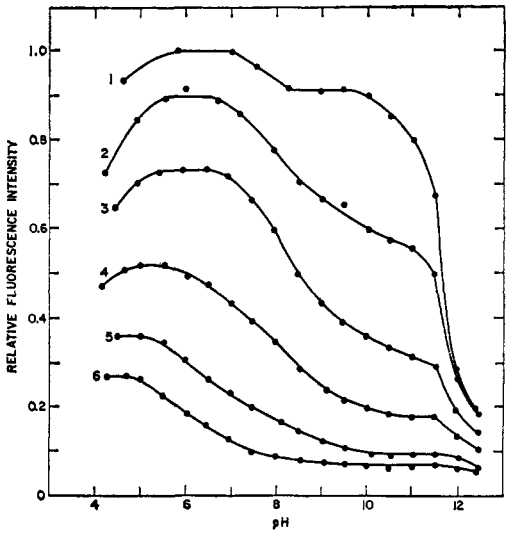

Figure 1. Fluorimetric titrations of native (no. 1) and iodinated (no. 2–6) HSA. Iodoamino acid composition of these preparations is given in Table I. Protein concentration 0.01%, in 0.02 *M* lysine–0.1 *M* KCl. Fluorescence excitation 275 mμ, emission 350 mμ.

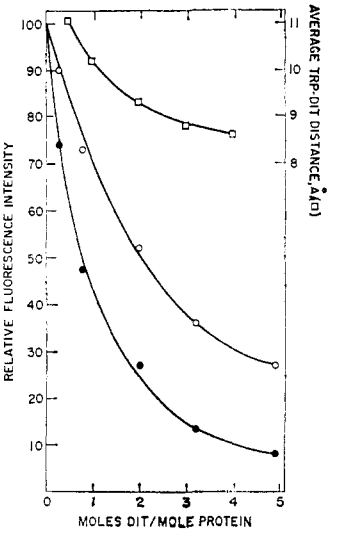

Figure 2. Relative fluorescence intensity of iodinated HSA as a function of diiodotyrosyl (DIT) content. Fluorescence of native HSA taken as 100: ○, maximum fluorescence intensity; ●, fluorescence intensity at pH 9; □, average tryptophanyl (TRP)–diiodotyrosyl (DIT) distance, calculated as described in text.

pH 10.5 (due in part to energy transfer from tryptophanyl to ionized tyrosyl residues), and a relatively small decrease in intensity between pH 7 and 9.[6] The maximum fluorescence intensity decreased significantly with increasing degrees of iodination. In order to demonstrate that the decrease in fluorescence was not due to the destruction of the tryptophanyl residue during iodination, the samples were also examined in 8 *M* urea, pH 6.0. In this solvent, all the iodinated samples had the same fluorescence intensity as native HSA. Evidently, energy transfer between the tryptophanyl and iodotyrosyl residues is completely eliminated in the unfolded state of the protein produced by 8 *M* urea. In other proteins, energy transfer between tryptophanyl and ionized tyrosyl residues is greatly reduced in 8 *M* urea.[6,21]

The decrease in maximum fluorescence intensity of iodinated HSA must be due to energy transfer from tryptophanyl to un-ionized iodotyrosyl residues since few, if any, of these residues are ionized at pH 5.[12] In Figure 2, the maximum fluorescence intensity of the iodinated HSA samples is shown as a function of their DIT content. This presentation ignores the contribution of MIT and thyroxyl residues to fluorescence quenching. However, since the critical transfer distance from tryptophan to DIT is greater than to MIT (see below), the DIT residues are probably the more important energy acceptors. The thyroxyl content of these preparations is negligible. Fluorescence intensity decreases approximately exponentially with increasing DIT content.

