Eur Biophys J (1996) 24: 284–292

ARTICLE

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Fourth derivative UV-spectroscopy of proteins under high pressure

II. Application to reversible structural changes

Received: 25 October 1995 / Accepted: 5 March 1996

Abstract The structural basis and the thermodynamics of pressure induced reversible spectral transitions in the fourth derivative ultraviolet absorbance spectra of proteins were analysed as described in the preceding paper. Three proteins were studied: adrenodoxin (a small iron-sulphur protein that serves as an electron donor for cytochrome P450scc), ribonuclease A, and methanol dehydrogenase (a tetrameric protein). Fourth derivative spectroscopy is used to probe important mechanistic aspects of these proteins. For adrenodoxin, the results suggest that one or two phenylalanines interact with the iron-sulphur redox centre. High pressure denaturation of ribonuclease leads to a molten globule like structure that also occurs as an intermediate in the high temperature induced denaturation process. This state is characterised by the local dielectric constant in the vicinity of tyrosines. Methanol dehydrogenase was found to be very stable towards pressure. High pressure appears to strengthen the interaction between the two α-subunits possibly through the increased interaction of four tryptophans with other aromatic amino acids.

Key words Adrenodoxin · Methanol dehydrogenase · Ribonuclease · Molten globule · Protein dissociation

1. Introduction

In general, protein structural changes in solution cannot be predicted from the classical concepts of physical chemistry mainly as a consequence of the difficulty of applying mechanistic schemes elaborated from chemical models to the complexity of proteins. This is so because physicochemical parameters such as pH or dielectric constant that can be measured in the bulk solution, are generally unknown in the local environment of functionally important amino acid residues of a protein. In the preceding paper we have described a technique based on fourth derivative UV spectroscopy that yields information about the dielectric constant in the vicinity of the aromatic amino acids. Here we use this method to study reversible protein structural changes that are induced by high pressure. In fact, within certain limits that are specific for a particular protein, pressure induced structural changes are often reversible and therefore allow the determination of the thermodynamic parameters (Heremans 1992). These parameters may then be used for a better understanding of the principles underlying protein stability.

Pressure influences chemical reaction equilibria that are characterised by a net volume change (Gross and Jaenicke 1994). The origin of the pressure effect is given by fundamental thermodynamics, relating the chemical potential μ to pressure p (van Eldik et al. 1989; Tauscher 1995).

$$\left(\frac{\partial \mu}{\partial p}\right)_T = V \tag{1}$$

Since at equilibrium the sum of the chemical potentials is zero, we have

$$\sum \nu_i \mu_i^0 + RT \sum \nu_i \ln a_i = 0, \tag{2}$$

where ν_i is the stoichiometric coefficient, μ_i^0 the standard chemical potential and $a_i = c_i^* \chi_i$, the chemical activity of compound i. Since the equilibrium constant K is defined as:

$$K = \prod_i a_i^{\nu_i}, \tag{3},$$

we obtain

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta V}{RT}. \tag{4}$$

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Accordingly, applying pressure to a chemical reaction such as reversible protein denaturation, will shift the equilibrium towards the state which has the smaller volume. This effect is sometimes attributed to the so-called Le Chatelier's principle (Mozhaev et al. 1994). It can be understood, as shown above, to be the consequence of the pressure effect on the chemical potential at equilibrium.

The reaction volume (ΔV) associated with pressure induced structural transitions of proteins can therefore be determined from Eq. (4) when the equilibrium is studied as a function of pressure. For kinetics, the same formalism holds for the activation volume, $\Delta V^{\ddagger}$. ΔV is the volume counterpart to the energetic terms ΔH and ΔS. It can give information about monomer – oligomer equilibria, the interaction of an enzyme with a specific ligand or the interaction with the solvation shell. Hemoproteins have been studied in particular by pressure perturbation techniques taking advantage of the fact that their chromophores absorb in the visible, and the ΔV and $\Delta V^{\ddagger}$ parameters have been determined for conformational equilibria and elementary reaction steps (Frauenfelder et al. 1991; Lange et al. 1994; Hui Bon Hoa et al. 1990).

Here we are interested in the pressure and temperature induced protein structural changes sensed by the ubiquitous chromophores phenylalanine, tyrosine and tryptophan. In contrast to fluorescence spectroscopy which concerns mainly the tryptophan environment, fourth derivative UV spectroscopy allows one to substantiate these transitions selectively by the quantitative determination of the mean dielectric constant in the vicinity of the aromatic amino acids. This approach is used here to study three proteins for which the UV spectrum is dominated by the contribution of a particular aromatic amino acid. These proteins are: adrenodoxin, an iron sulphur protein and electron donor for cytochrome P450scc (13.1 kDa, 4 phe, 1 tyr), ribonuclease A (13.7 kDa, 3 phe, 6 tyr) and methanol dehydrogenase (MEOH-DH) {152 kDa, 48(46) phe, 70 (52) tyr, 40 trp} Since the complete amino acid composition of MEOH-DH from *Methylophaga marina* is not known, we give here the composition of its homologous form from *Methylobacterium extorquens* (Nunn et al. 1989; Anderson et al. 1990). For a comparison, the known amino acid composition of the two large α-subunits of MEOH-DH from *Methylophaga marina* is indicated in parenthesis. For each of these proteins we recorded the UV spectrum as a function of pressure and temperature, and computed the fourth derivative spectrum.

