Resonance Energy Transfer between Cysteine-34 and Tryptophan-214 in Human Serum Albumin. Distance Measurements as a Function of pH†

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ABSTRACT: The single cysteine residue (Cys-34) of human serum albumin was modified with the organic mercurial [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate. Introduction of this chromophore into the protein results in the quenching of the protein tryptophan fluorescence spectrum due to energy transfer from the tryptophan residue to the mercurial. Since human albumin contains only a single tryptophan, it was then possible to calculate distances between the mercurial bound at Cys-34 and Trp-214 under various conditions. This distance contracted during the course of the N → F transition, being 34–35 Å in the N conformation (pH 6–7.5) and 29.9 Å in the F conformation (pH 3.6). The distance increased substantially during the course of the F → E transition occurring between pH 3.6 and pH 1.9 and was found to be nearly 37 Å at pH 1.9. The distance between Cys-34 and Trp-214 was found to undergo a slight contraction during the N → B transition occurring between pH 7.0 and pH 9.0. At pH 8.5–9 where the protein is predominately in the B form, the distance was found to be slightly more than 31 Å.

In the 1950s and 1960s Foster and co-workers showed that serum albumin undergoes a remarkable reversible change in conformation upon binding protons (Foster, 1977, 1960). In the pH range from 2 to 5, techniques such as optical rotation, UV[1] absorption, viscosity, sedimentation, NMR, etc., showed that the protein undergoes large changes in its secondary and tertiary structure. The reversible conformational changes occur in two steps. The first step between pH 4.5 and pH 3.5 is called the N → F transformation (the N form exists at higher pH), whereas the second step between pH 3.5 and pH 2 is called the F → E transformation (or acid expansion). Hydrodynamic studies indicate that the overall change from pH 5 to 2 involves a considerable expansion of the molecule, while optical studies indicate that the molecule loses a considerable amount of α-helix in the expansion.

While serum albumin has a single polypeptide chain, it is thought to be divided into three separate domains (Brown, 1977; Peters, 1975; Foster, 1977). The restrictions introduced by the presence of 17 disulfide bonds in the amino acid sequence clearly show that the structure contains three small double loops and six large double loops. The three domains of serum albumin have been pictured as forming a linear array each consisting of very similar repeating units: residues 1–190 (domain I), 191–382 (domain II), and 383–582 (domain III). It is thought that the N → F transition reflects the mutual separation of each of these domains from one another, while the F → E transition has been pictured as a disruption of the individual domains into a long viscous molecule.

Between pHs 7 and 9, serum albumin undergoes another conformational change termed the N → B transition (Leonard et al., 1963; Zurawski & Foster, 1974). Harmsen et al. (1971) concluded that as many as 10 histidine side chains are masked in the N form and become titratable in the B form. Modification of the single sulfhydryl group of serum albumin with a spin-label showed that the environmental changes near the sulfhydryl group are very similar in the N → F and N → B transitions (Cornell & Kaplan, 1978a,b). However, optical studies have indicated that the N → B transformation is a more subtle conformational change than is the acid expansion.

Human serum albumin contains a single sulfhydryl group located at Cys-34 in domain I and a single indole ring located at Trp-214 in domain II (Brown, 1977). The fluorescence emission of serum albumin at 340 nm, when excited at 295 nm, is due primarily to the single tryptophan. The introduction of a chromophore at Cys-34 which absorbs where Trp-214 emits affords the possibility of energy transfer between the two chromophores which can then be used to calculate the distance between Cys-34 and Trp-214 by using the Förster theory (Stryer, 1978). Once this distance is determined, more significant questions can be answered such as how these distances change during the acid expansion, during the neutral transformation, or in the presence of molecules which bind serum albumin under physiological conditions. The present report is a study of the effect of pH on the Cys-34–Trp-214 distance. The effect of small molecules on the distance will be reported subsequently.

