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Unusual Difference Spectra of Proteins Containing Tryptophan. II. Studies with Proteins*

V. S. Ananthanarayanan and C. C. Bigelow

ABSTRACT: An attempt is made to interpret unusual difference spectra observed with various tryptophyl-containing proteins in a consistent fashion working from studies made with model compounds. It is found that an analysis of the behavior of the hitherto ignored extremum around 300 mμ in these difference spectra can yield information about the electrostatic environment of tryptophyl residues in the native form of the proteins and about small local conformational changes around these residues which take place under certain conditions in some of these proteins.

Proteins containing tryptophyl residues exhibit, under certain conditions, an atypical extremum or shoulder around 300 mμ in their difference spectra. Figures 1 and 2 of the preceding paper and Figure 1 of this paper give examples of such unusual difference spectra obtained by us with β-lactoglobulin A. From the literature one can cite observations made on the proteins listed in Table I.

Studies made with model compounds (see Ananthanarayanan and Bigelow, 1969) have revealed that the unusual nature of these difference spectra probably arises from the presence of a weak long-wavelength band in the absorption spectrum of the indole chromophore and the characteristic behavior of this minor band toward changes in the chromophore's environment. Use is made of these results in this paper to interpret the unusual difference spectra of various tryptophyl-containing proteins.

Materials and Methods

β-Lactoglobulin A was prepared as outlined in the preceding paper. Lysozyme was a three-times-recrystallized sample from Worthington. Chymotrypsinogen was a Worthington sample. Other reagents were the same as described in the preceding paper (Ananthanarayanan and Bigelow, 1969).

Difference spectral measurements were carried out with a Cary Model 15 spectrophotometer using matched silica cells. pH measurements were made on a Radiometer pH meter.

Results and Discussion

The reader is referred to Figures 1 and 2 of the preceding paper (Ananthanarayanan and Bigelow, 1969) which show some of the unusual difference spectra obtained with β-lactoglobulin A when it undergoes denaturation by organic solvents. In Figure 1 of this paper are shown the difference spectra of β-lactoglobulin A denatured thermally and by urea. In both cases a distinct negative shoulder at about 300 mμ is found to be present along with the minima at 284 and 292 mμ.

Figure 2 shows the difference spectra of chymotrypsinogen, pepsin, and bovine albumin after they have been denatured by urea or Gu·HCl. A faintly discernible negative shoulder around 298 mμ was obtained in the case of chymotrypsinogen in 6 M urea whereas a broad positive maximum centered around 300 mμ was found in the case of pepsin in 7.2 M Gu·HCl. In both cases minima were found at 292.5 and 283–285 mμ. With bovine albumin there was practically zero difference absorp-

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TABLE I: Unusual Difference Spectra Reported in the Literature.

Protein	Experimental Conditions	Reference Conditions	Reference
	A. Examples Where $\Delta\epsilon_{300}$ is Positive		
Pepsin	Denatured, pH 7.4	Native, pH 5.8	Edelhoch (1958)
Lysozyme	84°	25°	Foss (1961)
Avidin	Complex with biotin	Free protein	Green (1962)
Soybean trypsin inhibitor	pH 9.0, 77°	pH 6.5, 3°	Wu and Scheraga (1962)
Lysoyzme	3.8 M Gu·HCl	3.2 M Gu·HCl	Hamaguchi and Kurono (1963a)
Lysoyzme	10 M urea	8 M urea	Hamaguchi and Kurono (1963a)
Lysozyme	8 M LiCl	6.4 M LiCl	Hamaguchi *et al.* (1963)
Lysozyme	32% 2-chloroethanol	Water	Hamaguchi and Kurono (1963b)
Lysoyzme	80% methanol	Water	Kurono and Hamaguchi (1964)
Lysozyme	9 M urea, pH 2.8	Water, pH 6.7	Steiner (1964)
Lysozyme	Complex with glycol chitin	Free protein	Hayashi *et al.* (1964)
α-Amylase	pH 6.4	pH 0.6	Imanishi *et al.* (1964)
Carbonic anhydrase	8 M urea	Buffer	Edsall *et al.* (1966)
Lysozyme	Cationic detergent	Buffer	Hayashi *et al.* (1968)
	B. Examples Where ϵ_{300} is Negative		
Trypsin	8 M urea	Buffer	Smillie (1959)
Bovine serum albumin	Dodecyl sulfate	Buffer	Bigelow and Sonenberg (1962)
α-Lactalbumin	pH 3.8	pH 5.6	Kronman *et al.* (1965)
Trypsin	78°	16°	Lazdunski and Delaage (1965)
Liver alcohol dehydrogenase	pH 2.7	pH 6.9	Blomquist (1967)
Bovine serum albumin	Anionic detergents	Water	Polet and Steinhardt (1968)
Bovine serum albumin	Fatty acid anions	Water	Reynolds *et al.* (1968)
Bovine serum albumin	pH 4	pH 5.6	Sogami and Foster (1968)
Bovine serum albumin complexed with dodecanoate	30% methanol	Water	Zakrzewski and Goch (1968)

