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THE ULTRAVIOLET FLUORESCENCE OF PROTEINS

I. THE INFLUENCE OF pH AND TEMPERATURE*

ROBERT F. STEINER AND H. EDELHOCH

Naval Medical Research Institute, National Naval Medical Center and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

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SUMMARY

The intensity of the ultraviolet fluorescence of a group of extensively studied, highly purified proteins has been investigated as a function of pH and temperature. In those proteins whose structures are believed to be invariant to pH, the effects of pH were generally small at pH values acid to the ionization of their tyrosyl groups. In the latter pH zone all proteins showed significant quenching of fluorescence which presumably resulted from the radiationless transfer of energy from tryptophanyl to ionized tyrosyl residues. In the pH zones where configurational transitions (reversible or irreversible) have been demonstrated by other procedures, these structural changes resulted in modifications in fluorescent intensity in all of the proteins studied. The influence of temperature on protein fluorescence in pH zones of thermal stability has been delineated in the case of several proteins. Deviations from a characteristic temperature dependence of fluorescence was observed in those pH zones in which the protein was known to undergo thermal denaturation. It would appear therefore that the observation of fluorescence intensity of protein solutions should provide a rapid and sensitive means of detecting and monitoring changes in protein structure.

INTRODUCTION

The property of ultraviolet fluorescence, which is shared by virtually all proteins, is conferred by the presence of tryptophan or tyrosine[1-3]. The emission of the third fluorescent amino acid, phenylalanine, has not been detected in native proteins. However, if both amino acids are present, even in disproportionate numbers, it has been shown by WEBER[2] and by TEALE[3] that the emission spectrum is predominantly that of tryptophan. Even in the case of human serum albumin, which contains but a single tryptophan, a relatively sophisticated analysis is required to identify the contribution of tyrosine to the fluorescence[4].

The basis for the suppression of tyrosine fluorescence in the presence of trypto-

Abbreviation: DNS, 1-dimethylaminonaphthalene-5-sulfonyl chloride.

* The opinions and assertions contained herein are those of the writers and are not to be constructed as expressing the views of the Navy Department or the Naval Service at large.

phan is imperfectly understood. It has been possible to rule out the importance of radiationless energy transfer from tyrosine to tryptophan as a process competitive with respect to direct emission[2]. Since the proteins to be discussed in the present paper all contain tryptophan and as measurements of emitted intensity have been confined to the peak of the tryptophan fluorescence spectrum (340–350 mμ), the observations reported here will be essentially concerned with tryptophan fluorescence alone.

TEALE[3] has found wide variations in the apparent quantum yields of tryptophan fluorescence in different proteins. This and the further observation that the quantum yields were markedly altered in the presence of high concentrations of urea and propane-1,2-diol leave little doubt that the intensity of fluorescence of the tryptophan residues of proteins is a sensitive function of their environment. To be sure, the importance of solvent effects remains to be assessed. However, the wide variation in both sign and magnitude of the change in quantum yield for different proteins suggests that the nature of the tryptophan environments in the original intact protein is the controlling factor. Indeed, the rather similar spectral distributions and quantum yields of fluorescence for a number of proteins in 8 M urea[3] is understandable in terms of the transition to a more open structure occurring in this solvent, with a resultant loss of those specific features of the tertiary structure which influence the tryptophan fluorescence in the intact protein.

Further evidence for the dependence of the tryptophan fluorescence upon the detailed molecular organization of the protein comes from the pronounced changes occurring in the presence of detergents[5]. Here also, part of the effect may be non-specific, arising from the binding of detergent[5].

The present paper will be confined to the effects of pH and temperature upon the tryptophan fluorescence of a series of proteins, with particular attention to structural transitions which are known to occur from other procedures. The only group present in unconjugated proteins whose absorption spectrum overlaps the emission band of tryptophan to a sufficient extent to permit quenching by a radiationless energy transfer is the ionized form of tyrosine ($\lambda_{max} = 295$ mμ). Quenching by this mechanism can obviously be of importance at alkaline pH. It is not, however, the sole factor as WEBER[6] has found that tryptophan itself undergoes quenching at pH's above 11. The process has been attributed to the formation of a transient complex of hydroxyl ions with the excited tryptophan molecule.

It is not difficult to list the various factors which would be expected to be of significance in determining the pH dependence of the tryptophan fluorescence of proteins. They are as follows: (a) The degree of shielding of the tryptophans from the solvent. This will depend upon the extent of embedding of these groups in the protein interior and upon the porosity of the protein. The extent of shielding from the solvent will also govern the degree of protection from collisional or other quenching by H^+ or OH^- ions. TEALE[3] has observed for a number of proteins an increase in the apparent quantum yield of tryptophan emission with decreasing dielectric constant of the solvent, which consisted of water and propane-1,2-diol mixtures. This indicates that at least some of the tryptophan groups of the examples cited must be accessible to solvent and suggests that a non-polar environment tends to increase the quantum yield. (b) The importance of non-radiant energy transfer to ionized tyrosine. This will depend upon the numbers and mutual positions of the two groups[7,8].

