Changes in Ultraviolet Absorption Produced by Alteration of Protein Conformation

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SUMMARY

Transfer of an aromatic chromophore from the interior of a protein into the water solvent upon denaturation of the protein produces an absorption change throughout the ultraviolet region, 215 to 320 nm, approximately 6 times the magnitude of that produced by transfer of the same chromophore from 20% ethylene glycol into water. In the 230 nm wave length region, the transfer of an indole chromophore produces an absorption change approximately 7 times that produced by the transfer of a phenolic chromophore; the chromophores of the amino acids cystine, histidine, and phenylalanine produce only slight changes in absorption.

Difference spectra for protein denaturation were calculated by summation of the 20% ethylene glycol perturbation difference spectra of the amino acids, and multiplication by the factor of 6. Between 215 and 320 nm, difference spectra produced by acid, autolysis, or urea denaturation closely resemble calculated ones, but are red shifted 2 to 3 nm near 280 nm and 4 to 10 nm near 230 nm. Thus, the absorption changes observed near 230 nm for globular proteins result primarily from changes in the environment of the aromatic chromophores indole and phenol. The helix to coil transition and the denaturation-produced solvent perturbation of the amide group must contribute less than 10% of the absorption changes observed near 230 nm.

Changes in ultraviolet absorption of proteins in the 230 nm region are observed when proteins are denatured or undergo conformational changes. Glazer and Smith (1, 2) first reported these absorption changes, and suggested that they were produced by helix to coil transformations in the proteins. Studies of the absorption changes in the n → π* absorption bands of synthetic polypeptides and proteins (3-5) indicate that proteins would have to contain large amounts of α helix to account for the changes in absorption observed in the 230 nm region. However, some globular proteins, such as chymotrypsinogen, contain relatively small amounts of α helix (6) but produce substantial absorption changes in the 230 nm region upon denaturation (2). Also, changes in absorption are occasionally observed in the absence of changes in optical rotation, as in the case of ovomucoid (7). Furthermore, many investigators, notably Eisenberg and Edsall (8) and Edsall, Mehta, Myers, and Armstrong (9), have presented evidence that the absorption changes of proteins at 230 nm are proportional to absorption changes observed near 280 nm, where the peptide bond does not absorb. At these longer wave lengths, the absorption changes on denaturation are known to be due only to changes in the environment of the aromatic chromophores of the protein (10, 11).

A protein can be regarded as a solvent for the chromophores buried within it. Denaturation, in which buried chromophores are transferred from the interior of the protein into the water solvent, can be regarded simply as a change in the environment of these chromophores—in effect, as a transfer from one perturbing solvent to another. With the use of the known refractive indices of solvents, semiquantitative calculations can be made which give the relative magnitude of the perturbation produced.

Experimentally, the determination of the absorption changes accompanying the denaturation of a protein can be carried out by determining (a) the difference in absorption between a chromophore in water solution and the same chromophore in a solution of a perturbant, (b) the factor relating the amount of perturbation produced by the perturbant to the amount of perturbation produced by the interior of the protein. To account for absorption changes in terms of a transfer of chromophores from one perturbing solvent to another, it was necessary to establish a “standard transfer” for the chromophores, upon which numerical results could be based. The most convenient solvents for this purpose were water, the solvent for the chromophores of denatured proteins, and 20% (v/v) ethylene glycol. Ethylene glycol at this concentration appears to have little effect on protein conformation (12) and does not specifically interact with the chromophores of proteins (13). This experimental approach to the interpretation of the absorption changes of proteins on denaturation is based almost entirely on the method and technique of solvent perturbation developed by Herskovits and Laskowski (14).