All the iodinated HSA preparations show a decrease in fluorescence in the pH range of iodotyrosyl ionization. Samples 2 and 3 (Figure 1) contain relatively little DIT, and consequently the major decrease in their fluorescence occurs in the pH range of MIT ionization (pH 8–10). Samples 5 and 6, which contain more DIT, are largely quenched in the region of DIT ionization (pH 5–9).[12] The quenching between pH 5 and 10 is not due to a structural change in the protein, since the major transition in both native[22] and iodinated HSA[12] occurs above pH 10.5. (It is assumed that the minor transition occurring between pH 7 and 9 does not modify the structure importantly). Figure 2 also shows the fluorescence intensity of iodinated HSA at pH 9, relative to native HSA at the same pH. At this pH, most of the DIT and relatively few of the MIT residues are ionized.[12] The relatively greater quenching at pH 9 than at pH 5 reflects the larger critical transfer distance for energy transfer from tryptophanyl residues to ionized than to un-ionized DIT residues.

The rapid fall in fluorescence between pH 11 and 12 in samples 1 and 2 corresponds to the region of tyrosyl ionization,[22] and is not due to base quenching. HSA is highly unfolded above pH 10.5, as shown by the elimination of electrostatic effects between the tyrosyl groups,[22] increased viscosity,[23] and decreased polarization of fluorescence.[24] Consequently, the strong quenching that takes place between pH 11 and 12 arises either from energy transfer to ionized tyrosyl groups or exposure of the tryptophanyl residues to the solvent or both.

The use of fluorescence quenching as a measure of energy transfer requires the assumption that all quenching in the presence of the energy acceptors is due to energy transfer. Tryptophanyl fluorescence in proteins can be strongly affected by changes in protein conformation. However, at the levels of iodination used in this study, the structure of HSA is unchanged, as measured by viscosity, optical rotatory dispersion, immunologic reactivity, and solubility. Although minor structural changes can not be excluded, certainly there are no gross differences in the structure of native and iodinated HSA.

Fluorescence of Iodinated Thyroglobulin. The iodination of thyroglobulin in aqueous solution at pH 9.35 results almost in the exclusive synthesis of DIT from

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tyrosyl residues, with very little change in the MIT and thyroxyl content (Table I).[25] Moreover, the synthesis of almost 40 mol of DIT/mol of thryoglobulin has no significant effect on its structure, as shown by viscosity, optical rotation, reactivity toward antinative thyroglobulin antibody, and solubility.[9] The tryptophan fluorescence of native and iodinated thyroglobulin was measured at pH 5.0 and 10.0, the limits of DIT ionization,[8] in aqueous solution and in 9 *M* urea (Figure 3). In both solvents, a linear decrease in fluorescence with increasing DIT content was observed; the decrease was slightly greater at pH 10.0 than at pH 5.0.

The quantum yield of tryptophan fluorescence in thyroglobulin is much greater in 9 *M* urea solutions than in water. The fluorescence enhancement due to urea alone, in the absence of DIT (obtained by extrapolation of the data in Figure 3 to zero DIT content) is about 100%. In native thyroglobulin, this enhancement occurs gradually between 3 and 9 *M* urea, and is approximately correlated with changes in viscosity and in ultraviolet difference spectra.[13] Neutral solvent perturbation studies of either tryptophan absorption[25] or fluorescence[26] indicate that relatively few (<25%) of the tryptophanyl residues are exposed to the solvent in aqueous solution at neutral pH; in 9 *M* urea, all the tryptophanyl residues are exposed to the solvent.[26] Exposure to a more polar environment in model tryptophan compounds leads to decreased fluorescence. The increase in thyroglobulin fluorescence in 9 *M* urea therefore suggests that specific intramolecular interactions quench tryptophan fluorescence of native thyroglobulin in aqueous solution.

The twofold enhancement in fluorescence in 9 *M* urea could be due to the loss in energy transfer to the five thyroxyl residues. Evidence suggesting that these residues have little or no influence on tryptophan fluorescence in native thyroglobulin is that their ionization (pK = 7.6)[8] has almost no effect on emission intensity (Figure 3). It would appear that either there are too few of these groups relative to the number of tryptophanyl residues or they are remotely situated from most of the tryptophanyl residues. The latter possibility is intrinsically more reasonable for a large molecule like thyroglobulin than for the smaller human serum albumin molecule.

