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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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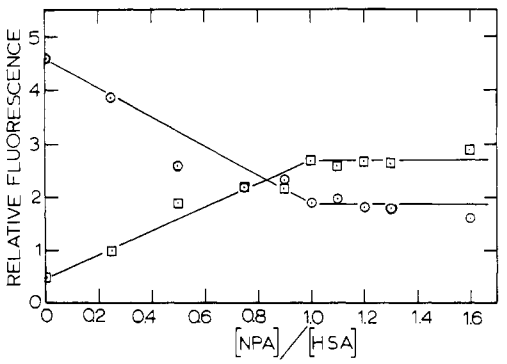


FIGURE 1: Reaction of *p*-nitrophenyl anthranilate with human serum albumin. Various concentrations of NPA were allowed to react with HSA for 7.0 h in 0.1 M phosphate at pH 8.0 at 25 °C. After reaction the samples were dialyzed against three 100 volumes of 0.1 M phosphate at pH 7.4. (Circles) Decrease in tryptophan fluorescence, λ_{ex} 295 nm, λ_{em} 348 nm. (Squares) Increase in anthraniloyl fluorescence, λ_{ex} 295 nm, λ_{em} 410 nm.

Preparation of anthraniloyl-Tyr-411-HSA was accomplished at pH 8.0 by the addition of a 1.25 molar excess of *p*-nitrophenyl anthranilate (NPA) to HSA in 0.1 M phosphate buffer. NPA was dissolved in acetonitrile so that less than 1% acetonitrile was added to the protein solution. The reaction was allowed to proceed for 7 h after which the preparation was dialyzed against 0.1 M phosphate at pH 7.4 to remove the *p*-nitrophenol produced. Spectral analysis of the dialysate and of the modified protein preparation indicated the complete removal of *p*-nitrophenol by this procedure.

Double-labeled HSA containing both the anthraniloyl and the azomercurial chromophores was prepared by first modifying Cys-34 with DPM followed by modification of Tyr-411 with NPA as described above. Experiments showed that the order of modification of Tyr-411 or Cys-34 was unimportant.

Spectral Measurements. All fluorescence emission, excitation, and polarization spectra were recorded on the Spex Fluorolog spectrofluorometer with the methodology described in the preceding paper (Suzukida et al., 1983). An excitation wavelength of 295 nm was used for all distance measurements between tryptophan-214 and either the azomercurial at Cys-34 or the anthranilate group at Tyr-411, since excitation at this wavelength minimizes any contribution to the emission spectrum by tyrosine residues. For measurement of the Tyr-411–Cys-34 distance, the anthranilate group was excited at 360 nm.

Circular dichroic spectra were obtained with a Cary Model 60 spectropolarimeter with a Model 6001 circular dichroism attachment .

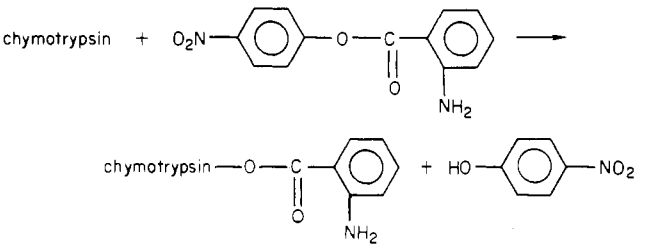
Quantum yields of HSA at various pHs were obtained by the method described in the preceding paper (Suzukida et al., 1983). Solutions of tryptophan [ϕ = 0.20 (Burstein et al., 1973)] and quinine sulfate [ϕ = 0.55 (Melhuish, 1955)] were used as standards for the protein and anthraniloyl fluorescence, respectively.

The Förster theory of through-space energy transfer was used to determine the distances between the chromophores. The details of the use of the theory can be found in the preceding paper (Suzukida et al., 1983).

Results and Discussion

Chemical Modification of Tyr-411. Although human serum albumin contains 19 tyrosine residues, evidence indicates that there is only one tyrosine which is highly reactive. Sanger (1963) found that HSA reacts with diisopropyl fluorophosphate (DFP), a serine protease inhibitor, but unlike serine proteases, the site of alkylphosphorylation in HSA was a tyrosyl hydroxyl group. When the amino acid sequence and composition of HSA was established (Behrens et al., 1975), this site was recognized to be Tyr-411. Means & Wu (1979) confirmed that alkylphosphorylation takes place at Tyr-411 and, furthermore, found that it is also the site of acetylation by *p*-nitrophenyl acetate (NPhOAc). Prior reaction of HSA with DFP completely inhibited its subsequent reaction with *p*-nitrophenyl acetate. Means & Wu (1979) established that tyrosine-411 has an unusually low pK_a of 8.3, accounting for its preferential reactivity. Fehske et al. (1980) also showed that Tyr-411 is nearly 20-fold more reactive with tetranitromethane than any other tyrosine side chain in HSA.

Some time ago Haugland & Stryer (1967) demonstrated that *p*-nitrophenyl anthranilate could be used at pH 6.8 to introduce the fluorescent anthraniloyl moiety into chymotrypsin (or trypsin) at the active serine-195 via the following reaction:



Haugland & Stryer (1967) noted that *p*-nitrophenyl anthranilate did not react with bovine serum albumin under their conditions (pH 6.8). However, since this reaction is very similar to the *p*-nitrophenyl acetate reaction with serum albumin, we reasoned that *p*-nitrophenyl anthranilate may react with the protein at higher pH.