It can in general be expected to play a role only in the zone of tyrosine ionization. Radiationless transfer to iodotyrosine may also be important when this residue is present[8]. (c) The action of particular neighboring groups, whose influence may depend upon their state of ionization. The occurrence of any transformation in structure would be expected to result in important modifications in some or all of th[illegible]. Indeed, when due care is taken to allow for trivial effects, ultraviolet fluorescence should provide a potential means of detecting such molecular events.

EXPERIMENTAL

Methods

Measurements of fluorescence intensity were made with an Aminco spectrofluorometer which had been modified so as to permit control of temperature. For this purpose a hollow cell-holder, through which water could be circulated, was substituted for that supplied with the instrument. The cell-holder was equipped with entrance and exit slits of 1 mm width. The use of either of two constant-temperature baths, set at 3° and 70°, permitted rapid variation of cell temperature from 15–60°. Temperature was measured directly in the cell, using a thermistor probe of 1 mm dia., encased in a polyethylene sheath. This was supplied by the Yellow Springs Instrument Co.

The existence of considerable unsteadiness in the instrument necessitated a careful attention to controls. In measurements of pH dependence the temperature was normally held at 25°. Alternate readings were made upon a solution at a given pH and upon a reference solution at constant pH. In the case of thermal profiles alternate readings were made upon a solution of varying temperature and a control solution maintained at 25°. The time required for a reading was insufficient to permit any appreciable warming of the control. While the control was being read the other solution was kept in a second external cell-holder, through which water from the same bath circulated.

The concentration of the proteins was held low (0.1 g/l) to avoid any complications arising from attenuation of the beam. The absorbancy at 280 mμ of the solutions measured was never greater than 0.15 in a 1.00-cm² cell.

With the Aminco instrument the monochromatization is dependent upon the slit width. The slits utilized by us were sufficient to reduce the background of scattered or reflected light to a negligible quantity. Normally an excitation wavelength of 290 mμ was employed and observations were made at 340 mμ. With labelled proteins, the excitation wavelength was near the absorption peak of the label, *i.e.*, at 340 mμ for DNS conjugates.

Measurements of ultraviolet absorbancy were made with a Beckman DU spectrophotometer, equipped with a thermostatted cell compartment. Measurements of pH were made with a Leeds and Northrup pH meter, using miniature electrodes. Beckman buffers at pH 4.00, 7.00 and 10.00 were used to calibrate the pH meter.

Titrations were normally performed in the same quartz cuvettes in which the fluorescence measurements were made. Small amounts of HCl or KOH were added to protein solutions at neutral pH to obtain the desired pH. Corrections were made for volume changes.

Materials

Crystalline bovine serum albumin, lysozyme and bovine γ-globulin were purchased from Armour Laboratories. The latter preparation contained about 90% of about 7 S and the remainder was a faster component of about 9 S which is usually present in alcohol-fractionated (Fraction II) γ-globulin preparations. Crystalline pepsin, trypsin, and chymotrypsinogen were purchased from Worthington Biochemical Corp. Crystalline β-lactoglobulin (Form A) was a gift from Dr. P. PFUDERER. Thyroglobulin was prepared from calf-thyroid tissue by a differential centrifugation procedure[10]. Iodination was performed in 8 M urea at pH 9.0. The preparation and properties of iodinated thyroglobulin derivatives are described in separate reports[11]. The iodinated thyroglobulin preparations were dialyzed for several days to remove iodide and other salts and buffers.

All reagents used were of analytical grade. Urea was recrystallized from alcohol. Glass-redistilled water was used in the preparation of all solutions. DNS was purchased from the California Foundation for Biochemical Research. Fluorescent conjugates of DNS were prepared by the method described in earlier publications[12,13]. The concentration of coupled dye was determined from absorbancy measurements at 340 mμ (ref. 12).

RESULTS AND DISCUSSION

I. Effect of pH

Lysozyme. This enzyme provides an example of a protein whose molecular conformation is believed to be invariant to pH, at least within the limits between 2 and 12 (see refs. 14–16). Consequently the dependence of tryptophan fluorescence on the state of ionization of lysozyme can therefore be evaluated. The intensity of lysozyme fluorescence does not depend on pH between about pH 6.0 and 8.5. A uniform minor decrease occurs between pH 5 and 2 (Fig. 1). Changes in the ultraviolet difference spectrum of tryptophan (blue shift) in lysozyme have been reported in this pH zone[15]. Both of these effects presumably result from the influence of the state of ionization of the carboxyl groups of lysozyme.

At pH values alkaline to about 8.5, a major quenching of fluorescence occurs with increasing pH (ref. 17). The quenching is in approximate accord with the ionization of tyrosine, as determined by spectrophotometric analysis[14,16]. The reversibility of the alkaline branch of the pH dependence of fluorescence indicates that the environments of the tryptophans are unchanged by an alkaline cycle between neutrality and pH 12. Similar reversibility was found in the titration curve of lysozyme and precludes any irreversible change in structure in this region[14].

The data for lysozyme illustrate three features, found to recur for many proteins, which depend primarily on the state of ionization of the protein. (1) The quenching in the carboxyl titration zone, which appears to be non-specific; (2) the constancy of intensity in the neutral pH range where imidazoles normally ionize; (3) the alkaline quenching which usually parallels the ionization of the tyrosyl residues. The behavior described above is to be expected in configurationally stable proteins. Where transitions occur additional modifications in fluorescence will be superimposed on the above.