tion at 300 mμ and the major difference minimum occurred at 287.5 mμ.

In the case of lysozyme, it has been observed (Hamaguchi and Kurono, 1963a,b; Hamaguchi *et al.*, 1963) that troughs occur around 300 mμ in the difference spectra produced by adding salts like Gu·HCl, LiCl, and NaBr even before the denaturation of the protein set in. We have observed the same behavior using KCl. However, no significant extremum around this wavelength was observed by Kurono and Hamaguchi (1964) in the difference spectrum of lysozyme in nondenaturing concentrations of an organic solvent like dioxane. Figure 3 shows the behavior of the 300-mμ extremum in the difference spectra of lysozyme in Gu·HCl and dioxane before and after denaturation using the data of Hamaguchi and Kurono (1963a,b).

The results of experiments with model compounds discussed in the preceding paper (Ananthanarayanan and Bigelow, 1969) together with the data available on the unusual difference spectra of proteins (Table I) not only enable us to interpret these difference spectra but allow us to draw certain important conclusions regarding the native conformations of these proteins and the changes occurring in them. We shall elaborate this statement by taking a few specific examples but the arguments put forth also apply to other proteins which are not discussed in detail here.

As a first example, the difference spectra of β-lactoglobulin A by organic solvents (Ananthanarayanan and Bigelow, 1969) and by urea or temperature (Figure 1) exhibit a large blue shift near 300 mμ when the protein is denatured. It is well known that denaturation of a protein brings about the transfer of groups buried in the interior hydrophobic regions of the native molecule to the relatively polar surroundings and that this is responsible for the blue shift of the major bands at 280 and 290 mμ of the tryptophyl chromophore. Such a transfer, from hydrophobic interior to polar exterior, would be expected to cause only a small red shift of the minor band around 300 mμ as we have shown in our studies with indole or tryptophan (Ananthanarayanan and Bigelow, 1969). The large blue shift which is actually observed, comparable in size with that of the major bands, cannot therefore be due to such a transfer. Our model compound studies showed that changes in the electrostatic environment of tryptophyl residues can bring about

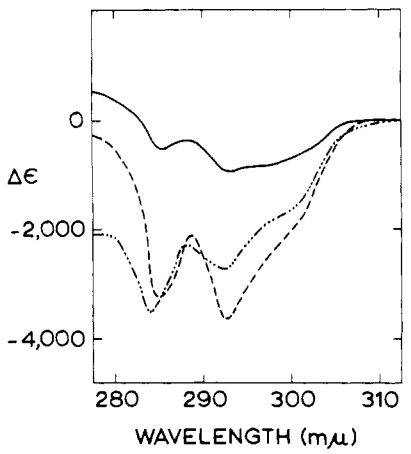

FIGURE 1: Difference spectra of β-lactoglobulin A. ———, in 4.5 M urea at 25°, pH 3.0, $\mu = 0.1$. — —, in 8.1 M urea at 25°, pH 3.0, $\mu = 0.1$. — — —, at 90.5°, pH 3.5, $\mu = 0.1$. In each case the reference solution was at the same pH as the experimental solution, at 25°, and with $\mu = 0.1$.

