THERMAL DIFFERENCE SPECTRA OF AROMATIC AMINO ACIDS IN WATER

Alan COOPER
Department of Chemistry, Yale University, New Haven, Conn. 06520, U.S.A.

Received 28 August 1974

1. Introduction

Recent studies of the temperature difference spectra of lysozyme [1] and ribonuclease [2] in aqueous buffers over an extensive temperature range have demonstrated significant spectral changes in the 230–300 nm range prior to denaturation of the proteins. In particular isosbestic points in the difference spectra at temperatures below denaturation have been cited as evidence for the existence of two conformational states of the enzymes below the denaturation temperature [1,2]. In view of the potential importance of these observations to the understanding of protein conformation in solution it seemed advisable, despite previous studies [3–8], to reinvestigate the effects of temperature on the U.V. spectra of the appropriate free chromophores in water. Thermal difference spectra are reported here for aqueous solutions of the *N*-acetyl ethyl esters of tryptophan, tyrosine and phenylalanine over a 5–65°C temperature range. It is shown that the effect of temperature on the free aromatic residues is qualitatively similar to that observed in proteins prior to denaturation, including the presence of apparent isosbestic points. It is further shown that the apparent isosbestic points are a manifestation of small, thermally induced wavelength shifts in the chromophore absorption bands.

2. Materials and methods

N-acetyl ethyl esters of L-tryptophan, L-tyrosine and L-phenylalanine (Mann Research Labs., New York) were used without further purification. Solutions for difference spectroscopy were made up to give a maximum absorbance of about 1.0 in the 260–290 nm range. Tyrosine ester was dissolved in 0.1 N HCl to suppress potential proton ionisation, otherwise the solvent was double-deionised water.

Thermal difference spectra were determined in 1 cm quartz cuvettes, fitted with Teflon stoppers, using a Cary 14 spectrophotometer with 0–0.1 A slidewire and individually thermostatted cuvette holders. Typically the reference cuvette was maintained at 25°C while the sample cuvette, containing an identical solution, was varied from 5 to 65°C (± 0.2°C). At least two difference spectra were recorded at each temperature and the sample was periodically returned to 25°C to check reversibility. Parent spectra were recorded for each solution at 25°C against water, using the 0–1.0 A slidewire.

3. Results and discussion

Figs. 1–3 illustrate the thermal difference and parent spectra in water of the Trp, Tyr and Phe chromophores, respectively. Notable features of the difference spectra include (a) an apparently monotonic perturbation of the spectra with increasing temperature, and (b) the appearance of at least two isosbestic points for each chromophore, i.e. points at which all spectra coincide to indicate wavelengths for which the extinction coefficient of the chromophore is apparently independent of temperature. The difference spectra are typical of small red shifts together with, at least in the case of phenylalanine, small intensity changes of the chromophore bands with increasing temperature.

Comparison of the tryptophan results with the published difference spectra for lysozyme in the pre-denaturation range [1] shows strong similarity between