Ovalbumin. The alkaline branch of the pH profile of fluorescence intensity for this protein is somewhat different from the other cases considered. In the absence of

added electrolyte the intensity is essentially constant between pH 5 and pH 10.5 (Fig. 2). Alkaline quenching begins to be appreciable only above the latter pH. Thus the quenching curve is displaced by several pH units to the alkaline as compared with that of lysozyme. If back-titration to neutrality is made from pH 12.0, only a marginal degree of hysteresis is observed (Fig. 2). If the reversal is made from pH 12.6 the divergence between the forward and reverse branches is much greater. Indeed recovery is incomplete under these conditions even at neutral pH.

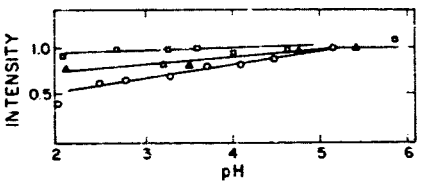

Fig. 1. The pH dependence of fluorescence intensity in the acid region for three proteins in water whose structures are independent of pH under these conditions. O—O, ovalbumin; Δ—Δ, pepsin; □—□, lysozyme. Unless otherwise specified the following conditions apply in all figures: (1) The wavelengths of activation and emission are 290 and 340 mμ, respectively. (2) The temperature is 25.0°. (3) The protein concentration is close to 0.10 g/l. (4) No salts or buffers have been added to the water solution of the protein.

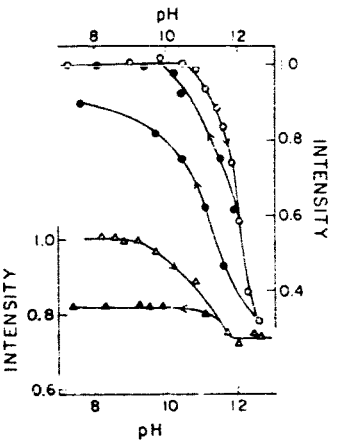

Fig. 2. The alkaline pH dependence of fluorescence intensity for ovalbumin in water (O, ●) and in 8 M urea (Δ, ▲). Filled points represent reversals. Intensities are not the same in water and urea solvents. Both have been normalized to 1.00 at neutral pH values.

To some degree these results are predictable in terms of the ionization of the tyrosyl groups in ovalbumin. In this protein CRAMMER AND NEUBERGER[18] showed that only about two of the tyrosyl groups have normal pK values. The rest of the groups ionizes at pH values greater than about 12 and shows major hysteresis effects on back-titration. The ionization of these latter groups is associated with the denaturation of the protein.

The pronounced hysteresis between the forward and reverse branches of the alkaline cycle to pH 12.6 (Fig. 2) is consistent with the occurrence of a major and irreversible structural change at pH's above 12. If radiationless energy transfer to ionized tyrosine is a dominant factor in the alkaline quenching, it is probable that the hysteresis arises in part from the normalization of tyrosine ionization resulting from this structural transition.

It is of interest that the hysteresis between the forward and reverse branches of an alkaline cycle persists in 8 M urea (Fig. 2). The failure of this denaturing solvent to render the alkaline pH profile reversible suggests that a fraction of the original structure is refractory to its action at neutrality but becomes labile upon exposure to high pH under these conditions.

It is at present uncertain whether any structural change occurs for ovalbumin at acid pH. YANG AND FOSTER[19,20] have reported that only very minor increases in specific viscosity and optical rotation occur at low pH. The intensity of fluorescence

of ovalbumin shows a significant decrease between pH 5 and 2 (Fig. 1). This is entirely reversible. If the difference spectrum at pH 2.55 in water is determined with respect to a reference solution at pH 5.1, a negative peak at 292 mμ is observed[21]. This peak arises from the tryptophan residues and is indicative of an influence of the state of ionization of the protein upon the electronic structure of these groups. The question of whether some subtle structural change shares the responsibility for the acid quenching with a non-specific pH effect must be left open for the present.

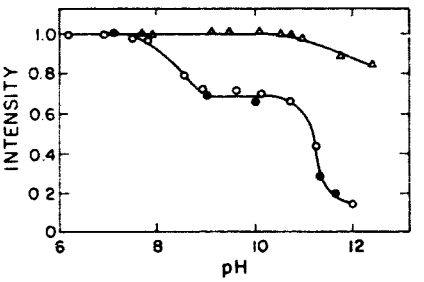

Fig. 3. The alkaline pH dependence of fluorescence intensity for bovine serum albumin in water (○, ●) and in 8 M urea (△, ▲). Filled points represent reversal from pH 12 (H_2O) and 12.5 (8 M urea).