2. Materials and methods

Ribonuclease-A (EC 3.1.27.5) was from Sigma, St. Louis, methanol dehydrogenase (MEOH-DH) was isolated from *Methylophaga marina* as described previously (Janvier et al. 1992) and adrenodoxin was isolated from bovine adrenal cortex (Suhara et al. 1972; Lange et al. 1988). The final enzyme concentrations were: adrenodoxin, 50 μM, ribonuclease-A, 73 μM, and methanol dehydrogenase, 3.6 μM. Guanidinium chloride (biochemistry grade) from Merck, Darmstadt, was prepared as a 7.5 M stock solution in water. For protein denaturation studies guanidinium chloride was added to the protein solution to a final concentration of 6 M in 50 mM potassium phosphate, pH 7.4. For pressure studies adrenodoxin was dissolved in potassium phosphate buffer, 50 mM, pH 7.0. The pK of this buffer is known to decrease as a function of pressure – about 0.8 units at 100 MPa (van Eldik et al. 1989). In spite of this inconvenience we used this buffer since adrenodoxin is very stable in phosphate, and it is also not possible to determine the variation of the local pH of adrenodoxin as a function of pressure. Ribonuclease was dissolved in HCl, pH 2.0. The buffer for MEOH-DH was 10 mM MOPS, pH 7.0. The pK of this buffer does not vary significantly as a function of pressure.

The spectral recording conditions, the pressure equipment and the derivation procedure (shift method) were those described in the preceding paper. The mean derivation windows for the UV-spectra of adrenodoxin, ribonuclease A and MEOH-DH were those that are optimal to observe the changes in the prevailing aromatic amino acids, i.e. 1.3 nm (Phe), 2.6 nm (Tyr) and 1.6 nm (Trp) respectively. For the visible spectrum of adrenodoxin we chose a derivation window of 10 nm. Spectra taken at different temperatures and pressures were corrected for the thermal expansivity (Kell 1973) and the compressibility (Gibson and Loeffler 1941) of water. The thermodynamic parameters were determined by fitting the data to the appropriate function by using the Marquardt-Levenberg algorithm with a Sigmaplot based program.

Enzyme activities were determined as follows. Adrenodoxin, 5 μM in 50 mM phosphate buffer pH 7.4, was incubated at 25 °C, with adrenodoxin reductase, 0.5 μM, NADPH, 1 mM and cytochrome *c*, 10 μM. The reduction kinetics of cytochrome *c* were followed at 550 nm. MEOH-DH was incubated in 10 mM CHES buffer, containing 50 mM ammonium chloride and 3 mM methanol at pH 10.0 with Wurster's Blue, 70 μM. The kinetics of the decoloration of the dye were followed at 612 nm ($\varepsilon = 12\,700\ M^{-1}\ cm^{-1}$). The hydrolytic activity of ribonuclease was assayed according to Irie (1965) with cytidine 2′, 3′ cyclic monophosphate sodium salt, 0.5 g/l in 10 mM sodium acetate pH 5.2, containing 0.1 M NaCl. The kinetics were recorded at 290 nm at 37 °C.

3. Results

a. Adrenodoxin

Figure 1 illustrates the power of fourth derivative spectroscopy: the zero-order UV spectrum only shows a very small undulation around 265 nm, while the fourth derivative spectrum reveals the characteristic spectral features of phenylalanine (see the preceding paper), even in the presence of large absorbance bands originating from the iron-sulphur centre and one tyrosine. An increase of pressure

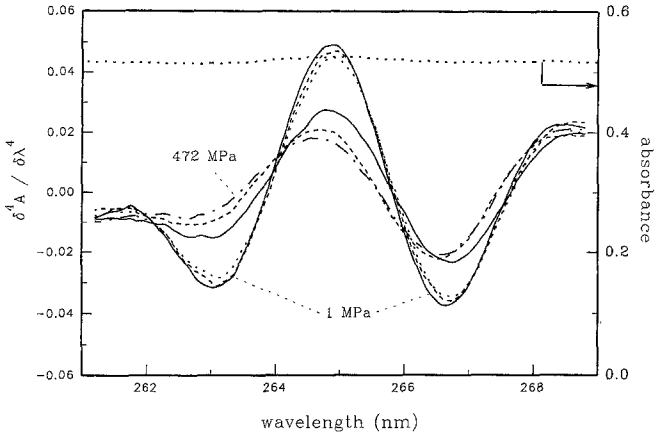

Fig. 1 Effect of pressure on the fourth derivative UV-spectrum of adrenodoxin. Zero-order spectrum (*dotted*) at 1 MPa, *right hand scale*; fourth derivative spectra at 1, 102, 202, 303, 392 and 472 MPa