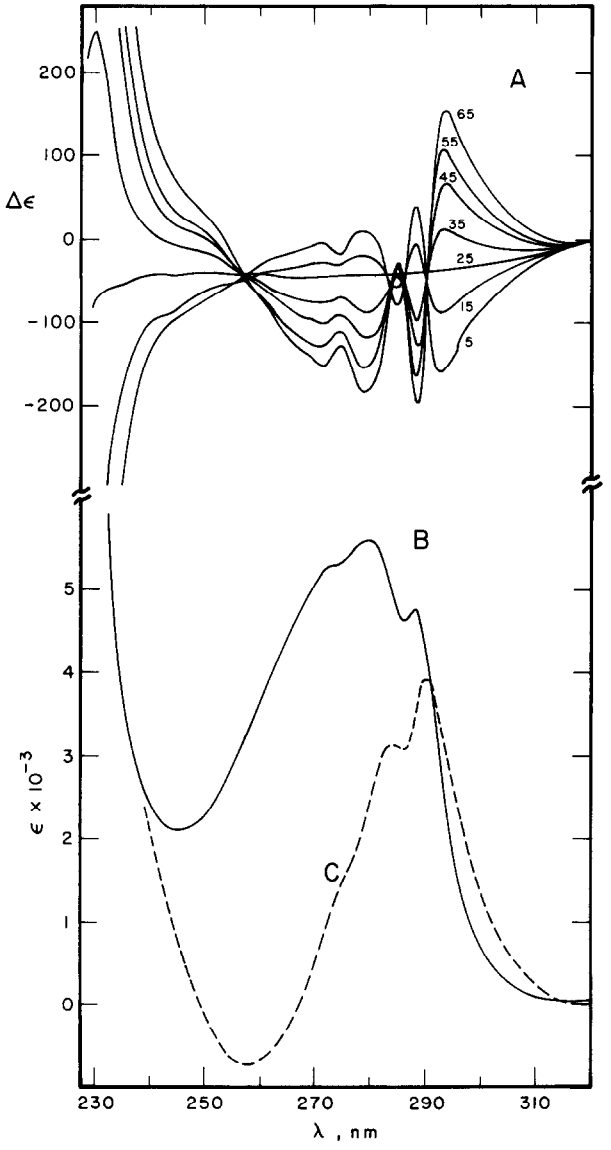

Fig. 1. *N*-acetyl-L-tryptophan ethyl ester in water. A: superimposed thermal difference spectra for 10°C intervals in the 5–65°C temperature range, reference cuvette at 25°C. Converted to molar absorbance differences using literature values of molar extinction coefficients [11]. B: parent spectrum versus water, 25°C. C: numerically integrated 45°C difference spectrum, arbitrary units.

the two. Thus, both the enzymes and the free chromophore undergo apparent red shift with increasing temperature and demonstrate apparent isosbestic points at about 256 and 291 nm for the enzyme, compared to about 257 and 289 nm for the free tryptophan (fig. 1). Similarly, ribonuclease, which contains no tryptophan, shows isosbestic points at 256 and 285 nm [2], compared to 251 and 283 nm for the free tyrosine chromophore (fig. 2). Phenylalanine shows two pairs of apparent isosbestic points at about 251 and 257 nm respectively, together with an apparent increase in overall intensity with increasing temperature. It is unlikely, however, that this would contribute significantly to protein difference spectra

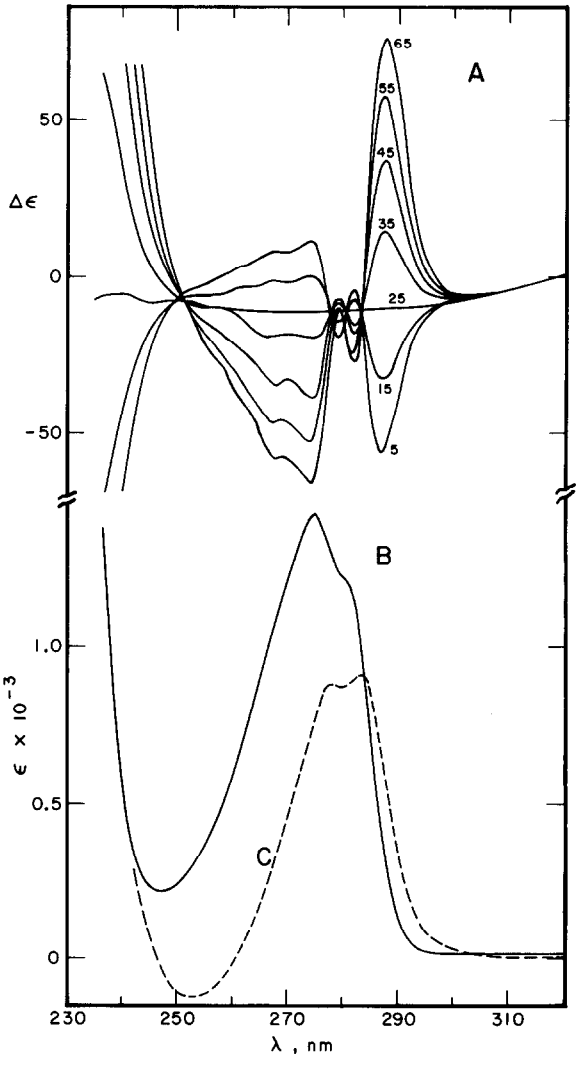

Fig. 2. *N*-acetyl-L-tyrosine ethyl ester in 0.1 N HCl. Details as in fig. 1.