Materials and Methods

Serum Albumin. Crystallized human serum albumin (Pentex, lot no. 36) from Miles Laboratories was used. Crystalline HSA was defatted before use by a slightly modified version of Chen's charcoal procedure (Chen, 1967). According to this procedure a 10% solution of the commercial product was prepared in deionized, distilled water. The solution was centrifuged for 15 min at 20 000 rpm on a Beckman J-21B certrifuge. Charcoal (Norrit), which had been previously washed with distilled, deionized water and dried (approximately 75% of the dry weight of the protein), was then added to the solution. The pH of the suspension was adjusted to 2.75 with 0.5 M HCl by dropwise addition with stirring. The acidified suspension was stirred for 1 h at 5 °C and was then centrifuged at 20 000 rpm for 15 min (or longer if necessary) to remove the charcoal. The pH of the serum albumin solution was adjusted to 5.6 with 0.1 M NaOH added dropwise with stirring. At this stage the protein was dialyzed at 5 °C over a 24-h period against four changes of the appropriate buffer.

Bovine serum albumin as isolated usually has 40–50% of the Cys-34 sulfhydryl groups oxidized in a disulfide bond with

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[1] Abbreviations: UV, ultraviolet; NMR, nuclear magnetic resonance; HSA, human serum albumin.

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cysteine and glutathione. We consistently found that defatted human serum albumin has only about 20% of the sulfhydryl groups available for reaction with Ellman's reagent (Ellman, 1959). Thus we used the method suggested by Foster (1977) and Janatova et al. (1968) to prepare protein (mercaptalbumin) which was enriched in free sulfhydryl group content and free of oligomers. Typically we found that one pass through the DEAE-Sephadex column resulted in an enriched preparation that contained 50–70% free sulfhydryl groups.

Protein concentrations were determined by using the absorbance at 278 nm where the 1% extinction coefficient for human serum albumin is 5.3 (Blauer et al., 1970). Absorption measurements were made on a Cary 14 or a Perkin-Elmer 320 spectrophotometer.

Modification of Serum Albumin Sulfhydryl. Modification of the sulfhydryl group of serum albumin with the organic mercurial [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate was carried out by using a slightly modified procedure of Horowitz & Klotz (1956). These workers found that the reaction of the mercurial was specific for the sulfhydryl group of bovine serum albumin when the reaction was carried out in 0.1 M glycine at pH 9.6 even when as much as a 4–5-fold excess of mercurial was added over sulfhydryl. Under these conditions it was found that more than 1 equiv of mercurial was bound to the human protein. Titration of the protein with increments of the mercurial clearly showed an equivalence point where enough mercurial had been added to fully modify the sulfhydryl group. Further addition of mercurial resulted in additional noncovalent binding of mercurial to the protein, which could not be removed by dialysis, but which could be removed upon treatment of the protein with charcoal. Thus the following procedure was adopted to modify the protein with the mercurial. A methanol solution of the azomercurial was measured by microburet into a flask, and the methanol was evaporated over a stream of nitrogen. A stoichiometric amount of sulfhydryl-free human serum albumin in 0.1 M glycine, pH 9.6, was then added to the flask, and the contents were stirred for 15 h in the dark. A control flask containing the same amount of protein but without the mercurial was treated the same way. After modification, aliquots were taken and adjusted to the desired pH with 0.3 M HCl from a microburet. All samples were then diluted to the same concentration with 0.1 M glycine buffer of the appropriate pH. Sulfhydryl analysis indicated the complete loss of free sulfhydryl groups from the samples treated with the mercurial. Analytical ultracentrifugation confirmed the absence of dimers in the mecurial-modified protein.

