Second Derivative Spectroscopy of Enolase at High Hydrostatic Pressure: An Approach to the Study of Macromolecular Interactions†

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ABSTRACT: Second derivative spectroscopy in the ultraviolet region of proteins has been used to study the polarity of the regions surrounding tyrosine residues. We show here that it can also be a tool to study the degree to which proteins associate and that it can be effectively combined with hydrostatic pressure in order to evaluate equilibrium dissociation constants and reaction volumes. Hydrostatic pressure causes yeast enolase to dissociate. Clear changes in the second derivative spectra of enolase were observed as pressure was increased. At enolase concentrations of about 20 μM, the midpoint of the transition is about 1800 bar. All aspects of the transition are reversible up to 2700 bar. It is likely that the transition observed is the result of enolase dimers dissociating into monomers. The second derivative spectra indicate that one or more tyrosine residues is in an unusually polar environment in the dimer, an environment that is less polar in the monomer. Three tyrosines (6, 11, 130) are near the dimer interface. Tyrosines 6 and 11 are pointing into the water-filled crevice between the subunits and are close to several immobilized waters. All three are close to a network of intersubunit salt bridges and hydrogen bonds. We believe that the average tyrosine polarity in the dimer reflects the exposure of these tyrosines to immobilized water and the fixed dipole of the salt bridge. The water in the crevice between the subunits should be more mobile in the monomer; the salt bridge does not exist in the monomer. In contrast to the behavior of native enolase under pressure, the same protein in guanidine hydrochloride shows no obvious changes with pressure. Similarly, the small protein hen egg-white lysozyme shows no change in the second derivative as a function of pressure.

Hydrostatic pressure is a powerful tool for perturbing the structure and function of proteins and other biological molecules [see Balny et al. (1989) and Gross and Jaenicke (1994) for recent reviews]. Its power derives from two aspects of the technique: (1) it is a relatively mild and readily reversible perturbant, and (2) analysis of pressure effects provides information on volume changes, compressibilities, and, in some cases, the type of interactions being perturbed (Morild, 1981; Heremans, 1982, 1987). Hydrostatic pressure acts on any closed system to drive a reaction in the direction of a smaller volume. For equilibria between proteins or between proteins and other large biological molecules, our current view is that as these structures come together, there is frequently the expulsion of water (Jaenicke & Lauffer, 1969) and/or the creation of small packing defects (Weber & Drickamer, 1983). Hydrostatic pressure drives association/dissociation reactions by constricting small voids (Weber & Drickamer, 1983) in the system or by altering the hydration state of the molecular surfaces (Kornblatt et al., 1993).

In order to study the effects of any procedure on a given protein, it is necessary to have something to measure that is indicative of the protein's structure. When the protein has an intrinsic chromophore, the task is somewhat easier than when it is does not (Kornblott et al., 1988; Hui Bon Hoa et al., 1982; Fisher et al., 1985). If denaturation is being studied, light scattering or visual inspection is frequently useful (Bridgeman, 1914). For any protein, it is frequently difficult to determine whether pressure has brought about dissociation of multimeric proteins into subunits, whether it has brought about conformational changes, or whether it has had no influence on the protein [see Gross and Jaenicke (1994) for a review]. The studies of Gregorio Weber (Weber, 1993; Weber & Drickamer, 1983) have effectively applied fluorescence−anistropy as well as steady state−measurements to elucidate pressure effects. The requirements for polarization measurements under pressure are substantial and cannot be performed in most of the laboratories that have pressure equipment. Steady-state measurements using tryptophan as the probe are sometimes useful but often not. Attaching fluorescence probes to proteins has to perturb the overall three-dimensional structure of the protein and hence its stability. X-ray crystallography (Kundrot & Richards, 1987, 1988) and high-resolution NMR (Samarasinghe et al., 1992; Jonas, 1992) give unequivocal information but are not easily carried out in most laboratories. Clearly, there is a need for structural probes that can be easily and routinely used under pressure.

