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Thermal Perturbation Difference Spectra of Proteins Containing Tryptophyl Residues*

Jake Bello

ABSTRACT: The thermal perturbation difference spectral method was applied to the study of the exposed tryptophyls of chicken lysozyme and α-chymotrypsinogen. In 6 M guanidinium chloride both proteins as well as their derivatives having reduced disulfides gave spectral results indicating substantially complete exposure of tryptophyls. In aqueous buffer the method indicates 3.5–4 exposed tryptophyls in lysozyme compared with about 0.9 reactive toward Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide). The thermal perturbation spectrum of α-chymotrypsinogen contains an additional extremum (303 nm), not found for lysozyme or for the model, *N*-acetyltyrosinamide. The spectra of the reduced proteins are markedly different from those of the native proteins.

The spectrum of α-chymotrypsinogen is dependent on the manner in which temperature changes are carried out. The spectrum of the model compound is strongly dependent on the solvent.

Thermal perturbation difference spectra are generated when solutions of identical composition, but at different temperatures are examined spectrophotometrically. We have reported earlier on the use of the thermal perturbation method for the estimation of exposed tyrosines of RNase (Bello, 1969a,b), for the study of water structure in mixed solvents (Pittz and Bello, 1970), and for qualitative indication of preferential hydration of tyrosine in a mixed solvent (Pittz and Bello, 1969). Cane (1969) has also presented an independent development of this technique. We now present data on *N*-AcTrp-NH_2[1] and on the tryptophyl-containing proteins, chicken egg lysozyme, bovine α-chymotrypsinogen, and, in passing, β-lactoglobulin. We shall see that tryptophan gives results that are much more complex than those of tyrosine.

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[1] Abbreviations used are: *N*-AcTrp-NH_2, *N*-acetyltryptophanamide; *N*-AcTyr-NH_2, *N*-acetyltyrosinamide; RCAM, disulfides reduced and alkylated with carboxamidomethyl groups; Gu·HCl, guanidinium chloride.

Materials and Methods

Materials. *N*-AcTrp-NH_2 was purchased from Mann Laboratories, lysozyme from Calbiochem and from Worthington, β-lactoglobulin from Pentex, Inc., and α-chymotrypsinogen from Calbiochem. *N*-Methylacetamide and *N*,*N*-dimethylacetamide were redistilled under vacuum.

Methods. Thermal perturbation spectra were taken as described earlier (Bello, 1969a,b) with a Cary 15 spectrophotometer. The base line was adjusted with both samples at 27°. The reference cuvet was kept at 27°, while the other was brought to 3°. Spectra were taken on solutions containing 0.025 M ammonium acetate (pH 6.1). The low solubility of α-chymotrypsinogen and RCAM-α-chymotrypsinogen at pH 6.1 required that the pH be lowered (with glacial acetic acid) to pH 4.2, and 3.6, respectively. Water contents were estimated by loss of weight over P_2O_5 under vacuum.

Reductions of disulfides were done with a 25-fold excess (over disulfides) of Cleland's reagent (dithiothreitol) in 8 M urea–0.1 M Tris-acetate (pH 8.0) for 3 hr, followed by treatment with 5% more than the stoichiometric quantity of iodoacetamide, while the pH was maintained at 7–7.5 by addition of NaOH, until the pH no longer decreased. The product was filtered through Sephadex G-25 (elution with 0.05 M acetic acid) and freeze-dried.

Reactions with Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide) were carried out at the same pH as the thermal difference spectra. Protein (3–4 mg) in 30 ml of water was treated with 1.5 ml of 0.06 M, or 1.0 ml of 0.1 M, Koshland's reagent in dried acetone, added in 20–30 increments. The pH was maintained (±0.5 pH) by additions of 0.1 M NaOH after each increment of reagent. The reaction mixture was filtered through Sephadex G-25. Analyses were carried out spectrophotometrically at pH 4.6 and 11 according to Horton and Koshland (1967). The spectra of 2-hydroxy-5-nitrobenzyl alcohol at pH 4.6 and 11 agreed with those reported by Horton and Koshland. RCAM-α-chymotrypsinogen presented some difficulties which are described under Results.