Spectral Measurements. Fluorescence measurements were made with a Spex Fluorolog spectrophotofluorometer equipped with a 450-W Xe lamp, a thermoelectrically cooled red-sensitive photomultiplier tube, and a SCAMP accessory. All measurements were made at 25 °C by using photon counting in the ratio mode with a thermostated cell compartment with 10-nm excitation and emission slits. Spectra were obtained by using 3 × 3 mm cells with absorbances less than 0.15 in a 1-cm cell to avoid inner-filter effects. The SCAMP microprocessor was used to determine the area under the emission peaks, the emission maxima at selected wavelengths for the calculation of the spectral overlap integrals, and the efficiency of energy transfer. An excitation wavelength of 295 nm was used throughout to minimize the contribution of the tyrosine residues to the emission.

Quantum yields of HSA at various pHs were obtained by comparing fluorescence intensities of the samples with those of standard solutions (having known quantum yields) under identical conditions of pH, temperature, etc. The quantum yields were calculated as follows:

$$\frac{\phi_x}{\phi_{st}} = \left(\frac{F_x}{F_{st}}\right)\left(\frac{A_{st}}{A_x}\right) \tag{1}$$

where ϕ_x, F_x, and A_x represent the unknown quantum yield, fluorescence intensity, and absorbance (at the same wavelength), respectively, and ϕ_{st}, F_{st}, and A_{st} are the corresponding parameters for the standard. The standards used in this case were solutions of tryptophan at various pHs. The quantum yields for these standards were determined, in turn, by comparison with a standard tryptophan solution at pH 6, for which a quantum yield of 0.20 has been reported (Burstein et al., 1973). The absorption and fluorescence spectra of all standards and protein solutions are very similar.

Theory. Förster derived equations for the through-space transfer of electronic excitation energy from a fluorescent chromophore to an absorbing chromophore by means of a dipole–dipole mechanism (Förster, 1948). Verification of this theory for the measurement of molecular distances has been found for both organic and inorganic chromophores [see Stryer (1978) for review].

The efficiency of such energy transfer from the fluorescent donor to an acceptor is given by

$$E = \frac{R_0^6}{R^6 + R_0^6} \tag{2}$$

where R is the distance between the donor and acceptor chromophores and R_0 is the distance at which energy transfer between the two chromophores is 50% efficient. R_0 (in Å) may be calculated by the following relationship (Förster, 1966):

$$R_0^6 = \frac{9000 \ln 10 \kappa^2 \phi J}{128 \pi^5 n^4 N} \tag{3}$$

where κ^2 is the orientation factor for the transition dipole moments of the donor and acceptor, ϕ is the quantum yield of the donor in the absence of acceptor, n is the refractive index of the medium, and N is Avogadro's number. The spectral overlap integral, J, representing the overlap of the acceptor absorbance and donor fluorescence normalized to unity is

$$J = \frac{\int_0^\infty \epsilon_A F_D \lambda^4 \, d\lambda}{\int_0^\infty F_D \, d\lambda} \tag{4}$$

In eq 4, ϵ_A is the molar extinction coefficient of the acceptor, and F_D is the relative fluorescence intensity of the donor at each wavelength λ. Calculations showed that essentially identical overlap integrals were obtained whether J was summed at 1-, 5-, or 8-nm intervals. We routinely calculated J by summing over 5-nm intervals between 310 and 450 nm.

Results

Figures 1 and 2 show the fluorescence of human serum albumin at pHs 1.9, 3.6, and 5.0 and pHs 7.0, 8.0, and 9.0, respectively. The upper curve in each part of the two figures represents the fluorescence spectrum of the native protein, whereas the lower curve represents the fluorescence spectrum of human serum albumin which has been modified by the reaction of the sulfhydryl group of Cys-34 with [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate. In each case introduction of the azomercurial dye has resulted in a quenching of the protein fluorescence. There are only two mechanisms that could account for this quenching. One is due to energy transfer between tryptophan and the azomercurial,