Second derivative [see Ragone et al. (1984) and references cited therein] and fourth derivative spectroscopy (Butler, 1979; Padros et al., 1982) in the ultraviolet region have the

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potential to detect subtle differences in the environments of the aromatic residues of proteins. Their application to the study of proteins under pressure would increase substantially the number of proteins that could easily be studied without modification. Neither spectroscopic technique has been applied to proteins under pressure even though several laboratories carry out both hydrostatic pressure experiments and second derivative spectroscopy (Fisher & Sligar, 1985; Di Primo et al., 1990). Until recently, the ability to easily and routinely treat data so as obtain a reliable derivative spectrum has been limited but is now within the capacity of most laboratories.

In order to determine the usefulness of this technique, we have applied it to yeast enolase. Previous studies have shown that this dimeric enzyme can be dissociated by pressure (Paladini & Weber, 1981); the dissociation is accompanied by changes in the tryptophan emission spectrum. Polarization of the intrinsic tryptophan fluorescence also changes. Other studies have shown that the dissociation of pig muscle enolase can be monitored by fourth derivative spectroscopy (Kulig & Wolny, 1988). We show here that derivative spectroscopy is a powerful tool for analyzing the effects of pressure on protein−protein interactions.

MATERIALS AND METHODS

Yeast enolase and lysozyme were purchased from Sigma as were Tris, *N*-acetyltryptophan ethyl ester, and *N*-acetyltyrosyl ethyl ester. Bistris, $MgCl_2$, and EDTA were from Fluka. MES and 2-phosphoglyceric acid were from Boehringer.

All enolase experiments were carried out in a buffer containing 25 mM MES, 25 mM Tris, and 0.1 mM EDTA, with (MTME) or without (MTE) 1 mM $MgCl_2$, pH 7.0. The pH of this buffer system, like other amino-based systems, is relatively insensitive to hydrostatic pressure. All enzyme solutions were dialyzed overnight against either MTME or MTE; the dialyzed solutions were centrifuged at 20 000 rpm in order to ensure clarity. The solutions were then diluted to about 20 μM enolase (ϵ = 81 000 M^{-1} cm^{-1}) (Brewer et al., 1978) and used directly for the pressure experiments. Enzymatic activities were monitored before and after the pressure experiments. An aliquot of the 20 μM enolase was diluted into MTME that contained 1 mM 2-phosphoglyceric acid. PEP production was monitored at 240 nm at 25 °C (Kornblatt & Klugerman, 1989).

Lysozyme was dissolved in 20 mM Bistris/0.1 M KCl, pH 7.0. The solution was passed through a 0.45 μm filter in order to ensure optical clarity.

UV spectra were obtained with a Cary 219 spectrophotometer interfaced to a high pressure bomb; the apparatus has been described (Hui Bon Hoa et al., 1982). For the purpose of the work described here, the only modification was to the pressure/bomb assembly: the pressure transmitting fluid, pentane, exited the pressure developing press and was filtered through an activated charcoal column, the internal dimensions of which were 0.5 cm × 5 cm. The filtered pentane then entered the bomb. The ends of the column were blocked with filter cartridges designed for use with 5 mL Eppendorf pipetters; filter tips for cigarettes would probably work just as well but were not tested. The special stainless-steel column was purchased from Nova (Switzerland) and was rated to 4000 bar. The filtered pentane had an absorbance of less than 0.002 at 240 nm. The charcoal filter removed the contaminants from 200 to 400 mL of pentane coming from the press before its capacity was exhausted.

Pressure experiments were carried out in a square quartz cuvette with an optical path of 0.5 cm. The bomb was thermostated at 15 °C. Data were collected with the program SPLAB of Dr. Dmitri Davidov. In a typical experiment, a buffer base line was obtained by scanning between 260 and 310 nm. A total of 127 data points were taken for each of 10 scans. The 10 scans were averaged and smoothed. The smoothing routine grouped the three points before and after the point to be treated (seven points total). The best curve was drawn through the seven points, and the point in question was assigned the value given by the smoothing routine. The entire 127 points were similarly treated. The resulting curve constituted the smoothed base line. The enzyme was treated the same way. Ten scans at 1 bar were averaged. The smoothed base line was subtracted from the average. The resulting spectrum was smoothed. The second derivative was taken using a window of 0.4 nm and the resulting second derivative smoothed once more.