The anthraniloyl group in NPA does not fluoresce in aqueous solution since its fluorescence is completely quenched when coupled to *p*-nitrophenol. However, upon reaction of NPA with proteins, the *p*-nitrophenolate ion is released, and the anthraniloyl fluorescence is greatly enhanced. The reaction can be followed by the increase in absorbance at 410 nm due to *p*-nitrophenolate release, by the increase in absorbance at 350 nm, by the increase in fluorescence at 412 nm, and by the decrease in protein fluorescence at 342 nm due to incorporation of the anthraniloyl moiety into the protein. Figure 1 shows the concentration dependence of the reaction of NPA with HSA at pH 8.0. In accordance with the experiments of Haugland & Stryer (1967), we find very little incorporation of the anthranilate at pHs below 7. At higher pHs, however, there is considerable reaction of NPA with albumin. Titration of HSA with NPA at pH 8.0 showed that only 1 mol of NPA reacted with HSA as judged by the increase in the anthranilate fluorescence or by the decrease in the protein emission. At pH 8.5 and 9.0 additional anthraniloyl groups are incorporated, presumably due to reaction at other tyrosine residues.

Figures 2 and 3 show the time course of the reaction of NPA with HSA. These data show a rapid quenching of the protein fluorescence (Figure 2A) and a rapid increase in anthraniloyl absorption (Figure 3) when HSA and NPA are mixed, followed by a slower reaction monitored by the release of *p*-nitrophenolate (Figure 3) that takes nearly 7 h for completion. Figure 4 shows the same time course as Figure 2B with an excitation wavelength of 295 nm which results in the fluorescence of both the tryptophan and anthraniloyl groups. Note that in the absence of NPA, the protein fluorescence band does not go through the isoemissive point near 380 nm (Figure 4). These data are all consistent with a rapid binding of NPA to the protein within a few seconds followed by a much slower reaction of NPA with the hydroxyl group of Tyr-411

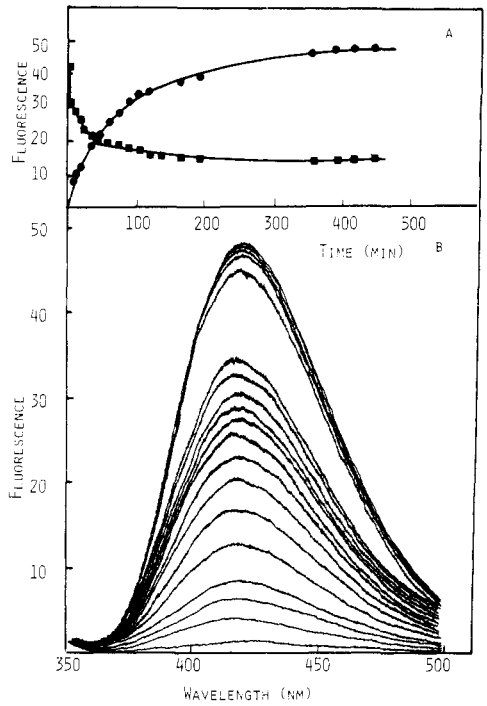


FIGURE 2: Time dependence of the reaction of *p*-nitrophenyl anthranilate with human serum albumin. One equivalent of *p*-nitrophenyl anthranilate was mixed with 2.0×10^{-5} M HSA in 0.1 M phosphate, pH 7.4. (A) Plot of the anthranilate emission at 412 nm (circles) and the HSA emission at 342 nm (squares) vs. time; λ_{ex} 295 nm (B) Anthranilate emission spectrum at the times shown in (A); λ_{ex} 360 nm.

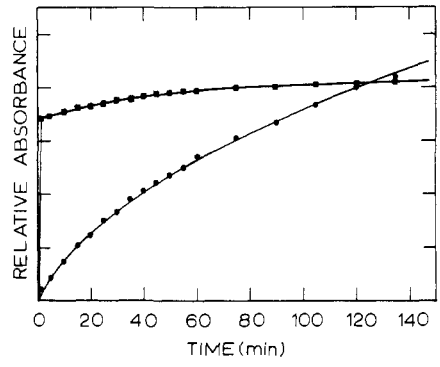


FIGURE 3: Time dependence of the reaction of *p*-nitrophenyl anthranilate with human serum albumin. Conditions as in Figure 2. Squares refer to anthranilate absorption at 342 nm, and circles refer to *p*-nitrophenol absorption at 405 nm. Although these wavelengths are not the absorption maxima, they allow separation of anthranilate absorption from nitrophenol absorption. These data represent the first 140 min of the reaction shown in Figure 2.

that is accompanied by the release of the *p*-nitrophenolate anion.