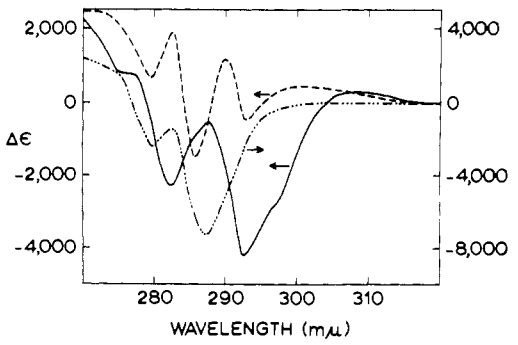

FIGURE 2: Difference spectra of various proteins. ———, chymotrypsinogen in 6 M urea, pH 2.7 (reference solution in water at pH 3.2). — —, pepsin in 7.2 M Gu·HCl, pH 4.35 (reference solution in water at pH 6.0). — — —, bovine serum albumin in 7.2 M Gu·HCl, pH 4.6 (reference solution in water at pH 6.0).

much larger shifts of the minor band than changes in the polarity of the medium. It thus seems reasonable to infer that when $\Delta\epsilon_{300}$ has a significant value in a denaturation process, it is due to changes in the electrostatic environment of one or more of the tryptophyl residues of the protein. Employing the data obtained with tryptophan as our guide we can conclude that a blue shift of the minor band (giving a negative value of $\Delta\epsilon_{300}$) would result when the total net charge around a tryptophyl chromophore is made more positive or less negative. Such a change in the electrostatic environment of tryptophyl residues in a protein could of course be imagined to accompany denaturation. In the native protein, some of the tryptophyl residues could be near charged groups. As discussed by Donovan *et al.* (1961) the electrostatic effect of the charges could be transmitted to the chromophores through the medium. When the protein is denatured, however, this effect would practically vanish because of the (nearly) random conformation assumed by the denatured protein, which results in the separation of charged groups from the chromophores. Transmission of charge effects through the peptide backbone is still possible in the denatured state but this effect has been shown by Donovan *et al.* (1961) to be relatively weak compared with that which may be transmitted through the solvent.

We thus conclude that in native β-lactoglobulin A some of the tryptophyl residues, which one would think would be "exposed" (that is, on the surface of the molecule) are found in close proximity to negatively charged groups, that is, carboxylate groups. During denaturation the close juxtaposition of the tryptophyl(s) and the carboxylate(s) is destroyed. The separation of these two types of side chain groups can give rise to a blue shift of the minor band, and therefore to a negative value of $\Delta\epsilon_{300}$.

Figure 2 shows the difference spectra observed when chymotrypsinogen is denatured by urea, and pepsin and bovine serum albumin by Gu·HCl. These three denaturation processes show the three sorts of difference spectra that one can expect to find.

Bovine serum albumin shows no apparent effect at all at 300 mμ when it is denatured by Gu·HCl although there is a clear denaturation blue shift due to tyrosyl residues. The absence of any significant effect at 300 mμ indicates that the two tryptophyl residues are in an electrostatically neutral environment in the native molecule. Significant negative values of $\Delta\epsilon_{300}$ have been observed with serum albumin when it binds anionic detergents (Bigelow and Sonenberg, 1962; Polet and Steinhardt, 1968) which shows the tryptophyl residues are near the binding sites.

Chymotrypsinogen shows a distinct negative shoulder at 300 mμ on denaturation by 6 M urea, the difference spectrum is quite similar in this regard to that seen when β-lactoglobulin A is denatured by urea (Figure 1). Thus we conclude that the tryptophyl residues of chymotrypsinogen are in a predominantly negatively charged environment in the native molecule. On the other hand, the positive value of $\Delta\epsilon_{300}$ observed when pepsin is denatured by Gu·HCl is consistent with separation of its tryptophyl residues from positive charges.

