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Thermal transitions in the structure of tubulin

Environments of aromatic aminoacids

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Abstract. The environment of aromatic aminoacids in the thermal transition of brain tubulin has been studied by several spectroscopic techniques (Fourth Derivative, Difference Absorption, Fluorescence and Circular Dichroism), in order to study its denaturation. An irreversible, temperature-induced, structural transition was found at around 48 °C. In order to establish the relative degree of hydrophobicity of tubulin aromatic residues, before and after the thermal transition, difference and fourth derivative absorption spectra at different temperatures were compared with spectra of tyrosine and tryptophan model compounds in different media. It was found that at high temperatures, tubulin acquires a partially denatured stable state, with a significant amount of residual structure still preserved. This state is characterized by a general increase of the exposure of tyrosine residues to the medium, while the environment of tryptophans becomes more hydrophobic.

Key words: Tubulin – Structural transition – Hydrophobicity – Thermal denaturation – Fourth derivative spectrophotometry – Difference absorption – Fluorescence – Circular dichroism

Introduction

Tubulin is a conformationally flexible protein which assembles to form microtubules. Highly purified unassembled tubulin ages very rapidly under standard solution conditions (Prakash and Timasheff 1982; Andreu and Timasheff 1982), though its stability can be enhanced by liganding or by the addition of cosolvents (Na and Timasheff 1981). The three-dimensional structure of tubulin is unknown because crystals of the α-β tubulin heterodimer (Mr 100000), appropriate for high resolution X-ray diffraction, have not been grown. The amino acid sequences of the α and β tubulin chains have, in general, weak secondary structure potentials (Ponstingl et al. 1982). Unassembled tubulin contains roughly 25% α helix and 40% β-pleated sheet (Lee et al. 1978; Andreu et al. 1986; Audenaert et al. 1989). Porcine brain tubulin has a total of 8 tryptophan and 36 tyrosine residues (Ponstingl et al. 1982). Tryptophan residues were reported to be essential for microtubule assembly (Maccioni and Seeds 1982) and are related to the binding of the assembly inhibitor colchicine (Andreu and Timasheff 1982; Hastie 1989). On the other hand, it was reported that heat-denaturated tubulin could poison microtubule assembly, by interaction with the native protein (Maccioni 1983).

In order to learn about the stability of tubulin it is necessary to characterize its denaturation. The development of sensitive spectroscopic methods has allowed newer insights in protein structure and/or function. While UV difference absorption spectrophotometry has been used extensively in studies on the structure of proteins for many years (Donovan 1973), the application of UV fourth derivative spectrophotometry to this field is relatively new (Butler 1979; Padrós et al. 1982). Its main advantage lies in its higher resolution of the individual contribution of each aromatic amino acid, over classical absorption spectrophotometry. It is better able to resolve the individual contribution of each aromatic amino acid, thus revealing information on the average degree of hydrophobicity of their environments, with more precision (Padrós et al. 1982, 1984; Duñach et al. 1983). The changes in the mean environment of tyrosine and tryptophan residues in the process of thermal denaturation of tubulin have been characterized by these two techniques, together with fluorescence and CD spectroscopy. The results are presented in this report.

Materials and methods

a) Sample preparation

Tubulin was purified from calf brain and stored in liquid nitrogen or at −70 °C, as described previously (Weisenberg et al. 1968; Lee et al. 1973; Andreu and Timasheff

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1982). The buffer used in all experiments (PG buffer) was 10 m*M* sodium phosphate, 0.1 m*M* GTP, pH 7.0. The tubulin concentration used in our experiments was measured by UV spectrophotometry, using an extinction coefficient of $1.16\ g^{-1} \cdot l \cdot cm^{-1}$.

Model compounds, N-acetyl-L-tyrosine ethyl ester (AcTyrOEt) and N-acetyl-L-tryptophan amide (AcTrp NH_2) were purchased from Sigma Chemical Co. and dissolved in several solvents of different hydrophobicity degrees (water, ethanol, ethyl acetate and dimethylformamide).

b) Spectroscopic methods

UV absorption spectra of tubulin in PG buffer, were recorded in a Perkin-Elmer model 320 spectrophotometer, connected to an Olivetti 240 microcomputer. This spectrophotometer includes a microprocessor for automatic storage and processing of data.