In order to determine whether or not the anthraniloyl group was covalently attached to HSA, the complex was extensively dialyzed in separate experiments against water, phosphate buffer (pH 7.4 and 8.0), acetate buffer (pH 4.0), 0.01 M HCl, and 8.0 M urea. In all cases the absorption and emission spectra after dialysis were essentially identical or only slightly altered due to the denaturing conditions employed. These experiments are consistent with covalent attachment of the anthraniloyl moiety to the protein. As judged by spectral measurements, the HSA–anthraniloyl complex was stable for several weeks. In addition, sedimentation velocity experiments showed that at a protein concentration of 2.0×10^{-5} M at pH 8.0 in 0.1 M phosphate buffer the HSA–anthraniloyl complex

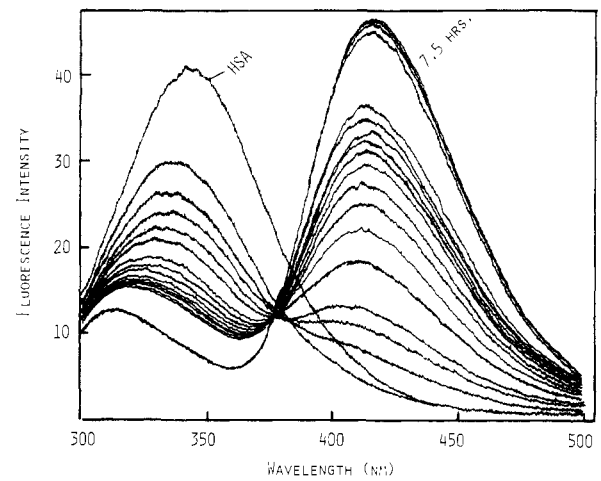


FIGURE 4: Time course of the reaction of *p*-nitrophenyl anthranilate with human serum albumin. Conditions are those in Figure 2. The emission spectrum was obtained by excitation at 295 nm rather than 360 nm as in Figure 2B. The curves correspond to the times shown in Figure 2A.

was present exclusively as a monomer (data not shown). Thus anthraniloyl-HSA, like our native HSA, is entirely monomeric, and the incorporation of the anthranilate group does not cause any dimerization of the protein under the experimental conditions employed.

The experiments discussed above provide evidence that a single anthraniloyl moiety is incorporated into HSA by reaction with NPA at pH 8.0. It is important now to address the question of whether or not Tyr-411 is the site of modification. It was mentioned earlier that Sanger (1963) isolated a pentapeptide containing (diisopropylphosphoryl)tyrosine from a tryptic hydrolysate of HSA pretreated with DFP. This tyrosine residue was later identified as Tyr-411 in the amino acid sequence proposed by Behrens et al. (1975). Means & Wu (1979) confirmed these results and further showed that Tyr-411 is the site of acetylation with NPhOAc. In addition, prior acetylation of Tyr-411 inhibited the incorporation of DFP into the protein.

On the basis of the above data, it was decided to see if both NPhOAc and DFP are competitive inhibitors of the NPA reaction with human serum albumin. In one experiment serum albumin was incubated with a 10-fold molar excess of NPhOAc to prepare acetylated HSA and block the Tyr-411 (Means & Bender, 1975). The Tyr-411-acetylated HSA was then allowed to react with 1 equiv of NPA as described earlier. The fact that no anthraniloyl fluorescence at 412 nm was observed demonstrated that acetylated HSA does not react with NPA at pH 8.0. This suggests that the reaction of *p*-nitrophenyl anthranilate with human serum albumin involves Tyr-411. A small difference between native HSA and acetyl-HSA fluorescence was observed when the samples were excited at 280 nm rather than 295 nm. This difference must be due to the loss of energy transfer from Tyr-411 to Trp-214 upon reaction with NPhOAc, since the protein fluorescence at 342 nm is due to tryptophan emission plus energy transferred to the tryptophan from the tyrosine residues. The native HSA and acetyl-HSA fluorescence spectra are the same when excited at 295 nm where only tryptophan fluorescence is observed (data not shown).

When DFP was used as a competitive inhibitor of the reaction of NPA with human serum albumin, similar results were obtained as shown in Table I. Preincubation of HSA with ^{14}C-labeled DFP resulted in incorporation of 0.93–0.95 mol of DFP/mol of protein. If the protein was first modified

Table I: Competitive Inhibition of the Reaction of *p*-Nitrophenyl Anthranilate with Human Serum Albumin by Diisopropyl Fluorophosphate

sample	counts per minute observed[a]	counts per minute theoretical[b]	DFP/HSA[c]
DIP-HSA[d]	6979	7507	0.93
	7128	7490	0.95
	12428	13225	0.94
anthraniloyl-HSA[e]	512	7507	0.073
	627	13342	0.045
	705	14043	0.050

[a] Counts observed after reaction with DFP followed by dialysis. [b] Theoretical counts that would be observed if 1 mol of DFP reacted covalently per mol of albumin. [c] Ratio of observed to theoretical counts (mole of DFP per mole of albumin). Protein concentration = 2.0×10^{-5} M. [d] Prepared by incubation of HSA with 7-fold molar excess of DFP in 0.1 M phosphate buffer, pH 8.0, followed by dialysis. The three experiments correspond to three different isotope distributions. [e] Prepared by incubation of HSA with a 1.25-fold molar excess of NPA in 0.1 M phosphate, pH 8.0, followed by dialysis and then treated with DFP as in footnote *d*.

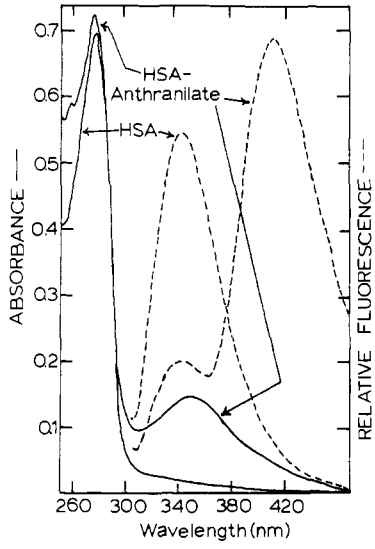


FIGURE 5: Absorption and fluorescence spectra of human serum albumin (HSA) and anthraniloyl-HSA. Conditions as in Figure 2. Excitation wavelength was 295 nm.

with NPA and then allowed to react with [^{14}C]DFP, the incorporation of DFP was virtually eliminated. Since DFP has been shown to specifically modify Tyr-411, these experiments clearly show that Tyr-411 is also the same site of reaction for *p*-nitrophenyl anthranilate to form anthraniloyl-HSA.