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EXPERIMENTAL PROCEDURE

Materials—Rabbit muscle fructose diphosphate aldolase (EC 4.1.2.7), obtained from Sigma sedimented in a single boundary ($s_{av, m} 7.70 \pm 0.10$ at 0.8 mg per ml) with the sedimentation coefficient reported by Stellwagen and Schachman (15). Sedimentation equilibrium at 8,000 rpm gave a linear plot of $\ln A_{350}$ versus $x^2$. The calculated molecular weight was 159,000, in agreement with that reported by Kawahara and Tanford (16). Ovomucoid was purified by the method of Davis, as described earlier (7). Ethylene glycol, Eastman White Label, was selected from several lots for low ultraviolet absorption. Distilled water used for preparation of solutions was checked for absorption at short wave lengths against an air path reference. Amino acids and derivatives were obtained from Mann.

Methods—Spectra, difference spectra, and fluorescence spectra were obtained at room temperature (22°) with a Cary model 15 spectrophotometer and with a Turner model 210 spectrophotofluorometer. Absorption cells with a path length of 1 cm were used throughout. The absorbance of solutions of amino acids and proteins used for difference spectra was less than 2 optical density units at all wave lengths for which difference spectra are reported. Precautions were taken to avoid errors due to stray light, fluorescence of samples, or other artifacts. These precautions included variation of slit width to ensure resolution of the difference spectra and verification of Beer’s Law to ensure absence of errors from stray light. In addition, the Turner model 210 was used as a spectrophotometer to obtain difference spectra (see below). In measuring solvent perturbation differences, the tandem cell arrangement of Herskovitz and Laskowski (17) was not employed when the Cary model 15 was used. This spectrophotometer, unlike the Cary model 14 and the Turner model 210, has separate detectors for the reference and sample beams. Because it is impossible to match the spectral response curves of these two detectors exactly, the base-line (zero absorbance as a function of wave length) of the spectrophotometer depends on the slit width. The instrument is designed to operate at constant signal at the reference detector, and thus the slit width increases as the absorption of the reference increases. To duplicate the changes in slit width which occur during the scan of a difference spectrum, a separate scan was obtained, with the reference material in both the sample and reference cells, placed in their respective compartments, but with all other instrumental adjustments identical. This zero baseline scan was used for calculations, and was different from the base-line obtained for a water-water scan when there were appreciable changes in slit width. The absorption of perturbant was determined with respect to water in a separate experiment, and was subtracted from the difference spectrum after the latter was corrected for the base-line.

Concentrations of amino acids were determined from the absorption of the solutions, with the absorption coefficient reported by Mihalyi (18), or by preparing the solutions by weight. Concentrations of ovomucoid and aldolase were determined with the use of the optical factors reported previously (7, 19). Spectra and difference spectra are reported in terms of molar absorption coefficient ($\epsilon$) and molar difference absorption coefficient ($\Delta \epsilon$) or optical density ($D$) and optical density difference ($\Delta D$).

![Fig. 1](image-url)  
**Fig. 1.** Excitation and emission spectra of $4 \times 10^{-4}$ M tyrosine in water and in 29% (v/v) ethylene glycol (E.G.) at 22°. A Turner model 210 spectrophotofluorometer was used, with 25-Å excitation and emission band widths. The excitation spectrum of tyrosine in 29% ethylene glycol was determined with fluorescence at 305 nm. Both emission spectra were measured with excitation at 225 nm.

![Fig. 2](image-url)  
**Fig. 2.** Beer’s Law plot for the short wave length difference spectrum of tyrosine produced by 20% ethylene glycol perturbation. $\bigcirc$, data obtained with the 0 to 1 absorbance scale of the Cary model 15; $\bigtriangleup$, data obtained with the 90 to 110% transmission scale of the Turner model 210.
RESULTS

