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ARTICLE

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Fourth derivative UV-spectroscopy of proteins under high pressure

I. Factors affecting the fourth derivative spectrum of the aromatic amino acids

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Abstract A tunable fourth derivative UV absorbance method based on a variable spectral shift has been developed and compared to the Savitzky-Golay method and the analytical derivative. The parameters of the method were optimised for the analysis of the UV absorbance spectra of the aromatic amino acids to quantify the effect of decreasing solvent polarity on their fourth derivative spectra. The wavelength of the highest maximum (λ_{max}) (for tyrosine and phenylalanine) or the amplitude of the highest maximum (A_{max}) (for tryptophan), were shown to depend linearly on the dielectric constant of the solvent, ranging from water to cyclohexane. The only effect of pressure in the 1 to 500 MPa range is a small decrease in the fourth derivative amplitude. This method appears therefore as a suitable tool to evaluate changes of the dielectric constant in the vicinity of the aromatic amino acids in proteins which undergo pressure induced structural changes.

Key words Tryptophan · Tyrosine · Phenylalanine · Dielectric constant · Solvent

1. Introduction

There is growing evidence now that the key for the understanding of enzyme function may be found in the conformational flexibility of proteins (Karplus and McCammon 1981). In fact, proteins may be seen as very complex systems, in thermal equilibrium between many conformational states and substates, and interacting with solvent and other solute molecules (Ehrenstein et al. 1991; Frauenfelder et al. 1991). Clearly, the understanding of enzyme mechanisms requires the study of both energetic and structural features associated with protein conformational changes. The mechanism of these changes can be studied by a perturbation of the protein conformational equilibrium. In contrast to chemical and thermal perturbation, a change in pressure is an elegant way to perturb a conformational equilibrium since it does not change the chemical composition of the solution or its energy (Butz et al. 1988; Morild 1981; Gross and Jaenicke 1994; Lange et al. 1994). Furthermore, when the pressure does not exceed a certain critical value (depending on the protein), its effects are reversible (Heremans 1992). In contrast, increasing the temperature often results in irreversible thermal denaturation.

Any chemical reaction that is accompanied by a change in volume is pressure sensitive and the technology to perform experiments under high pressure (equilibrium and fast kinetics) has been developed (see Mozhaev et al. 1996, for a recent review). Many reactions of proteins have been found to be pressure dependent such as changes in quaternary structure, conformational equilibria, the specific interaction with ligands or the interaction with the solvation shell.

While it is relatively easy to characterise conformational equilibria and elementary reaction steps of proteins when chromophores absorbing in the visible region are present, it is more difficult to obtain protein structural information from bands absorbing in the ultraviolet region, since here many overlapping individual electronic transitions broadened by their vibronic structure are found. Not only the three aromatic amino acids phenylalanine, tyrosine and tryptophan, but also cysteine, histidine and UV components of visible chromophores such as the heme group contribute to the ultraviolet absorbance of proteins resulting in rather structureless spectra. Yet, a better resolution can be achieved, by calculating the second or the fourth derivative of the spectra (Talsky 1994). Second derivative spectroscopy is the more widely used method (Demchenko 1986; Ichikawa and Terada 1977, 1979, 1981;

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Ragone et al. 1984; Bray and Clarke 1995; Mach and Middaugh 1994), in particular for the detection of structural changes in the environment of tyrosine residues (Fisher and Sligar 1985, Kornblatt et al. 1995). Fourth derivative spectroscopy has the advantage that a maximum in the original spectrum corresponds to a maximum in the derivative spectrum. Furthermore, the fourth derivatives are more selective for narrow bands than the second derivatives, and they enable a more detailed study of the environment of all three aromatic amino acids (Mozo-Villarias et al. 1991; Padros et al. 1984 a, b).