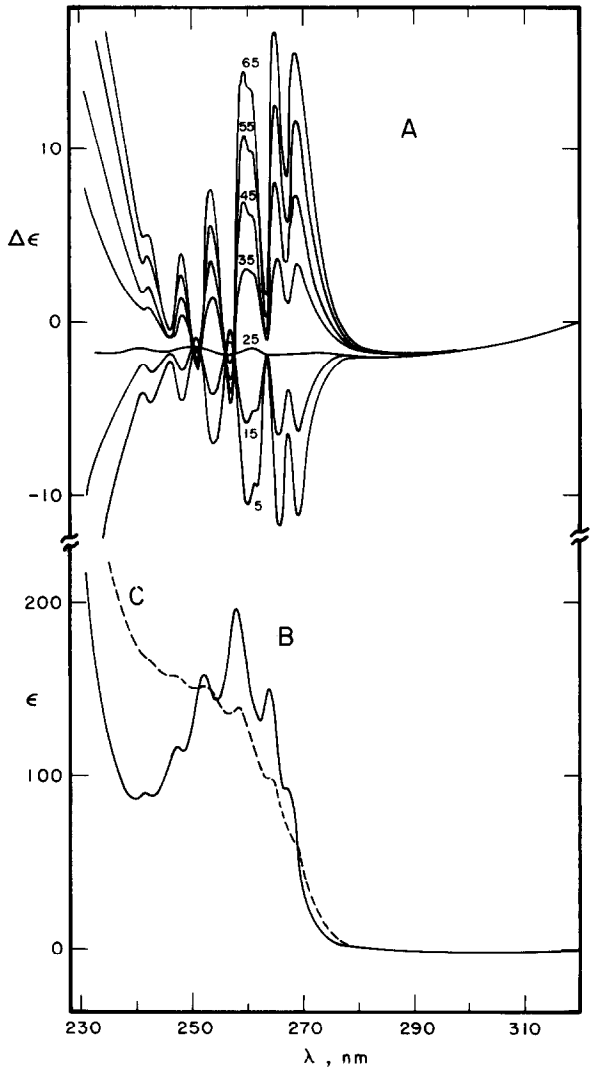

Fig. 3. *N*-acetyl-L-phenylalanine ethyl ester in water. Details as in fig. 1.

due to the relatively low molar extinction of phenylalanine.

Conventionally, isosbestic points in perturbation spectra imply an equilibrium between two distinct species in the solution, the equilibrium being shifted by the perturbation. This interpretation led Privalov et al. [1,2] to propose two distinct states for lysozyme and ribonuclease prior to denaturation. Unfortunately the observation here of similar effects of temperature on the free chromophores must cast doubt on this. It is not inconceivable that the *N*-acetyl ethyl esters of the aromatic amino acids should have two, temperature dependent chromophoric states in water (e.g. due to different molecular conformation, molecular association in solution or, in the case of Trp and Tyr, change in extent of water hydrogen bonding to the indole nitrogen or phenolic groups), though it seems coincidental for the different chromophores to show the same effect. There is, however, an alternative explanation. The absorption spectra of aromatic groups in the 240–300 nm range are made up of a series of overlapping π-π^* absorption bands [3]. Perturbation of these spectra by change in temperature or environment might be described in terms of a change in position, shape or intensity of one or more of the overlapping bands. Assume, for the moment, that by some undefined process, temperature perturbation gradually shifts the position of the absorption bands by an amount $\Delta\lambda(T)$, from $\epsilon(\lambda)$ to $\epsilon(\lambda + \Delta\lambda)$, without significant change in shape or intensity. Under these conditions the difference spectrum is given by $\Delta\epsilon(\lambda) = \epsilon(\lambda + \Delta\lambda) - \epsilon(\lambda)$, which, to first order in a Taylor series expansion, approximates to $\Delta\epsilon(\lambda) \simeq$