Measurements of difference spectra in the short wave length region are susceptible to greater error from experimental artifacts than the same measurements carried out at longer wave lengths. Fig. 1 shows (a) that the fluorescence of tyrosine is increased 40% by the addition of 20% ethylene glycol, and (b) that the fluorescence is approximately 5 times greater when the tyrosine solution is excited at 225 m\(\mu\) than when it is excited at 280 m\(\mu\). In the Cary spectrophotometers, light fluoresced by the sample can reach the detector, although the geometry of the optics for the detection of this fluoresced light is poor. Mihalyi (20) indicates that only about 1/7 of the fluorescence reaches the detector in the Cary model 14. To ensure that fluorescence did not introduce artifacts in these experiments, perturbation difference spectra of tyrosine, which fluoresces more efficiently than proteins (21, 22), were measured using the Turner model 210 as a double beam spectrophotometer. In this instrument the samples are placed between monochromators, so that any light fluoresced by the samples is dispersed by the second monochromator before it reaches the detector (23). Fig. 2 shows that the absorption differences measured with the Turner instrument gave the same difference extinction coefficient for the 20% ethylene glycol perturbation of tyrosine as did measurements made with the Cary model 15.

Fig. 3, the result of a typical experiment in which the 20% ethylene glycol perturbation of tryptophan was determined (see "Methods"), shows that the perturbation of indole chromophores in the short wave length region is over 8 times that in the longer wave length region. Figs. 4 and 5 show the 20% ethylene glycol perturbation difference spectra of tyrosine, tryptophan, cystine, histidine, and phenylalanine. At longer wave lengths, these difference spectra agree with those previously reported (19, 24). Beer's Law was verified for all maxima shown, except that only one of the maxima near 260 m\(\mu\) in the phenylalanine perturbation difference spectrum was so checked.

One way to determine the number and type of chromophores released from a protein interior when it is unfolded is to measure the number of chromophores exposed to solvent in both the native and the denatured protein. In carrying out these measurements, by the solvent perturbation method of Herskovits and Laskowski (14), it has been found useful to match the observed perturbation difference spectra with calculated perturbation difference spectra obtained by summation of the difference spectra determined separately for tyrosine and tryptophan (19). In Fig. 6, perturbation difference spectra for native aldolase (neutral pH) and unfolded aldolase (acid pH) are compared with difference spectra obtained by summation of the difference spectra determined separately for tyrosine and tryptophan (19). In Fig. 7, observed perturbation difference spectra at shorter wave lengths are similarly compared with calculated perturbation difference spectra obtained by summation of the difference spectra presented in Figs. 4 and 5.

The perturbation difference spectrum of unfolded aldolase shown in Fig. 7 bears a strong resemblance to difference spectra...
between native and denatured proteins obtained in this wave length region (2). In Fig. 8, the similarity of these difference spectra is shown. The difference spectrum for the acid denaturation of aldolase is compared with the 20% ethylene glycol difference spectrum calculated for the perturbation of 46 - 18, or 28 phenolic and 12 - 2, or 10 indole chromophores. It is also compared with the difference between the experimentally determined perturbation difference spectra of acid-unfolded and of native aldolase. From this figure it is evident that transfer of a chromophore from the interior of a protein into the water solvent

![Image](http://www.jbc.org/content/244/8/1964/F1.large.jpg)

**Fig. 6.** Perturbation difference spectra of rabbit muscle aldolase produced by 20% ethylene glycol, in the longer wave length region. The observed spectra (-----) are compared with calculated perturbation difference spectra (--), calculated for 46 moles of tyrosine plus 12 moles of tryptophan (at pH 1) and for 18 moles of tyrosine plus 2 moles of tryptophan (at pH 6). The perturbation difference spectra were determined at a concentration of $1.70 \times 10^{-4} \text{ M}$, with the use of the 0 to 0.1 absorbance scale of the Cary model 15. The molecular weight of aldolase was taken as $1.58 \times 10^6$ (16).

![Image](http://www.jbc.org/content/244/8/1964/F2.large.jpg)

**Fig. 7.** Perturbation difference spectra of rabbit muscle aldolase produced by 20% ethylene glycol, in the shorter wave length region. See the legend to Fig. 6 for details.