The derivatives are usually computed by shifting a spectrum for a given wavelength difference (called the derivation window) and its subsequent subtraction from the original spectrum. The derivation window is a very important feature of this technique, since it determines the spectral amplitude and resolution of the derivative (Butler 1979). Its judicious choice therefore allows one to study selectively certain electronic transitions, according to their spectral band widths. In contrast to many commercial spectrophotometers which impose fixed values for this parameter, we used a tunable method, allowing the optimisation of the necessary compromise between spectral amplitude and resolution for the aromatic amino acids. Here we report a critical analysis of the parameters of the shift method applied to a mathematical model and a comparison with derivatives obtained either analytically or with the method of Savitzky and Golay (1964). This provides the basis for the application of the method to the analysis of the solvent and pressure effects on the fourth derivative spectra of the aromatic amino acids. It will be shown that this method offers a simple way to evaluate the polarity in the vicinity of the aromatic amino acids, and in the accompanying paper it is used to study pressure induced protein structural changes.

2. Materials and Methods

The acetyl-O-ethylesters of phenylalanine (Ac-Phe-OEt), tyrosine (Ac-Tyr-OEt) and tryptophan (Ac-Trp-OEt) were from Sigma, St. Louis. Guanidinium chloride (for biochemistry) was from Merck, Darmstadt. All solvents were of UV-grade quality. Ac-Phe-OEt was dissolved in the different solvents as a 10 mM stock solution and used at a final concentration of 1.67 mM. Ac-Tyr-OEt and Ac-Trp-OEt were dissolved in ethanol as 50 mM and 25 mM stock solutions. Their final concentrations in the different solvents were 0.3 mM and 0.15 mM, respectively.

Baseline corrected UV spectra were recorded in the double beam mode with a Cary 3 E (Varian) spectrophotometer which has a high wavelength reproducibility (SD <0.02 nm). For phenylalanine the bandwidth was 0.5 nm, the data acquisition time 1.5 s per data point and the data interval was 0.05 nm. For tyrosine and for tryptophan the bandwidth was 1.0 nm, the data acquisition time 1.0 s and the data interval was 0.1 nm. The fourth derivatives were computed by the spectral shift method (see below) with a Sigma-plot based program using the spectra saved in ASCII-format.

For all experiments we used a thermostated (–50 °C to +50 °C) high pressure cell (total volume 15 ml) which was designed in our laboratory and built by S.O.F.O.P. (Rodez, France). The cell core was made of Marval X12 special steel from Aubert et Duval (Neuilly sur Seine, France), allowing experiments up to 500 MPa. Around this core an envelope of the same steel was added in which the thermostating alcohol circulated. The geometry of the assembly was miniaturised so that the cell could be placed in the sample compartment of the Cary 3 E spectrophotometer. The windows were made of synthetic sapphire ($\varnothing = 10$ mm, h = 8 mm) from RSA (Jarrie, France) which has a transmittance at 250 nm of 50% for a light path of 8 mm. The windows were placed directly upon the optical obturators. The tightness was assured by the reversible deformation of a polyurethane seal, which was placed between the external face of the obturator and a metallic counterpart. Pressure was generated by a manual pump (700 MPa) and controlled by a metallic gauge type PR851C from Top Industries (Dammarie les Lys, France). The working pressure was measured by an Infinity Indicator, Model Infcp from Newport Electronique (Trappes, France). The pressure vector, water, was mediated through stainless steel capillaries (outer diameter 1/16 inch, inner diameter 0.2 mm). Protein samples were measured in a 2 ml quartz cuvette which was closed with a Dura-Seal polyethylene stretch film maintained by a rubber O-ring. The stability of the temperature was ±0.2 °C. At 500 MPa the stability of the pressure was ±1 MPa.

Our derivation procedure is based on the method suggested by Butler (1979). In this method, the original spectrum is shifted over a small distance, $\Delta\lambda$, to longer wavelengths. The calculated difference between the shifted and the original spectrum is then taken as the first derivative spectrum (but shifted $\lambda/2$ to the red). Derivatives of higher order are obtained by repeating this procedure.