In all experiments, a series of 1 bar spectra were collected after which additional series were collected at pressure. Between the application of pressure and the start of data collection, we allowed 10 min for the samples to come to equilibrium. During this time, we occasionally monitored the spectra. During the first minute, there were very small fluctuations caused by the slow rate at which the pentane entered the bomb. After 1 or 2 min at pressure, there were no systematic fluctuations in the absorption spectrum of the protein. With the exception of a noisy signal that resulted from a long optical path through the bomb and a slit of 0.5 nm, the first and last spectra in a series were identical. On occasion, we monitored pressurized samples over a period of several hours (before and after lunch) and once monitored the same sample overnight; the 10 min spectra were the same as the 10 h spectra.

Molecular modeling of yeast enolase was performed on an IBM RISC 6000 running the Molecular Simulations Inc. software QUANTA and CHARMm. The coordinates used for the visualization and measurements were from the Brookhaven Protein Data Bank (file PDB3ENL).

RESULTS

In order for a probe to be useful for the study of a process, the probe must give an accurate indication of the process and not of some other side event. The process here is subunit dissociation as the result of hydrostatic pressure. For the second derivative of the UV spectrum to be a useful probe, the spectra of tyrosine and tryptophan must be more or less invariant with pressure. Additionally, the spectra of native monomeric proteins must be more or less invariant with pressure so long as the monomeric proteins are in their native conformations.

The first constraint, that the spectra of tyrosine and tryptophan must be invariant with pressure, is met; the data are presented in Figure 1. *N*-Acetyltryptophan ethyl ester (Figure 1A) and *N*-acetyltyrosine ethyl ester (Figure 1B) have been submitted to pressures between 1 and 3000 bar. Within

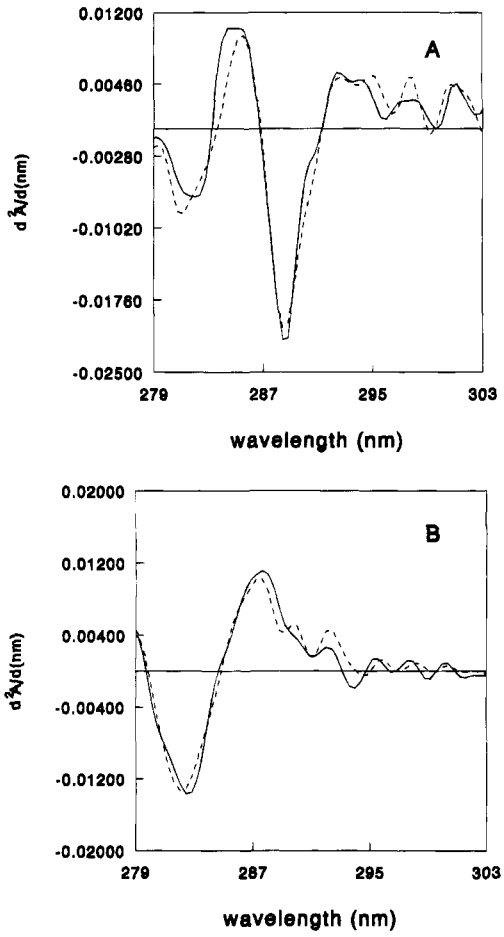

FIGURE 1: Influence of hydrostatic pressure on *N*-acetyltryptophan ethyl ester (A) and *N*-acetyltyrosine ethyl ester (B). The two compounds (ca. 0.1 mg/mL) were dissolved in 20 mM Bistris, pH 7.0. The tryptophan second derivatives are shown at 1 bar (solid line) and 1200 bar (dashed line). The tyrosine derivatives are shown at 300 bar (solid line) and 1200 bar (dashed line).

experimental error, the spectra at the two limits are more or less superimposable for tryptophan. In the tyrosine spectrum, there is a small shift between 1 and 300 bar; the spectra are constant above 300 bar.

The second constraint, that the spectrum of a monomeric protein must be invariant with pressure so long as the protein is in its native conformation, has been met. Figure 2 shows the second derivative spectrum of hen egg white lysozyme at 1 bar. Two factors contribute to the shape of the curve: (1) the ratio of tyrosine to tryptophan and (2) the polarity of the environment around the tyrosines (Ragone et al., 1984). These determine the overall ratio between the peak to trough distances labeled a and b in the figure. We are able to measure this ratio (r) to $\pm 10\%$ accuracy. As can be seen in Table 1, r does not change between 1 and 3000 bar.