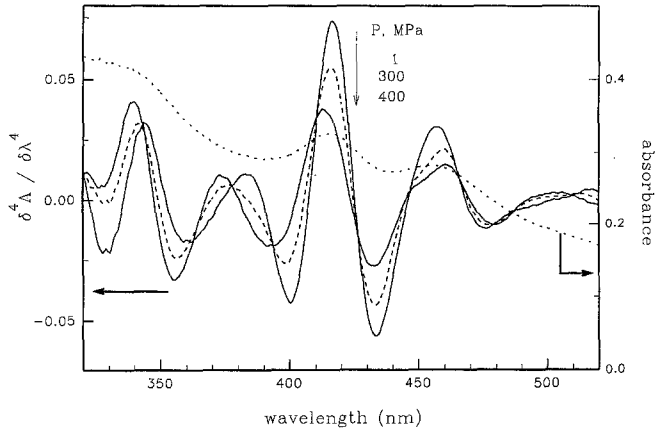

Fig. 2 Effect of pressure on the fourth derivative visible-spectrum of adrenodoxin. Zero-order absorbance spectrum at 1 MPa (data interval 0.5 nm), *right hand scale*; derivative spectra (*left hand scale*) computed with a derivative window of 10 nm and a linear smoothing of 13 data points

from 1 to 472 MPa results in a strong decrease of the amplitude and a small hypsochromic effect (from 264.85±0.025 to 264.55±0.025 nm) of the fourth derivative. These spectral changes were fully reversible. At these high pressures the enzyme was still active: At 400 MPa the cytochrome *c* reduction rate was even increased by a factor of 17.

The visible spectrum of adrenodoxin is dominated by the contribution of the iron-sulphur (Fe_2-S_2) centre which has a maximum at 417 nm. Since the pressure effects on this spectral band appear to be relatively small, the fourth derivatives are also useful here since they are insensitive to baseline shifts. As shown in Fig. 2, a pressure increase from 1 to 400 MPa results in a diminution of the amplitude and a marked blue shift (from 416.5±0.25 to 413.0± 0.25 nm) of λ_{max}. The spectral transition has clear isosbestic points even though the effect is not fully reversible, since after depressurization only 72% of the original optical density at 417 nm and 78% of the enzyme activity is recovered. In contrast, the action of guanidinium chloride (6 M) or high temperature (75 °C) leads to a completely irreversible decoloration in the visible spectral region and a complete loss of activity. Therefore only pressure induced spectral changes were studied here.

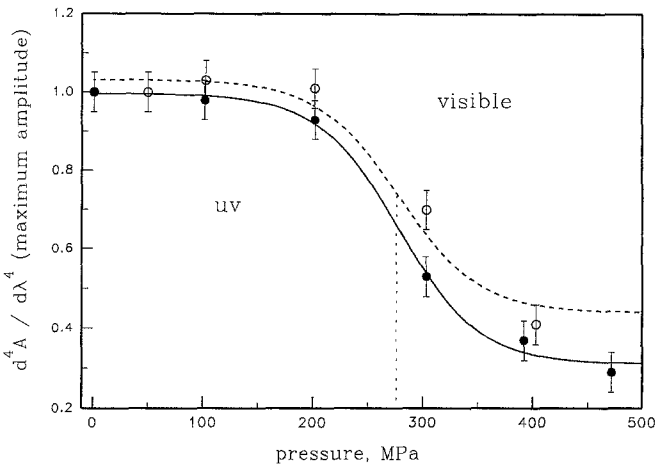

Fig. 3 Pressure effect on the amplitude of the fourth derivative UV and visible spectra of adrenodoxin. The normalised amplitude (relative to that at atmospheric pressure) of the visible band (417–435 nm) is compared to that of the UV band (264.85–263.25 nm). The *broken* and *solid lines* are the respective fits through the data

In Fig. 3 the pressure induced UV and visible spectral transitions are compared. The amplitude of the fourth derivatives (corrected for the volume contraction) was fitted with a simple two state model

$$l \xleftrightarrow{K} h$$

with

$$K = \frac{h}{l} \tag{5}$$

where l and h denote the structural states at low and high pressure. Since for the fourth derivatives the Lambert-Beer law holds, i. e. the amplitude is proportional to the concentration, we obtain

$$A = \frac{A_l - A_h}{1+K} + A_h \tag{6}$$

where A_l and A_h denote the amplitudes of the pure l and h state.

Since

$$K = e^{-\left(\frac{\Delta G_{p=0} + p\Delta V}{RT}\right)} \tag{7}$$

where $\Delta G_{p=0}$ is the free energy change at zero pressure, the relation of the fourth derivative amplitude as a function of pressure can be expressed as

$$A = \frac{A_l - A_h}{1 + e^{-\frac{\Delta G_{p=0} + p\Delta V}{RT}}} + A_h . \tag{8}$$

Fitting the UV data with this model gives the following values for the thermodynamic parameters at 25 °C and 0.1 MPa: $\Delta G^0 = \Delta G_{p=0} + p\Delta V_{0.1\ \mathrm{MPa}} = 18.3 \pm 1$ kJ/mol and $\Delta V = -66 \pm 5$ ml/mol. To know whether the visible spectral transition reflects the same process, we imposed the same values of these parameters to the fit of the visible transition, and only the spectral amplitude values of the pure *l* and *h* forms were allowed to vary. As can be seen in Fig. 3, the fit is quite satisfactory, i.e., the visible data can be fitted with the same parameters as the UV data.

