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Pressure-induced molten globule state of cholinesterase

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Abstract The denaturing effect of pressure on the structure of human butyrylcholinesterase was examined by gel electrophoresis under pressure and by 8-anilino-1-naphthalene sulfonate (ANS) binding. It was found that the fluorescence intensity of bound ANS is increased by pressure between 0.5 and 1.5 kbar and that the hydrodynamic volume of the enzyme swells when pressures around 1.5 kbar are applied. These findings indicate that pressure denaturation of butyrylcholinesterase is a multi-step process and that the observed transient pressure-denatured states have characteristics of molten globules.

Key words: Cholinesterase; Molten globule; Pressure; Electrophoresis

1. Introduction

Cholinesterases are ubiquitous enzymes primarily involved in cholinergic transmission [1]; they are the main target of organophosphate poisons [2]. Since allosteric effectors acting as destabilizers of cholinesterase structure are considered to facilitate oxime-mediated reactivation of phosphylated cholinesterases, understanding the mechanisms of ligand-induced conformational changes of cholinesterases is expected to stimulate research leading to new drugs against organophosphate poisoning.

Recently it has been found that acetylcholinesterase (EC 3.1.1.7) undergoes molten globule (MG) transitions upon certain chemical modifications or under mild denaturing conditions [3–5]. MG states are the general compact intermediates in protein folding that can be obtained under mild denaturing conditions [6]. These intermediates have a hydrodynamic radius 10–20% greater than that of the native conformation; although they retain most of their secondary structure, they lose their tertiary structure and patches of hydrophobic residues become solvent-exposed (for reviews on MG, see [7,8]). Hydrostatic pressure is a mild denaturant. Below 3–4 kbar, pressure does not cause extensive conformational changes in proteins [9–11]. Several lines of evidence indicate that moderate pressure-induced denatured states of proteins resemble the MG states [12–14]. Pressure in the range of 1-3.5 kbar has been found to inactivate butyrylcholinesterase (EC 3.1.1.8; BuChE) and to induce its partial unfolding [15]. So, it was of interest to determine whether the pressure-induced partially unfolded state of BuChE was MG like. In this paper, a pressure-induced increase in the hydrodynamic volume of BuChE and the fluorescence of ANS (8-anilino-1-naphtalene sulfonate) upon binding provide evidence for the existence of MG intermediates between native and unfolded states.

2. Materials

2.1. Enzyme preparation and chemicals

The tetrameric form (G_4; M_r = 340 kDa) of butyrylcholinesterase (EC 3.1.1.8) was highly purified from human plasma. The enzyme concentration was 1 mg/ml as determined by the bicinchoninic acid method (BCA kit, Pierce, Rockford, IL, USA). 1.61 mM ANS (anilino naphtalene sulfonate, ammonium salt) was in disolved 10 mM Tris-HCl buffer, pH 7.5.

2.2. Electrophoresis under hydrostatic pressure

High-pressure electrophoresis were performed in polyacrylamide capillary gel rods (Ø/ = 1 mm) of different acrylamide concentrations (T = 4–6.5%) under pressures up to 3 kbar (1 bar = 0.1 MPa) in a thermostated high pressure vessel with electrical connections [16]. There was no stacking gel. Samples (4 μl of enzyme diluted 1000-fold in running buffer containing tracking dye) were loaded on to the gels. Electrophoresis gels were pre-equilibrated at the desired pressure for 10 min. Electrophoreses were carried out at 10°C and constant intensity (0.3 mA/gel) for 15 min under pressure. 8.26 mM Tris/0.1 M glycine buffer, pH 8.3 was used as running buffer. After removal from capillary tubes, gels were stained using butyrylthiocholine iodide (1 mM) as the substrate for enzyme activity according to the method of Karnovsky and Roots [17]. Analysis of the stained microgels was performed using a video densitometer (Vilber Lourmat, Marne-la-Vallée, France) to determine the protein and tracking dye (Bromophenol blue) migration distances.

The construction of Ferguson plots [18] log m against T, allowed the determination of K_r, the retardation coefficient, according to the equation

$$\log m = \log m_0 - K_r T$$

where m is the relative protein mobility (ratio of the migration distance of BuChE to that of Bromophenol blue). Assuming sphericity of the protein, K_r is related to the molecular size according to the equation

$$K_r^{1/2} = c\,(R + r) \text{ or } K_r \approx c(3V/4\pi)^{2/3}$$

where R is the equivalent molecular radius and V the hydrodynamic volume, c is an experimental constant and r is the radius of the polyacrylamide fiber.