As suggested by Butler (1979), we used two types of noise suppression. Firstly, the successive derivatives were computed by using slightly different $\Delta\lambda$ values. This technique results in an effective noise averaging. Therefore, the indicated values of $\Delta\lambda$ represent mean values. For instance, a $\Delta\lambda$ of 1.6 means that for the first to the fourth derivative wavelength shifts of 1.5, 1.6, 1.7 and 1.6 nm were used. Secondly, the resulting derivative spectra were convoluted with a block shape function with a window of 1.05 nm for phenylalanine, 0.9 nm for tryptophan and 1.1 nm for tyrosine.

3. Results

a. The shift method

For a better understanding of the spectral parameters in derivative spectroscopy, let us inspect the derivatives of a pure Gaussian type spectrum. As shown in the Appendix,

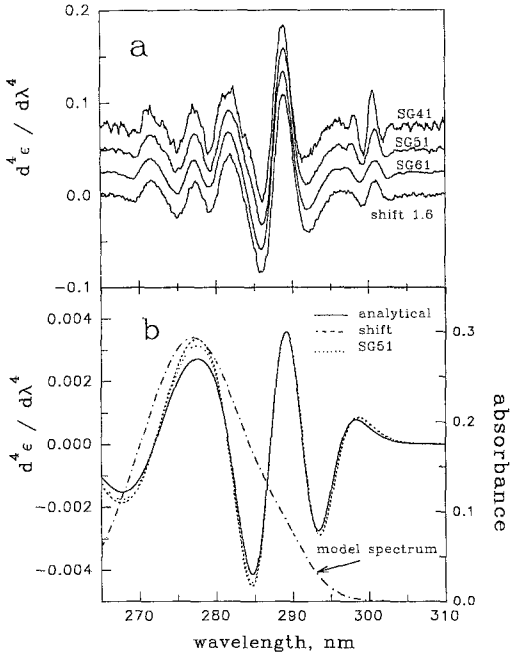

Fig. 1 a, b Comparison of fourth derivative spectra obtained by different methods. **a** Fourth derivative spectrum of tryptophan acetyl-O-ethylester in water; the Savitzky-Golay method with 41, 51 and 61 points (SG41 to SG61) is compared to the shift method (successive shifts of 1.5, 1.6, 1.7, 1.6 nm); **b** model spectrum contructed of two Gaussian bands (*right hand scale*). The parameters were $A_{m,1}=0.293$, $\lambda_{m,1}=277$ nm, $w_1=16.013$ nm for the first band and $A_{m,2}=0.030$, $\lambda_{m,2}=289$ nm, $w_2=7.771$ nm for the second band. The analytical solution is compared to the Savitzky-Golay (*S.G.*) and the shift method

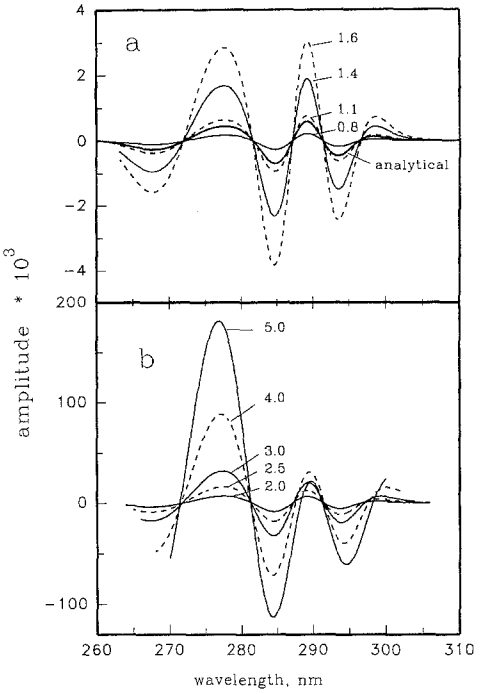

Fig. 2 a, b Influence of the derivative window ($\Delta\lambda$) on the fourth derivative spectrum. Model spectrum of Fig. 2. In **a** and **b** the mean derivation window (shift) was increased as indicated in the figure. For instance 1.4 means that the successive derivatives (first to fourth) were taken with a $\Delta\lambda$ of 1.3, 1.4, 1.5 and 1.4 nm