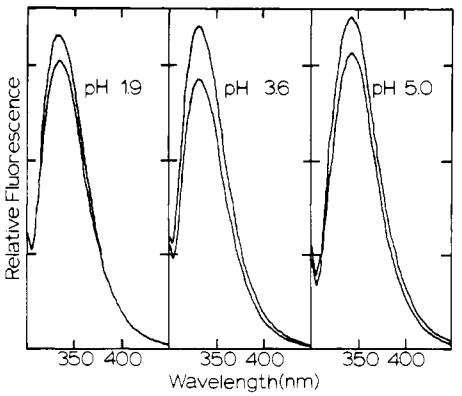

FIGURE 1: Quenching of human serum albumin emission by covalently bound [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate at pH 1.9, 3.6, and 5.0. In each case the upper spectrum is the fluorescence spectrum of unmodified HSA while the lower spectrum is the emission spectrum of the modified protein. Protein concentration was 10 μM and contained 56% free (upper) or modified (lower) Cys-34 sulfhydryl groups. Excitation was at 295 nm. Buffer was 0.1 M glycine.

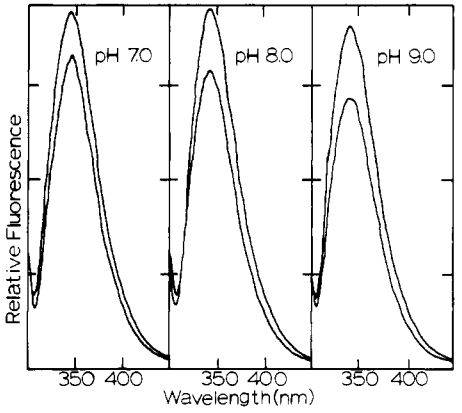

FIGURE 2: Quenching of human serum albumin emission by covalently bound [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate at pH 7.0, 8.0, and 9.0. Conditions as in Figure 1.

and the other is due to a conformational change in the protein upon modification of Cys-34 that results in an environmental change in Trp-214. Modification of the sulfhydryl by a group that does not absorb in the spectral region where tryptophan fluoresces does not affect the fluorescence spectrum of the protein (Foster, 1977). Thus the quenching of the fluorescence observed at each pH upon reaction with the mercurial dye is due to resonance energy transfer. The fluorescence spectra shown in Figures 1 and 2 together with the absorption spectra of the same samples (Figures 3 and 4) were used to calculate the overlap integrals by using eq 4.

The spectra presented in Figures 1–4 were obtained with a human serum albumin preparation that was enriched to 56.0% free sulfhydryl groups, and consequently the protein samples had only 56.0% of the protein bound with mercurial. In order to calculate proper overlap integrals, it was necessary to calculate what the absorption spectra of the modified protein would be at the various pHs if 100% of the sulfhydryl groups were modified. Similar corrections were necessary to calculate the efficiencies of energy transfer for the completely modified protein. The quantum yields of the samples were calculated as described under Materials and Methods. Using the refractive index of water (1.33) and assuming an orientation factor of $^2/_3$ (see Discussion) we calculated the critical distances (R_0) at each pH. From these values, the distance between the mercurial chromophore at Cys-34 and indole ring of Trp-214 was calculated at each pH. The data from several

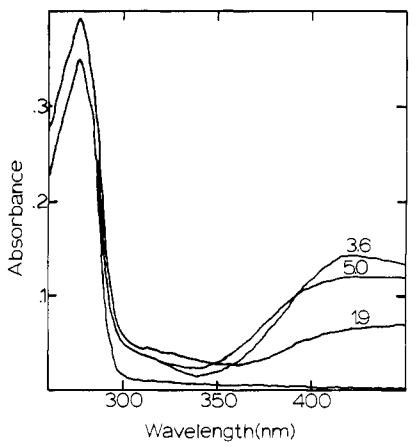

FIGURE 3: Absorption spectra of [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate modified human serum albumin at pH 1.9, 3.6, and 5.0. The absorption spectrum of unmodified HSA is also shown for comparison. The concentration of HSA was 10 μM and contained 56% of modified Cys-34 in 0.1 M glycine buffer. Absorption spectra were taken in 1.0-cm cells.