Discussion

Critical Transfer Distances. Table II presents the critical distances for the transfer of electronic excitation

Table II. Critical Energy Transfer Distances R_0, in Å

	Donor			
	Tryptophan		Tyrosine	
Acceptor	Un-ionized[a]	Ionized[a]	Un-ionized[a]	Ionized[a]
Tyrosine	...	11.2	8.9	15.2
Monoiodotyrosine	7.9	15.7	13.4	17.3
Diiodotyrosine	10.5	20.4	14.9	18.8
Thyroxine	15.4	23.3	17.4	18.6
Tryptophan	7.2		14.7	

[a] Un-ionized and ionized refer to phenolic hydroxyl of acceptor.

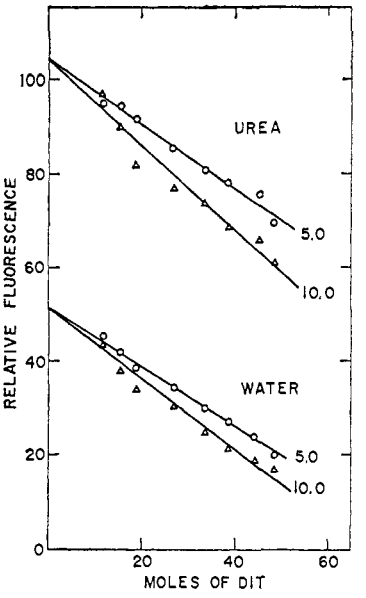

Figure 3. Relative fluorescence intensity of iodinated thyroglobulin in aqueous and 9 *M* urea solutions as a function of diiodotyrosyl (DIT) content: ○, pH 5; △, pH 10.

energy from tryptophan and tyrosine to various acceptor amino acids, calculated as described in Material and Methods. As there is no overlap between the fluorescence emission spectrum of tryptophan and the absorption spectrum of un-ionized tyrosine, energy transfer between them is precluded. Both iodination and ionization lead to a red shift and an intensification of the tyrosyl absorption spectrum, resulting in increasing overlap with the tryptophanyl fluorescence spectrum, and increasing possibility of energy transfer. The critical transfer distances increase with increasing iodination, and increase further on ionization of the phenolic hydroxyl groups.

Energy transfer can also occur between two un-ionized tyrosyl residues. Iodination and ionization of the acceptor tyrosine result in larger transfer distances. The calculated critical transfer distances are large enough so that energy transfer would be expected to occur between many of these donor–acceptor pairs in proteins. In fact, transfer from tryptophanyl to ionized tyrosyl,[6,21] and from tyrosyl to ionized,[27] un-ionized,[28] and iodinated[29] tyrosyl residues has been observed in proteins.

The efficiency of energy transfer as a function of the distance between donor and acceptor may be calculated from the relationship

$$E = 1/[(R/R_0)^6 + 1]$$

where R is the distance between donor and acceptor, and R_0 is the critical transfer distance. Using this equation, the ratio of the efficiency of energy transfer from tryptophan to ionized DIT, compared to un-ionized DIT, as a function of the distance between tryptophan and DIT, was calculated; the results are plotted in Figure 4. From the data in Figures 2 and 3, giving relative fluorescence intensity in the presence of ionized and un-ionized DIT, an "average" distance between tryptophanyl and DIT residues in the two

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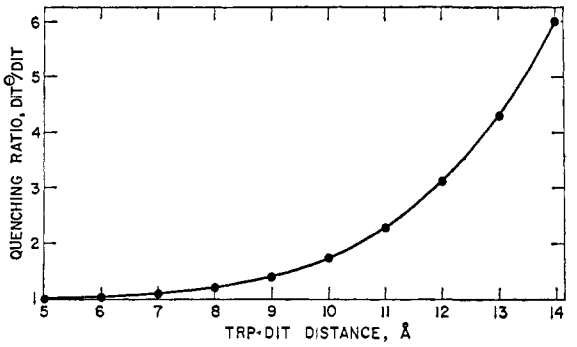