the bandwidth plays a very important role. In fact, the signal amplitude is inversely proportional to the nth power of the bandwidth, where n is the order of the derivative. This results in a selective resolution enhancement which improves with increasing order of the derivative. Thus, compounds with a narrow spectral bandwidth (like the UV bands of aromatic amino acids) are advantaged with respect to wider bands (from other amino acids and chromophores such as flavine or porphyrine). However, the amplitude decreases at higher derivatives. For example, for a linewidth of 5 nm, the spectral amplitude decreases to 22% and 15% for the second and the fourth derivative. The fourth derivative appears to be a reasonable compromise between resolution enhancement and decreased signal-to-noise ratio. With respect to the second derivative, it has the additional advantage that its extrema have the same sign as those of the original spectrum.

Since the mathematical expression of a real spectrum is not known, it is not possible to compute its analytical derivative. Several approximation methods have been proposed: the use of analogue filters (Ernst 1966), the method of Savitzky and Golay (1964) and corrected by Steinier (1972), which is a spectral convolution with a mathematically derived function, and the shift method (Butler 1979). The resolution and the signal-to-noise ratio of the Savitzky-Golay method depend on the number of spectral points per derivation window. As shown in Fig. 1 a for the fourth derivative spectrum of tryptophan, the Savitzky-Golay method with a derivation window of 51 data points gives results that are comparable to the shift method performed with a mean wavelength shift of 1.6 nm. To judge the degree of distortion caused by the different methods, the fourth derivatives obtained by the shift and the Savitzky-Golay method of a model spectrum composed of two gaussians are compared with the analytical solution in Fig. 1 b, which clearly shows only small deviations.

For the computation of the fourth derivative by the shift method, the derivation window, $\Delta\lambda$, is a crucial parameter. As pointed out by Butler, the amplitude decrease is proportional to $\Delta\lambda^4$ for small $\Delta\lambda$ values. Figure 2 a illustrates this effect for $\Delta\lambda$ values from 0.8 to 1.6 nm, corresponding to a fifth of the bandwidth w_2. As shown in Fig. 2 b, the derivative spectrum becomes strongly distorted (change of the relative amplitudes of the two maxima) when $\Delta\lambda$ becomes greater than one third of the spectral bandwidth. In the search for the best $\Delta\lambda$ values that should be used to obtain the fourth derivative spectra of the different aromatic amino acids we looked therefore for a compromise between the achievement of an adequate signal-to-noise ratio and the amount of spectral distortion. We found empirically that the following $\Delta\lambda$ values are suitable for the aromatic amino acids: 1.3 nm (Phe), 2.6 nm (Tyr) and 1.6 nm (Trp). The fourth derivatives of phenylalanine, tyrosine and tryptophan using the $\Delta\lambda$ values are depicted in Fig. 3. Whereas the fourth derivatives of phenylalanine and tryptophan can be readily distinguished, the bands of tyrosine are partly masked by those of tryptophan.

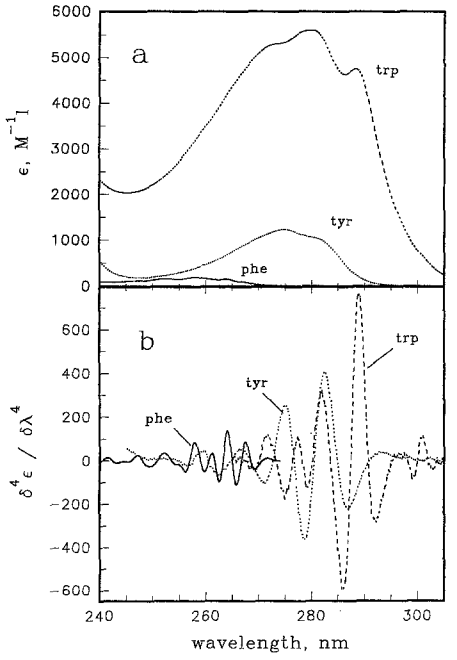

Fig. 3 a, b UV spectra of phenylalanine, tyrosine and tryptophan. Acetyl-O-ethylesters in water. **a** Zeroth order spectra, **b** fourth derivative spectra. The spectral conditions and the parameters used for the derivatives are described in the Materials and methods section