b. Ribonuclease A

At pH 2 and 25 °C, an increase of pressure up to 500 MPa results in a smaller amplitude of the fourth derivative band and in a blue-shift (Fig. 4) from 285.7±0.05 to 283.5±0.05 nm. This transition is fully reversible with clear isosbestic points, indicating an equilibrium between two structural states of the protein. Assuming that this spectral shift reflects a change in the dielectric constant sensed by the tyrosines, we can, as described in the preceding paper, quantify this transition in physico-chemical terms: the observed shift of 2.2 nm in the fourth derivative spectrum corresponds to an increase of the mean dielectric constant from 25 to 59. The effect of high pressure on the fourth derivative spectrum is similar to that obtained with 6 M guanidinium chloride (at pH 7.4, result not shown) which is known to produce an unfolded protein state (Tamura and Gekko 1995) indicating that high pressure may also induce an unfolded denatured state. The pressure effect can also be described by a simple two-state model between the native ($\varepsilon_{tyr} = 25$) and the denatured ($\varepsilon_{tyr} = 59$) state. The pressure dependence of the maximum amplitude (285.7±283.5 nm) fitted to Eq. (8) (Fig. 6a), yielded the thermodynamic parameters of this structural transition: $\Delta G^0 = 10.3 \pm 0.5$ kJ/mol and $\Delta V = -52 \pm 2$ ml/mol with a transition midpoint at 196 MPa. A comparable volume change was measured by Brandts et al. (1970) ($\Delta V = -45$ ml/mol) by normal (zero order) UV spectroscopy.

The denaturation of ribonuclease by high temperature appears to be more complicated, as shown in Fig. 5. The fourth derivative reveals three transitions as a function of temperature, denoted as *I*, *II* and *III*, in the following scheme:

$$n' \xleftrightarrow{I} n \xleftrightarrow{II} d \xleftrightarrow{III} d'$$

Below 30 °C the position of λ_{max} (285.7) does not vary significantly, but the amplitude at this wavelength decreases. An isosbestic wavelength is found at 283.1 nm for transition *I*. From 30 °C to about 48 °C (transition *II*) the derivative spectrum is blueshifted to 283.7, an effect similar to that caused by increasing the pressure. For this transition the isosbestic wavelength is 284.5 nm. Above 48 °C (transition *III*) the amplitude decreases and the maximum shifts to 283.9 nm. The determination of the thermodynamic parameters is therefore more difficult. As shown in Fig. 6b, it is possible to eliminate at least one of the three transi-

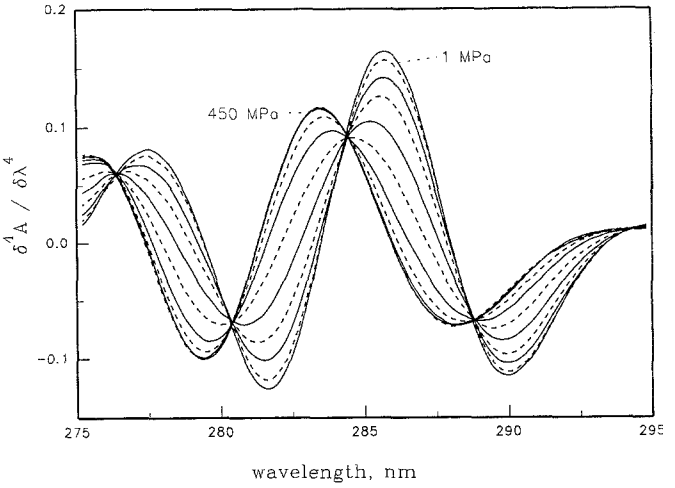

Fig. 4 Effect of pressure on the fourth derivative UV-spectrum of ribonuclease A. Ribonuclease A, 73 μM, at pH 2 and 25 °C

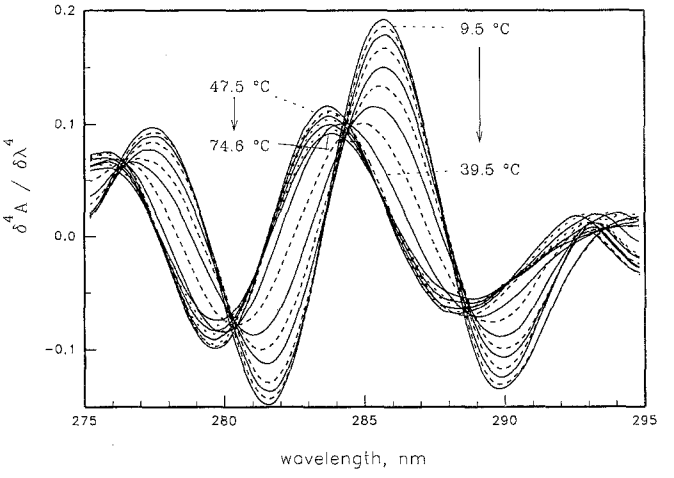

Fig. 5 Temperature effect on the fourth derivative UV-spectrum of ribonuclease A. Ribonuclease A, 73 μM, at pH 2 and 1 MPa

tions by analysing the data at the isosbestic wavelengths. Thus at 285.5 nm we see only transitions *II* and *III*, and at 283.1 nm only transitions *I* and *II*. Transition *II*, which can be fully observed, was fitted with a two-state and model between a native *n* and a denatured *d* state as in the case of denaturation by pressure. For that purpose, the term $\Delta G_{T=\mathrm{const.}} = \Delta G_{p=0} + p\Delta V$ in Eq. (7) was replaced by the corresponding $\Delta G_{p=\mathrm{const.}} = \Delta H - T\Delta S$. In order to take into account transitions *I* and *III*, which are accessible only in part, we assume in a first approximation that at 283.1 nm the derivative amplitude of state *d*, and at 285.5 nm the derivative amplitude of state *n* decrease linearly as a function of temperature. Hence for the fits we used Eqs. (9) and (10):