Difference absorption spectra at each temperature were obtained by automatic subtraction of the stored spectrum at 20 °C from the spectrum at this particular temperature. Fourth derivative spectra were calculated by the spectrophotometer microprocessor. The derivative interval was 4 nm, with a slit width of 1 nm, a time constant of 1 s and a scan rate of 30 nm/min. Temperature was increased at 5 °C intervals, allowing 15 min for each temperature to stabilize before recording the spectrum. Absorption spectra for both tyrosine and tryptophan model compounds were obtained in media of different hydrophobicity degrees. Fourth derivative spectra of these model compounds were calculated and stored in a microcomputer and were later added in order to generate combination spectra whose main peaks are coincident with those found for tubulin at different temperatures. The characterization of these spectra is described in detail elsewhere (Padrós et al. 1982; Duñach et al. 1983).

Fluorescence spectra for tubulin were collected in a FICA 55 Mk2 double beam spectrofluorimeter. Excitation wavelengths were 280 nm (for tyrosines and tryptophans) and 295 nm (for tryptophans). Excitation and emission bandwidths were 2.5 nm and 7.5 nm respectively. Circular dichroism experiments were performed in a Roussell-Jouan Dichrograph II, employing a thermostated 10 mm cell for near-ultraviolet measurements and a 0.1 mm cell for experiments done in the far-ultraviolet.

Results and discussion

a) Absorption spectra

We have characterized the thermal perturbation of tubulin by means of difference absorption spectra, obtained as described in the previous section (Fig. 1). As can be seen in this figure and in Fig. 3b, no turbidity in the region of 310–340 nm was observed in the course of the temperature increase, which indicates that no non-specific aggregation took place due to denaturation of the samples. Figure 2 shows the increase of absorption difference vs.

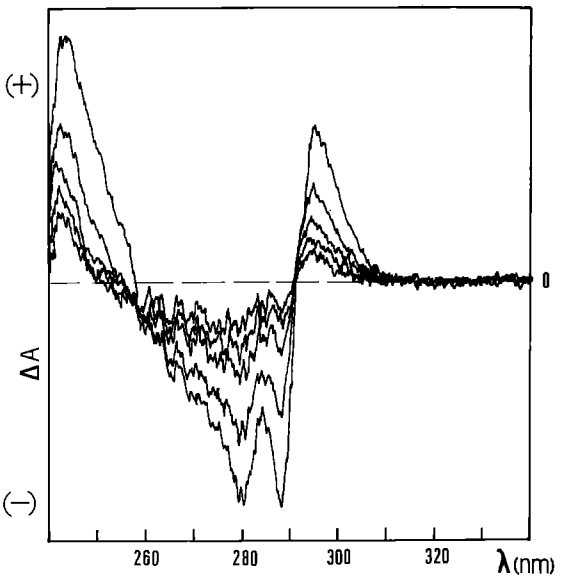

Fig. 1. Characteristic examples of Absorption Difference Spectra of a 20 μM solution of tubulin in PG buffer, at several temperatures. From outermost to innermost, the traces correspond to spectra taken at 50°, 45°, 40°, 35° and 30 °C. The minimum at 288 nm is due to contributions of both tyrosine and tryptophan residues, whereas the peak at 295 nm is due entirely to Trp