![Image](http://www.jbc.org/content/244/8/1964/F3.large.jpg)

**Fig. 8.** Comparison of the observed acid difference spectrum of aldolase (-----) with calculated difference spectra obtained in two different ways: --, difference between the 20% ethylene glycol perturbation difference spectra for aldolase in acid solution and in neutral solution, multiplied by a factor of 6.8; -- -- --, 29% ethylene glycol perturbation difference spectrum of 28 moles of tyrosine plus 10 moles of tryptophan, calculated from the data of Fig. 4 and multiplied by a factor of 6.0.

results in a perturbation similar in kind to, but about 6 times the magnitude of, the perturbation of that chromophore by 20% ethylene glycol. This has been noted previously for difference spectra obtained at longer wave lengths (19), but appears to be applicable at shorter wave lengths, also.

**Fig. 9.** Difference spectrum produced by the autolysis of pepsin, observed by Glazer and Smith (2) (-----), compared with a calculated difference spectrum (-----) obtained by multiplying the 20% ethylene glycol perturbation difference spectrum of 3 moles of tyrosine and 1.5 moles of tryptophan by a factor of 6.0.
absorption changes observed by Eisenberg and Edsall (8) for the interior of the protein into water solvent. Coincidentally, the unfolding of human serum albumin and reduced carboxymethyl-albumin by acid and by urea (12, 200 at 236 nm and 3,800 at 287 nm) were proportional to the concentration of the absorbing agent, as is likely at the low concentrations used here (but which was not checked), then the measured difference in absorption between these solutions, although too low by a constant factor, would appear to obey Beer's Law. For equal displacements of their spectra, tryptophan would tyrosine.

In Fig. 10, the acid difference spectrum of ovomucoid (which contains no tryptophan) is compared with a difference spectrum calculated for the transfer of 6 phenolic chromophores from the interior of the protein into water solvent. Coincidentally, the absorption changes observed by Eisenberg and Edsall (8) for the unfolding of human serum albumin and reduced carboxymethyl-albumin by acid and by urea (12, 200 at 236 nm and 3,800 at 287 nm) are nearly identical with the absorption changes shown in Fig. 10.

**DISCUSSION**

**Important Experimental Factors**—Both the Cary model 15 and the Turner model 210 have been used in these experiments to rule out artifacts arising from solution conditions or from limitations in design of the apparatus. Ordinarily, adherence to Beer's Law (the linear dependence of absorption upon concentration) is a sufficient criterion for eliminating experimental artifacts. However, in the present experiments, the solution containing the perturbing fluorescence more strongly than the solution which contains no perturbant, as Fig. 1 shows. If this fluorescence were proportional to the concentration of the absorbing agent, as is likely at the low concentrations used here (but which was not checked), then the measured difference in absorption between these solutions, although too low by a constant factor, would appear to obey Beer's Law. The use of the Turner instrument to check the results obtained with the Cary model 15 insures that this particular error has not been introduced into these measurements. However, there were some small differences between the perturbation difference spectra obtained with these two instruments. The maxima in the difference spectra of the amino acids were occasionally displaced as much as 1 μm from one instrument to the other, although the molar difference absorption coefficients were in agreement in magnitude. In the 240 to 250 nm region, where the curvature in the spectrum of tyrosine and tryptophan is large but the absorption small, the Turner instrument recorded the difference spectrum as slightly negative (apparent Δε soak ≈ -30 for tyrosine). The essentially zero value recorded in this region by the Cary instrument has been assumed to be correct, and the results obtained with the Turner instrument have been tentatively ascribed to the use of the large (fixed) slit width of 25 A in this region of large change in the curvature in the spectrum of the amino acids tyrosine and tryptophan. Gibson (26) has discussed the relation between the slit width and the curvature (d²ε/dX²) of the absorption spectrum in terms of the magnitude of the error in the absorption measurement.