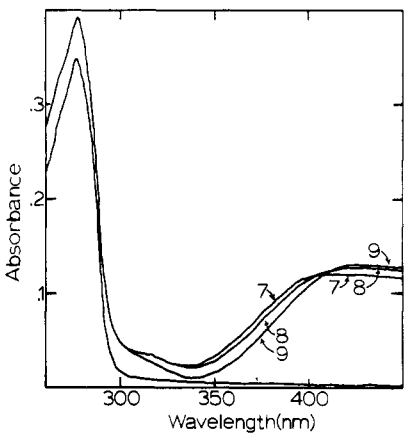

FIGURE 4: Absorption spectra of [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate modified human serum albumin at pH 7.0, 8.0, and 9.0. Conditions as in Figure 1.

repeat experiments are tabulated in Table I.

Discussion

The distance values listed in Table I are subject to several experimental errors including errors in the measurement of the overlap integral, the quantum yield, the extent of quenching, and the percent of sulfhydryl content. All of these errors would be expected to cause a total error which would cause the calculated value of R to oscillate about the true value from experiment to experiment. However, a potential systematic error lies in the value used for κ^2, the orientation factor.

One of the uncertainties of most energy transfer distance measurements comes from the value assumed for the orientation factor, κ^2. Most investigators assume a random orientation of the donor and acceptor transition dipoles resulting in a value of $^2/_3$ for κ^2. However, since κ^2 can range between 0 and 4, depending upon the orientation, there can be a considerable error in using κ^2 as $^2/_3$ in the absence of outside information, although no serious discrepancy from the value of $^2/_3$ has been reported for measurements in solution. Lakowicz & Weber (1980) and Munro et al. (1979) have determined that near neutral pH the single tryptophan of human serum albumin undergoes considerable rotation during the unquenched excited-state lifetime of the protein over the temperature range from 25 to 43 °C. Fluorescence polarization experiments on the tryptophan emission of HSA at

Table I: Effect of pH on the Distance between Trp-214 and Cys-34 in Human Serum Albumin

expt[a]	pH	efficiency of energy transfer, E	quantum yield, Q	overlap integral, $J \times 10^{-15}$ (cm^3 M^{-1})	critical distance, R_0 (Å)	distance between Trp-214 and Cys-34, R (Å)	average distance[b]
1	1.9	0.15	0.16	10	26	35	37 ± 1
2		0.12	0.14	10	26	36	
3		0.09	0.14	12	26	39	
3		0.11	0.15	12	26	37	
1	3.6	0.31	0.18	11	27	31	30 ± 1
2		0.36	0.15	13	27	30	
3		0.41	0.15	12	27	28	
3		0.31	0.14	12	26	30	
5		0.30	0.15	12	27	31	
1	5.0	0.23	0.21	16	29	36	36 ± 1
2		0.25	0.18	16	29	34	
3		0.20	0.17	16	28	36	
3		0.19	0.17	17	29	36	
5		0.20	0.19	16	29	36	
1	6.0	0.24	0.20	17	29	36	35 ± 1
2		0.31	0.19	18	29	33	
1	6.5	0.28	0.19	17	29	35	34 ± 1
2		0.29	0.18	17	29	34	
1	7.0	0.27	0.23	17	30	35	35 ± 1
2		0.34	0.19	18	29	33	
3		0.23	0.17	17	29	35	
3		0.23	0.18	17	29	36	
5		0.26	0.18	17	29	34	
1	8.0	0.33	0.23	16	30	34	33 ± 1
2		0.37	0.19	16	29	32	
4		0.27	0.16	15	28	33	
5		0.34	0.18	16	29	32	
1	8.5	0.43	0.22	16	29	31	31
2		0.40	0.19	16	29	31	
1	9.0	0.40	0.20	17	29	31	31 ± 1
3		0.35	0.18	16	29	32	
3		0.29	0.16	15	28	32	
5		0.39	0.17	16	28	30	