The Denaturation Red Shift at 300 mμ. The denaturation of lysozyme has been studied in great detail by difference spec-

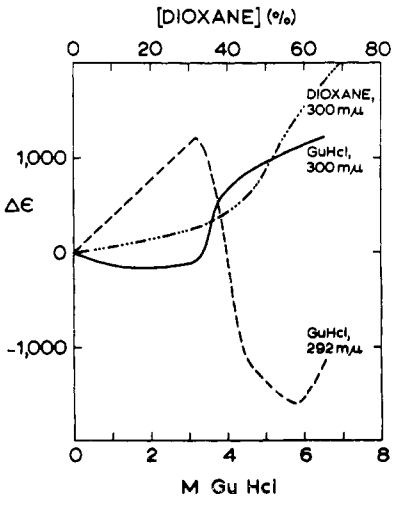

FIGURE 3: Plots of $\Delta\epsilon_{292}$ and $\Delta\epsilon_{300}$ against the concentrations of dioxane and Gu·HCl for lysozyme. The data have been taken from the papers of Hamaguchi and Kurono (1963a,b).

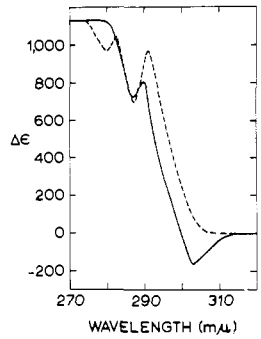

FIGURE 4: Difference spectra of lysozyme in 2.4 M LiCl. ———, pH 6.1 (reference solution at pH 6.1). ——, pH 0.5 (reference solution at pH 1.0).

troscopy, especially by Hamaguchi and coworkers (see Table I). These authors have employed a wide variety of denaturants in their studies. The difference spectra they have obtained with some of the denaturants show positive and others negative $\Delta\epsilon$ values at 300 mμ *before denaturation* (Table I and Figure 3); this will be discussed further below. However, all of them show a large positive peak around this wavelength once the protein is denatured (Table I). (The native protein in water at the appropriate pH and ionic strength is the reference solution in each case.) Similar results have been reported by Steiner (1964) using urea and Gu·HCl as denaturants.

Proceeding along the same line of argument used for β-lactoglobulin A, we would conclude that the tryptophan residues in the native lysozyme molecule are, on the whole, in an environment whose net charge is positive; in other words, there may be a preponderance of positively charged groups near the tryptophyl residues. An examination of stereoscopic photographs of the three-dimensional model proposed for this enzyme from X-ray analysis by Blake *et al.* (1967) and of a large-scale drawing of the same model (Phillips, 1966) reveals that this is probably the case. We are indebted to D. C. Phillips for a private communication concerning these relationships.

The side-chain nitrogen atom of Trp-62 is within 3.5 Å of the guanidinium group of Arg-61, and appears to be not much farther from Arg-73. None of the other indoles are as close as this to positive charges but several (Trp-63, Trp-11, and Trp-123) have a C–ϵ atom within about 5 Å of lysyl side chains (67, 116, and 33, respectively) (D. C. Phillips, private communication). The only indole side chain which appears to be close to a carboxyl side chain is Trp-108 which is near Glu-35, which is however thought to have a pK of 6.8.

Thus it appears that the indole side chains of four of the six tryptophyl residues of the lysozyme molecule are in fact located in space near positively charged groups from other side chains. And therefore the interpretation of the "denaturation red shift" at 300 mμ, given above, is consistent with the data available from X-ray crystallography.