Results

The thermal perturbation spectra of *N*-AcTyr-NH_2 and *N*-AcTrp-NH_2 in aqueous buffer are shown in Figure 1. The difference spectra for the two model compounds are of generally similar shape, but differing in detail. We shall deal mostly with the extremum at 292 nm for *N*-AcTrp-NH_2. Since the positions of the extrema depend on the environment of the chromophore, it will be convenient to name three of them as extrema A, B, and C, as indicated in Figure 1. The overlapping of the tyrosyl and tryptophyl spectra at the λ_B and λ_C regions will produce difficulties in the estimation of either of these residues in the presence of the other. At λ_A, the situation is simpler. While the 292- and 287-nm extrema for the two models overlap, $\Delta\epsilon_A$ for *N*-AcTrp-NH_2 is three times as great as $\Delta\epsilon_A$ for *N*-AcTyr-NH_2. Therefore, unless there is a preponderance of tyrosine over tryptophan in the protein, the major contribution to $\Delta\epsilon_A$ is expected to come from tryptophyl residues. Perhaps better, at 300 nm $\Delta\epsilon$ for *N*-AcTrp-NH_2 is 0.48 that of $\Delta\epsilon_A$, but zero for *N*-AcTyr-NH_2. In proteins λ_A for both chromophores shifts to the red. In Figure 1 are difference spectra for a mixture

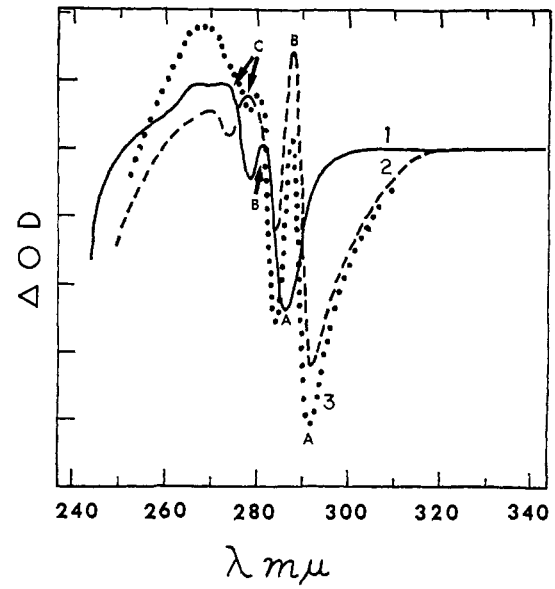

FIGURE 1: Thermal perturbation difference spectra of model compounds. (1) *N*-AcTyr-NH_2, 0.1 mg/ml; (2) *N*-AcTrp-NH_2, 0.05 mg/ml; and (3) *N*-AcTyr-NH_2, 0.1 mg/ml and *N*-AcTrp-NH_2, 0.05 mg/ml. Each unit on the ΔOD axis is 0.01 ΔOD.

of *N*-AcTrp-NH_2 and *N*-AcTyr-NH_2. $\Delta\epsilon_{300}$ was found to be constant (−77 to −83) at all ratios studied, from zero to 7 moles of *N*-AcTyr-NH_2/mole of *N*-AcTrp-NH_2. The deviations from the average value showed no systematic trend. The sum of the thermal perturbation spectra of *N*-AcTyr-NH_2 and of *N*-AcTrp-NH_2 in water and 6 M Gu·HCl agreed well with the spectra of the mixtures, indicating no complex formation. Beer's law was obeyed for concentrations of *N*-AcTrp-NH_2 and *N*-AcTyr-NH_2 in the range studied. Concentrations higher than 0.1 mg/ml of the former or 0.3 mg/ml of the latter were not used, to avoid large slit widths.

In Figure 2 are shown the thermal perturbation difference spectra of lysozyme, α-chymotrypsinogen, and β-lactoglobulin. Although the spectrum of lysozyme, which contains six tryptophyls and three tyrosyl residues (Jollés *et al.*, 1963; Canfield, 1963), resembles that of *N*-AcTrp-NH_2, there are differences in detail. λ_A is at 294 nm, instead of 292 nm. Extremum A for the protein is broader than for *N*-AcTrp-NH_2. The ratio of $\Delta\epsilon_{300}$ to $\Delta\epsilon_{292}$ (λ_A) for *N*-AcTrp-NH_2 is 0.48, while for lysozyme the ratio of $\Delta\epsilon_{302}$ (shifted 2 nm, because λ_A is 294 nm) to $\Delta\epsilon_{294}$ is 0.67. In the effective denaturant, 6 M Gu·HCl, the corresponding ratio, $\Delta\epsilon_{303}$ to $\Delta\epsilon_{295}$ (λ_A is 295 nm) is 0.73–0.76 for *N*-AcTrp-NH_2 and for the proteins.

α-Chymotrypsinogen and β-lactoglobulin give spectra with negative extrema at 303 nm. In difference spectra of proteins induced by acid, organic solvents, or detergents, extrema or shoulders have been observed near 300 nm (Ananthanarayanan and Bigelow, 1969a,b, and references cited there), which have been attributed to interactions of indole and carboxylate groups.

We investigated reduced-carboxamidomethylated proteins with the results shown in Figure 2. No extremum was seen at 303 nm for RCAM-α-chymotrypsinogen, but, instead, an extremum with a minimum at 293 nm, and with $\Delta\epsilon_{301}$/

TABLE I: Thermal Perturbation Spectral Data in 6 M Gu·HCl.