[a] Experiments 1, 2, 3, 4, and 5 were done on albumin containing 56%, 63%, 51%, 70%, and 66% free sulfhydryl, respectively. [b] Arithmetic mean with standard deviation.

various pH values (data not shown) indicated that the rotational freedom enjoyed by the tryptophan at neutral pH was also seen at pHs between 1.9 and 9.0. The effect of this rotation is to substantially reduce the uncertainty in the value of κ^2 assumed. We have no direct information as to the rotational mobility of the mercurial. However, indirect information about the azomercurial rotational freedom may come from the spin-label work reported by Cornell et al. (1981). Cornell et al. concluded that the sulfhydryl group of human albumin is located in a crevice nearly 10 Å deep. Earlier, Cornell & Kaplan (1978a) had shown that the N → F transition was not discernible with short spin-labels (~4–6 Å long). However, this transition was discernible when longer spin-labels were used (~9.3 Å). An increase in the rotational freedom of the longer spin-labels was observed in the F conformation as compared to the N conformation. The rotational correlation time of the longer spin-labels attached to the sulfhydryl of HSA was on the order of 2 ns at neutral pH while that of the shorter spin-labels was ~12 ns. This shorter correlation time is more than an order of magnitude faster than the rotational relaxation time of the protein, indicating relatively free movement of the label. The mercurial we have used to modify Cys-34 has an overall length of ~12 Å from the mercaptide bond, and thus this chromophore may enjoy as much rotational freedom as the spin-labels. Thus in our calculations at all pHs, a value of κ^2 of $^2/_3$ was used.

It is important to note that both the absorption spectra of the acceptor at Cys-34 and the fluorescence spectra of the donor (Trp-214) change with pH. Thus an independent overlap integral must be calculated for each pH in order to use the Förster method to calculate the distance at each pH.

The distances shown in Table I were made on preparations that contained from 51 to 70% of Cys-34 modified with the azomercurial. At first glance it might appear that distance calculations made on preparations not containing fully modified Cys-34 would increase the ambiguity of the distance calculations. As was mentioned under Materials and Methods, however, corrections were made for incomplete modification of Cys-34. The fact that we measure essentially the same distance for preparations containing from 50 to 70% modified Cys-34 is good evidence that this is a valid practice.

The native or N form of serum albumin is stable over the pH range from 5 to 7. The distance calculated between the modified sulfhydryl (Cys-34) and Trp-214 varies from 36 to 34 Å over this pH range (Table I). The fact that the pH 5 distance (36 Å) is slightly greater than the pH 6.5 distance (34 Å) suggests that this variation (2 Å) is representative of our experimental error in these measurements. This is also consistent with the errors shown for repeat experiments in Table I.

At pH 3.6 where the protein is in the F form (as verified by circular dichroism measurements; data not shown) we

measure a distance of 30 Å, a decrease of 6 Å from the native form. It is surprising that the distance between the two chromophores actually decreases in going from the N to the F forms of the protein, since many measurements have indicated an overall expansion of the protein through this pH transition. On the other hand, Bloomfield (1966) concluded that there was little change in the overall dimensions of the albumin molecule as the pH is lowered to 3.6. It is clear from our results that rather than an increase in the distance between the mercurial chromophore in domain I and the tryptophan chromophore in domain II of the protein during the N → F transition, there is instead a decrease in this distance. Any overall expansion of the protein due to the N → F transition must therefore occur in other portions of the protein, presumably between domains II and III. In fact Foster (1977), Hilak et al. (1974), and Reed et al. (1975) have concluded that the N → F transition occurs primarily in the carboxyl-terminal half of the molecule. This conclusion was based mainly on studies of enzymatic cleavages of serum albumin in the N and F states and upon subsequent studies of these peptide fragments.