2.3. ANS binding

ANS is a useful fluorescent probe to study protein conformational changes and denaturation processes as its fluorescence is enhanced when bound to solvent-exposed hydrophobic areas of proteins [19,20]. BuChE solubilized in the pressure pH-invariant buffer [21], 10 mM Tris- HCl, pH 7.5, was exposed to pressure for 1 h at 10°C. The pressure range applied was from 10^{-3} to 2.5 kbar. The final concentration of enzyme was 8 μg/ml, that of ANS was 100 μM. Fluorescence emission spectra of free and bound ANS were recorded after pressure release with a SFM 25 (Kontron) spectrofluorimeter. Spectra of bound ANS were corrected by subtraction of the fluorescence spectra of free ANS at each pressure.

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Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; MG, molten globule; ANS, 8-anilino-1-naphtalene-sulfonate.

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3. Results and discussion

3.1. Ferguson plot analysis

Fig. 1 shows polyacrylamide capillary gels of different acrylamide concentrations stained for activity of BuChE after electrophoresis at three different pressures. Construction of Ferguson plots at pressures varying from 10^{-3} to 3 kbar allowed determination of K_r at different pressures. The effect of pressure on K_r was determined by reploting K_r against pressure as shown in Fig. 2. K_r values were almost constant at pressures up to 1.25 kbar. Above this pressure K_r increased under pressures up to 1.5 kbar and then dropped. A transient increase in K_r suggests a pressure-induced conformational transition toward a compact state whose hydrodynamic radius is greater than that of the native enzyme. Swelling of compact partially denatured proteins is one of the characteristics of MG states. As stated by Silva and Weber, moderate pressures (1–2 kbar) can induce stable MG-like states [10]. To test the hypothesis of a pressure-induced MG state of BuChE, the effects of osmolytes, known to be stabilizing additives [22,23] were studied. First, we increased the concentration of glycine in the running buffer (41 mM Tris/0.5 M glycine, pH 8.3). Second, we added 1 M sucrose or 2 M sorbitol to the separating gel and samples. The presence of osmolyte/electrolyte at high concentrations modified the electrophoretic mobility according to the theory of electrophoresis of proteins. Interestingly, as expected, the effect of pressure on K_r was abolished or dramatically reduced: e.g. the maximum change in K_r between 1 kbar and 1.5 kbar of pressure was only 13% in the 0.5 M glycine buffer containing 1 M sucrose compared to a change in K_r of 36% in the 0.1 M glycine buffer. In the 0.5 M glycine buffer containing 2 M sorbitol no change in K_r was observed at pressures up to 2.5 kbar, suggesting that the conformational transition was abolished or shifted toward higher pressures. Moreover, above 1.5 kbar the degree of irreversible inactivation of BuChE in 0.5 M glycine buffer

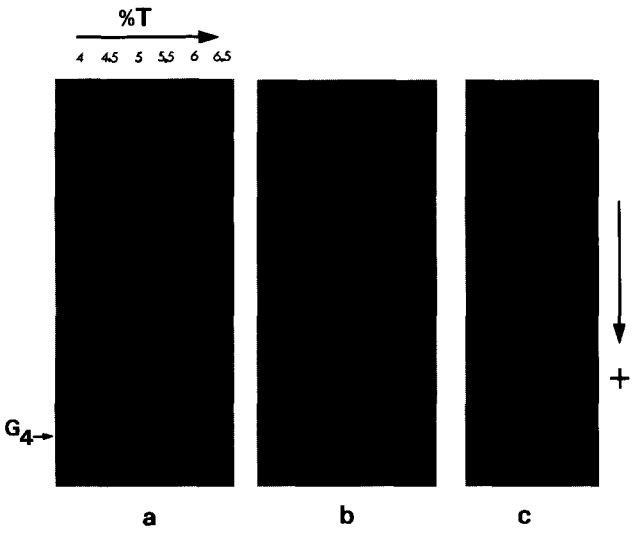

Fig. 1. Polyacrylamide gel electrophoresis of the tetrameric form (G_4) of BuChE in capillary rods of different acrylamide concentrations (T = 4–6.5%) under pressure (a, 10^{-3} kbar; b, 0.5 kbar; c, 1 kbar) at 10°C in 8.26 mM Tris/0.1 M glycine, pH 8.3. BuChE activity was revealed by the method of Karnovsky and Roots [17]. After staining the relative mobility of the enzyme was determined by video densitometry.