Solute	$\Delta\epsilon_{300}$	$\Delta\epsilon_A$[a]	$\Delta\epsilon_{300}$ (Protein) / $\Delta\epsilon_{300}$ (*N*-AcTrp-NH_2)	$\Delta\epsilon_A$ (Protein) / $\Delta\epsilon_A$ (*N*-AcTrp-NH_2)
N-AcTrp-NH_2	−73	−92		
Lysozyme	−404	−492	5.6	5.4
RCAM-lysozyme	−432	−510	5.9	5.6
α-Chymotrypsinogen	−560	−700	7.7	7.6
RCAM-α-chymotrypsinogen	−543	−682	7.4	7.4

[a] λ_A is 294–295 nm.

$\Delta\epsilon_{293}$ 0.45, similar to that for *N*-AcTrp-NH_2. For RCAM-lysozyme, the thermal perturbation spectrum showed no extremum at around 292–294 nm, but, rather, a large negative extremum at 303 nm. The positive extremum B for RCAM-lysozyme is 3.5 times as large as that of native lysozyme. This could obscure a small negative extremum around 292 nm.

In a solvent which effectively unfolds proteins we expect the number of exposed tryptophyls found by thermal perturbation spectra to be near the total number of tryptophyls. In 6 M Gu·HCl tyrosyl residues have an extremum A at 288 nm and do not contribute to $\Delta\epsilon_A$ of tryptophyls. The ratio of $\Delta\epsilon$ for the protein to $\Delta\epsilon$ for the model compound is taken as the number of exposed tryptophyls. Thermal perturbation data in 6 M Gu·HCl are shown in Table I and the difference spectra are shown in Figure 3. In this solvent, α-chymotrypsinogen showed 7.6 and 7.7 exposed tryptophyls at 295 and 303 nm, respectively. (All of the solutes had λ_A at 294.5 ± 0.5 nm.) These results are in fairly good agreement with the expected value of 8 (Hartley, 1964). For RCAM-α-chymotrypsinogen, the number of exposed tryptophans is somewhat lower than for α-chymotrypsinogen.

The results for lysozyme in 6 M Gu·HCl are slightly lower than expected. If a 10% correction for apparent low protein

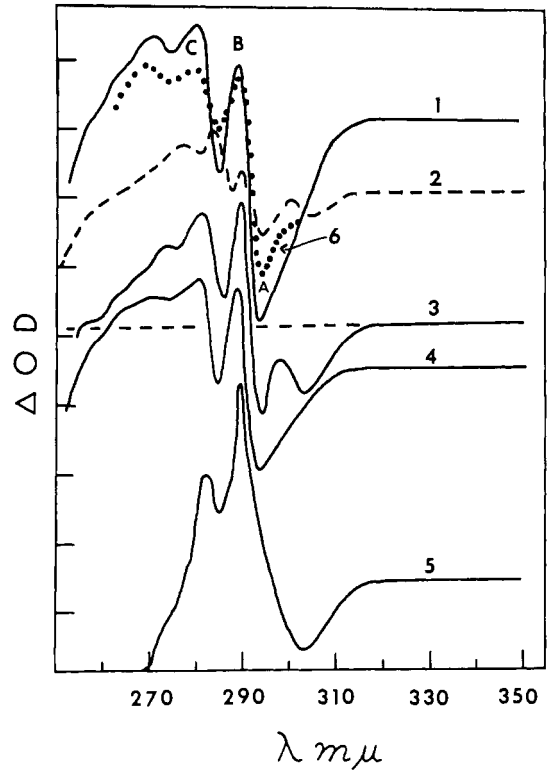

FIGURE 2: Thermal perturbation difference spectra of proteins. (1) Lysozyme, pH 6.1; (2) β-lactoglobulin; (3) α-chymotrypsinogen; (4) RCAM-α-chymotrypsinogen; (5) RCAM-lysozyme; and (6) lysozyme, pH 4.1. All concentrations about 1 mg/ml. Each unit on the ΔOD axis is 0.01 ΔOD. Spectrum 6 coincides with spectrum 1 above 302 nm.