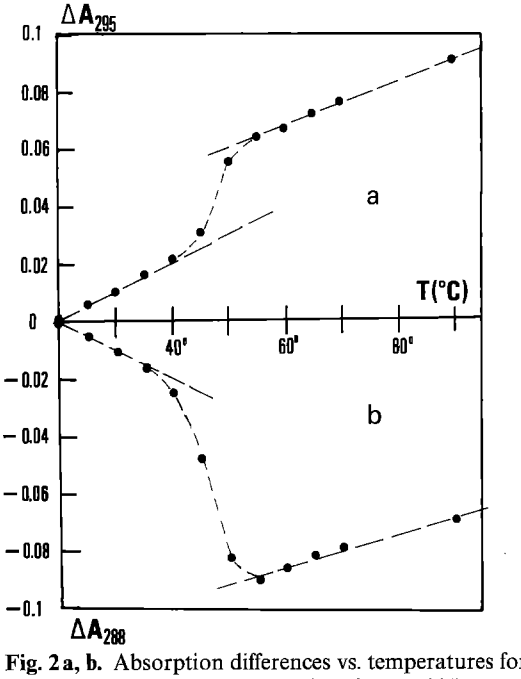

Fig. 2a, b. Absorption differences vs. temperatures for a solution of 20 μM tubulin in PG buffer. **a** Plot of ΔA at 295 nm vs. temperature. The slopes of both linear parts give an indication of the degree of exposure of Trp residues to the solvent, when compared with slopes obtained in free Trp solutions (see text). **b** Plot of ΔA at 288 nm vs. temperature. Since both tyrosine and tryptophan residues contribute to this peak, it is not possible to unambiguously determine the degree of exposure of either type of residue

temperature for both wavelengths, 295 nm (a) and 288 nm (b). These wavelengths correspond to the major peaks in the spectra of Fig. 1. At 295 nm, the absorption difference peak is almost entirely due to tryptophan residues, whereas the peak at 288 nm is due to both tryptophan and tyrosine residues (Donovan 1973). Figure 2a

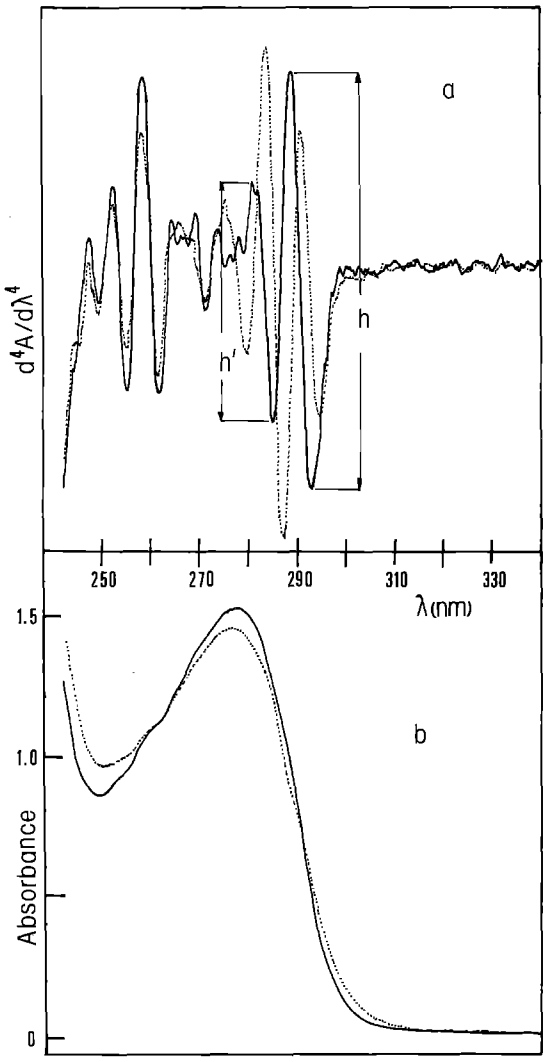

Fig. 3. a Fourth derivative spectra of a solution of 20 μM tubulin in PG buffer, at 5 °C (continuous line) and 65 °C (dotted line) in arbitrary units, that correspond to the absorption spectra, represented in **b**. The parameter R is defined as the ratio of the amplitudes of the two main peaks h/h'. A red shift at high wavelengths can be observed between both spectra, as well as a variation in the parameter R, as a reflection of changes in the shape of the spectra at these wavelengths