Distance Measurements between Trp-214 and Tyr-411. The absorption spectrum of anthraniloyl-HSA is shown is Figure 5. The anthraniloyl group absorbs in the 310–450-nm region with a maximum peak at 350 nm. The molar extinction coefficient for the attached chromophore at 350 nm is 6.3×10^3 for HSA compared to a value of 4.0×10^3 found by Haugland & Stryer (1967) for the anthraniloyl group in chymotrypsin.

The absorption spectrum of the anthraniloyl chromophore extensively overlaps the fluorescence emission of the lone tryptophan of HSA (Figure 5), creating the possibility of fluorescence energy transfer between these two chromophores which should allow distance measurements to be made between Trp-214 and Tyr-411 by using the Förster energy transfer theory (Förster, 1948). And indeed, the emission at 342 nm of anthraniloyl-HSA when excited at 295 nm is quenched by ~63% compared to the emission of the native protein.[2] There is a concomitant increase in the emission at 412 nm for the anthraniloyl-HSA (Figure 5). The 342-nm peak is due to the fluorescence of HSA (λ_{ex} 295 nm), while the 412-nm emission arises from the anthraniloyl chromophore.

At this point it is important to establish to what extent Trp-214 emission is affected by chemical modification of Tyr-411 which may have nothing to do with energy transfer. For example, it is possible that modification of Tyr-411 produces a conformational change in the protein which results in a quenching of Trp-214 fluorescence. This would make calculations of distances between the two chromophores in error, since the amount of quenching of Trp-214 is assumed to be only due to energy transfer from Trp-214 to anthraniloyl-Tyr-411. Therefore, the emission characteristics of Trp-214 were examined when Tyr-411 was modified with *p*-nitrophenyl acetate and diisopropyl fluorophosphate. Since neither of these derivatives absorb in the spectral region where Trp-214 emits, any changes in Trp-214 emission upon their incorporation into the protein should be due to changes in the tryptophan environment. In both cases the emission of Trp-214 was unaffected by tyrosine modification when the protein was excited at 295 nm (data not shown). There were small changes in the emission spectra of the modified derivatives when the protein was excited at 280 nm as might be expected due to the fact that the tyrosine residues as well as the tryptophan residue contribute to the emission via tyrosine to tryptophan energy transfer when excited at this wavelength. This is in agreement with the difference spectrum observed by Means & Wu (1979) for DIP-HSA. Since the incorporation of the small acetyl group or the bulky diisopropylphosphoryl group at Tyr-411 results in no change in Trp-214 emission, it is unlikely that the quenching of Trp-214 emission by the anthraniloyl group results from a mechanism other than energy transfer.

Table II shows the data and results of distance calculations between Trp-214 and Tyr-411 for a number of experiments. Each entry in Table II represents a complete, independent experiment; i.e., for each row entry, a new preparation of HSA was defatted and modified with NPA, and the spectral data were measured. The efficiency of energy transfer between Trp-214 and Tyr-411 is about 63%, and the distance between the two residues is about 25 Å.

Distance Measurements between Trp-214 and Cys-34. The single sulfhydryl side chain of Cys-34 was modified as described earlier (Suzukida et al., 1983), with the exception that the modification with [4-[[*p*-(dimethylamino)phenyl]azo]-phenyl]mercuric acetate was carried out at pH 8.0 in 0.1 M phosphate buffer rather than at pH 9.6 in 0.1 M glycine buffer. The titration of HSA with the azomercurial dye (DPM), followed by the absorption spectrum of the bound dye, is linear, indicating stoichiometric binding of 1 mol of the azomercurial dye/mol of free sulfhydryl. Addition of excess mercurial dye results in small additional increases in absorbance. However, this is apparently due to noncovalent binding of the mercurial dye to the protein since the excess dye can be removed by treatment with charcoal. This was also confirmed by protein emission studies. These experiments are in agreement with White et al. (1973), who also observed additional noncovalent binding of the azomercurial at concentrations higher than necessary to modify the sulfhydryl group.

[2] The percent quenching of the protein fluorescence was obtained by resolving the protein fluorescence from the anthranilate fluorescence. The validity of the resolution was demonstrated by the fact that the percent quenching calculated was independent of the wavelength region used for the calculation.