Effect of State of Carboxyl Groups on $\Delta\epsilon_{300}$ for Lysozyme. It was pointed out that when lysozyme at neutral pH is treated with nondenaturing concentrations of salts like guanidine hydrochloride, LiCl, LiBr, and NaBr (Hamaguchi and Kurono, 1963a; Hamaguchi *et al.*, 1963), there occurs a small blue shift of the minor band and a red shift of the major bands (see Figure 3). The effect on the major bands has of course been frequently observed, and it appears to be a consequence of the increase in refractive index of the medium caused by the addition of the salts (Bigelow and Geschwind, 1960). The blue shift of the minor band was however a surprise; it was not consistent with the results we obtained with the model compounds (Ananthanarayanan and Bigelow, 1969). It was therefore suspected that the effect observed in this protein could be due to the binding of cations to carboxylate groups.

The argument in favor of this is as follows. We know that there is a small negative value of $\Delta\epsilon_{300}$ observed when lysozyme is titrated from pH 5.04 to 1.15 (Donovan *et al.*, 1961), but there is no evidence that this is caused by a conformation change. In fact it appears to be due to the reduction of negative charge in the vicinity of one or more indole groups (as we mentioned above the indole of Trp-63 has three carboxylate groups nearby). The shift of the minor band is observed when the carboxylate groups react with protons. One imagines that binding cations to the carboxylate groups would have the same *spectral* effect.

The spectral effect observed at 292 mμ is more complicated (Figure 3). The effect of neutralizing negatively charged groups will cause a blue shift of the major band as well as the minor band. But there is a much bigger refractive index caused red shift of the major band, leading to a net red shift. Since there is no significant refractive index caused shift of the minor band, we find a (small) net blue shift.

One way to test the argument immediately presents itself. The solvent effect observed at 300 mμ, so the interpetation goes, is not really a bulk solvent effect, but is due, rather, to specific interaction of cations and charged carboxylate groups. If that is correct, then the same salts should give qualitatively different effects if they are added to the protein at low pH—when the carboxylate groups are already protonated. We therefore measured the effect of 2.4 M LiCl on lysozyme at pH 9 (the reference solution was also at pH 1). The difference spectrum in this case was "normal," that is, there was no trough around 300 mμ (Figure 4), which is consistent with out interpretation. Neither urea (Hamaguchi and Kurono, 1963a) nor dioxane (Hamaguchi and Kurono, 1963b; see Figure 3) show negative values of $\Delta\epsilon_{300}$ before denaturation at neutral pH, which is to be expected if negative values are caused only by binding cations to carboxylate groups.

It should be pointed out here that Hamaguchi and Kurono (1963b) *did* find a negative $\Delta\epsilon_{300}$ in the solvent effect of 2-chloroethanol on lysozyme. However, in these experiments the 2-chloroethanol contained hydrochloric acid which was not removed. Therefore, although the reference solutions were at an unstated but presumably "neutral" pH, the solutions containing the 3-chloroethanol were at a pH between 2.6 and 3.6. In other words the lysozyme in the experimental solution had all its carboxyl groups in the protonated form, and the protonation itself and not just the addition of 2-chloroethanol, was likely responsible for the negative values of $\Delta\epsilon_{300}$ which was observed by Hamaguchi and Kurono.

The variation of $\Delta\epsilon_{293-295}$ with changes in the pH of solutions of lysozyme was studied both by Donovan *et al.* (1961) and by Ogasahara and Hamaguchi (1967), and some of these results deserve comment. Donovan *et al.* plotted $\Delta\epsilon_{295}$ *vs.* pH between pH 1 and 9 and showed how to correct the higher pH values for tyrosyl ionization. They showed that the indole spectra changed in two steps, with pK's near 3.15 ($\Delta\epsilon_{295} \sim -550$) and 6.20 ($\Delta\epsilon_{295} \sim -300$) (we give negative values for $\Delta\epsilon$, which would apply as the pH was shifted downward).

Donovan *et al.* showed that both of these shifts in indole absorption were due to titration of carboxylate groups, since neither step was observed with methylated lysozyme. This can be seen from the Phillips model (1966) to be very reasonable—both the α-amino group and the single histidyl residue (position 15), which might conceivably have pK's near 6.2, are distant from all of the six tryptophyl residues.