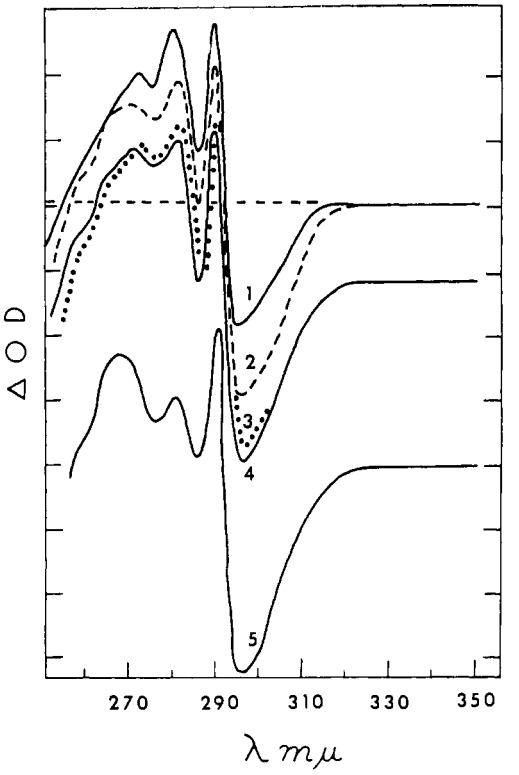

FIGURE 3: Thermal perturbation difference spectra in 6 M Gu·HCl. (1) *N*-AcTrp-NH_2, 0.05 mg/ml; (2) lysozyme, 1 mg/ml; (3) RCAM-α-chymotrypsinogen, 1 mg/ml; (4) α-chymotrypsinogen, 1 mg/ml; (5) RCAM-lysozyme, 1 mg/ml. Each unit of ΔOD is 0.01 ΔOD.

TABLE II: Thermal Perturbation and Koshland's Reagent Data.

Solute	pH	$\Delta\epsilon_{300}$	$\Delta\epsilon_A$	$\Delta\epsilon_{302}$ (Protein)[a] / $\Delta\epsilon_{300}$ (*N*-AcTrp-NH_2)	$\Delta\epsilon_A$ (Protein) / $\Delta\epsilon_A$ (*N*-AcTrp-NH_2)	Trp Modified[b]
N-AcTrp-NH_2	6.1[c]	−80	−160			
Lysozyme	6.1	−269	−493	3.5[d]	3.2[d]	0.9[e]
Lysozyme	4.1	−210	−402	2.7[d]	2.6[d]	
α-Chymotrypsinogen	4.2		−320		2.4[d]	2.4[f]
RCAM-α-chymotrypsinogen	3.6	−390	−870	5.0[d]	5.5[d]	7.7[f]
RCAM-lysozyme	6.1					6.5[g]

[a] At 302 nm for lysozyme and 301 nm for RCAM-α-chymotrypsinogen. [b] Koshland's reagent. [c] $\Delta\epsilon$ for *N*-AcTrp-NH_2 is the same at both pH 6.1 and 4.1. [d] Including correction for volume change; see text. [e] Ratio of reagent to Trp-35 and -70. [f] Ratio of reagent to Trp-110. [g] Ratio of reagent to Trp-70.

content is included, we obtain 6.2 and 5.9 exposed tryptophans in 6 M Gu·HCl. This 10% correction is based on the following considerations. ϵ_{282} of the lysozyme was 36×10^3, about 10% below the value calculated from the amino acid composition. This, in itself, is not conclusive. However, ϵ_{282} in 6 M Gu·HCl (to eliminate effects arising from buried groups) was found to be 12% greater for RCAM-lysozyme than for unmodified lysozyme, after allowing for the weight of the carboxamidomethyl groups. This suggests that some inert impurity was removed. Thus, a reasonable correction brings the number of exposed tryptophyls very close to theory. Even without the correction, the agreement is rather satisfactory. (α-Chymotrypsinogen and RCAM-α-chymotrypsinogen in 6 M Gu·HCl had ϵ_{282} values within 2% of each other; therefore no correction was made.)

In Table II are the calculated numbers of exposed tryptophyls for lysozyme, α-chymotrypsinogen, and RCAM-α-chymotrypsinogen in aqueous buffer. No calculation was made for RCAM-lysozyme because the spectrum is so different from that of the model compound. The value of exposed tryptophyls in lysozyme is 3.5 at 302 nm and 3.2 at λ_A. (302 nm was used for the protein because λ_A is 2 nm to the red of λ_A for *N*-AcTrp-NH_2.) These include a correction of 0.1 residue, arising from the volume contraction of the cold solution resulting in a contribution to $\Delta\epsilon$ from buried chromophores, estimated from burial of one-third of the chromophores, from ϵ of the direct spectrum and from the thermal coefficient of expansion of water. (The use of a more accurate estimate for buried chromophores would have a negligible effect.) If the 10% correction described above is included, the exposed tryptophyl estimates are 3.9 and 3.5 at 302 nm and λ_A, respectively, compared with 4.2–4.8 found by Williams *et al.* (1965), by solvent perturbation. Another lot of lysozyme had ϵ_{max} 31×10^3. The number of exposed tryptophans for this lot was less in the same proportion as the ϵ_{max} values. (The lysozyme used by Williams *et al.*, contained 5.2 residues of tryptophan. The values of exposed tryptophyls found by Williams *et al.*, are adjusted here to a tryptophyl content of 6 residues/molecule.)