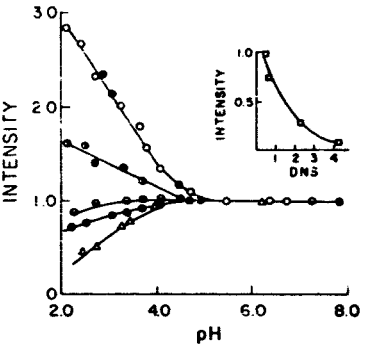

Fig. 4. The acid pH dependence of fluorescence intensity for DNS conjugates of bovine serum albumin (0.20 g/l) in water. △, unsubstituted; ◐, 0.46 DNS groups per molecule; ⊖, 0.53 DNS; ◑, 2.3 DNS; ○, 4.4 DNS; ●, 4.4 DNS, reversal from pH 2.1. Inset: effect of degree of labeling on relative fluorescence intensity at pH 6.0 in water. The abscissa represents the number of DNS groups conjugated per molecule of protein.

Non-conjugated bovine serum albumin. The pH dependence of fluorescence intensity between pH 2 and 12 can be discussed in terms of several distinguishable zones, as has already been reported[17]. In the absence of electrolyte two essentially constant regions, between pH 4.0 and 7.5 and between pH 9 and 10.5, are separated by a zone in which the intensity changes by about 30% (Fig. 3)*. The pH range of this quenching corresponds to that in which major changes in the binding of dyes and calcium ions have been reported[22,23]. On the basis of the binding studies, KLOTZ *et al.*[22] suggested that a molecular transition occurs in bovine serum albumin in this pH zone in spite of the fact that none was evident from those methods commonly employed to detect changes in the molecular structure of proteins. WILLIAMS AND FOSTER[24] have found a minor perturbation in the spectra of an anthracene conjugate to serum albumin in this pH region. It would appear therefore that fluorescence-intensity measurements may be capable of detecting relatively minor conformational changes where other methods are insensitive.

* The intensity of fluorescence of two different samples of purified human serum albumin was also examined as a function of pH. However, a pronounced divergence in behavior of the two in the pH region 4–2 was observed. One sample (supplied by L. RODNEY which had been through a Dintzis-type ion-exchange column) showed a significant (20%) exaltation in this zone. The other (supplied by D. GOODMAN, purified by extraction with 5% glacial acetic acid–isooctane) showed a minor (10%) quenching. While their behavior in the alkaline region was qualitatively similar to that of bovine albumin, no discussion of human albumin will be attempted here because of this irreproducibility.

Above pH about 11.5 the fluorescence intensity shows a further decrease with increasing alkalinity (Fig. 3). This quenching occurs in the pH region of tyrosyl ionization[25] and results, at least in part, from a non-radiative transfer. In 8 M urea, the fluorescent change associated with the transfer mechanism is preserved, although a considerable diminution results in the degree (Fig. 3). The transition occurring between pH 7.5 and 9 in water is presumably eliminated by the unfolding that takes place in 8 M urea.

As has been found in the case of other parameters, the fluorescence-intensity changes occurring between pH 2 and 12 are reversible. In the acid pH zone a notable fall in intensity occurs between pH about 4 and 2 (Fig. 4)[17], the degree of this quenching being much more pronounced than in the case of pepsin or lysozyme. An increase in ionic strength reduces the magnitude of the effect[17]. While the zone of acid quenching corresponds to that of the well-studied acid structural transition, it is not feasible to attribute it entirely to this factor, in view of the similar (although smaller) quenchings which occur in other proteins whose structures are believed to be invariant to pH, or almost so, in this region. In all probability what is observed is the resultant of the non-specific effect of carboxyl neutralization and the change in the environment of the tryptophans accompanying the acid structural change. It is presumably the latter component which is suppressed by an increase in ionic strength, in view of the smaller optical rotatory, fluorescence-polarization, viscosity and sedimentation changes observed for high levels of electrolyte[12,20,26].

Conjugated bovine serum albumin. Conjugation of bovine serum albumin with DNS produces a marked quenching of the tryptophan fluorescence when measured at neutral pH at 340 mμ (inset, Fig. 4). However, concomitant with the quenching observed at 340 mμ, there appears a fluorescent band with a peak near 520 mμ which corresponds to that of DNS fluorescence. As seen in Fig. 4, the tryptophan fluorescence of the conjugates is very dependent on the pH below about 5. The acid quenching of serum albumin is progressively replaced by an exaltation whose magnitude increases with increasing degree of substitution. These effects may be understood in the following way. Since the absorption band of the DNS chromophore (λ_{max} approx. 340 mμ) overlaps the tryptophan emission band extensively, energy transfer must be an important factor in the quenching observed at neutral pH (ref. 7). The partial repression of this transfer in acid solutions is probably a consequence of the inflation of the albumin molecular domain, which would be expected to increase the average separation of the tryptophan and DNS groups[20,26,27]. The proportionality of the efficiency of transfer to the reciprocal sixth power of the separation of absorbing and emitting groups[7,8] renders this phenomenon very sensitive to structural modifications.

The quantum yields and pH profiles of the fluorescence of DNS itself will, of course, depend upon the specific environments of the labels and hence upon the degree of substitution. Nevertheless, the alkaline pH profiles of DNS conjugates spanning a wide range of levels of substitution show certain features in common. In all cases a zone of gradual increase of fluorescent intensity with pH is terminated by an abrupt drop at about pH 11.5. Fig. 5 illustrates this for several conjugates with different degrees of labelling. This pH is indeed very close to the region of extensive structural change, as reflected by changes in fluorescence polarization and in the electrostatic free-energy factor deduced from potentiometric experiments[12,25].