Table II: Distance Measurements between the Three Chromophores in Modified Human Serum Albumin[a]

efficiency of energy transfer E	quantum yield, ϕ	overlap integral, $J \times 10^{-15}$ (cm^3 M^{-1})	critical distance, R_0 (Å)	distance, R (Å)
		(a) Trp-214 to Tyr-411 Distance		
0.687	0.246	10.0	27.4	24.0
0.546	0.194	10.0	26.4	25.6
0.529	0.194	7.3	26.0	25.5
0.633	0.243	9.5	27.1	24.8
0.598	0.243	9.2	27.0	25.2
0.656	0.243	11.4	28.4	25.6
0.655	0.243	11.5	28.4	25.7
0.665	0.243	11.6	28.4	25.5
0.657	0.243	12.0	28.6	25.8
0.655	0.243	11.6	28.4	25.5
			av:	25.6 ± 0.6
		(b) Trp-214 to Cys-34 Distance		
0.410	0.212	14.9	28.9	30.8
0.405	0.219	14.9	29.1	31.0
0.320	0.219	14.9	29.1	33.0
0.305	0.212	14.9	28.9	33.0
0.360	0.220	14.9	29.0	31.9
0.360	0.231	14.9	28.6	31.6
0.438	0.243	14.4	29.5	30.9
0.393	0.243	14.4	29.5	31.9
0.440	0.243	16.4	30.1	31.6
0.429	0.243	16.5	30.2	31.8
			av:	31.9 ± 0.8
		(c) Tyr-411 to Cys-34 Distance		
0.892	0.178	59.0	35.4	25.0
0.900	0.178	59.0	35.4	24.7
0.800	0.220	61.8	36.5	29.2
0.848	0.198	63.2	36.0	27.0
0.866	0.173	38.3	32.8	23.0
0.927	0.174	40.3	34.1	21.8
0.906	0.219	49.2	35.6	24.5
0.852	0.180	49.2	34.4	25.9
			av:	25.2 ± 2.1

[a] Cys-34 and Tyr-411 were modified as described in the text. All spectral measurements were obtained at pH 7.4 in 0.1 M phosphate on protein that was 2.0×10^{-5} M. Excitation was at 295 (tryptophan fluorescence) or 360 nm (anthranilate fluorescence). Overlap integrals were calculated between 310 and 450 nm (tryptophan) or 375 and 500 nm (anthranilate).

As Suzukida et al. (1983) demonstrated, the azomercurial absorption overlaps the Trp-214 emission and results in energy transfer between the two chromophores. Table II shows data for energy transfer experiments and distances between the two chromophores at pH 7.4. The mean distance between Trp-214 and the azomercurial at Cys-34 is 31.8 ± 0.8 Å at pH 7.4 in 0.1 M phosphate. This compares to values of 34.5 ± 1.1 Å at pH 7.0 and 33.2 ± 1.3 Å at pH 8.0 obtained by modifications in glycine buffer at higher ionic strength (Suzukida et al., 1983). The relatively small differences between these measurements may be due to the different buffers employed; however, they are close to the experimental error in the measurements.

Distance Measurements between Tyr-411 and Cys-34 (Double-Labeled HSA). The single sulfhydryl at Cys-34 was modified as described above. The absorption spectrum of the azomercurial-HSA overlaps the fluorescence spectrum of the anthraniloyl-HSA (Figure 6), suggesting the possibility of energy transfer between these two residues. And indeed Figure 6 also shows that the incorporation of the azomercurial into anthraniloyl-HSA (double-labeled HSA) results in quenching of the anthraniloyl fluorescence. The efficiency of energy

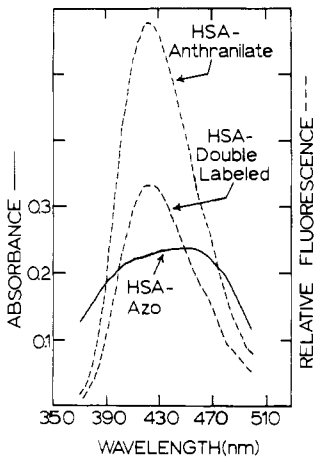


FIGURE 6: Absorption spectrum of azomercurial-HSA (—) and fluorescence spectra of anthraniloyl-HSA and double-labeled-HSA (---). Conditions are those in Figure 2. The sulfhydryl content was 0.62 mol/mol of HSA and λ_{ex} 360 nm.

transfer between the two chromophores was on the order of 80–90% after a correction was made for the fact that only 62% of the sulfhydryl groups were free for reaction with the mercurial.

Table II shows data for measurements of distances between anthraniloyl-Tyr- 411 and azomercurial-Cys-34. The mean distance between these two residues is 25.2 Å. This distance shows the greatest fluctuation from experiment to experiment of the three distances measured. Presumably the reason for this fluctuation lies with the sensitivity in the distance calculated on the efficiencies measured at very high efficiencies of energy transfer.

Orientation Factor. The major assumption made in the distance calculations between Cys-34, Trp-214, and Tyr-411 is that κ^2, the orientation factor in eq 3 (Suzukida et al., 1983), is $^2/_3$. This value of κ^2 corresponds to the case where emission of the donor and absorption of the acceptor, over their range of spectral overlap, are each characterized by a single transition moment which is rotating rapidly with respect to the excited-state lifetime of the donor. As Suzukida et al. (1983) argued, this appears to be the case for the Trp-214 as the donor chromophore since independent experiments have shown that Trp-214 has considerable rotational mobility during its excited-state lifetime at 25 °C (Munro et al., 1979; Lakowicz & Weber, 1980). Suzukida et al. (1983) also argued that the mercurial at Cys-214 should have considerable rotational mobility.