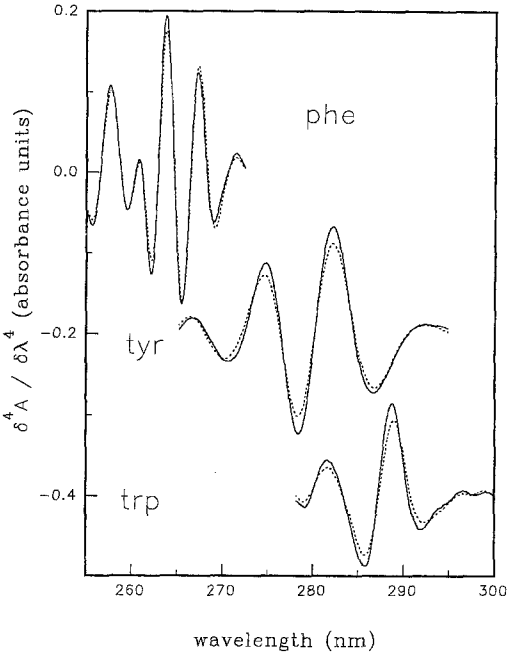

Fig. 5 Pressure effect on the fourth derivative spectra od the aromatic amino acids. Acetyl-O-ethylesters of phenylalanine, tyrosine and tryptophan. *Solid line:* 1 MPa, *broken line:* 450 MPa. The spectra of tyrosine and tryptophan are down-shifted. All spectra were corrected for the solvent compressibility

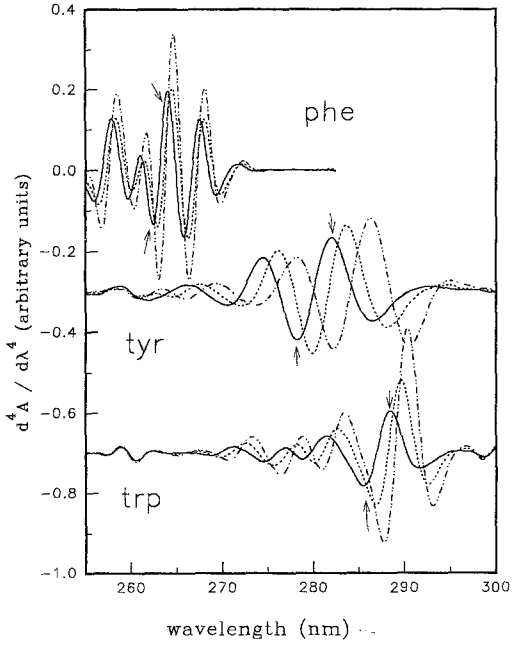

Fig. 4 Effect of ethanol on the fourth derivative UV-spectra of the aromatic amino acids. Acetyl-O-ethylesters of phenylalanine, tyrosine and tryptophan in water (——), 50% ethanol (- - - -) and 100% ethanol (– · · –). The minima and maxima used in Table 1 to determine the derivative amplitude are indicated for the aqueous solvent. The spectra of tyrosine and tryptophan are displaced for clarity

b. Solvent effect on the fourth derivative UV-spectra of the aromatic amino acids