Bovine γ-globulin. The fluorescence intensity does not change significantly between pH 3.5 and 9. Above pH about 9 the usual alkaline quenching commences. However, beginning at about pH 11.5, time-dependent increases in intensity are observed (Fig. 6) which, upon back-titration to neutrality, increase even further. Similar time-dependent changes are found at pH's below 3.5, which are also irreversible (Fig. 6). The pH dependence of fluorescence exaltation parallels those of loss in solubility (denaturation) and increase in specific levorotation[28]. It is of interest that time-dependent changes in the intensity of fluorescence of a DNS conjugate of γ-globulin first begin to occur at approximately the same alkaline pH at which the natural fluorescence shows time effects[29]. The pH-stability zone of γ-globulin therefore would not appear to be materially affected by the conjugation of dye. Polarization of fluorescence measurements indicates that bovine γ-globulin is stable between pH about 4 and 9 and shows minor, reversible changes between pH 9 and 11. By all criteria the transitions occurring in the acid and alkaline regions (below pH 3 and above pH 11) show only partial reversibility. Reversal of pH to a neutral value leads to extensive aggregation which obviously precludes precise analysis of the degree of reformation of the native structure[28].

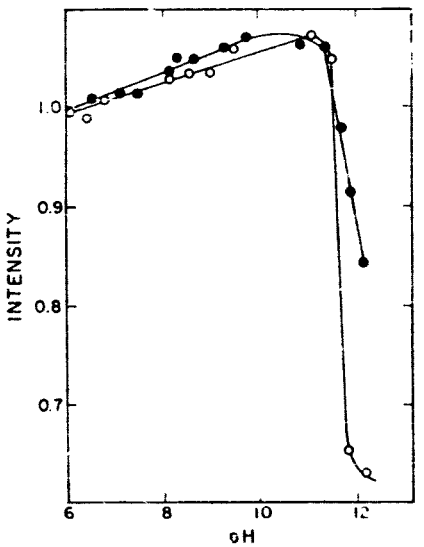

Fig. 5. The alkaline pH dependence of the visible fluorescence intensity for DNS conjugates of bovine serum albumin in H_2O. The wavelengths of activation and emission are 340 and 520 mμ, respectively. O, 0.46 DNS groups per molecule; ●, 2.3 DNS.

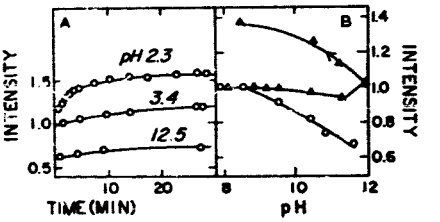

Fig. 6. Right: the pH dependence of fluorescence intensity for bovine γ-globulin in 0.10 M KCl (O) and in 8 M urea (△, ▲). The data in 0.10 M KCl showed time effects at pH values alkaline to 11. Filled points represent reversals. Left: effect of pH on the kinetics of fluorescence-intensity changes in 0.10 M KCl.

Major exaltation in fluorescence results also from an alkaline cycle in 8 M urea (Fig. 6). Consequently at least part of those structural characteristics of native γ-globulin, which result in partial suppression of tryptophan emission, must be refractory to this level of urea at neutral pH. The combined action of urea and alkaline pH is required to disrupt this residual structure and permit a major exaltation in fluorescence. The observation is consistent with other measurements which indicate an important loss in structure under these conditions[28,29].

β-Lactoglobulin. In contrast to the proteins discussed above, β-lactoglobulin in water shows a minor variation in tryptophan-emission intensity between pH's 7 and 9.5 which is completely reversible (Fig. 7). A molecular transition has been postulated

to occur in this pH zone, based on modifications in the optical rotation, sedimentation and proton-binding properties of β-lactoglobulin[30]. The fluorescence change is rather marginal in this case.

The fluorescence-intensity curve shows a continuous decline at pH values greater than about 10. Back-titration to neutral pH values results in an exaltation in intensity. Irreversible denaturation is known to occur at alkaline pH values. The effects of the structural changes associated with denaturation are obviously masked by the alkaline quenching due to transfer to ionized tyrosine in this pH region.

The acid branch of the pH profile of intensity shows a major exaltation at pH's below about 6 (Fig. 7).

Pepsin. In the absence of added electrolyte an irreversible denaturation of pepsin occurs in the pH range 7.5–8.5. The pH dependence of rate is very steep, the velocity of denaturation changing from a negligibly slow to an extremely rapid value in less than a pH unit[31,32]. An increase in ionic strength serves to displace the denaturation zone to lower pH's.

The molecular changes accompanying the denaturation process are unusually drastic in this case. Indeed the product has many of the characteristics of unorganized polyelectrolytes, including the contraction of the molecular domain by increasing ionic strength, as indicated by a marked drop in intrinsic viscosity[31].