Klotz & Ayers (1957) noticed small changes in the absorption of the bound azomercurial as albumin passed through the N→F conformation. And as shown in Figure 3 we also observed spectral changes between pH 5.0 and 3.6. These changes are not associated with the protonation of the tertiary nitrogen, since Klotz & Ayers (1957) showed that the pKa of this group in BSA is near 1.9. Thus clearly there are changes in the environment of the sulfhydryl when the protein undergoes the N→F transformation. Cornell & Kaplan (1978a,b) have interpreted these changes as being due to an opening of the crevice which contains Cys-34.

On the other hand tryptophan-214 also undergoes changes in environment during the N → F transition. Chen (1966) first noticed a decrease in intensity and a blue shift of the emission maximum of Trp-214. Several other groups have confirmed these results [see Foster (1977)], concluding that the changes in fluorescence of the tryptophan result from a shift of this residue from an aqueous environment in the N form to a more hydrophobic one in the F form. Sogami & Agura (1972) using solvent perturbation spectroscopy observed that one of the two tryptophan residues (presumably that which corresponds to Trp-214 in HSA) became buried and inaccessible to ethylene glycol during the N → F transition. One explanation for the 5–6 Å decrease in distance we observe between the mercurial and Trp-214 when the protein undergoes the N → F transformation is that Trp-214 is moving closer to domain I at the same time, presumably, that it is moving farther away from the residues in domain III. However, because we see changes in spectral properties of the mercurial during the N → F transition, we cannot rule out the possibility that the mercurial also moves closer to domain II during this transition.

As the pH is lowered to 1.9 from pH 3.6, the distance between the sulfhydryl and the indole ring increases by 7–37 Å. This is in accord with a variety of earlier experiments (see introduction) that indicate that the protein undergoes considerable unfolding as the pH is lowered to produce the E form.

As the pH is increased from 7.0 to 9.0, there seems to be a small (~3 Å), but nevertheless reproducible, contraction in the distance between the mercurial chromophore attached at Cys-34 and Trp-214. This is in accord with hydrodynamic measurements, since the N → B transition appears to be accompanied by a more subtle conformational change than those which characterize the acid transitions.

Cornell & Kaplan (1978b) suggested that the N → F and N → B transitions were very similar in that the sulfhydryl group moves to a less restricted environment during both transitions. Zurawski & Foster (1974) also noted similarities between the two transitions. Our data suggest that there is a contraction between Trp-214 and Cys-34 during both the N → F and N → B transitions. However, this change is more dramatic in the case of the N → F than the N → B transition.

From models that Brown (1977) has proposed for human serum albumin, the distance of 35 Å between Cys-34 and Trp-214 that we measure seems to fit with either the parallel or antiparallel subdomain model for the three-dimensional structure of the protein.

Energy transfer measurements have been used in a large number of systems to measure distances between two chromophores. Most of these measurements have been done under a single set of experimental conditions and have not been used to measure distance changes under varying conditions. However, as shown here and previously (Khan et al., 1978) it is possible to use this technique to measure changes in distances under various conditions such as different temperatures or pHs. The absolute distances measured here may have certain errors associated with them as discussed earlier, but the *changes* in the distance observed at different pHs are more accurate since some of the same errors involved in all calculations will be self-compensating. We are currently extending these studies to examine how the small molecule ligands which are known to bind HSA such as fatty acids, steroids, and other drugs affect the distance between the chromophores in serum albumin. Preliminary data indicate there are rather dramatic changes in these distances when certain metabolites are bound to human serum albumin.

Registry No. Cys, 52-90-4; Trp, 73-22-3; [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate, 19447-62-2.