$$\frac{\partial \epsilon(\lambda)\,\Delta\lambda}{\partial\lambda}$$

Thus, for small perturbations, the difference spectrum would be proportional to the first derivative of the parent spectrum [3]. It follows immediately that apparent isosbestic points (i.e. $\Delta\epsilon = 0$ for all temperatures) would appear in the difference spectrum whenever $\frac{\delta\epsilon(\lambda)}{\delta\lambda} = 0$, i.e. at maxima or minima in the spectrum of the bands being perturbed.

Examination of figs. 1–3 shows that, in general, isosbestic points in the difference spectra do coincide with maxima or minima in the parent spectra. However, not every turning point in the parent spectra gives rise to an isosbestic point, presumably because not all the absorption bands within a particular spectrum are perturbed by change in temperature. If the above assumption is valid integration of a difference spectrum should regenerate that part of the parent spectrum which is undergoing perturbation. Numerically integrated difference spectra for each of the aromatic amino acid residues are given in figs. 1–3. In the case of tryptophan (fig. 1) the integrated spectrum corresponds closely to the ^1L_b band of the

indole chromophore [9] suggesting that the major ^1L_a band is relatively unaffected by temperature. Similarly for tyrosine (fig. 2) the integrated difference spectrum seems to correspond to the minor band which appears as a shoulder at about 280 nm in the complete spectrum. In the case of phenylalanine (fig. 3) integration of the thermal difference spectrum is not entirely valid due to intensity changes in addition to the red shift. However, the integrated spectra do show features which correspond closely to each of the peaks in the parent spectrum. For mono-substituted benzenes the ^1L_a band appears at lower wavelengths [10] so, once again, it appears to be the ^1L_b transition which is most affected by temperature.

Thus, the major affect of increasing temperature on the spectra of aromatic residues in water is a small, monotonic red shift in certain of the chromophore absorption bands which, in the case of tyrosine and tryptophan may be estimated to be about 0.01 nm/°C. It must be concluded that similar affects are responsible for the observations on proteins in the pre-denaturation range [1,2] and that the appearence of isosbestic points is a consequence of small, monotonic red shifts rather that unique changes in protein conformation. This is not to deny that such processes might occur, but a more detailed quantitative analysis of the protein difference spectra would be required to distinguish the specific effects of conformational changes from the general effect of temperature on the aromatic chromophores.

It might be pointed out here that a temperature induced red shift in water is contrary to what one might expect from simple variations in solvent polarity with temperature [5,10].

Acknowledgements

This work was supported in part by research grants to Professor J. M. Sturtevant from the National Institutes of Health (GM-04725) and the National Science Foundation (GB-23545).

References

[1] Khechinashvili, N. N., Privalov, P. L. and Tiktopulo, E. I. (1973) FEBS Lett. 30, 57–60.
[2] Tiktopulo, E. I. and Privalov, P. L. (1974) Biophys. Chem. 1, 349–357.
[3] Wetlaufer, D. B. (1962) Adv. Protein Chem. 17, 303–390.
[4] Bello, J. (1969) Biochem. 8, 4542–4550.
[5] Pittz, E. and Bello, J. (1970) Trans. Farad. Soc. 66, 537–545.
[6] Bello, J. (1970) Biochem. 9, 3562–3568.
[7] Leach, S. J. and Smith, J. A. (1972) Int. J. Protein Res. 4, 11–19.
[8] Elödi, P. and Lakatos, S. (1973) Eur. J. Biochem. 36, 45–52.
[9] Strickland, E. H. and Billups, C. (1973) Biopolymers 12, 1989–1995.
[10] Jaffé, H. H. and Orchin, M. (1962) Theory and Applications of Ultraviolet Spectroscopy, Wiley, N.Y.
[11] Handbook of Biochemistry (Sober, H. A., ed.) (1968) p. B-18, Chemical Rubber Co.