shows two linear regions of increase of absorption difference, separated by a transition zone between 40° and 55 °C, centered around 48 °C. The transition is irreversible. The linear regions (20 °C–40 °C and 55 °C–90 °C) have been interpreted as due to a direct effect of temperature on the chromophores (Nicola and Leach 1976). However, the sigmoidal increase between 40 °C and 55 °C is interpreted as being due to a transition towards a more hydrophobic environment for tryptophan residues (Donovan 1973). The slopes of both linear parts, typically positive at this wavelength, are: 33.8 $M^{-1} \cdot cm^{-1} \cdot K^{-1}$ (from 20° to 40 °C) and 27.0 $M^{-1} \cdot cm^{-1} \cdot K^{-1}$ (from 55° to 90 °C) respectively. One can make an estimate of the average degree of exposure of these tryptophan residues, by relating these values to the values of slopes reported in the literature for tryptophan derivatives as model compounds in free solution, once volume expansion effects have been taken into account. These values range from 6.3 $M^{-1} \cdot cm^{-1} \cdot K^{-1}$ to 7.1 $M^{-1} \cdot cm^{-1} \cdot K^{-1}$ (Nicola and Leach 1976; Bello 1977). We estimated the number of Trp residues exposed by the ratio of the slope obtained for the protein, over the slope found for tryptophan models in solution. Before the transition takes place ($T<40$ °C) this ratio lies around 5.0 (which counts for 63% of the total 8 tryptophan residues in tubulin), whereas at high temperatures ($T>55$ °C) one estimates only as many as about 4.0 exposed residues (50% of all tryptophans in tubulin). This means that after the transition takes place, about 1 tryptophan residue less (13%) is exposed. Obviously, these values are only average values over all tryptophan residues and over all possible degrees of exposure to the solvent, so they give no information on the exposure of individual residues.

The same experiment was monitored at 288 nm, as shown in Fig. 2 b. At this wavelength, tryptophan aminoacids in free aqueous solutions are known to display a negative slope when the temperature is raised, whereas tyrosine aminoacid solutions yield positive slopes (Nicola and Leach 1976; Bello 1977). We interpret the negative slope at low temperatures observed in tubulin, followed by a negative sigmoidal transition, as meaning that tryptophan residues are the predominant contributors to the absorption difference. After the thermal transition takes place however, the slope becomes positive. This indicates that some tyrosine residues, during the thermal transition, become more exposed to the solvent, thus becoming the main contributors to the difference spectra.

b) Fourth derivative spectra

As indicated in the Introduction, fourth derivative spectrophotometry is a sensitive tool to monitor changes in the environment of aromatic residues. Particular attention has been paid to the region of the spectrum between 270 nm and 300 nm in order to detect possible changes in tyrosine and tryptophan environments.

Examples of fourth derivative spectra of tubulin are shown in Fig. 3 for both 5 °C and 65 °C. These temperatures correspond to situations before and after the transition. One salient feature due to the thermal transition is a red shift observed in the longest wavelength minimum, attributed to tryptophans (Duñach et al. 1983), going from 293 nm to 295 nm as the temperature increases (shown in Fig. 4 a).

We have compared our fourth derivative spectra of tubulin with computer-generated spectra obtained by adding spectra from tyrosine and tryptophan models in media of different hydrophobicity. The proportion of tyrosine over tryptophan in these computer-generated model compound spectra was selected to be the same as in tubulin, 35/8. We found that high temperature spectra of tubulin such as those at 65 °C, were similar to those obtained from an addition of the individual spectrum of tyrosine in water plus the spectrum of tryptophan in dimethylformamide, keeping the same molar proportion of these aminoacids as in tubulin. These first two minima were found to be positioned at 295 nm and at 288 nm, as