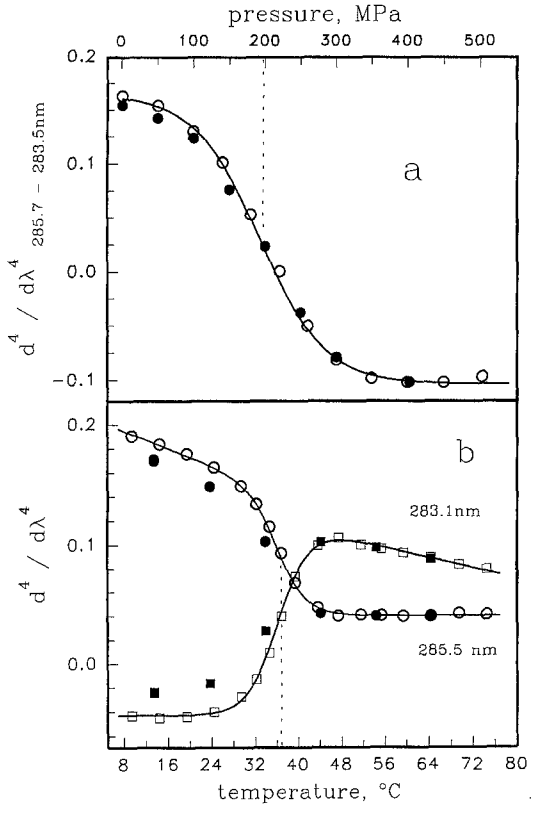

Fig. 6 a, b Amplitude of the fourth derivative spectrum of ribonuclease A in the course of denaturation by pressure and temperature. *Open symbols* reflect the results for the increase in pressure or temperature, and *closed symbols* a decrease in pressure or temperature. The fits (*solid lines*) are shown for the experiments at increasing pressure or temperature. **a** Pressure dependence of the maximum amplitude (trough to peak). **b** Temperature dependence of the amplitudes at 283.1 nm and 285.5 nm

at 283.1 nm

$$A = \frac{A_n - A_d + mT}{1 + e^{\frac{\Delta S}{R} - \frac{\Delta H}{RT}}} + A_d - mT \qquad (9)$$

at 285.5 nm

$$A = \frac{A_n - A_d - qT}{1 + e^{\frac{\Delta S}{R} - \frac{\Delta H}{RT}}} + A_d \qquad (10)$$

where m and q are the temperature coefficients, and A_n and A_d are the intrinsic derivative amplitudes of states n and d. As can be seen in Fig. 6b, the fits are quite satisfactory. The thermodynamic parameters of the n to d transition at 283.1 nm (285.5 nm) are $T\Delta S_{298} = 265$ kJ/mol (283 kJ/mol) and $\Delta H = 275$ kJ/mol (294 kJ/mol), with a transition midpoint at 36.7 °C. Obviously, the parameters obtained at the two isosbestic wavelengths are not significantly different. The free energy calculated for the thermal transition was $\Delta G^0 = 10$ (11) kJ/mol, is equal to the ΔG^0 value of the pressure induced denaturation. As can be seen in Fig. 6, the denaturation by high temperature is not fully reversible, in contrast to the denaturation by pressure.

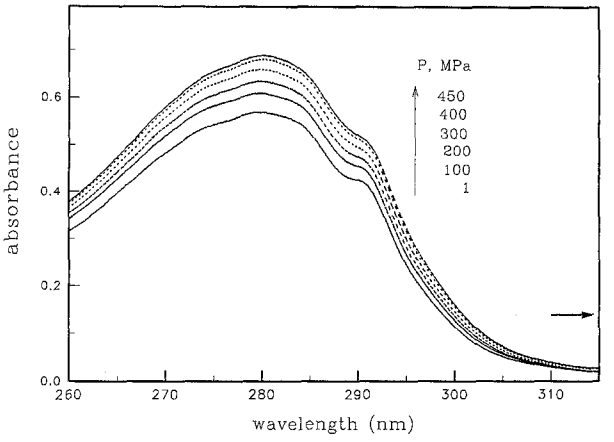

Fig. 7 Effect of pressure on the zero-order UV-spectrum of MEOH-DH

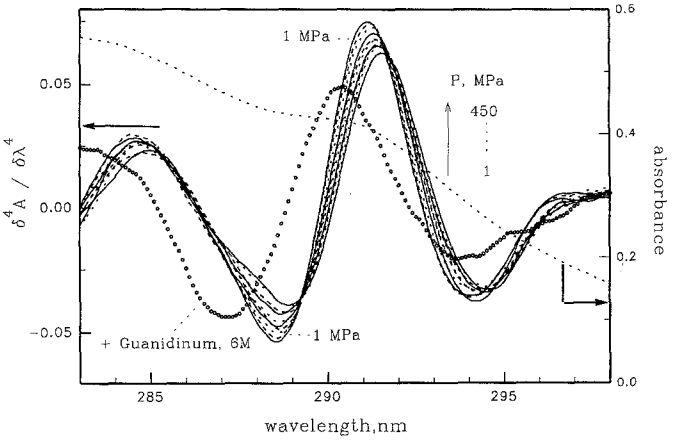

Fig. 8 Effect of pressure on the fourth derivative spectrum of MEOH-DH. The pressure was increased from 1 to 450 MPa in steps of 50 MPa. The derivative spectrum of MEOH-DH denatured by the action of guanidinium chloride is shown by *open circles*. For comparison, the original zero-order spectrum is shown as a *broken line* (*left hand scale*)