As shown in Table 1, the successive decrease of the solvent polarity and hydrogen bond forming capacity when going from water to cyclohexane results in significant changes of the fourth derivative spectra of the three aromatic amino acids. The decrease of the solvent polarity results in a general increase of the amplitude and in a shift of the derivative bands to longer wavelengths. However, these two spectral features do not show the same solvent dependence for the three aromatic amino acids. An example is given in Fig. 4 where the effect of ethanol is shown. Replacing the aqueous solvent by ethanol strongly increases the amplitude of the phenylalanine and tryptophan bands, whereas it changes only slightly the position of the phenylalanine bands and moderately those of tryptophan. On the other hand, the fourth derivative bands of tyrosine show a marked bathochromicity, but their amplitudes are only slightly affected. Table 1 presents the solvent effects of 6 different solvents of decreasing polarity. All three aromatic amino acids show a continuous shift of the fourth derivative λ_{max} to longer wavelengths. To analyse amplitude effects, Padros et al. (1982) and Duñach (1983), have introduced a geometric parameter. This parameter, which was already used by Servillo et al. (1982) for the second derivatives, varies to some extent for tyrosine in different solvents. It is of no use however for phenylalanine and tryptophan, where it does not vary significantly between the different solvents. We propose to use two other parame-

Table 1 Solvent effects on the fourth derivative spectra of aromatic amino acids

Solvent	Dielectric constant, ε_r	phe		tyr		trp	
		λ_{max}	ΔA_{rel}	λ_{max}	ΔA_{rel}	λ_{max}	ΔA_{rel}
Water[a]	80.4	262.45	1.00	282.0	1.00	288.4	1.00
50% ethylene glycol[b]	64.5	263.00	1.36	283.4	0.97	290.2	1.26
50% ethanol[c]	52.4	262.90	1.03	283.8	1.22	289.6	1.65
Ethanol[a]	24.3	263.10	1.84	286.2	1.27	290.3	2.84
Diethyl ether[a]	4.34	263.25	1.87	286.3	3.03	291.0	3.26
Cyclohexane[a]	2.02	263.25	1.72	287.7	1.06	290.4	4.09

λ_{max} = wavelength of the highest peak, ΔA_{rel} = relative maximum amplitude (see Fig. 4) with respect to the amplitude in aqueous solvent. The dielectric constants were taken from [a] the Handbook of Chemistry and Physics (1974), [b] Douzou (1977), [c] the mean value of ε_r between pure water and pure ethanol

ters: first the position of the highest maximum, and second, the relative maximum amplitude with respect to a standard condition (aqueous solvent). The first parameter is most useful for tyrosine, the second one for phenylalanine and tryptophan.

c. Pressure dependence of the aromatic amino acid fourth derivative-spectra

As shown in Fig. 5, pressure does not modify significantly the fourth derivative UV spectrum of the aromatic amino acids up to 500 MPa in aqueous solvent. After correction for the solvent compressibility, the only effect of pressure was a small decrease of the amplitudes of the phenylalanine and tyrosine spectra. In addition, the tryptophan spectrum was red shifted by less than 0.2 nm. The same experiments carried out in ethanol, gave very similar results.

Discussion

Second and fourth derivative absorbance and fluorescence spectroscopy have been employed by many scientists to examine structural changes of proteins (as well as nucleic acids) accompanying their reactions or their denaturation (Padros et al. 1984 a; Talsky 1994; Restall et al. 1986; Garriga et al. 1995). The common approaches (shift or Savitzky-Golay) depend much on the choice of the parameters, such as the derivation window with respect to the spectral bandwidth and the convolution window. Therefore, although the computation of the derivatives has become easy nowadays, it is not always clear whether the resulting spectra can be used to arrive at physically sound conclusions. So when we use fourth derivative spectroscopy to study the effect of pressure on proteins, we need to know whether the method used to obtain the derivatives is free of artefacts and which structural information can be deduced from the spectral changes observed at high pressure.

The application of the fourth derivative method is only meaningful, when the results are close to the analytical derivative of the corresponding mathematical function. Only in that case we can control the filtering effect of the fourth derivatives and be sure that the Lambert-Beer law still holds. We have shown that when the parameters are judiciously chosen, the shift and the Savitzky-Golay method lead to very similar results which are nearly identical to the analytical solution. The advantage of the shift method is its ease and its tunable control through the size of the $\Delta\lambda$ parameter, which can be adapted to the analysis of a particular component, allowing it to be distinguished from other components in the mixture according to its spectral bandwidth.