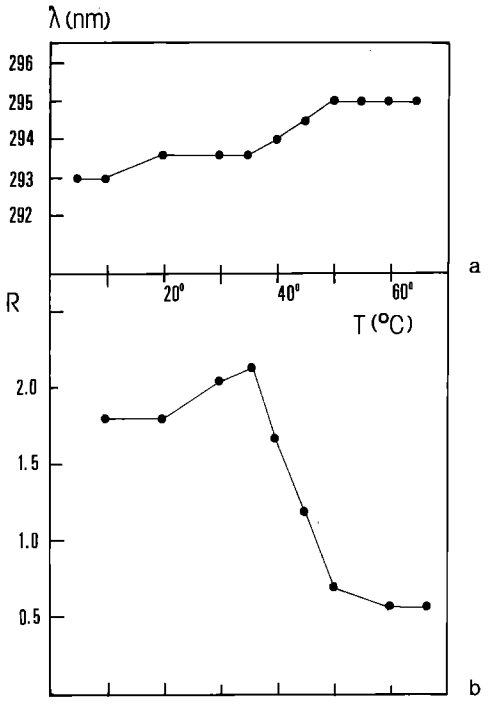

Fig. 4. **a** Dependence of the red shift observed in the longest minimum in the fourth derivative spectra, with temperature, indicating that tryptophans undergo a transition towards more hydrophobic environments. **b** Variation of R with temperature, indicating that a major conformational change is taking place in the protein

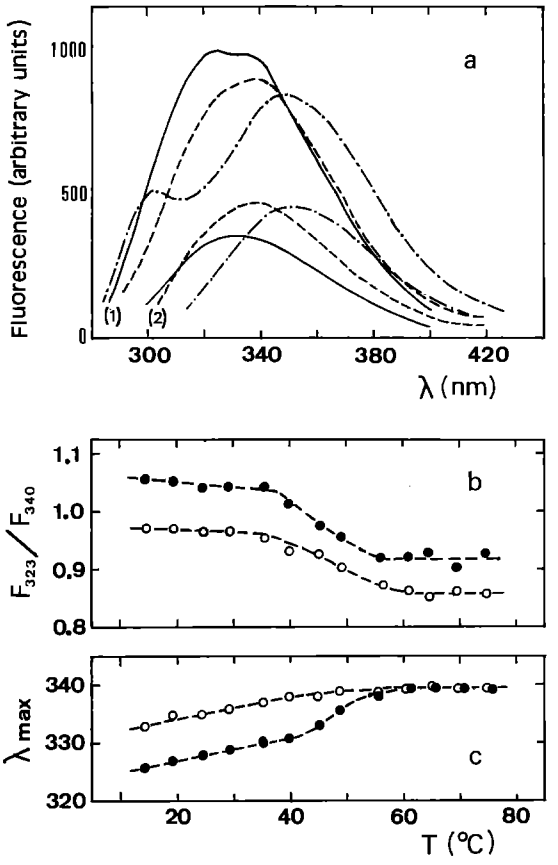

Fig. 5a–c. Temperature-induced changes of the intrinsic fluorescence of tubulin. **a** Fluorescence emission spectra of 0.2 mg/ml tubulin in PG buffer, employing excitation and emission bandwidths of 2.5 and 7.5 nm respectively. Spectra (1) were obtained by excitation at 280 nm and spectra (2) were obtained by excitation at 295 nm, which is selective for tryptophan residues. Continuous lines: emission spectra at 25 °C. Dashed lines: emission spectra at 65 °C, amplified 2×. Dashed-dot lines: emission spectra of tubulin in 6 M guanidinium hydrochloride at 25 °C, amplified 3×. **b** Ratio of emission intensities as 323 nm (F_{323}) and 340 nm (F_{340}) versus temperature. Filled circles: excitation at 280 nm. Empty circles: excitation at 295 nm. **c** Position of the emission maxima versus temperature. Symbols are as in **b**

in tubulin. Parameter R (Duñach et al. 1983), defined as the ratio of the relative height of these peaks, h/h' (see Fig. 3a), can be used to compare the tubulin spectrum at a given temperature with that from computer-generated models. Values of R for different combinations of model compounds in a variety of different hydrophobic media, have been found to range from 0.18 to 1.8 in the literature (Padrós et al. 1982; Duñach et al. 1983). Although the specific physical significance of R has not been ascertained, changes in this parameter have been interpreted as reflecting conformational changes. In our experiments, R has been plotted vs. temperature (Fig. 4b), showing a sharp decrease from 35° to 50 °C. From 50 °C to 90 °C, R was found to be about 0.59–0.61, close to the value of 0.63 obtained with the above mentioned combination of tyrosine in water plus tryptophan in dimethylformamide.