The data above were obtained at pH 6.1. At pH 4.1 lysozyme showed a shoulder at 300 nm (Figure 2). $\Delta\epsilon_A$ was −402, yielding a value of 2.9 exposed tryptophyls, including the 10% correction and the 0.1 residue correction for thermal contraction. ($\Delta\epsilon$ for *N*-AcTrp-NH_2 was the same at pH 4.1 and 6.1.)

For α-chymotrypsinogen, no estimate based on *N*-AcTrp-NH_2 can be made at 302 nm, but at λ_A, we estimate 2.4 exposed tryptophyls, including a correction of 0.4 for the volume contraction of the solution on the assumption that two-thirds of the chromophores are buried. Williams *et al.* (1965) found 2.4 to 4.4 exposed tryptophans depending on the choice of perturbant and model compound. (These values were calculated from the fractional exposures given by Williams *et al.*, multiplied by 8.) Their lowest value, which corresponds with ours, was obtained with the perturbant Me_2SO and a mixture of *N*-AcTrp-NH_2 and *N*-AcTyr-NH_2 as the model.

At the same pH (4.2) in the absence of acetate (pH adjusted with hydrochloric acid) the thermal perturbation difference spectrum of α-chymotrypsinogen was the same as in acetate buffer, indicating that the 303-nm extremum does not arise from interaction with the buffer.

For RCAM-α-chymotrypsinogen (at pH 3.6 because of insolubility at higher pH), we find about 5.5 exposed tryptophyls, including the corrections for volume change. RCAM-α-chymotrypsinogen is not fully soluble in the buffer we used. Filtration through a Millipore filter (0.8 μ) gave 0.46 of the native ϵ_{280}. This factor was used in calculating $\Delta\epsilon$ in Table II. The result in Table II may not be representative of the total protein. Also, aggregates may be present. This result for RCAM-α-chymotrypsinogen indicates that tryptophyls of the modified protein are largely, but not completely exposed.

Turning to the correction needed for the contribution of buried tryptophan, arising from changes in volume of the protein, we confront the difficulty of finding a suitable model solvent. The marked effect of environment on the thermal perturbation spectra are illustrated in Figures 4 and 5. In the solvents of Figure 4, extrema are seen near 300–303 nm, with small $\Delta\epsilon$ values of around −40 to −50. Potassium formate was used because of nearly total absence of hydrophobic character and its high solubility. For *N*-AcTyr-NH_2 in 16 M potassium formate, λ_A is shifted from 287 to 289 nm with no change in $\Delta\epsilon_A$, while λ_B and λ_C are shifted 3 nm to the red with a 2.5-fold increase in $\Delta\epsilon$.

The mixture of 80% *N*-methylacetamide–20% *N*,*N*-

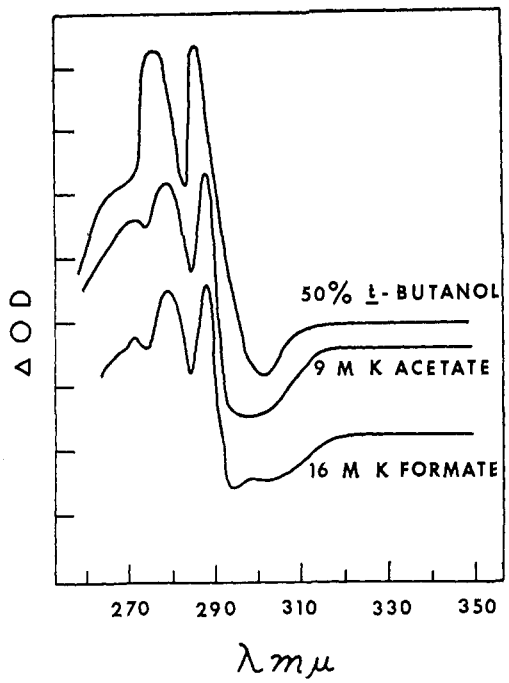

FIGURE 4: Thermal perturbation difference spectra of *N*-AcTrp-NH_2 in three solvents at pH 6.3. Concentrations are 0.05 mg/ml.

dimethylacetamide was chosen as a crude model for the interior of a protein because of its polar and apolar groups, peptide model and hydrogen-donating and -accepting ability. (*N*-Methylacetamide cannot be used alone because its freezing point is too high.) In 50% acetic acid λ_A for *N*-AcTrp-NH_2 is shifted from 292 to 296 nm, and $\Delta\epsilon_A$ falls to one-third that in water, while $\Delta\epsilon_B$ rises to twice that in water. In the solvents studied, at a constant wavelength of 292 nm, $\Delta\epsilon_A$ for *N*-AcTrp-NH_2 varies from -160 in water to $+100$ in the amide mixture. These results make it extremely difficult to estimate the contribution of buried tryptophyls to $\Delta\epsilon$. They also point to possible differences in spectral responses of partially buried tryptophyls. Even if we could find a solvent that adequately represents the interior of a protein, we must then know if a temperature change will change the environment of a chromophore in the interior of a protein in the same way as in the solvent. Probably not, since solvent molecules have more freedom than protein groups.