c. MEOH-DH

As shown in Fig. 7, raising the pressure to 450 MPa results in a hyperchromicity of the UV absorption spectrum of MEOH-DH. This apparent increase of the absorbance coefficient is mainly due to the compressibility of the aqueous solvent. To give an idea, the volume of water diminishes by 7% when the pressure is raised to 200 MPa and by 12.7% at 450 MPa according to Gibson and Loeffler (1941) who determined the pressure dependent parameters of the Tait equation. In addition to the effect on the concentration, we sometimes observed small effects on the baseline. They possibly arise from small changes in the geometry of the light path due to reversible deformations of the sapphire windows or from changes in the refractive index. It is therefore difficult to analyse the relatively small

pressure induced changes of the zero-order absorbance of MEOH-DH. As shown in Fig. 8, a clear spectral transition with isosbestic points becomes apparent when the fourth derivative spectra are superimposed. Obviously, the main peak of the fourth derivative spectrum corresponds to the shoulder at 291 nm, which is characteristic for the tryptophan absorption. This main peak is shifted by 0.4 nm, from 291.1 to 291.5 nm at 450 MPa. Again, if a change in the polarity of the environment is the main cause, this red shift indicates a decrease of the mean dielectric constant in the vicinity of tryptophans. However, when compared to the fourth derivative spectra of tryptophan in pure solvents, the position of λ_{max} indicates a very low polarity of the environment of tryptophan in MEOH-DH (comparable to that of diethyl ether) which is even more decreased by pressure. Its absolute value is too low to permit a reliable determination of the dielectric constant by the method described previously. This indicates that for MEOH-DH, in addition to the dielectric constant other parameters are governing the spectral changes. Concomitant with the red shift a decrease of the derivative amplitude is observed at high pressure. The spectral transition is fully reversible and at high pressure, MEOH-DH retains its activity. In fact, at 400 MPa the speed of decoloration of Wurster's blue is accelerated by a factor of 4.5, which confirms and extends the data obtained by Heiber-Langer et al. (1992) in the 1 to 200 MPa range.

After depressurization to atmospheric pressure, MEOH-DH entirely conserves its enzymatic activity. This is not the case, however, after a high temperature treatment: an exposure to 75 °C irreversibly inactivates the enzyme completely. Furthermore, the turbidity of the sample starts to increase above 50 °C, where the enzyme aggregates. Similarly, the action of guanidinium chloride results in irreversible protein denaturation accompanied by a blue shift (see Fig. 7) characteristic of the exposure of tryptophan to water, i.e. to a higher local dielectric constant (Yanari and Bovey 1960) as occurs in a fully denatured protein.

4. Discussion

In the preceding paper we have described a fourth derivative spectroscopic method that allows one to quantify protein structural changes induced by elevated temperature or pressure. In fact, from a comparison in various solvents, the method correlates changes in the UV spectrum of the aromatic amino acids with the dielectric constant in their vicinity. One must keep in mind, however, that sometimes other processes, such as the formation of charge-transfer complexes may also contribute to the spectral changes. To test the applicability of our method, we chose three very different proteins: adrenodoxin (for phenylalanine), a small (14 kD) protein which contains an iron sulphur (2 Fe-S 2) cluster that absorbs in the visible light, ribonuclease A (for tyrosine), which has been studied extensively as a model for protein unfolding and refolding processes, and MEOH-DH (for tryptophan), which is a relatively large tetrameric protein. For the three proteins the pressure induced changes of the zero-order absorbance spectrum were composed of spectral shifts, small baseline shifts and a hyperchromicity due to the solvent compression. The spectral analysis was further complicated owing to the overlapping of the UV bands and to the presence (for adrenodoxin and MEOH-DH) of chromophores absorbing in the visible region. The selective resolution of the fourth derivative method (Demchenko 1986; Duñach et al. 1983; Mach and Middaugh 1994) allowed us to avoid most of these problems, and clear spectral transitions with isosbestic points were revealed, providing us with the following structural information for these three proteins.

a. Adrenodoxin

This protein is part of the electron transfer chain of the cytochrome P450scc catalysed conversion of cholesterol to pregnenolone. It is thought to act as an electron shuttle between a reductase and P450scc (Coghlan and Vickery 1991). There are strong indications that electron transfer is mediated by the π electrons of aromatic amino acids, and since Beckert et al. (1994) have found that tyrosine is not involved, the four phenylalanines are potentially involved. Indeed, Greenfield et al. (1989) obtained evidence by NMR that one phenylalanine is functionally related to the iron-sulphur cluster, since the reduction of adrenodoxin causes a phenylalanine related shift in the NMR. Two-dimensional NMR data allowed Miura and Ichikawa (1991) to propose a structure where Phe 43 is close to the iron-sulphur centre. Finally, Palin et al. (1994) showed by site-directed mutagenesis that Phe 59 is essential for electron transfer.