It was also found that the spectrum obtained by addition of a spectrum of tyrosine plus a spectrum of tryptophan (always in a 35/8 mol/mol proportion), both in a mixed medium of ethyl acetate and water in about equal parts, was the one that most resembles that of tubulin at low temperatures (from 5 °C to 45 °C), in terms of the position of the peaks in the spectra. The position of the first two minima were found, in this case, at 293 nm and at 287 nm, whereas in tubulin they are found at 293 nm and at 285 nm respectively. In this case the value of R found was 1.14, which is not as close as the values obtained for tubulin at low temperatures ($\sim$1.5). However, these values support the result obtained in the previous paragraph, namely that the average tyrosine environment tends to change into a more polar nature when the protein is heated, whereas some tryptophan residues in tubulin undergo an average change from a moderately hydrophobic environment towards a more hydrophobic one.

c) Fluorescence

Figure 5a shows fluorescence spectra of tubulin, excited at either 280 nm or at 295 nm (this last one being a selective excitation of tryptophan) for two temperatures, 25 °C and 65 °C. At both excitations, the displacement of the emission maximum towards longer wavelengths was observed between 25° and 65 °C. Figure 5b shows the variation of the ratio of fluorescence intensities at 323 nm over 340 nm with temperature. Figure 5c shows the displacement of the emission maximum vs. temperature. The most salient feature in Fig. 5b and c is the pronounced change in the environment of tubulin fluorophores between 35 °C

and 55 °C, which can be interpreted as reflecting the denaturation of the protein. The midpoint of this change is 48° ± 1 °C (Fig. 5 b), which is essentially coincident with the midpoint of the transition monitored by differential absorption (Fig. 2). Preliminary differential scanning calorimetry results indicate two thermal transitions in tubulin, centered at 48 °C and 52 °C respectively (M. Menéndez, personal communication).

In the final heat denaturated state there is a single emission maximum at 340 nm. In this state, tryptophan residues are not exposed to the solvent and therefore, the tubulin chains are not completely unfolded. This is clearly indicated by the comparison of the heat denaturated spectra to the spectra in 6 M guanidinium hydrochloride (Fig. 5 a) and in 1% SDS (Andreu and Muñoz 1986). In the latter spectra the tryptophan emission maximum is near 350 nm and a separate emission peak of tyrosine at 302 nm appears, indicating total exposure of the tryptophan residues to the solvent and the disappearance of the tyrosine energy transfer to tryptophan and/or quenching, which are characteristic of the folded state (Lakowicz 1983). The moderate red shift of the fluorescence maximum observed in tubulin upon heating may be interpreted as a direct effect of temperature on the fluorophores, rather than on the conformation of the protein. This effect is similar to that observed in the thermal perturbation of the chromophores observed by difference absorption spectroscopy (linear parts in Fig. 2), as can be seen in Fig. 5 c. This figure shows that there is no abrupt transition of the maximum upon heating when the sample is excited at 295 nm. At this wavelength, only Trp is excited. There is a clear transition for the maximum when the sample is excited at 280 nm. At this wavelength both Tyr and Trp are excited. Demchenko and Ladokhin (1988) observed a similar moderate red-shift upon heating melittin. These authors showed that this red-shift may be due to the relative changes of the relaxation time and the excited life-time of the fluorophores, becoming closer to each other.

d) Circular dichroism

The near ultraviolet circular dichroism spectrum of native tubulin (Fig. 6 a), which is mostly sensitive to tertiary structure in the environment of the protein chromophores, is very similar to the spectrum reported by Lee et al. (1978) for this protein preparation. Notice that the near ultraviolet circular dichroism of tubulin depends on the preparation procedure (Clark et al. 1981). The spectrum of the heat-denaturated tubulin (Fig. 6 a, dashed line), indicates strong loss of assymmetry, i.e. much less structure in the environment of the chromophores. Nevertheless, a significant amount of structure is conserved, as revealed by the band present in the 250 nm–290 nm region of the spectrum.