Ogasahara and Hamaguchi (1967) repeated some of the measurements of Donovan *et al.* (1961) and their difference spectra should also be discussed. As lysozyme is titrated from pH 6.9 to 0.9 both the major band and the minor band are blue shifted, as expected, giving negative extrema at 293 and 300 mμ. However in the first of the two steps (pK = 6.2) it is clear that the difference spectra of Ogasahara and Hamaguchi are peculiar in that $\Delta\epsilon_{300}$ is larger (absolutely) than $\Delta\epsilon_{293}$, which is just opposite to what has been seen with tryptophan (Donovan *et al.*, 1961; Ananthanarayanan and Bigelow, 1969). In the second step (pK = 3.15) this situation becomes more normal with $\Delta\epsilon_{293} > \Delta\epsilon_{300}$. It is not now possible to be certain about the cause of the abnormal behavior with the pK of 6.2, but one possibility is that besides the blue shift due to carboxylate protonation, the major band undergoes a red shift due to a change in the polarity of the environment of one or more of the indole groups—that is due to the occurrence of a very small local conformation change which accompanies the protonation. It should be pointed out that no unequivocal evidence for such a conformation change exists elsewhere in the literature.

Ogasahara and Hamaguchi (1967) also measured difference spectra for lysozyme at various low pH values in 1.6 and 3.6 M Gu·HCl. The addition of 1.6 M Gu·HCl causes no significant change in the behavior of lysozyme at low pH—even at pH values below 1 the molecule appeared to exist in its native conformation. However, when the Gu·HCl concentration was raised to 3.6 M acid denaturation occurred with a pK of about 3.2. It was demonstrated by a sizable blue shift of the main indole band ($\Delta\epsilon_{292} \sim -3000$).

Not only did the 3.6 M Gu·HCl allow denaturation to occur, but it also abolished the small step in $\Delta\epsilon_{292}$ which had been seen around pH 6.2 in water and in 1.6 M Gu·HCl.

When data for $\Delta\epsilon_{300}$ in 3.6 M Gu·HCl were plotted against pH they showed some interesting results. First, there were a large positive value of $\Delta\epsilon_{300}$ during denaturation ($\sim$1000) and second, there was a sizable negative value of $\Delta\epsilon_{300}$ preceding denaturation (*i.e.*, at higher pH values). In others words observation of $\Delta\epsilon_{300}$ *does* show the effect of neutralizing a carboxylate group just as it did in water, even though observation of $\Delta\epsilon_{292}$ does not. The reason for this complicated behavior is not obvious at the moment.

We have spent considerable effort in discussing the absorption changes at 300 mμ observed when lysozyme is denatured or acidified, and it might be helpful to summarize our conclusions here. Our data show that the major indole band and what we consider to be a probable minor indole band give very different information about the environment of the indole groups. The major band responds to changes in the polarity or the electrostatic nature of the environment; the minor band is nearly unaffected by changes in the polarity, but is quite sensitive to changes in electrostatic environment.

When the medium is changed without denaturing the protein the major band shifts to longer wavelengths as the solvent's refractive index increases and this is true whether the additive is a salt or an organic solvent. While many of our data come from experiments with one or two proteins, they are presumably applicable to all in which similar situations prevail.

The studies on lysozyme discussed above point out the important distinction between the behavior of the major bands and the minor band in the difference spectra. Thus when titration of a carboxyl group takes place, all the bands show a blue shift; any slight conformational change occurring during the titration causes a red shift of the major bands with negligible effect on the minor band. When denaturation of the protein takes place, the major bands experience the denaturation blue shift which is mainly the result of change in solvent polarity, whereas the minor band undergoes a denaturation red shift which is mainly the result of change in the electrostatic environment near the tryptophyls.