The effects of hydrostatic pressure on yeast enolase are quite different (Figure 3). Between 1 and 2700 bar, there is a decrease in the first peak to trough distance (a) and an increase in the second (b); r decreases from a value of 1.5 (1 bar) to 0.86 (2700 bar). Two complete sets of spectra (enolase vs pressure) were obtained, and the second derivatives were calculated at each pressure. The normalized data are presented in Figure 4. Between 1 and 1000 bar, there is little detectable change in the second derivative. There is substantial scatter in the data, but there are a sufficient number of points to allow unequivocal interpretation. Above

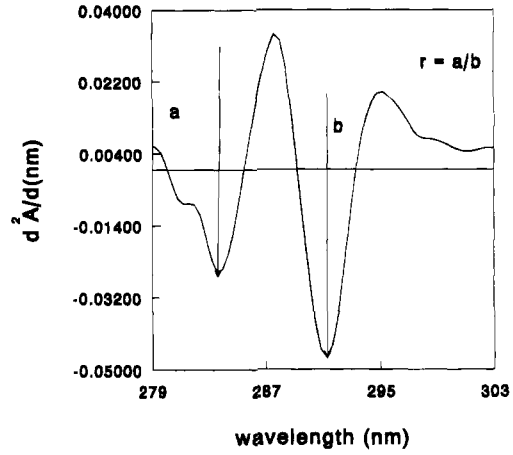

FIGURE 2: Second-derivative spectrum of lysozyme, at 1 bar. The vertical lines, a and b, mark the two peak to trough distances used to calculate r.

Table 1: The Second Derivative of Lysozyme Is Almost Independent of Pressure

pressure	r
1 bar	0.89, 0.88, 0.78
1 kbar	0.97
2 kbar	0.99
3 kbar	0.92

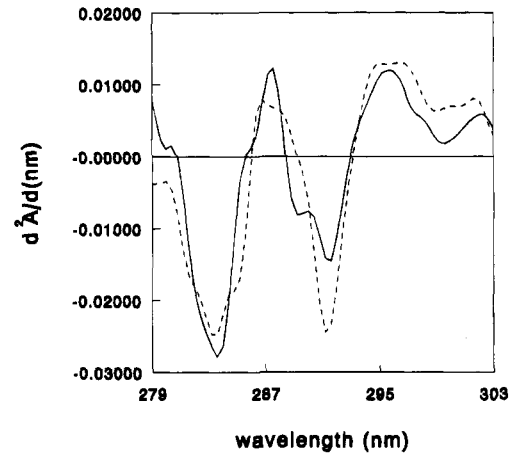

FIGURE 3: Effect of hydrostatic pressure on the second derivative spectra of yeast enolase. Solid line, enolase at 1 bar; dashed line, enolase at 2700 bar.

1200 bar, there is a gradual diminution in the second derivative with a lower plateau showing up at pressures above 2200 bar.

The spectral changes induced by pressure are completely reversible within 1 or 2 min and stable over long periods. The spectrum at any given final pressure was totally independent of the direction of approach; a 1 kbar spectrum was the same regardless of whether it was approached from 1.2 kbar or 800 bar. There was never any indication of slow processes occurring. Activities before and after pressure have been measured, and there is no loss under any of the conditions shown.

What is pressure doing to enolase? Enolase has been denatured with guanidine hydrochloride, and the second derivative as a function of pressure has been determined (Figure 5). The value of r for the denatured protein (1.69) is slightly higher than that of the native enzyme and does not vary with pressure over a range of 3000 bar. The effects