Figure 4. Efficiency of energy transfer from tryptophan (TRP) to ionized (DIT^-) as compared to un-ionized (DIT) diiodotyrosine, as a function of the tryptophan-diiodotyrosine distance, calculated as described in text.

proteins can be estimated. These "averages" are strongly weighted in favor of the closest DIT residues, and do not at all include those DIT residues too far away to quench tryptophanyl fluorescence. These calculations assume that the distances between tryptophanyl and DIT residues do not change with increasing iodination or with DIT ionization. The structures of HSA and thyroglobulin, as examined by several criteria, are essentially unchanged by the levels of iodination in the preparations used here. Further, there are no major structural changes in either HSA or thyroglobulin in the pH range of DIT ionization.

The "average distance" between the DIT residues and the single tryptophanyl residue in HSA decreases slightly with increasing iodination. The average distance between the tryptophanyl residue and the first DIT residue formed is close to 10 Å. This value is close to the critical transfer distance of un-ionized DIT. Consequently a quenching of 50% of tryptophan emission is expected. The observed value is about 30% (Figure 1). At a distance of 10 Å, ionized DIT would be expected to quench tryptophan emission almost completely. Only about 55% quenching is observed (Figure 1). A value of K^2 of the orientation factor in the Forster equation less than $^2/_3$ could account for the low quenching efficiency, though other factors cannot be excluded. The distances calculated between tryptophan and DIT decrease from about 10 to 8.5 Å as the DIT content increases from 1 to 4 mol/mol of protein. It is clear that none of these residues can be within 8.5 Å of the tryptophan residue since tryptophan emission would be 80% quenched at this distance by a single residue. In addition all the residues must be within 16 Å or they would have negligible effect.

The distance between donor and acceptors in HSA is greatly increased in 8 *M* urea: in this solvent, there is no quenching of tryptophanyl fluorescence by un-ionized DIT residues. Thus, no DIT residues can be within several residues of the tryptophanyl residue along the polypeptide chain.

Steiner, *et al.*,[30] have recently studied the binding of thyroxine by HSA by the method of fluorescence quenching. HSA has one binding site with very high affinity for thyroxine; at pH 7.4, where the bound thyroxine is ionized, the binding constant is 1.6×10^6. The binding of 1 mol of thyroxine/mol quenches about 55% of the HSA fluorescence. This suggests that the strong thyroxine binding site is about 22 Å from the tryptophanyl residue in HSA.

The average distance between the tryptophanyl residues and the DIT residues in thyroglobulin does not change with increasing DIT content. This distance is about 8 Å in water, and only increases to about 9 Å in 9 *M* urea. Major structural elements persist in thyroglobulin in 9 *M* urea, as shown by fluorescence polarization measurements. These interactions are more completely eliminated in 6 *M* guanidine.[31] The failure to observe a large increase in the average distance may result from the close proximity of the large number of tryptophanyl and iodotyrosyl residues along the polypeptide chain, or from numerous clusters of residues which remain in 9 *M* urea.

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Communications to the Editor

Two Modes of Formation of Carbonium Ions from Olefins and Suitable Electron Acceptors

Sir:

Electron acceptors, *e.g.*, $SbCl_5$, convert polynuclear aromatic hydrocarbons into the respective radical cations which were identified by their esr[1] and optical[2] spectra. It has been claimed[3] that analogous electron-transfer processes produce radical cations from vinyl or vinylidene monomers and the latter initiate cationic polymerization. The initiation should resemble the anionic process;[4] radical cations are expected to dimerize into dicarbonium ions or to react with excess of monomer and yield dimeric radical cations.

To investigate the course of the initiation, we have chosen 1,1-diphenylethylene (D) as the "monomer." Its head-to-tail polymerization is prevented by steric hindrance, although the dimerization is feasible.[5] Hence, studies of the initiation in this system are not complicated by the subsequent polymerization.[5]

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