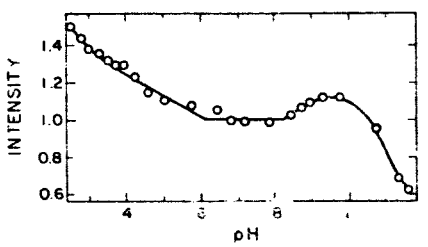

Fig. 7. The pH dependence of fluorescence intensity for β-lactoglobulin in 0.01 M KCl. The acid and alkaline branches were obtained independently by titrating solutions from pH 6.0. The data between pH 6 and 9 were reversible.

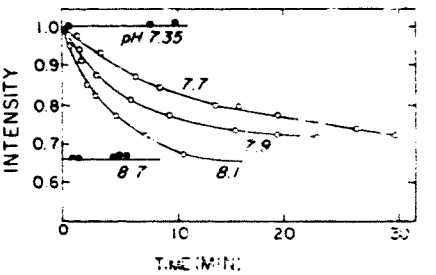

Fig. 8. The effect of pH on the kinetics of the alkaline transition of pepsin. All solutions contained 2.5 mM Tris and 2.5 mM KCl.

The fluorescence intensity of pepsin shows a minor decrease at pH's above 4 (Fig. 1). Inasmuch as no evidence exists for any structural change in this zone of pH, it is logical to attribute the observed minor quenching to non-specific factors, as in the cases of ovalbumin and lysozyme. The acid ultraviolet difference spectrum is similar to that observed in the latter two cases[33].

In the absence of added electrolyte a major and time-dependent quenching is found in the pH zone 7.5–8.5 (Fig. 8). At higher pH's, beginning at about 10.5, a further quenching occurs, which is not time-dependent and which probably corresponds to the usual alkaline quenching. Back-titration from a pH above the transition zone shows a major divergence between the forward and reverse branches[17].

It seems clear that the time-dependent quenching at pH 7.5–8.5 is a consequence of the irreversible denaturation occurring in this same range of pH. The dependence of quenching rate upon pH parallels the pH profile of inactivation under the same

conditions sufficiently closely to leave little doubt as to the relatedness of the two processes.

In contrast to the case of many of the other structural transitions dealt with in this paper, the degree of fluorescence quenching shown by pepsin in the denaturation zone is very dependent upon the ionic strength. At an ionic strength of 0.20 the denaturation quenching is almost abolished[17]. Above pH about 9 a notable decrease occurs which reflects a quenching by energy transfer to ionized tyrosine residues[17].

It does not seem likely that the denaturation quenching of pepsin is a direct consequence of the ionization of several anomalous carboxyl groups which accompanies the process[32], inasmuch as the sign of the change is opposite to that occurring upon ionization of carboxyls in other proteins (Fig. 1). It appears most likely to be a direct result of the loss in molecular organization *per se*, which causes a major modification in the environment of the tryptophans. The suppression of the quenching at higher ionic strengths is almost certainly a consequence of the shrinkage of the molecular domain occurring under these conditions. The return to a more compact state apparently restores to some degree an environment resembling the original.

Thyroglobulin. The fluorescence intensity of unmodified native thyroglobulin, of low iodine content, does not vary in the neutral pH zone (Fig. 9). A quenching occurs above pH about 10 which parallels the ionization of tyrosine. The reverse branch of an alkaline cycle is displaced below the forward branch (inset, Fig. 9). Some degree of hysteresis can be accounted for by the shift in pK of the tyrosines accompanying the alkaline denaturation. In addition, the disruption of molecular organization found at pH about 12 is incompletely reversed on back-titration to neutrality[34]. Consequently the failure to recover the initial intensity after neutralization presumably reflects the altered environment of the tryptophans.

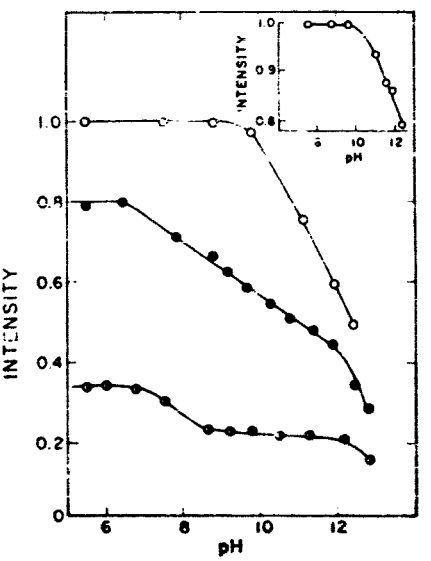

Fig. 9. The alkaline pH dependence of fluorescence intensity for thyroglobulin of varying degree of iodination. ○, native thyroglobulin; reacted with 55 moles (●) and 220 moles (●) of iodine per mole of protein. Inset: relative intensity of native thyroglobulin at pH 7.8 after 30 min at indicated pH. Solvent is 0.01 M Tris and 0.01 M KCl.