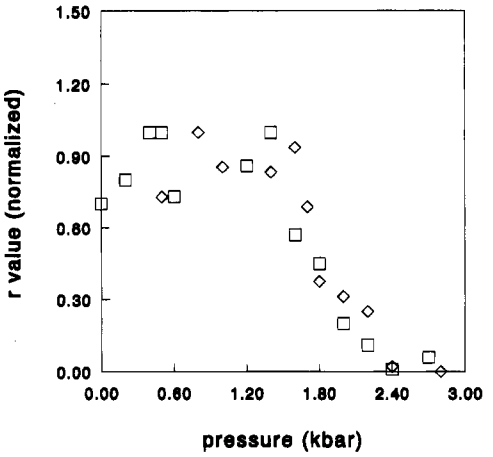

FIGURE 4: Effect of hydrostatic pressure on the parameter, r, of the second derivative spectrta of yeast enolase. The value of r was calculated for each second derivative spectrum. Data from two complete experiments (squares and diamonds) were combined and normalized by adjusting the highest value of r in each set to 1.00 and the lowest value equal to 0.0.

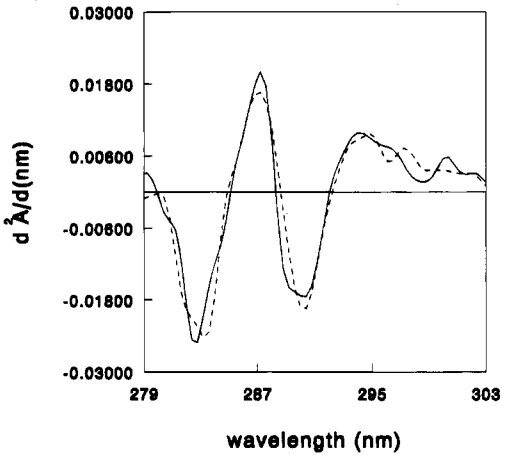

FIGURE 5: Effect of hydrostatic pressure on the second derivative spectra of denatured enolase. Solid guanidine hydrochloride was added to a sample of yeast enolase in MTME to give a final concentration of 6 M. The spectra shown were recorded at 1 bar (solid line) and 3000 bar (dashed line).

of pressure on the second derivative spectrum of enolase are totally different from the effects of denaturation.

It is known that yeast enolase can be dissociated into inactive subunits by a combination of high salt and excess EDTA (Brewer et al., 1978; Brewer & Weber, 1968). Figure 6 shows the second derivative spectra of enolase in 1 M KCl, at 1 bar, plus and minus excess EDTA. The addition of 1 M KCl to enolase has no measurable effect; the enzyme is dimeric and has the same second derivative spectrum as in its absence. The addition of excess EDTA dissociates the enzyme (Brewer et al., 1978); r decreases from 1.5 to 0.83. This change is the same as that produced by pressure.

Assuming that pressure is dissociating enolase and that the limiting r values represent the dimeric and monomeric forms of the enzyme, our results can be used to calculate dissociation constants (Paladini & Weber, 1981; Kornblatt et al., 1982). Only data from pressures greater than 1000 bar are used. At lower pressures, the equilibrium is changing, but the overall equilibrium constant still heavily favors the dimer. From the plot of ln K_D vs pressure (Figure 7), we calculated ΔV, the reaction volume, for dissociation to be -110 ± 30 mL/mol.

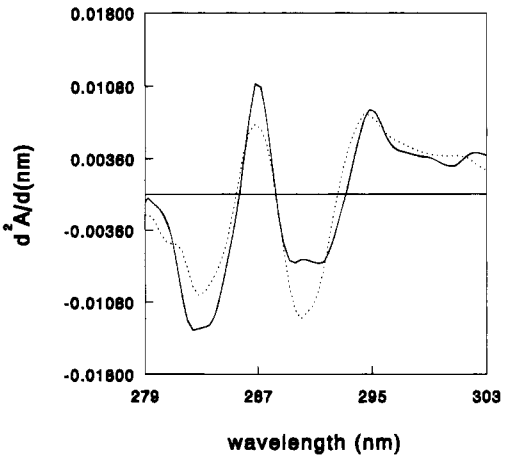

FIGURE 6: Second-derivative spectra of the dimeric and monomeric forms of yeast enolase. Dialyzed yeast enolase was diluted into MTME containing 1 M KCl. After 10 spectra had been recorded at 1 bar (solid line), EDTA was added to give a final concentration of 6 mM and 10 more spectra were recorded at 1 bar (dotted line).