The thermal perturbation spectrum of α-chymotrypsinogen is dependent on the manner in which the temperature is adjusted. If the temperature is brought quickly to 3°, spectrum a of Figure 6 is obtained. On raising the temperature to 11 and 17°, spectra b and c are obtained. On adjusting again to 27°, the original base line is not regained; a difference spectrum, d, is obtained, which becomes greater on standing overnight at 27°, spectrum e. If the temperature program is reversed and a fresh sample is cooled from 27 to 17, 11, and 3°, spectra f, g, and h are obtained. In one experiment after initial cooling to 3°, the sample was brought abruptly to 19°, and after 2 min the thermoregulator was readjusted to 17°. The resulting spectrum resembled f. Only for α-chymotrypsinogen was the spectrum dependent on the temperature program. For the others $\Delta\epsilon_A$ was linear with temperature and independent of the direction of temperature change.

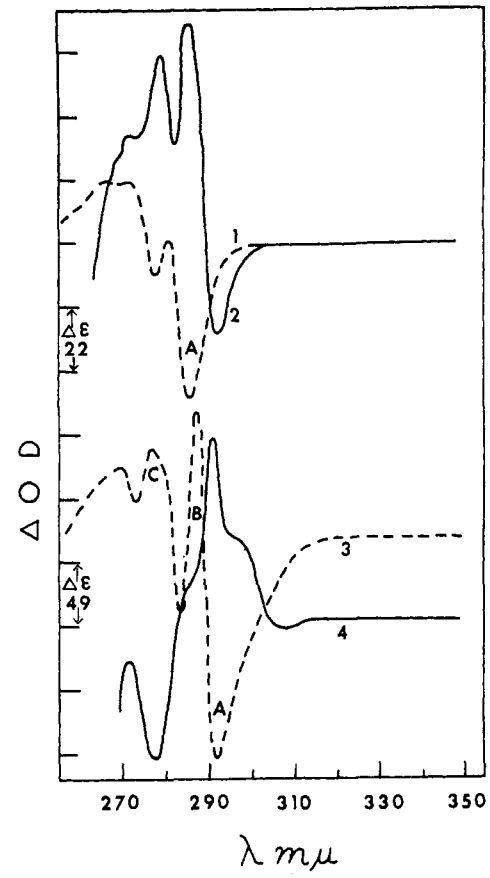

FIGURE 5: Thermal perturbation difference spectra in water and 80% *N*-methylacetamide–20% *N*,*N*-dimethylacetamide. (1) *N*-AcTyr-NH_2 in water, 0.1 mg/ml; (2) *N*-AcTyr-NH_2 in the above amide mixture, 0.1 mg/ml; (3) *N*-AcTrp-NH_2 in water, 0.05 mg/ml; (4) *N*-AcTrp-NH_2 in the above amide mixture, 0.05 mg/ml.

The spectra of Figure 6 suggest that on quick cooling to 3°, the 27° conformation is frozen in, but on cooling in smaller jumps and more slowly, conformational changes occur. That the 27° conformation is frozen in at 3° with little conformational change appears probable from the following considerations. On stepwise slow cooling to 3°, $\Delta\epsilon_{max}$ is 6000; $\Delta\epsilon_B$ and $\Delta\epsilon_C$ for the 3°, quick-cool spectra are about 600. Since the shapes of extrema B and C are more like those of *N*-AcTrp-NH_2 and *N*-AcTyr-NH_2 than like those of the lower part of Figure 6, the contribution of conformational change must be small. A conformational change would make $\Delta\epsilon_A$ smaller than if there were no change, resulting in a low value of exposed tryptophans.

We have reacted the proteins with Koshland's reagent for tryptophan (2-hydroxy-5-nitrobenzyl bromide). Reaction of lysozyme with Koshland's reagent modified 0.9 tryptophyl (Table II), with both 35 and 70 moles of reagent per mole of residue of tryptophan. Bewley and Li (1965) found that not more than 1 residue of Koshland's reagent was incorporated. The X-ray structure suggests that one tryptophyl (residue 62) is accessible for reaction (personal communication from Dr. Colin Blake, Laboratory of Molecular Biophysics, Oxford). RCAM-lysozyme apparently underwent modification of all of its tyrptophyls; the number of 2-hydroxy-5-nitrobenzyl groups was somewhat high. It was noted that the

spectrum at pH 11 was time dependent (about a 5% increase during 30 min); 8 M urea was used in the gel filtration (followed by refiltration with 0.05 M acetic acid). If urea was not used very high values were found. For the native proteins this effect was not noted.