Haas et al. (1978) have pointed out that when a chromophore has two or more electronic transitions which participate in energy transfer, the errors in using $\kappa^2 = {}^2/_3$ are substantially reduced to ±10% or less. The absorption spectrum of anthraniloyl-HSA (Figure 5) shows a peak centered at 350 nm which tails toward the red end of the spectrum. This non-Gaussian shape suggests that more than one electronic transition is present. The presence of at least two electronic transitions in the 310–450-nm region at anthraniloyl-HSA was confirmed by circular dichroic measurements. The circular dichroism spectrum of the labeled protein (Figure 7) shows that anthraniloyl-HSA is optically active in this wavelength region. The appearance of a negative Cotton effect centered at 340 nm and a positive Cotton effect centered near 390 nm confirms the presence of at least two electronic transitions. Since the overlap integral between Trp-214 and the anthranilate group at Tyr-411 is calculated between 310 and 450 nm, both transitions of anthranilate are involved in the calculations.

The effect of the presence of two or more transition dipoles

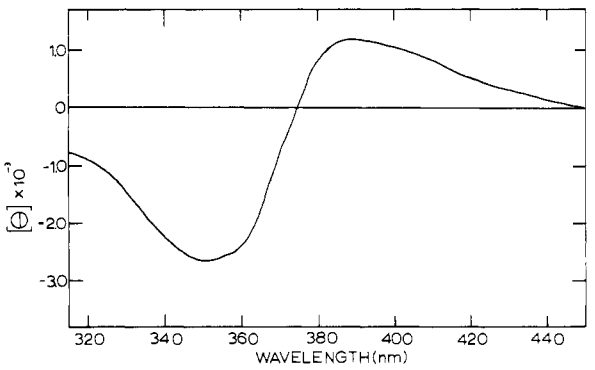


FIGURE 7: Circular dichroic spectrum of anthraniloyl-HSA. The spectrum was obtained in a 5.0-cm cell. Conditions are those of Figure 2. Molar ellipticity is expressed per mole of anthranilate.

in an observed spectrum is to cause depolarization of the fluorescence. Haugland & Stryer (1967) demonstrated this phenomenon for the anthraniloyl fluorescence in chymotrypsin. The emission anisotropy of the fluorescence was only 0.211 at 20 °C compared to a maximum value of 0.4. To eliminate depolarization of the fluorescence due to rotation of the protein during the fluorescence lifetime, they measured the anisotropy as a function of temperature and viscosity and by extrapolation of a plot of A^{-1} vs. T/η obtained a value for A_0, corresponding to the anisotropy of the rigid anthraniloyl group, of 0.304. Thus, even when the anthraniloyl group is completely rigid when bound to chymotrypsin, there is still ~25% depolarization, most likely due to multiple transition moments. If we assume that the anthraniloyl group has the same lifetime in HSA as in chymotrypsin, we can estimate a value of A_0 for HSA based on the slope of the T/η plot for chymotrypsin. Making the worst case assumption that HSA is rotating at the same rate as chymotrypsin, our value of $A = 0.174$ corresponds to a value of $A_0 = 0.233$. If, as is likely, the HSA molecule is rotating more slowly than chymotrypsin, the slope of the T/η plot will decrease, resulting in a still smaller value of A_0. Thus the value of 0.233 can be regarded as an upper limit for A_0, resulting in an upper limit of 0.313 for the polarization of the anthraniloyl group bound to HSA. A comparison of the polarizations of the tryptophan and anthraniloyl groups with those from the tables listed in Haas et al. (1978) suggests maximal limits in the error in R due to κ^2 of +24% and −13%. If we take the more probable error limits at half-height of the distribution function we, have ~ ±8%. Thus the polarization data tend to confirm the prediction of Haas et al. (1978) that a value of $\kappa^2 = {}^2/_3$ is reasonable for a chromophore having a multitransition moment absorption spectrum. It should be noted that any freedom of rotation experienced by the anthraniloyl chromophore independent of the protein rotation will reduce the error estimated by the above calculation still further.

By the same token, White et al. (1973) have shown that the azomercurial, when attached to serum albumin at Cys-34, becomes optically active in the spectral range between 330 and 500 nm. A positive Cotton effect is observed near 380 nm, and a negative one is observed near 450 nm, indicating that at least two electronic transitions are involved in energy transfer from the anthraniloyl group to the azomercurial. Thus, it appears that the use of $\kappa^2 = {}^2/_3$ is justified in both of these cases.

Anthraniloyl Environment. The absorption and emission maxima of anthraniloyl-HSA occur at 360 and 412 nm, respectively. This compares to absorption and emission maxima of anthraniloylchymotrypsin at 342 and 422 nm, respectively (Haugland & Stryer, 1967). By comparison of the spectrum of anthraniloylchymotrypsin with spectra of model anthranilate esters, Haugland & Stryer (1967) concluded that the anthraniloyl group was located in a highly polar environment. For example, methyl anthranilate has an emission maximum in water at 420 nm and in cyclohexane at 380 nm. On this basis our data also suggest that the anthraniloyl group is in a rather polar environment. Means & Wu (1979), on the basis of the ionic strength dependence of the reaction of Tyr-411 with DFP, concluded that charged groups were near Tyr-411. This would also account for the unusually low pK_a of Tyr-411.

HSA Structure. The amino acid sequence and the repeated patterns of loops formed by the cystine bridges of serum albumin have been used by Brown (1975, 1976, 1977) as the basis for predicting the three-dimensional structure of the protein. The basic structural unit of Brown's models is the subdomain, a troughlike structure formed by three helices of about 20 residues each. The three helices are covalently linked at one end by the double Cys bridge. The middle helix is antiparallel and makes contacts along its length with the helices on either side. The proline residues that occur at the tips of the five large loops form part of the bend that connect the two helices within the large loop. The troughlike geometry of the subdomain is based on the Cys-Cys sequence being helical so that there is 100° rotation between the linkages of the middle helix to the adjacent helices.