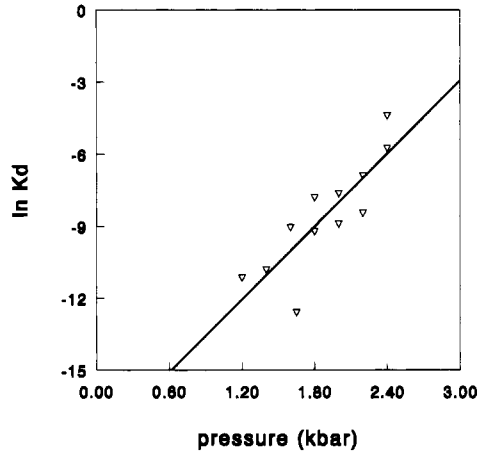

FIGURE 7: Effect of hydrostatic pressure on the dissociation constant for yeast enolase. K_D was calculated from the data in Figure 4; only data obtained in the range of 1200−2400 bar were used.

DISCUSSION

The UV spectrum of a protein consists of contributions from tryptophan, tyrosine, and phenylalanine. Since phenylalanine is well separated from that of the other two aromatics (Cantor & Schimmel, 1980) and has a small extinction coefficient, it need not be considered in this analysis. The absolute spectra of tryptophan and tyrosine overlap. They have comparable extinction coefficients, but the spectrum of tryptophan is considerably richer in detail. The second derivative of tyrosine has been reported to be sensitive to the polarity of its surroundings whereas that of tryptophan is considerably less so (Ragone et al., 1984). The manifestation of the tyrosine sensitivity is to shift the second derivative toward shorter wavelengths as the tyrosine moves from an apolar environment to a polar one. In the second derivative spectrum of a protein (Figure 2), the function which yields information on tyrosine polarity is the ratio of a to b. As tyrosine moves from a less polar environment to a more polar one, the ratio (r) becomes larger.

The major point to be made from this work is that second derivative spectroscopy is a useful tool for studying the effects of pressure on the interactions between protein subunits; it may also have application in studying interactions between dissimilar proteins. Additionally, the second de-

rivative spectrum of native yeast enolase has turned out to be exceedingly interesting in its own right. In the dimer, the average tyrosine appears to be in a highly polar environment, and, contrary to expectations, this environment appears to become less polar as the subunits dissociate. We will return to these major points.

It is necessary to restate that there are only minor effects on the spectra of tyrosine and tryptophan at the moderate pressures used here. Similarly, the spectrum of hen egg white lysozyme is also more or less constant between 300 and 3000 bar. These data are presented in Figures 1 and 2 and Table 1. It was necessary to verify that the spectra of the two amino acids were pressure-invariant but they are not good controls for a study of proteins; lysozyme is.

The effects of pressure on chicken lysozyme have previously been studied; below 2000 bar there is almost no detectable effect on its intrinsic (tryptophan) fluorescence (Li et al., 1976).

Kundrot and Richards (1987, 1988) have determined the crystal structure of lysozyme at 1000 bar. Their data show that most lysozyme atoms at pressure do not change by more than 0.2 Å. The structure reveals that the number of water molecules associated with lysozyme increases with pressure from 151 to 163 molecules. The very important point to emerge from these studies is that, in a protein whose structure does not change with pressure, the spectral properties of the aromatic residues also do not change with pressure (Figure 2, Table 1).

In contrast to lysozyme, pressure produced significant changes in the second derivative spectra of yeast enolase. Does this reflect dissociation? High KCl plus EDTA causes yeast enolase to dissociate as evidenced by sedimentation equilibrium experiments (Brewer & Weber, 1968). These conditions also produce changes in the intrinsic fluorescence of enolase (Brewer et al., 1978). The steady-state fluorescence and the polarization values indicate that there are two states that can be monitored: one characteristic of the dimer (moderate salt, little EDTA) and one characteristic of the monomer (high KCl plus EDTA). Paladini and Weber (1981) in their classical study of pressure effects on yeast enolase showed that both steady-state fluorescence and anisotropy changed as pressure was increased. The pattern for the fluorescent changes was the same as that found for dissociation as a result of high KCl and EDTA treatment (Brewer et al., 1978). They concluded that pressure was causing the protein to dissociate.