The results obtained here show that we can be confident that the shift method leads to reliable fourth derivative spectra so that we can attempt its use as a tool to interpret pressure induced spectral changes of proteins in structural terms. For that purpose, an understanding of the environmental effects of solvent and pressure on the spectra of the aromatic amino acids is required. The generally complex problem of the origin of solvent effects on UV-spectra (Gerrard and Maddams 1978 a, b; Yanari and Bovey 1960) is simplified in the case of the aromatic amino acids, where an exposure to a more hydrophobic environment (e. g. the interior of proteins) always leads to a spectral red shift. The relatively small red shift on the fourth derivative spectrum of the nonpolar benzene ring of phenylalanine can be explained by the action of dispersion forces between the solvent and the chromophore which lead to an energy decrease of its excited state (Reichardt 1990). These forces, which are always present, result in a small bathochromic effect whose magnitude depends on the refractive index of the medium. The solvent effects on the more polar phenol and indole moieties of tyrosine and tryptophan is more complicated. In addition to the dispersion forces, depending on the solvent, dipole-dipole and/or dipole-induced dipole interactions occur. It depends now on the respective solvation (and stabilisation) of their Franck-Condon excited state or their electronic ground state whether the result is a red or a blue shift (Reichardt 1990). Since in the case of tyrosine and tryptophan a decrease of solvent polarity always results in a bathochromic effect, it is mainly the energy level of the electronic ground state which is increased in a less polar solvent. Again, such an effect should depend on the refractive index of the solvent.

In an attempt to relate the spectral shift directly to the macroscopic properties of the solvent, Padros et al. (1982) fitted various parameters in a solvatochromic equation which takes into account the hydrogen-bond donor acid-

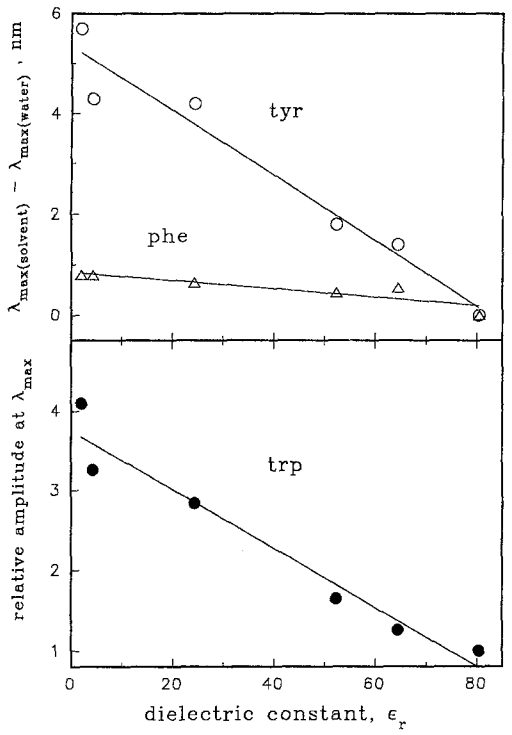

Fig. 6 Effect of the dielectric constant on the fourth derivative spectral properties of the aromatic amino acids. The solvents were those of Table 1

ity, the acceptor basicity and the solvent polarity. A satisfactory fit was obtained for tyrosine, but not for phenylalanine and tryptophan. Furthermore, no details were given on the definition of the polarity parameter. As shown in Fig. 6, for a variety of solvents ranging from water to cyclohexane, simple and adequate relationships between the solvent effect on the fourth derivative spectra of the aromatic amino acids and the dielectric constant can be found. The strong red shift of λ_{max} of tyrosine and the relatively weak shift of phenylalanine depend linearly on ε_r, while in the case of tryptophan, it is mainly the amplitude of the fourth derivative spectrum that depends on ε_r. An increase in the amplitude of the fourth derivative spectrum means a sharpening of the absorbance band in the zero-order spectrum. This may be explained by the fact that in tryptophan the two electronic transitions, from the ground state to the excited states, ^1L_a and ^1L_b, that contribute to the UV spectrum, behave differently as a function of solvation (Duñach et al. 1983). The main contribution to the fourth derivative spectrum is that of the ^1L_b transition which has the smaller bandwidth. However, owing to its larger dipole, the ^1L_a excited state is more sensitive to the solvent, and the relative diffuseness of this band in polar solvents is explained by inhomogeneous broadening (Muiño and Callis 1994).