Brown et al. (1979) suggested three plausible ways to pair the six subdomains of human serum albumin. The first arrangement consists of parallel subdomains, where the tips of the large loops are at one end and the double Cys bridges are at the other end of each domain. Since the first and third domains are connected to adjacent helices in the middle domain, the structure tends to be condensed and relatively spherical.

A second possible model proposed by Brown is one where the two subdomains are paired in an antiparallel orientation, i.e., with the tips of the large loops of one subdomain facing the double Cys-bridge end of the other subdomain. This structure results in a linear arrangement of the three domains.

A third structural arrangement was also considered. Since the molecule consists of six subdomains, it is possible that adjacent subdomains between the repeated homology units are paired. This would result in a two-domain model with the first and sixth subdomains unpaired. In this case, only the antiparallel orientation is possible.

Although all three models for the three-dimensional structure of HSA seem reasonable, there is no chemical evidence to favor any one structure over the other. However, a variety of hydrodynamic studies have been reported since 1947, and some favor the parallel model and others fit well with the antiparallel model. For example, Oncley et al. (1947) first described albumin as a prolate ellipsoid with the dimensions 140 × 38 Å. Bloomfield (1966) presented a model for bovine serum albumin at pH 3.6 as a covalently bonded trimer with two outer spheres of radius 19.0 Å and a middle sphere of radius 26.2 Å. Squire et al. (1968) reported that bovine serum albumin is a prolate ellipsoid with a major axis of 140 Å and a minor axis of 40 Å, i.e., an axial ratio of 3.0, which is consistent with Bloomfield's linear arrangement of the three spheres. A fluorescence polarization study of ANS binding to BSA was interpreted in terms of a molecule with an axial ratio of 4/1 (Anderson & Weber, 1969). Dielectric and birefringence relaxation time studies suggest that the BSA molecule is an ellipsoid with 138 × 46 Å dimensions (Moser et al., 1966). More recently Rosseneu-Motreff et al. (1973) and Wright & Thompson (1975) obtained values of 140 ± 3.7

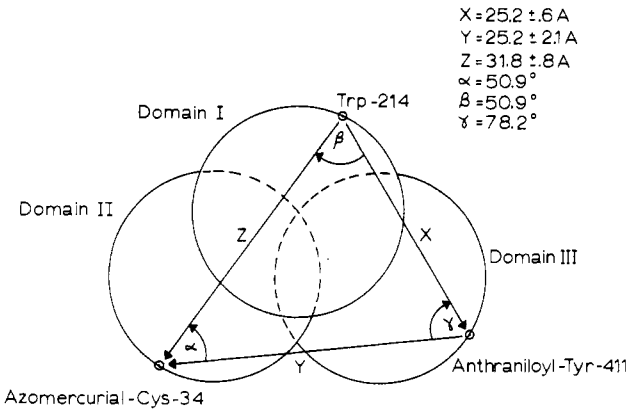


FIGURE 8: Schematic representation of the distances between Cys-34, Trp-214, and Tyr-411. See the text for discussion.

and 140.9 Å for the major axis and 34.8 ± 0.4 and 41.6 ± 3.6 Å for the minor axis for a prolate ellipsoid, respectively. Thus the antiparallel model fits well with all of the above physical measurements.

In contrast, however, Riley & Oster (1951) using low-angle X-ray scattering concluded that bovine serum albumin was not far from being spherical, and their data were best accommodated by assuming that the albumin molecule had a height and diameter of 45 and 49 Å, respectively. A spherical form for the human serum albumin molecule was assumed by Loeb & Sheraga (1956) as the model for their viscosity, sedimentation, and diffusion experiments. Slayter (1965) using electron microscopy concluded that the predominant form of the bovine serum albumin molecule at neutral pH was a globular particle approximately 60 Å in diameter and 45 Å high. More recently, Muroga et al. (1981) using the small-angle X-ray scattering technique reported that bovine serum albumin, when purified and defatted, has a radius of gyration of 31 Å at its isoionic point. When the net charge was 56 and 90, the radius of gyration was found to be 38 and 42 Å, respectively. These data are all more in accord with Brown's parallel model, where the shape of the molecule would be more nearly spherical.

Thus, the data available from the literature on the dimensions of the albumin molecule are conflicting. Some are in favor of an ellipsoid model, and others fit with a rather spherical model.

By examination of the amino acid sequence of HSA (Brown, 1977), one finds that Cys-34 is located in helix Y of domain I, Trp-214 is located in helix X of domain II, and Tyr-411 lies in helix X of domain III. The separation between domains I and II as measured by fluorescence energy transfer between Trp-214 and azomercurial-Cys-34 was on the order of 32 Å at pH 7.4. Similarly, the distance between domain II and domain III, as measured by the distance between Trp-214 and anthraniloyl-Tyr-411, was on the order of 25 Å at pH 7.4. A comparison of these two distances with the structures proposed by Brown et al. (1979) suggests that both the parallel and antiparallel models fit the data.

However, when we consider the distance between domain I and domain III, i.e., the distance between azomercurial-Cys-34 and anthraniloyl-Tyr-411, neither model fits the data very well. The antiparallel model of the albumin tertiary structure proposed by Brown & Shockley (1982) in which the domains are laid out in a linear array predicts a distance of some 80 Å between Cys-34 and Tyr-411. The parallel model proposed earlier by Brown et al. (1979), where the three domains are arranged in a triangular array, predicts a distance of approximately 55 Å. Since we calculate a distance at pH 7.4 of approximately 25 Å, the parallel model of Brown fits our data best, but the agreement is poor.