Our results show that high pressure induces a spectral transition in the visible region, caused by a conformational change at or near the iron-sulphur centre, affecting its electronic transition. Since the transition in the visible region parallels that of a similar transition in the UV that can be ascribed to phenylalanine, it seems most likely that the same conformational transition affects the environments of both the iron-sulphur cluster of at least one phenylalanine, supporting a structural relationship between the 2 Fe-S 2 cluster and phenylalanine.

The transition is rather steep, and the corresponding high value of ΔV (–66 ml/mol) indicates a major protein conformational change. The red shift of the position of λ_{max} in the derivative spectrum is larger than that found for Ac-Phe-OEt in the solvent of lowest polarity (see the preceding paper), and does not allow the determination of the local dielectric constant. A possible explanation for this unexpected red shift would be that at low pressure the π electrons of at least one phenylalanine interact with Fe_2-S_2 orbitals. This interaction would be weaker in the conformation prevailing at high pressure.

b. Ribonuclease A

Many authors have studied the mechanism of protein denaturation by taking ribonuclease as a model. Brandts et al. (1970) came to the conclusion that the denaturation of ribonuclease by low pH, high temperature and high pressure can be adequately described by a two-state model. Their results were then used by Hawley (1971) to construct the widely used pressure – temperature elliptic phase diagrams for the denaturation of proteins. More recently, many laboratories have provided evidence that protein unfolding (and refolding) is a more complicated process that proceeds via several intermediates. Working at low temperatures, Biringer and Fink (1988) and Lustig and Fink (1992 a, b) found that the kinetics of ribonuclease unfolding and refolding were compatible with a minimum of four phases. Similarly, Tamura and Gekko (1995) found that ribonuclease denaturation by guanidinium chloride cannot be described by a simple two state model. This is also supported by the recent ^{1}H NMR investigation of Zhang et al. (1995), whose data suggest that different regions of the protein do not unfold cooperatively, and by the dilatometric measurements of Ybe and Kahn (1994) who observed three kinetic phases of the volume change of ribonuclease upon refolding. On the other hand, Kiefhaber and Baldwin (1995) obtained evidence from the kinetics of hydrogen bond breakage that the overall unfolding of ribonuclease by guanidinium is triggered by a single rate-limiting step after the breakdown of an intermediate state (Kiefhaber et al. 1995).

Our results show a clear distinction between the denaturation by pressure and temperature. The pressure induced transition can be described by a simple two-state model. In contrast, the temperature induced transition proceeds through a minimum of three steps which are each characterised by isosbestic points. The second step (from 30 °C to 48 °C) results in a spectrum that is closely similar to that obtained at high pressure. The derivative spectrum at 48 °C may therefore reflect a similar or the same protein structure as that obtained at 25 °C at 400 MPa, characterised by a maximum at 283.5 nm, that corresponds to a local dielectric constant of $\varepsilon = 59$ (see the preceding paper). This indicates that for this intermediate state some parts of the protein are still in a relatively non-polar environment.

What may be the nature of this intermediate? Two recent papers strongly suggest that the action of pressure on ribonuclease leads to a partially unfolded state. The FT-IR data of Takeda et al. (1995) indicate that this state has a native-like secondary structure, but it permits solvent access to internal protein regions. In addition, Zhang et al. (1995) observed certain histidine ^{1}H NMR resonances in cold-denatured and pressure-denatured ribonuclease which they assigned to a folding intermediate state. The results obtained by both methods (FT-IR and ^{1}H NMR) are consistent with a "molten globule" like nature of this intermediate. A partially denatured protein is called a "molten globule" if it has a compact, native-like secondary structure with little or no rigid tertiary structure (Ptitsyn, 1992). The presence of molten globule type intermediates for ribonuclease has also been inferred from recent thermal denaturation studies (Sosnick and Trewhella 1992; Lustig and Fink 1992 a; Seshadri et al. 1994; Tamura and Gekko 1995), as well as from chemical denaturation studies (Kiefhaber et al. 1995).

Although the concept of the molten globule state is not yet well defined (Ewbank and Creighton 1991), a compact conformation which conserves a certain amount of secondary structure appears to be a general intermediate in the course of protein denaturation (Foguel and Silva 1994; Vidugiris et al. 1995). Pressure appears to be particularly suitable for the study of these states as it leads to a stable intermediate without additional protein unfolding in the pressure range from 350 to 500 MPa, while protein denaturation by temperature generally induces further structural transitions. In addition, part of the process occurring at temperatures above 48 °C is irreversible, possibly due to chemical modification of the primary structure (Zale and Klibanov 1986).

The reason for the occurrence of a stable unfolding intermediate at high pressure may be found in the time dependent volume changes occurring in the course of a protein denaturation. In their pioneering work, Ybe and Kahn (1994) showed that the refolding of completely denatured ribonuclease is characterised by an initial rapid positive volume change which corresponds to the establishment of the secondary structure followed by two slower volume changes, namely a negative (–63 ml/mol) one, attributed to proline isomerization, and a positive (+53 ml/mol) one. The latter volume change could well correspond to the mirror reaction we induced by high pressure since, starting from the native enzyme, we measured a ΔV of –52 ml/mol. These authors suggested that this denaturation step is mainly due to a rearrangement of the tertiary structure for packing defects. The important increase of the dielectric constant we observe near the tyrosines indicates, however, that hydrophobic solvation plays an important role. One may imagine that either the hydrophobic residues migrate from the inside of the protein to its surface, or the protein structure becomes less rigid and the solvent penetrates into the interior. Structural changes of hydration water may, in addition, contribute to the properties of the pressure induced unfolding intermediate (Yamaguchi et al. 1995).