In the past, several attempts have been made to relate solvent induced spectral shifts to the refractive index, n_r, and thus to ε_r ($n_r = \varepsilon_r^{1/2}$), for instance the semiclassical approach of Bayliss (1950) that relates the spectral shift to $(n^2-1)/(2n^2+1)$, which is successful in certain ranges of solvent polarity. For the more polar solvents, alternative relations have been proposed (Zipp and Kauzmann 1973).

However, until now, the theoretical background of solvent induced spectral shifts is not quite clear. The linear relationship with ε_r that is found in the present investigation is astonishingly simple. Maybe the increased spectral resolution of our method was necessary to reveal this relationship. Moreover, this phenomenon did not depend critically on the choice of the derivation window, suggesting therefore that it reflects a real physico-chemical property of these chromophores. However, our limited empirical approach does not provide a theoretical explanation, and, as discussed above, spectral changes in the UV may be caused by various factors. Therefore, even if it is possible that not all solvents will fit this linear relationship, the empirical ε_r dependency observed in the series of solvents used in this study gives us a scaling tool allowing the fourth derivative spectral shifts and amplitudes of the amino acids in proteins to be tentatively interpreted in terms of the polarity of their environment.

As reported by Zipp and Kauzmann (1973), pressure usually has a bathochromic effect. This is explained by the pressure induced increase in solvent density, d, which is related to the refractive index, n, by the Lorentz-Lorentz equation

$$(n^2-1)/(n^2+2) = Kd$$

where the constant K depends on the solvent polarizability. However, owing to the rather small solvent compressibility in the pressure range up to 500 MPa the pressure induced shifts of the fourth derivative spectra are negligible for phenylalanine and tyrosine and relatively small for tryptophan. It is more difficult to interpret the small pressure induced decrease of amplitude of the fourth derivative spectra of the aromatic amino acids. A possible explanation is that the aromatic rings of these model compounds interact more strongly with each other under pressure. This interaction would affect their electronic transitions. But evidently, in proteins the intrinsic pressure effects on the fourth derivative spectra of the aromatic amino acids can be expected to be small in comparison to the spectral changes due to the pressure induced protein conformational changes which result in a modified polarity of their environment. In the accompanying paper we report the use of fourth derivative spectroscopy to examine pressure induced conformational changes of proteins.

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Appendix

1. The analytical second and fourth derivatives of a Gaussian absorption spectrum of the type

$A = A_m \cdot e^{-\left(\frac{\lambda-\lambda_m}{a}\right)^2}$ where A_m is the maximum amplitude

and $a = \frac{w}{2\sqrt{\ln 2}}$

is a parameter proportional to the linewidth, w, at $A = A_m/2$

are: $\frac{\partial^2 A}{\partial \lambda^2} = \frac{2A_m}{a^2} e^{-\left(\frac{\lambda-\lambda_m}{a}\right)^2} \left[2\left(\frac{\lambda-\lambda_m}{a}\right)^2 - 1\right]$

and $\frac{\partial^4 A}{\partial \lambda^4} = \frac{4A_m}{a^4} e^{-\left(\frac{\lambda-\lambda_m}{a}\right)^2} \left[4\left(\frac{\lambda-\lambda_m}{a}\right)^4 - 12\left(\frac{\lambda-\lambda_m}{a}\right)^2 + 3\right]$

It follows that the amplitudes at λ_m are

$\frac{\partial^2 A}{\partial \lambda^2}_{(\lambda_m)} = \frac{-8A_m \ln(2)}{w^2}$ and $\frac{\partial^4 A}{\partial \lambda^4}_{(\lambda_m)} = \frac{192A_m \ln(2)^2}{w^4}$

for the second and fourth derivatives.

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