Figure 8 shows a schematic representation of the distances we have determined between the chromophores in domains I, II, and III. Once these distances are determined, the angles between lines joining the chromophores may be calculated from simple geometric relations. These angles are also shown in Figure 8.

Brown favors the antiparallel arrangement of helices as a model of serum albumin structure since this best fits with the dimensions of 140 × 40 Å obtained from a variety of hydrodynamic measurements. This model is attractive from several standpoints, the most important being that it predicts channels in each domain where fatty acids or other ligands may bind (Brown & Shockley, 1982). It may be possible, however, to fashion an alternate arrangement of the helices within a subdomain or between domains which would still preserve the essential features of Brown's model, but which would better fit our distance measurements.

Registry No. NPA, 19176-60-4; Trp, 73-22-3; Tyr, 60-18-4; Cys, 52-90-4.

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Interaction of Bovine Blood Clotting Factor Va and Its Subunits with Phospholipid Vesicles†

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ABSTRACT: Thrombin-activated factor Va and factor Va subunit binding to large-volume vesicles was investigated by a technique based on the separation by centrifugation of phospholipid-bound protein from the bulk solution. This technique allows the direct measurement of free-protein concentration. It is concluded that the phospholipid binding site on factor Va is located on a basic factor Va subunit with M_r 80 000 (factor Va-LC). The effects of phospholipid vesicle composition, calcium concentration, pH, and ionic strength on the equilibrium constants of factor Va- and factor Va-LC–phospholipid interaction were studied. Factor Va and factor Va-LC binding to phospholipid requires the presence of negatively charged phospholipids. It is further demonstrated that the following occur: (a) Calcium ions compete with factor Va and factor Va-LC for phospholipid-binding sites. (b) The dissociation constant of protein–phospholipid interaction increases with the ionic strength, whereas the maximum protein-binding capacity of the phospholipid vesicle was not affected by ionic strength. (c) The dissociation constant for factor Va–phospholipid interaction depends on pH when the vesicle consists of phosphatidic acid. It is concluded that factor Va–phospholipid interaction is primarily electrostatic in nature, where positively charged groups on the protein directly interact with the phosphate group of net negatively charged phospholipids. The results suggest that factor Va, like factor Xa and prothrombin, has the characteristics of an extrinsic membrane protein.

The prothrombinase complex catalyzes the proteolytic conversion of the zymogen prothrombin to the serine protease thrombin. The components that constitute the complex are factor Xa (the catalytic component), calcium ions, phospholipid, and factor Va [see Jackson & Nemerson (1980) for a review]. Factor Va, which is derived by limited proteolysis of a single-chain precursor of M_r 330 000, functions as a cofactor (Esmon, 1979; Nesheim & Mann, 1979). The effects of factor Va and phospholipids on the kinetic parameters of prothrombin activation have given insight in the mode of action of nonenzymatic cofactors in prothrombin activation (Rosing et al., 1980).

Knowledge of the molecular details of the assembly of the prothrombinase complex is of importance in order to understand the mechanism of prothrombin activation at a phospholipid–water interface. From the studies reported by Nelsestuen and co-workers, a detailed model of the prothrombin- and factor Xa–membrane complexes can be drawn (Lim et al., 1977; Resnick & Nelsestuen, 1980; Wei et al., 1982).

The nature of the factor Va–phospholipid interaction has still to be disclosed. In most previous studies, gel filtration has been used to elucidate the factor V(a)–phospholipid-binding characteristics. On the basis of these qualitative studies, it appeared essential to have net negative charged phospholipids in the membrane for factor V–membrane interaction (Subbaiah et al., 1976). In addition, the possibility that the binding of factor V to membranes is mediated by calcium ions can be ruled out (Greenquist & Colman, 1975; Subbaiah et al., 1976). In essence, those observations were confirmed in a study that utilizes factor V of high purity and a method of analysis that yields quantitative equilibrium-binding data (Bloom et al., 1979). It was suggested that factor Va–membrane interaction is nonelectrostatic in nature. The role of phosphatidylserine in the protein–lipid nonelectrostatic interaction could not be explained (Bloom et al., 1979).

The purpose of this study is to establish the mode of action of acidic phospholipid in the factor Va–phospholipid interaction. We utilized large-volume unilamellar vesicles, which can be separated from the bulk solution by centrifugation, for determination of binding parameters of the factor Va- and factor Va subunit–phospholipid interactions. The binding technique presented here is a valuable tool in the study of the effect of phospholipid composition, pH, ionic strength, and calcium on factor Va–phospholipid interaction.

Materials and Methods

S 2238[1] and S 2337 were purchased from AB Kabi Diagnostica. Soybean trypsin inhibitor (type I-S), *Echis carinata*

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[1] Abbreviations: S 2238, D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride; S 2337, *N*-benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-L-glycyl-L-arginine-*p*-nitroanilide hydrochloride; RVV-X, factor X activator purified from Russell's viper venom; RVV-V, factor V activator purified from Russell's viper venom; Tris, tris(hydroxymethyl)aminomethane; STI, soybean trypsin inhibitor; EDTA, ethylenediaminetetraacetic acid; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphatidic acid; LC, light chain; HC, heavy chain.

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