Iodination of the tyrosines in thyroglobulin has a profound effect upon its fluorescence (Fig. 9). In addition to a major fall in emission intensity which accompanies iodination, a progressively larger fraction of the overall quenching is displaced to less alkaline values (Fig. 9). These findings are explicable in terms of the ionization and spectral changes produced by the iodination of tyrosine. The pK of the tyrosine phenolic hydroxyl group decreases from 10.1 to 8.2 for monoiodotyrosine and to 6.4 for diiodotyrosine[35]. The absorption maxima of the unionized forms increase from 275 to 283 and 287 mμ, respectively[36]. The peaks of absorption of the ionized forms occur at 293, 305 and 311 mμ for tyrosine and its two iodinated derivatives, respectively[35,36]. The spectral overlap of the absorption bands of the iodotyrosines with the emission band of tryptophan leads to the prediction that radiationless energy transfer to iodotyrosines, as a process competitive with respect to emission[7-9], should increase in importance with increasing extents of iodination and also that the pH zone of variation of quenching should be shifted toward neutrality. This is in accord with what is observed.

II. Thermal dependence

There has been observed in all cases a monotonic decrease in fluorescence intensity with increasing temperature if no structural change occurs in the range of temperatures studied[37]. In most cases the variation is almost linear. If the structure is invariant to temperature, the curve is completely reversible. In general, a thermally induced transition can be reflected by (a) a change in slope of the quenching curve, (b) the appearance of irreversibility, (c) time-dependent intensity changes.

Lysozyme. The thermal quenching curve for lysozyme is nearly linear between 15° and 55° at both pH 2.3 and pH 6.4. This is in harmony with the absence of any reported change in the structure of lysozyme in this pH zone and temperature range[14,16,19]. Thus uncomplicated fluorescent behavior would be expected. It is possible that the thermal quenching results from enhanced collisional deactivation by the solvent of tryptophans in the excited state. If this is the correct mechanism, it follows that some fraction of the tryptophan residues must be accessible to solvent.

γ-Globulin. At pH 7 there is no evidence for any change in the molecular organization of purified rabbit antibody at temperatures up to 60°. Thus it is not surprising to find that the quenching of fluorescence is completely reversible and almost linear with respect to temperature[37]. However, at pH 11.2 in 0.1 M KCl, the behavior of bovine γ-globulin is quite different. Beginning at about 35° the slope of intensity *versus* temperature becomes increasingly less negative and becomes positive above 50°. Upon cooling from 60° the reverse half cycle shows a major exaltation over the forward curve. The behavior of rabbit γ-globulin at pH 11.2 is similar to that of rabbit antibody at pH 11.8 (ref. 37).

Pepsin. The characteristic denaturation of pepsin[31,38] can be brought about thermally as well as by an alteration of pH. The effect of an increase of temperature is to lower the pH at which the rate of transformation begins to be appreciable, and *vice versa*.

In 2.5 mM KCl, 2.5 mM Tris buffer (pH 5.9) the intensity falls linearly between 15° and 55°. At pH 6.1 the thermal profile of intensity again shows a linear decrease until about 50°. At higher temperatures a zone of rapid fall of intensity with temperature is reached. With increasing pH the transition region moves to progressively

lower temperatures until, at pH 7.5 and above, denaturation is complete at the lowest temperatures examined and linearity is regained. The data closely resemble that reported previously at somewhat lower ionic strength[37].

Upon cooling from a temperature above the transition zone the initial thermal profile is not retraced. Instead the reverse curves are in fact essentially superimposable upon the thermal profile for the completely denatured protein at pH 7.5. As in the case of the alkaline denaturation occurring at room temperature, the quenching is suppressed by the presence of a high concentration of added electrolyte (*i.e.*, 1 M KNO_3).

Chymotrypsinogen. It has been known since the work of EISENBERG AND SCHWERT[39] that α-chymotrypsinogen at pH 2 undergoes a reversible thermal denaturation beginning at about 35°. The criterion employed to study the kinetics and equilibria of the transformation was the loss in solubility in 1 M NaCl (pH 3.0), upon rapid cooling to room temperature. The above authors failed to find a change in any other molecular parameter that they investigated as a result of this transition.

At pH 7.0, the thermal-quenching profile of α-chymotrypsinogen shows only a continuous, nearly linear decrease in intensity with temperature, with no sign of a significant change in slope (Fig. 10). This is in harmony with the absence of any reported structural change below 60° under these conditions. At pH 1.9, however, a small but definite anomaly appears in the quenching curve. Zones of roughly linear intensity-decrease at low and high temperatures are separated by an intermediate flat region where the intensity is almost invariant to temperature (Fig. 10).

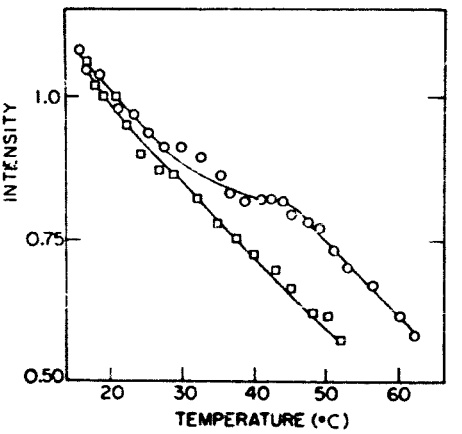

Fig. 10. Thermal dependence of fluorescence intensity for α-chymotrypsinogen in water at pH 7.0 (□) and pH 1.9 (○).