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Resonance Energy Transfer between Cysteine-34, Tryptophan-214, and Tyrosine-411 of Human Serum Albumin†

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ABSTRACT: Reaction of *p*-nitrophenyl anthranilate with human serum albumin at pH 8.0 results in esterification of a single anthraniloyl moiety with the hydroxyl group of tyrosine-411. The absorption spectrum of the anthraniloyl group overlaps the fluorescence emission of the single tryptophan residue at position 214. This study complements that of the preceding paper [Suzukida, M., Le, H. P., Shahid, F., McPherson, R. A., Birnbaum, E. R., & Darnall, D. W. (1983) *Biochemistry* (preceding paper in this issue)] where an azomercurial group was introduced at cysteine-34. Anthraniloyl fluorescence was also quenched by the azomercurial absorption at Cys-34. Thus measurement of resonance energy transfer between these three sites allowed distances to be measured between Cys-34 in domain I, Trp-214 in domain II, and Tyr-411 in domain III of human serum albumin. At pH 7.4 in 0.1 M phosphate the Trp-214 → Tyr-411, Tyr-411 → Cys-34, and Trp-214 → Cys-34 distances were found to be 25.2 ± 0.6, 25.2 ± 2.1, and 31.8 ± 0.8 Å, respectively.

Human serum albumin plays a special role in transporting metabolites and drugs throughout the vascular system. Literally hundreds of studies have been reported on the binding of small molecules to serum albumin. In many cases, binding of a small molecule at one site on serum albumin drastically affects the binding of a molecule at a distant site [see Vallner (1977) and Kragh-Hansen (1981) for reviews]. How ligands affect the binding of other ligands is for the most part still unclear.

Human serum albumin contains a single free sulfhydryl group at Cys-34, a single indole ring at Trp-214, and a particularly reactive phenolic side chain at Tyr-411, located in domains I, II, and III, respectively. We have chemically modified Cys-34 (Suzukida et al., 1983) and Tyr-411 of HSA[1] with chromophores suitable for resonance energy transfer studies. These modifications have allowed us to measure the distances between Cys-24, Trp-214, and Tyr-411 in domains I, II, and III using the resonance energy transfer theory of Förster (1948). Subsequently we will report how these distances are affected by the binding of various drugs and metabolites to HSA.

The preceding paper (Suzukida et al., 1983) has described the modification of Cys-34 with the azomercurial [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate (DPM). This modification has allowed distances to be measured between Trp-214 and Cys-34. Herein we report the modification of Tyr-411 with *p*-nitrophenyl anthranilate (NPA) which introduces the anthraniloyl moiety at the phenolic hydroxyl group. This fluorescent chromophore overlaps the fluorescence of Trp-214, allowing the calculation of the distance between Trp-214 and Tyr-411. When excited directly, the anthraniloyl-Tyr-411 can also transfer energy to the mercurial-modified Cys-34, permitting the calculation of the distance between these two chromophores.

Materials and Methods

Human serum albumin was obtained from Miles Laboratories (Pentex) and was defatted and enriched in SH content as described in the preceding paper (Suzukida et al., 1983). *p*-Nitrophenyl anthranilate and [4-[[*p*-(dimethylamino)-phenyl]azo]phenyl]mercuric acetate were obtained from Sigma Chemical Co.

The azomercurial-modified protein was prepared as previously described (Suzukida et al., 1983) with the exception that the modification was carried out at pH 8.0 in 0.1 M phosphate buffer to avoid exposure of the protein to the high pH of 0.1 M glycine at pH 9.6. After modification, the protein was dialyzed against 0.1 M phosphate buffer at pH 7.4 in which all subsequent spectral measurements were taken.

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[1] Abbreviations: DFP, diisopropyl fluorophosphate; HSA, human serum albumin; NPA, *p*-nitrophenyl anthranilate; NPhOAc, *p*-nitrophenyl acetate; DIP-HSA, diisopropylphosphoryl human serum albumin; DPM, [4-[[*p*-(dimethylamino)phenyl]azo]phenyl]mercuric acetate; ANS, 8-anilino-1-naphthalenesulfonate.

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