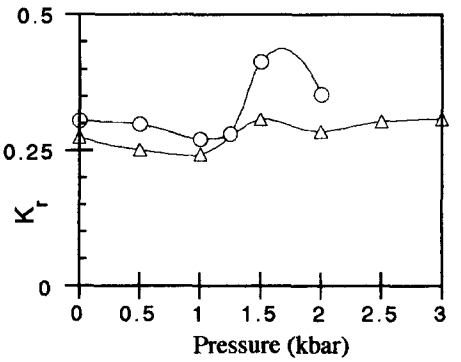

Fig. 2. Variation of the retardation coefficient (K_r of BuChE with pressure. (△) in 41 mM Tris/0.5 M glycine buffer pH 8.3 containing 1 M sucrose at 25°C; (○) in 8.26 mM Tris/0.1 M glycine pH 8.3 at 10°C.

was two times lower than in 0.1 M glycine buffer. These findings can be explained by the protective effect of osmolytes against the denaturing action of pressure. This stabilizing effect which is now well documented [14,24–26] may be interpreted in terms of preferential exclusion of osmolyte molecules from the enzyme surface [27]. The presence of the osmolyte shell strips water molecules of the hydration layer of the enzyme, which in turn counteracts the denaturing effect of pressure.

3.2. ANS binding

No significant pressure-induced changes in the spectra of free ANS spectra were observed at pressures up to 2.5 kbar. On the other hand, the fluorescence of ANS bound was to BuChE varied with pressure. As shown in Fig. 3 up to 1 kbar, as pressure was increased, the relative fluorescence intensity increased, at higher pressures the intensity decreased. Moreover, spectra were recorded overnight every hours after pressure release; they did not reveal any time evolution, indicating complete irreversibility of the native state → MG state transition. In this connection, it should be mentioned that low concentrations of guanidinium chloride (≈1.5 M) have been found to induce irreversible transition of AChE into MG state [4,28]. Since the increase in fluorescence of bound ANS is proportional to the number of ANS molecules bound to hydrophobic sites, our data indicate that hydrophobic groups of BuChE became progressively more solventexposed as pressure was increased. The increase of ANS binding during the folding/unfolding pathway of proteins has been attributed to the formation of MG states [8,20]. Thus, present ANS fluorescence data support the MG hypothesis for the pressure denaturation of BuChE. However, the fact that relative fluorescence maxima were at 1 kbar whereas $K_{r\,max}$ were observed at pressures of the order of 1.5 kbar indicates that pressure-induced denaturation of BuChE is a multistep process.

4. Conclusion

An increase in fluorescence of ANS bound to BuChE between 0.5 and 1.5 kbar of pressure and electrophoresis data showing a transient increase of the protein size between 1.25 and 1.5 kbar strongly suggest plurality of pressure-induced partially unfolded states of BuChE between 0.5 and 1.5 kbar. It should be remembered that the study of pressure denatura-

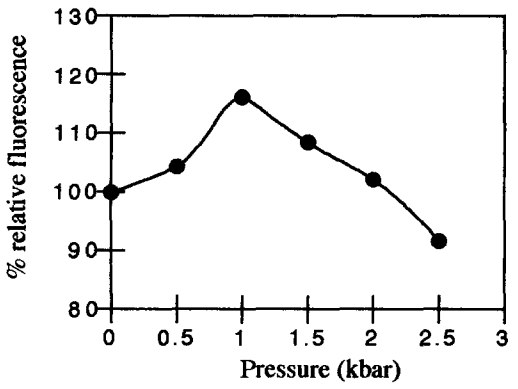

Fig. 3. Change in the fluorescence intensity of ANS in the presence of BuChE as a function of pressure. The enzyme in 10 mM Tris-HCl buffer pH 7.5 was exposed to pressure for 1 h at 10°C. Relative intensity of bound ANS (λ_{ex} = 358 nm; λ_{em} = 447 nm) was measured immediately after pressure release.

tion of BuChE by infrared spectroscopy under high pressure did not show any secondary structure changes below 3.5 kbar [29]. Moreover, in a previous work we found that pressure denatured BuChE has the tendency to aggregate [15], which is consistent with an extensive exposure of hydrophobic groups to the solvent. The tendency to aggregate was found to depend on the enzyme concentration and the exposure time to pressure. Electrophoresis showed that formation of aggregates of partially unfolded BuChE occurs when stacking gels were used – in stacking gels the protein concentration becomes very high in the moving disc – and after long exposure times ($t > 30$ min) to pressure prior to electrophoresis runs (unpublished), e.g. 12 h [15]. In the present study we operated under different conditions to overcome this complication. Therefore, the results we report confirm that denaturation of BuChE is a multistep process [15,30] and support the hypothesis that moderate pressures induce the sequential formation of at least two folding intermediates that may correspond to 'highly ordered' molten globule and molten globule states, respectively. The complete characterization of these intermediates is of outstanding interest. The high pressure denaturation approach to generate partially unfolded states of proteins has been applied to other wild-type and mutant cholinesterases (in preparation). Results are expected to shed light on both the folding process and the molecular basis of the stability of the enzymes of the cholinesterase family.

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