**Absorption Changes of Amino Acids**—From the solvent perturbation difference spectra of Figs. 4 and 5 it is clear that, except in cases of unusual protein composition, neither cystine nor histidine! will contribute much to the absorption changes observed for proteins at short wave lengths. Phenylalanine residues undergo substantial changes in absorption (comparable to those of tyrosine), but since their absorption is displaced about 10 μm to shorter wave length, the contribution to the absorption change near 290 μm will be relatively small. The double peak in the difference spectrum of phenylalanine at short wave lengths appears to be real—both peaks obey Beer's Law, and the absorption spectrum of phenylalanine shows three points of inflection between 230 and 210 μm.

A remarkable aspect of the results presented in Figs. 4 and 5 is the large change in absorption produced at shorter wave lengths when the indole chromophore is perturbed. For an equivalent transfer from perturbing solvent into water, one indole chromophore produces about as much change in absorption as seven phenolic chromophores. This difference is not unexpected. For a perturbation which produces a simple wave length displacement of the spectrum, the absorption difference between the displaced and the undispersed spectra is given by the product of the wave length shift and the slope of the absorption spectrum (27, 28).

\[
\Delta \varepsilon \approx \Delta \lambda \left( \frac{d \varepsilon}{d \lambda} \right)
\]

Although tyrosine and tryptophan have approximately the same absorption near 230 μm, the slope of the absorption spectrum of tryptophan is considerably greater than that of tyrosine (29). Thus, for equal displacements of their spectra, tryptophan would be expected to show a much larger absorption difference than would tyrosine.

**Absorption Changes of Proteins**—The acid difference spectrum of aldolase has been presented previously (15, 19, 30). The acid difference spectrum shown in Fig. 8 has been redetermined, and presented on the basis of molecular weight 189,000 (10). Beer's Law has been verified for the short wave length region. Stellwagen and Schachman (15) have presented evidence that the aldolase molecule is highly disorganized at acid pH, and previous perturbation difference spectra determined in the 270 to 300 nm region indicate that 2 indole and 4 or 5 phenolic chromophores are buried in native pepsin. This ratio of phenolic to indole chromophores (approximately 2:1) gives the best fit to the data of Glazer and Smith. However, the total change in absorption observed by Glazer and Smith is less than expected from complete liberation of 4 phenolic and 2 indole chromophores, and may be the result of incomplete autolysis.

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\[
\Delta \varepsilon \approx \Delta \lambda \left( \frac{d \varepsilon}{d \lambda} \right)
\]
220 μm wave length range have been interpreted on the basis that all chromophores are exposed to solvent at acid pH (19, 25). The observed solvent perturbation of unfolded aldolase in the 220 to 240 μm region (Fig. 7) is close to that calculated with the use of the perturbation difference spectra of the amino acids tyrosine and tryptophan, shown in Fig. 4.

The fact that the short wave length perturbation difference spectra of both the native and the unfolded protein can be satisfactorily accounted for by a consideration of the perturbation of only the side chain phenolic and indole chromophores strongly suggests that all changes in absorption produced by the unfolding of the protein are due to changes in the environment of these chromophores, and not (within experimental error) to changes in arrangement or environment of other portions of the protein molecule, in particular, its polypeptide backbone.

As discussed previously (19), determination of the difference between the solvent perturbation difference spectra of a protein in its unfolded and in its native conformations is equivalent to the determination of the solvent perturbation difference spectrum of those chromophores which are buried in the native protein. However, in the native protein, these chromophores are perturbed by a different “solvent,” their surroundings (the local protein interior). The protein is a stronger perturbant than 20% ethylene glycol appears to be reasonable. Bays and Ellodi (31) for dehydrogenase enzymes, on the basis of experiments similar to those presented here. Absorption changes resulting from the denaturation of proteins by urea can also be accounted for in the same way, since several instances have been presented in which urea denaturation produces essentially the same changes in absorption as denaturation by acid. For example, Eisenberg and Edsall (8) state that denaturation of albumin by 8 M urea gives the same ratio of ΔεH2O:ΔεD2O as treatment of albumin with acid does, and Stellwagen and Schachman (15) show that denaturation of aldolase by urea results in substantially the same absorption change as denaturation of aldolase by acid does.