Effect of Substrate Analog Binding on $\Delta\epsilon_{300}$ for Lysozyme. It is also of considerable interest to examine recent results of Imoto and Rupley (1968) who have been able to study the effect of binding tri-*N*-Ac-Glu[1] on the tryptophyl residues in the active centre (62, 63, and 108). By studying the complex formation also with two lysozyme derivatives in which residues 62 and 108 had been selectively destroyed, they attempted to determine the individual contributions to the difference spectra made by the three tryptophyl residues at the active center. (The assumption needed is that the individual contributions of the two residues which are uncharged in each derivative are the same as they were in the native molecule. Dr. Rupley, in a private communication has told us that in fact he believes Trp-62 and Trp-63 interact with the result that the assumption is really invalid. Thus the "individual difference spectra" must be interpreted with great caution.) Imoto and Rupley measured the difference spectra found when *N*-Ac-Glu binds to lysozyme or the derivatives, at pH 2, 5, and 8, and in all the difference spectra they found unusual absorption differences at 300 mμ. Their abstract concluded, "Available data on model systems do not account for the characteristics of the three tryptophans."

We believe the data we now have does allow an interpretation of some of the individual spectra of Imoto and Rupley. These investigators have been kind enough to send us some of these difference spectra in advance of publication, and some of these results will now be considered.

The difference spectra for Trp-108 are the largest ($\Delta\epsilon_{292} \sim 1200$), and the peaks at 285 and 293 mμ are positive, roughly independent of pH, and consistent with the removal of the residue from contact with the nonpolar environment. There is also a long-wavelength positive tail at pH 2, which becomes a shoulder at pH 5 and a distinct peak at 302 mμ at pH 8. The positive tail seen at pH 2 presumably means that as the complex forms Trp-108 is separated from positive charges (since no negative charges exist at pH 2). However as the pH is raised it is clear that one or more groups near Trp-108 is titrated (with most of the titration occurring between pH 5 and 8). The approach or creation of a negative charge near Trp-108 would account for the increase in $\Delta\epsilon_{302}$ without any large accompanying change in $\Delta\epsilon_{293}$. It has previously been suggested that Glu-35, which is quite close to Trp-108,

[1] Abbreviation used is: tri-*N*-Ac-Glu, the trimer of *N*-acetylglucosamine.

has a pK of about 6.2 (Blake *et al.*, 1967), and Imoto and Rupley also suggested that it might be implicated in the spectral effects from Trp-108. Our results show that the kind of change they observed can be reasonably accounted for from data on model systems.

The difference spectra for Trp-62 are interesting, giving positive values of $\Delta\epsilon_{284}$ and $\Delta\epsilon_{292}$, which are nearly independent of pH. As with Trp-108, this is consistent with burial or covering of Trp-62 in complex formation. At pH 2, $\Delta\epsilon_{301}$ is -250, at pH 5, -125, and at pH 8, $\Delta\epsilon_{303}$ is $+125$. That is, at low pH, Trp-62 appears to move closer to positive charges (which could be Arg-61 or Arg-73) when the complex is formed. At pH 8, Trp-62 appears to move closer to negative charges or farther from positive ones. Examination of the model photographs, however, does not indicate either of these possibilities is very likely, and it seems probable the positive $\Delta\epsilon_{303}$ arise from an interaction with one of the other Trp residues involved (J. A. Rupley, private communication).

The difference spectra for Trp-63 are small, irregular, and have negative values of $\Delta\epsilon_{303}$ at all pH values studied. The fact that the difference spectra are small, even though Trp-63 is bound to be affected by saccharide binding, shows that there are probably more than one spectral effect of different sign working, and it is far too risky to attempt at this stage to interpret the difference spectra it gives rise to.

Thus it appears that some, at least, of the unusual features of the difference spectra of Imoto and Rupley can be qualitatively understood in terms of the approach or removal of indole residues from charged groups. It will obviously require increased understanding of the individual difference spectra, and especially of interaction effects between the tryptophyl residues before more can be said.

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