For α-chymotrypsinogen the number of tryptophyls modified is in agreement with the thermal perturbation data. Reaction of RCAM-α-chymotrypsinogen with Koshland's reagent presented difficulties. Several methods of working-up the reaction mixture (gel filtration with dilute acetic acid or with 8 M urea, ether extraction or dialysis followed by Millipore filtration) caused loss of 70–80% of the protein.

Finally, we adopted the following procedure. The reaction mixture was dialyzed against daily changes of 0.016 M acetic acid for 3 days, then against 8 M urea overnight (which resulted in removal of some more yellow material), and finally against 0.016 M acetic acid. During the last dialysis most of the protein precipitated. Therefore, a useable spectrum could not be obtained for quantitation at pH 4.6. But at pH 11, all of the protein dissolved. The spectrum indicated 7.7 moles of Koshland's reagent/mole of protein, on the assumption that no protein was lost. The result with Koshland's reagent indicated that RCAM-α-chymotrypsinogen is much more unfolded than is the native protein, which is the expected case. The result with Koshland's reagent is substantially greater than the result of the thermal perturbation spectrum.

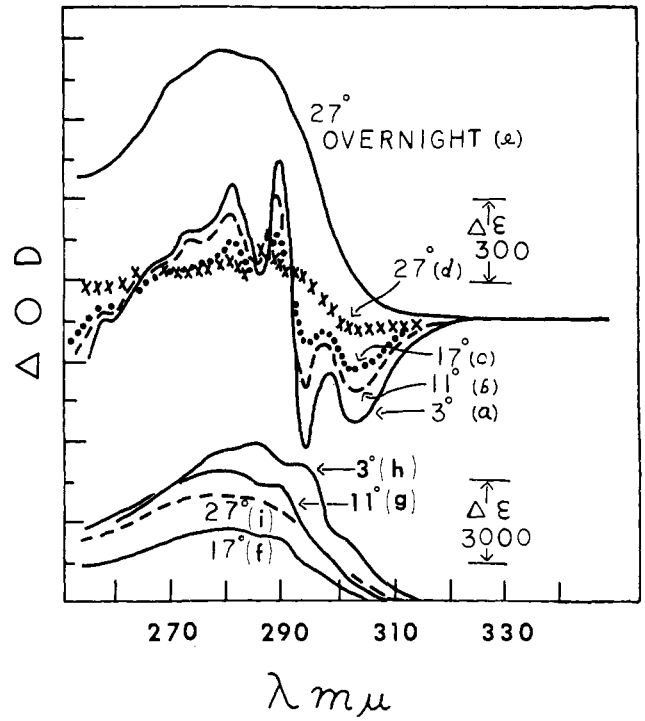

FIGURE 6: Dependence of thermal perturbation spectrum of α-chymotrypsinogen on temperature program. Lower spectra: temperature program downward; upper spectra: temperature program upward. Concentration is 1 mg/ml.

Discussion

The estimates of exposed tryptophyls in 6 M Gu·HCl are in reasonable agreement with expected values.

For lysozyme in aqueous buffer the estimate of exposed tryptophyls (made at about 300 nm) is about 0.5 residue lower than the average estimate from solvent perturbation spectra, a difference that is not necessarily significant. From the X-ray results shown by Phillips (1966), Browne *et al.* (1969), Harte and Rupley (1968), and a personal communication from Mrs. W. J. Browne, we estimate the total exposure of indole groups (including partial exposure, and without distinguishing nitrogen from carbon) to be about 2.5–3.5. Without a direct examination of the structural model we cannot be more precise. This estimate is lower than indicated by the thermal perturbation method or the solvent perturbation method.

Williams and Laskowski (1965) found, by oxidation with *N*-bromosuccinimide, that each tryptophyl of lysozyme contributes, with approximate equality, to $\Delta\epsilon/\epsilon$ with ethylene glycol as perturbant. This appears to be a high result in the light of the data from X-ray and Koshland's reagent. Oxidation of the first tryptophyl of lysozyme resulted in the loss of 40% of the spectral effect (with a polymeric perturbant), indicating that one tryptophyl is significantly different from the others. Perhaps this is the one that reacts with Koshland's reagent.

In view of the strong dependence of the difference spectrum of *N*-AcTrp-NH_2 on environment, it is, perhaps, remarkable that the difference spectrum of lysozyme is so similar to that of the model compound in water, since the X-ray data show that none of the tryptophyls of lysozyme is fully exposed and out of contact with other parts of the protein.