c. MEOH-DH

The pressure induced red shift of the fourth derivative spectrum indicates that the tryptophans of MEOH-DH are on the average in a less polar environment at elevated pressure. What are the protein structural features responsible for this effect? MEOH-DH from *Methylophaga marina* is an $\alpha_2\beta_2$ type tetramer with two large (66 kDa) α- and two small (10 kDA) β-subunits. Its three-dimensional structure is not yet known, but as a model may serve the X-ray structures of the very similar MEOH-DH's from *Methylobacterium extorquens* (Gosh et al. 1995) and *Methylophilus methylotrophus* (Xia et al. 1992). In MEOH-DH from *Methylobacterium extorquens* for tryptophans (Trp 44 and Trp 594 in each α-subunit) are situated at the interface be-

tween the α-subunits (C. Anthony, personal communication). Similarly, for MEOH-DH from *Methylophilus methylotrophus* we find Trp 44 and Trp 499 near the interface.

For oligomeric proteins an increase of pressure generally results in a shift of the equilibrium towards dissociation (Weber 1993; Foguel and Silva 1994; Gross and Jaenicke 1994; Sindern et al. 1995). This is so because of the smaller volume caused by electrostriction of charged residues and the hydration of hydrophobic residues upon exposure to the solvent. This would result in a blue shift in the UV spectrum of these residues upon pressure induced protein dissociation. Instead, up to 500 MPa, we observed an increasing red shift of the tryptophan like derivative spectrum suggesting that the environment of the tryptophans becomes less polar, which is incompatible with dissociation. The failure to dissociate under these conditions is indeed in part supported by the observation that MEOH-DH migrates as a single band in electrophoresis at 200 MPa with the same migration rate as at 0.1 MPa (Heiber-Langer et al.).

For an understanding of the structural basis of the observed spectral transition we have to take into account the eventual presence of water molecules in the crevice between the two α-subunits. In an elegant analysis using second derivative spectroscopy, Kornblatt et al. (1995) have shown for the pressure induced dissociation of dimeric enolase that some tyrosine residues are in a less polar environment after dissociation. They attributed this effect to the particular structure of immobilised water at the interface between the subunits at atmospheric pressure. It could be that for MEOH-DH we have a similar situation, where some tryptophans are in contact with immobilised water molecules. The breakage of this interaction at high pressure would not lead to a total protein dissociation, but it would explain the observed red shift. One can imagine a situation similar to the predissociated complex of the dimeric arc-repressor (Foguel and Silva 1994). This complex that still has a native-like secondary structure was shown to precede the pressure induced dissociation and denaturation of the molecule.

However, the absence of any indication for dissociation by high pressure electrophoresis, makes this hypothesis less likely for MEOH-DH. Instead, pressure may act to squeeze out eventual water molecules from the intersubunit crevice and to permit an interaction of tryptophans from one α-subunit with aromatic amino acids of the other α-subunit. Indeed, the stacking of aromatic residues is known to be strengthened by pressure (van Eldik et al. 1989). Support for this idea comes from the three-dimensional structure of MEOH-DH of *Methylobacterium extorquens* and *Methylophilus methylotrophus*. In the first, model, Trp 594 could interact with Tyr 449 and Trp 44 with Phe 510 or Tyr 586. In the second model, Trp 44 could come close to Phe 501. Although this interaction of aromatic amino acids may explain the stability of MeOH-DH against pressure dissociation, it is probably responsible for only a part of the relatively large red shift in the spectrum. It is therefore likely that several other tryptophans are involved in an increased interaction with non-polar amino acids in the protein.

It thus appears that, in contrast to the majority of the studied oligomeric proteins studied so far the quaternary structure of MEOH-DH is not weakened, but strengthened by pressure-possibly through interaction of aromatic amino acids. The extreme stability of the tertiary structure of MEOH-DH to pressure is also surprising. In addition, we have tried many conditions, varied the temperature, pH and the concentration of organic cosolvents, but in no case did we obtain a denaturation of MEOH-DH, and after release of the pressure, the enzyme was still active. In contrast, MEOH-DH was readily denatured (and inactivated) by an increase of temperature. This enzyme appears therefore to be an interesting model to study the structural prerequisites of the protein stability to temperature and pressure.

5. Conclusion

Application of the optimised high pressure fourth derivative UV spectroscopic method, described in the preceding paper, to the thermodynamic study of three selected proteins yielded valuable information. It provided insight into the electron transfer mechanism of an iron-sulphur protein, it permitted us to obtain some thermodynamic parameters of a molten globule like denaturation intermediate of ribonuclease, and to evaluate its local dielectric constant, and it could be used to explore pressure induced conformational changes and solvation of a tetrameric enzyme. We believe that this method may be used with profit to study temperature and pressure induced structural changes of many other proteins.

Acknowledgements The authors wish to thank Dr. Claude Balny for constant help and advice. The authors are grateful to INSERM (Poste Vert, V. M.) and INSERM/NWO (J. F.) for support by grants. Part of the work was performed in the framework of the COST D6 action and the INTAS 93-38 project.

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