Figure 6 b shows far ultraviolet spectra of 2.6 mg/ml tubulin at 25 °C (continuous line) and at 65 °C (dashed line). The change was not reversible upon cooling the sample. There was a 30% to 40% reduction in intensity at 220 nm (three experiments). The minima at 220 nm and

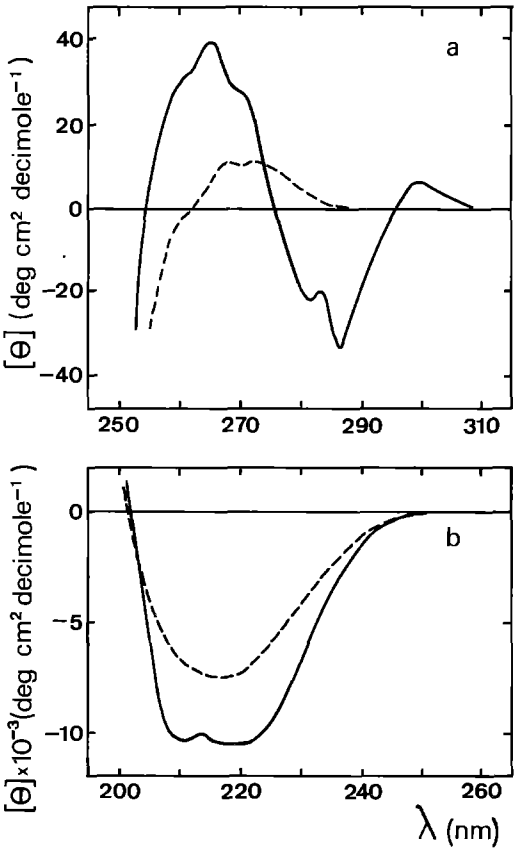

Fig. 6 a, b. Temperature-induced changes of the circular dichroism of tubulin in PG buffer: **a** near-UV spectra of tubulin at 1 mg/ml; **b** far-UV spectra of tubulin at 2.6 mg/ml. In both **a** and **b** continuous lines represent spectra at 25 °C, whereas dashed lines represent spectra at 65 °C

210 nm disappear, leaving a single minimum at 217 nm. These changes can be interpreted as an important reduction in α-helical content (roughly 15% less), leaving a significant proportion of β-sheet structure in the heat denatured state (Greenfield and Fasman 1969). For a detailed interpretation of a very similar tubulin circular dichroism change, see Andreu et al. (1986).

Conclusion

Our data show that tubulin, upon heating, acquires a stable structural state, different from its native state, in which part of its structure is retained, as revealed by the CD spectra. In this partially unfolded state the protein becomes more exposed to the solvent, as revealed by the behaviour of its tyrosine residues. However, we show that in this new structural state, the average Trp environment acquires an apparent increase in hydrophobicity. Kuznetsova et al. (1988), working on tryptophan residues in actin, reported results similar to those reported here. These authors found that in spite of an increase of intramolecular mobility, heated actin still retains the asymmetrical microenvironment of the tryptophan residues.

As indicated above, the lack of turbidity in our samples, regardless of the temperature, indicates that no non-

specific aggregates are formed. We can not rule out the formation of small oligomers which could contribute to the apparent tryptophan burial. However, this seems unlikely too, for such an oligomerization would have the same effect in "burying" tyrosines as well as tryptophans, contrary to our observations. Therefore, our results point to different denatured states of different tubulin domains, as monitored by the average changes in the tyrosine and tryptophan environments. Each tubulin monomer consists of three proteolysis resistant regions, roughly corresponding to the N-terminal, middle and C-terminal thirds of the peptide chain (De la Viña et al. 1988). The tyrosine residues are evenly distributed along the α- and β-tubulin sequences, while the tryptophan residues are absent from the middle fragments of each sequence (Ponstingl et al. 1982). Our results would be compatible with the middle region becoming more solvent-exposed (or most of the molecule) in the heat-denatured state, while the environment of the tryptophan residues in the C-terminal or N-terminal regions becomes more hydrophobic.

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