Does the second derivative of the UV absorption spectrum monitor the quaternary state of enolase? The second derivative spectra of enolase as a dimer and also under conditions that promote dissociation are shown in Figure 6. Enolase in buffer plus high KCl (the solid curve) has full activity and is primarily a dimer. The protein shows an absolute r value of about 1.5, about the same as that for the enzyme at 1 bar in the bomb but without KCl present. The protein dissociates when EDTA is added to the KCl-containing buffer (the dashed curve); the monomeric protein has a smaller r value (0.83), about the same as the protein at 2700 bar.

Inspection of Figure 4 shows that the midpoint for the pressure-induced transition is about 1800 bar, nearly the same as found by Paladini and Weber at similar concentrations. The value that we obtain for a reaction volume is -110 ± 30 mL/mol. This value is almost twice their value, but both numbers have substantial errors associated with them. Extrapolating our pressure vs ln K_D curve back to 1 bar, we obtain a value for K_D of 1.5×10^{-8} M. This figure compares favorably with that calculated by Brewer and Weber (1968) using fluorescence signals from the protein as well as activity measurements.

One thing is clear; pressures between 1 and 3000 bar are not bringing about the general unfolding of the enolase. Comparing the second derivatives in buffer and in 6 M guanidine hydrochloride shows that the average polarity of the tyrosine environment increases as the protein becomes chemically denatured. Pressures up to 3000 bar cause just the opposite effect. We take these combined data to mean that the second derivative spectra are monitoring the quaternary state of enolase. We believe that this technique would be useful for studying other oligomeric proteins.

The apparent polarity of the environment of the enolase tyrosines decreases as the protein dissociates. This is counter-intuitive. At 1 bar, the value 1.5 represents a rather high polarity for a protein. The majority of proteins with similar tyrosine to tryptophan ratios show r values that are considerably lower (Ragone et al., 1984). Using the method and assumptions of Ragone, we calculate that for a protein with a ratio of tyrosine to tryptophan of 9/5, the value of r observed for dimeric enolase would represent a degree of exposure (α) of the tyrosines of about 0.8; this value decreases to 0.4 as the protein dissociates. The latter value is quite reasonable judging from an analysis of the X-ray structure of Stec and Lebioda (1990).

In our opinion, an average exposure of 0.8 is probably far too high. Examination of the crystal structure shows that four of the nine tyrosines are buried within the monomer; three are near the surface, but not pointing into the solvent, and two are at the subunit interface. One would predict, therefore, a small value for α, as is observed for the dissociated enzyme. Moreover, it is not likely that a tyrosine going from one exposed position to another during a pressure experiment or on addition of KCl/EDTA would see diminished polarity. A potential solution to this paradox is that some of the tyrosines are in an environment that is more polar than that of bulk water. The crystal structure of yeast enolase includes 353 rigid waters (Stec & Lebioda, 1990); many of these are in the deep cleft between the subunits. Rigid water molecules behave as fixed dipoles and thereby increase the polarity of the crevice substantially over that of free water. What is most striking about the tyrosines and the fixed waters is that two of the tyrosines (residues 6 and 11) point into this crevice. A second feature is the network of intersubunit contacts that occur within this crevice. There is a complex salt bridge formed between the two subunits, consisting of arginine 414 and glutamate 417 on one subunit and glutamate 20, arginine 8, glutamate 22, and arginine 31 on the other subunit. In addition, between residues 2 and 20, there are a number of positions where either the backbone or the side chains are hydrogen bonded to the other subunit. Tyrosines 6 and 11 are in the midst of these contacts, and tyrosine 130 is within 5 Å of glutamate 417, close enough to sense the large dipole formed by the coming together of the subunits. Upon dissociation, these interactions are disrupted, and the water in the crevice would become mobile. The three tyrosines (6, 11, 130) go from an environment in the dimer that is icelike to one that is like bulk water.

Yeast enolase is not the only protein that has abnormally high r values. Cytochrome c in the oxidized state is totally normal as found by other workers. On reduction, there is a change in the crystal structure. A water molecule, water 166, becomes prominent. There is a concomitant change in the second derivative of the protein in the UV. R, in the reduced protein, becomes unusually high. This will be the subject of another report.

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