Inasmuch as the intermediate zone of transition corresponds fairly closely to that of the reversible denaturation, as reported by EISENBERG AND SCHWERT, it is reasonable to attribute it to an exaltation of fluorescence accompanying the continuous conversion of native to denatured protein. This exaltation is sufficient to compensate for the normal thermal quenching and results in the flat appearance of the transition zone. The linear decrease of intensity with temperature at higher and lower temperatures must represent the thermal quenching of native and denatured chymotrypsinogen, respectively.

CONCLUSIONS

It is clear from the limited data which have been collected that the quantum yield of fluorescence of the tryptophan residues depends in a complex way on the molecular structure and state of ionization of the protein. Among the generalizations which can be made is that proteins which contain tyrosyl residues show an important degree of quenching at alkaline pH. The zones of quenching appear to correspond approximately to those of tyrosyl ionization, thereby strengthening the idea that non-radiative energy exchange with the latter groups is an important factor in this process. However, this is not likely to be the sole factor in view of the observation of WEBER[6] that tryptophan itself is quenched by hydroxyl ion, a result which he has attributed to the formation of a transient complex with the excited state.

At present it is not possible to sort out completely the quenching due to transfer to ionized tyrosine from that arising from complex formation of hydroxyl with tryptophan since considerable overlap exists in their pH dependence. It is worthy of mention that they should be affected in opposite ways by any major loss of structural organization. If, as is usually the case, the latter is accompanied by some degree of expansion of the molecule, the average separation of tryptophans and tyrosines should be increased, resulting in a reduced efficiency of transfer[7,8]. On the other hand the more open structure of the unfolded molecule should result in greater accessibility to solvent and hence in enhanced susceptibility to quenching by complexing or collision with hydroxyl ion, barring of course some compensating solvent effect. The observation that the overall degree of alkali quenching is usually reduced in the presence of 9 M urea suggests that quenching through transfer may be the more important mechanism.

In spite of the complications described above which arise in alkaline solutions it is possible, in many cases, to resolve these from the effect of a true structural transition, especially if the latter is irreversible or time-dependent. Thus the major degree of divergence between the forward and reverse branches of the alkaline pH profiles of ovalbumin, γ-globulin, thyroglobulin, and pepsin are entirely consistent with the irreversible structural changes known to occur in these cases. The highly specific influence of the tryptophan environments is demonstrated by the dramatic differences in the above examples as to both the magnitude and sign of the irreversible changes in intensity resulting from an alkaline cycle.

Non-specific quenching appears to occur in the zone of carboxyl ionization for several proteins, whose structures are believed to be invariant to pH in this region. This is most logically regarded as arising from a vicinal perturbation of the electronic state of particular tryptophans by adjacent carboxyls. It is presumably analogous to the non-specific perturbation of the tryptophan absorption band of several proteins in this region. In the cases of pepsin, ovalbumin, and lysozyme the non-specific quenching occurring in the pH zone 2–5 is paralleled by the development of a characteristic difference spectrum in the tryptophan band at 293–295 mμ.

In the case of bovine serum albumin it is difficult to resolve the effects of the non-specific quenching from those resulting from the acid structural transition. However, the reduction in magnitude of the total quenching at high ionic strengths suggests that the structural transition likewise results in quenching. Ultraviolet difference spectra have been reported also with bovine serum albumin and have been correlated with structural modifications.

Three instances have been observed in which a definite enhancement of fluorescence occurs in the acid pH region. In each case it appears likely that a definite conformational change is responsible for the changes observed in intensity. The acid pH profile of the exaltation of fluorescence of bovine γ-globulin parallels so closely those of solubility, optical rotational, and molecular-kinetic changes that there can be little doubt that they reflect basically the same molecular events[28,29]. The question of precisely which molecular features result in the exaltation must remain open for the present. The enhancement observed in the case of β-lactoglobulin possibly is related to the dissociation of the molecule which occurs at acid pH values.

It is of considerable interest that the visible fluorescence of DNS conjugates of serum albumin displays an abrupt drop at a pH (about 11.6) close to that at which a major structural change, as indicated by hydrogen-ion titration[25] and fluorescence-polarization[12] data, occurs. It is known that the ionization behavior of DNS may be drastically modified when it is conjugated to serum albumin[26]. Similar behavior has been reported for γ-globulin, in both respects[28]. In addition, polarization of fluorescence data obtained on a considerable number of conjugated proteins indicate that the rotational freedom of the dye is largely, if not completely, that of the protein[12,13,29]. To explain this sensitivity of the fluorescence of the label to the molecular state of the protein itself, it is necessary to postulate a definite involvement of the DNS group in the protein tertiary structure. An obvious possibility is that an envelopment of the residue by the protein occurs, as was suggested by WILLIAMS AND FOSTER[24] for an anthracene label. In any event it is likely that the fluorescence of protein conjugates may provide an additional index of conformational changes.

In the absence of any structural transition, all of the proteins examined show a continuous drop in intensity with increasing temperature, the curve of relative intensity *versus* temperature being linear, or nearly so, between 15° and 60°. A similar thermal quenching, presumably arising from collisional deactivation by solvent, is known to occur in the case of tryptophan itself[21].

As in the case of the molecular events occurring at extremes of pH, thermally induced structural changes are readily recognizable from the character of the thermal-quenching curve. The transition is reflected by a marked change in slope in the critical temperature range.

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