From what is known about shifts of absorption spectra, the factor of 6 relating the shift produced by a protein to that produced by 20% ethylene glycol appears to be reasonable. Bays and Ellodi (32) suggests that the shift (in wave numbers) of a spectrum of a chromophore immersed in a solvent of refractive index n is given by

\[ \Delta \delta \text{ (vapor \rightarrow solvent)} = \text{constant} \cdot \mu^2 \left( \frac{n^2 - 1}{2n^2 + 1} \right) \]  

Here, the reference spectrum of the chromophore, from which the displacements are measured, is the vapor spectrum and \( \mu^2 \) is proportional to the transition probability, or absorption coefficient. If the denaturation shift in the absorption spectrum of a protein is assumed to arise from polarizability effects alone, it may be treated as the difference between two polarizability shifts, one for the chromophore placed within the protein, the other for the chromophore placed in the water solvent. If the transition probability can be considered constant, then only the term in \( n \) need be considered, and approximately

\[ \Delta \delta \text{ (water \rightarrow protein)} = \Delta \delta \text{ (vapor \rightarrow protein)} - \Delta \delta \text{ (vapor \rightarrow water)} \]

or

\[ \Delta \delta \text{ (water \rightarrow protein)} = \text{constant} \left( \frac{n^2 - 1}{2n^2 + 1} \right) \text{protein} - \left( \frac{n^2 - 1}{2n^2 + 1} \right) \text{water} \]

A similar relation would hold true for a perturbant such as ethylene glycol. The refractive indices of water, ethylene glycol, and n-heptane (a model for the hydrocarbon side chains of a protein) are 1.33, 1.43, and 1.40, respectively. Values of \( (n^2 - 1)/(2n^2 + 1) \) for these solvents are 0.171, 0.206, and 0.215, respectively. For n-heptane, the term in brackets on the right hand side of Equation 3 is thus 0.044. For 100% ethylene glycol, the corresponding term is 0.035. Thus, a protein interior might produce a perturbation 0.044/0.035 greater than 100% ethylene glycol, or roughly 6.3 times that produced by 20% ethylene glycol (assuming linearity of refractive index with concentration of ethylene glycol).

This agreement with experiment should be accepted only in a qualitative sense, since (a) the polarizabilities of the side chains of a protein vary according to their nature, sulfur-containing side chains being more polarizable than the others, (b) the individual chromophores in the same protein will differ in their local environments, and (c) the theoretical relation (Equation 2) above is based on many simplifying assumptions, which include treating the chromophore as an isotropic sphere within a continuous medium of refractive index n. Nevertheless, this calculation indicates that the relative perturbation of a chromophore by the protein, when compared to ethylene glycol, is of the correct order of magnitude. Differences between the environments of chromophores in different proteins ought to affect the character of the difference spectra produced on denaturation. A marked difference among the denaturation difference spectra of Figs. 8, 9, and 10 is the difference in wave length between the observed difference peak and the calculated one. This difference is about 4, 6, and 10 μm for pepsin, aldolase, and ovomucoid, respectively. Although the sulfur content of aldolase and pepsin is about the same, ovomucoid contains more than twice as much sulfur as either. Thus, the average chromophore environment in ovomucoid may be quite polarizable.

Since the discrepancies between the calculated and observed difference spectra for denaturation of these proteins are quite small, it appears that, as a generous estimate, no more than 10%
of the observed absorption change for these proteins in the 230
nm region can be due to changes in the absorption of the poly-
peptide backbone. This conclusion is likely to be general, but
exceptions should be expected for proteins which contain large
amounts of helix. In addition, anomalies (for example, $\Delta_{\text{obs}}$
$\Delta_{\text{cal}}$ ratios greater than 8) may be expected for proteins
which have unusual amino acid compositions, or in which denaturation
results in disulfide bond hydrolysis (34), ionization of phenolic,
sulfhydryl, or imidazole chromophores, or other chemical re-
actions such as Maillard reactions (35) in the case of glycopro-
teins.

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