The pH dependence found for lysozyme is interesting. Williams *et al.* (1965) found no pH dependence for the solvent perturbation spectrum of lysozyme. Donovan *et al.* (1961) found pH-induced difference spectra, which they attributed to local charge effects rather than to major conformational changes. Since solvent perturbation (Williams *et al.*, 1965) viscosity (Yang and Foster, 1955) and specific rotation (Yang and Foster, 1955; Jirgensens, 1958) are constant over the pH range we have used, it appears that thermal perturbation is a sensitive indicator of local conformational changes and/or charge effects.

The thermal perturbation result for RCAM-α-chymotrypsinogen is in some disagreement with the apparently complete reaction with Koshland's reagent. This disagreement may result from partial burial of some of the indole groups with exposure of the reactive portions, unfolding caused by chemical modification of some of the tryptophyls, or conformational fluctuations that expose tryptophyls that are, on a time average, buried. Perhaps RCAM-α-chymotrypsinogen contains some hydrophobic groupings.

We turn now to the proteins that have extrema at 303 nm. For α-chymotrypsinogen we obtained agreement between the values of exposed tryptophyls by thermal perturbation (at 294 nm) and by treatment with Koshland's reagent. This may be fortuitous because a partially buried tryptophyl may or may not have its reactive position exposed. An additional difficulty is the presence of the 303-nm extremum. If the 303-nm extrema of chymotrypsinogen and RCAM-lysozyme arise from similar tryptophyls, and if both the 294- and 303-nm extrema of the former arise from the same tryptophyls, we should expect to observe an extremum at 294 nm for RCAM-lysozyme. This is not observed. The

positive $\Delta\epsilon$ for RCAM-lysozyme at 294 nm may dominate a smaller negative $\Delta\epsilon$. If the 303- and 294-nm extrema of α-chymotrypsinogen arise from different tryptophyls, we should be able to calculate the number in each class if we had a suitable model. Taking *N*-AcTrp-NH_2 in *t*-butyl alcohol or potassium formate ($\Delta\epsilon$ −35) for the 303-nm model, the number of exposed tryptophyls calculated at 303-nm ($\Delta\epsilon$ −270) is 7.7. Combined with the estimate of 2.5 at 294 nm we obtain a total that is much too high. Clearly the model is not adequate. For α-chymotrypsinogen Williams and Laskowski (1965) found that oxidation of three tryptophyls by *N*-bromosuccinimide resulted in the linear decrease of $\Delta\epsilon/\epsilon$ to zero, with no further change in $\Delta\epsilon/\epsilon$ on oxidation of the remaining tryptophyls. We are puzzled by the finding of Williams and Laskowski that the tryptophyls that do not appear to contribute to the solvent perturbation spectrum are available to *N*-bromosuccinimide.

The extrema at 303 nm probably arise from tryptophyls that are not completely exposed but are in special environments. The data do not indicate a clear choice as to the nature of the environment, since both less polar (*t*-butyl alcohol) and ionic cosolvents (potassium formate) give rise to extrema at 303 nm for *N*-AcTrp-NH_2. Since potassium formate gives rise to extrema at both 294 and 303 nm, we must be cautious in attributing the 294- and 303-nm extrema of α-chymotrypsinogen to tryptophyls in different environments. But *N*-AcTrp-NH_2 in 16 M potassium formate may exist in a variety of environments which may give rise to more than one extremum.

Estimation of exposed tyrosine in the presence of tryptophan does not appear to be feasible at present. $\Delta\epsilon_B$ of lysozyme when referred to a calibration curve obtained from mixtures of *N*-AcTyr-NH_2 and *N*-AcTrp-NH_2, leads to an estimate of about zero exposed tyrosine, corrected for the volume effect. At λ_C we obtain an estimate of 1.2 exposed tyrosines. The X-ray data suggest that the three tyrosyls of lysozyme are partially exposed to the sum of about one residue. We do not attach much significance to our estimates of exposed tyrosines for three reasons: first, if wavelength shifts in the protein are different for the two chromophores (tyrosyl and tryptophyl), relatively large differences in $\Delta\epsilon$ will result since the extremum B is rather sharp; second, the contribution of the buried chromophores cannot be evaluated; and third, because the magnitude of the λ_B and λ_C regions vary in ways not yet understood.

In summary, tryptophyl-containing proteins give thermal perturbation spectra that lead to estimates of exposed tryptophyls which must be considered with caution and must be tested against other methods. Further investigation may provide more useful information.

One of the points to be investigated is the possibility that for α-chymotrypsinogen and β-lactoglobulin the 294- and 303-nm extrema arise from strongly shifted tyrosyl and tryptophyl, respectively.

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