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Effect of pressure on pulse radiolysis reduction of proteins

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Abstract

Pulse radiolysis experiments were performed on proteins under pressure. Whereas many spectroscopic techniques have shown protein modifications at different pressure ranges, the present measurements performed using the water radiolysis allowed to generate radical species and to study the mechanisms implied in their reactions with proteins. This work gives the first results obtained on the effects of pressure on the rate constants of the proteins reduction by the hydrated electron at pressures up to 100 MPa. The reaction with the hydrated electron was investigated on two classes of protein: the horse myoglobin and the mussel metallothioneins. We have successively studied the influence of the pH value of metmyoglobin solutions (pH 6, 7 and 8) and the influence of the metals nature (Zn,Cu,Cd) bound to metallothioneins. For both protein, whatever the experimental conditions, the pressure does not influence the value of the reduction rate constant in the investigated range (0.1 – 100 MPa).

Keywords: Pressure; Pulse radiolysis; Reduction; Protein; Hydrated electron

1. Introduction

Radical species can be produced by both normal and pathologic metabolic processes, exogenous chemicals (drugs, alcohol, smoking, metals, toxic compounds) and both UV and ionising radiations (γ, X, electron). When cellular protections (enzymes, antioxidants) against radicals are insufficient, under the sensitizing effect of oxygen, there is an uncontrolled production of these radicals and in particular reactive oxygen species [1]. The radicals which are unstable and highly reactive will react with their environment [2]. By reacting on macromolecules as DNA, proteins and lipids, radical species cause subcellular and cellular damage which can be implicated in a number of diseases [3–6]. Moreover, owing to their high reactivity, radicals can also be used as probes of both structural and dynamics modifications of macromolecules [7,8].

The water radiolysis is a powerful technique to generate the radical species and to study the involved mechanisms and the formed products in their reactions with macromolecules [9]. The water radiolysis and the physico-chemical properties of the radicals are well known [2]. The effects of different intervening factors on water radiolysis such as pH, presence of oxygen, temperature and pressure have been extensively described [10].

Besides the framework of biotechnological applications and notably food processing [11,12], the effects of temperature and pressure on biological macromolecules have been studied in the framework of fundamental research [11,13]. The effects of these thermodynamic variables are

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studied in two ways: either extreme conditions are used for the analysis of macromolecules properties of extremophilic organisms, or extreme conditions are used as perturbation techniques of physico-chemical properties of macromolecules [13]. So, numerous investigations on proteins structure, dynamics and activity have been developed under unusual conditions of pressure [13]. However, even if a pulse radiolysis study has showed the slow intra- and intermolecular electron transfer in the second time scale from ruthenium(II) ammine complexes to cytochrome *c* up to 150 MPa [14], no study has been reported on the use of radicals as probes of reactivity of proteins under pressure. In order to answer these questions, we give here for the first time the results of protein reactions with radicals generated by pulse radiolysis under pressure. These experiments have been performed with two proteins.

Myoglobin is the well-known protein which was a model of study in many pressure experiments using optical absorption [15–18], Raman [19], nuclear magnetic resonance [20–22], Fourier-transform infrared [23,24], small-angle neutron scattering [25] spectroscopies. These experiments have shown that pressures up to 300 MPa lead to modifications in the myoglobin molecule at the level of the active site [15–22] and the reorganisation of the secondary structure with an alteration of the electrostatic and hydrogen-bond array [23,24] without change in the tertiary structure of the protein [25].

Metallothioneins are small proteins (6–7 kDa) particularly rich in cysteine residues (30% of the total number of residues) which form two clusters having a great capacity to bind divalent metal ions (Me): Cys_9–Me_3 and Cys_{11}–Me_4. They chelate essential metals (Zn,Cu) and heavy metals (Cd, Hg) [26]. Another characteristic is the in vivo-inducibility of metallothioneins in response to heavy metals [26,27]. The hydrothermal environment of extremophilic organisms is characterized, among other physical and chemical extreme conditions, by high metal concentrations, high pressures and radioactivity. So, we used metallothioneins as a model to study the reactions of radical species with protein under pressure.

We have investigated the myoglobin and metallothioneins reactions with hydrated electrons provided with radiolysis of water obtained with an electron linear accelerator. Due to previous pulse radiolysis studies which have shown the metmyoglobin (Mb) reduction rate constant depends on the pH of the solution [28–30], the Mb reduction has been studied at three pH values. Otherwise, the metallothioneins (MeT) reduction rate constant was measured on two preparations: (Zn,Cu)-MeT and (Zn,Cu,Cd)-MeT solutions. The experiments were performed in the pressure cell (described in Materials and methods) designed and carried out in the laboratory. The pressure will be up to 100 MPa.

2. Materials and methods

2.1. Protein samples preparation

All chemical reagents and gases were of the highest purity available. Pure water was prepared using an Elix-5 and Alpha-Q Millipore system which delivers water with a resistivity of 18.2 MΩ.cm and a low total organic carbon better than 10 ppb. All protein samples were prepared and stored at 4 °C. Lyophilised horse-heart myoglobin (Sigma M-1882) was dissolved in pure water, dialysed three times against water to remove all the salts, dialysed against buffered solutions and centrifuged at $24000\times g$ during 10 min. For the pulse radiolysis experiments, bis-Tris and Tris buffers were chosen because their dissociation constants should not be altered by pressure [31]. Three buffered solutions were used: 1×10^{-3} mol dm^{-3} bis-Tris pH 6, 1×10^{-3} mol dm^{-3} bis-Tris pH 7 and 1×10^{-3} mol dm^{-3} Tris pH 8 (at 20 °C). The metmyoglobin solutions were checked by absorbance measurements in the visible region [32]. The Mb concentrations were adjusted by dilution in the appropriate buffer to $4–6\times10^{-5}$ mol dm^{-3}.

Metallothioneins were extracted and purified from the edible mussel *Mytilus edulis*. Mussels were collected and placed in aquariums for a period of acclimatization (72 h). To induce the de novo MeT biosynthesis, the sea water was enriched in 100 μg dm^{-3} $CdCl_2$ during 8 days [33]. As standards, mussels were maintained under the same conditions in sea water. *Mytilus edulis* (Zn,Cu,Cd)-MeT and (Zn,Cu)-MeT were prepared according to the previously described methods [26,34]. The first step was the homogenization of tissues. MeT being contained in the mantle, gill, digestive gland and kidney, the whole mussels were homogenized in 100×10^{-3} mol dm^{-3} Tris pH 8.1 (at 4 °C): first in a mixer and then in a glass-Teflon homogenizer. The homogenate was centrifuged at $24000\times g$ during 5 min. The next step was the heat treatment of supernatant. The supernatant obtained by centrifugation of the homogenate has been heated at 95 °C during 15 min to remove heat-unstable proteins and thereby leaving the high heat-stable metallothioneins intact in the soluble fraction. Lipids were removed with the insoluble fraction. The solution was centrifuged at $24{,}000\times g$ during 20 min. After a dialysis against water and against 1×10^{-3} mol dm^{-3} bis-Tris pH 7, the (Zn,Cu)-MeT and (Zn,Cu,Cd)-MeT bulk solutions were used for the pulse radiolysis experiments without further purification. So, the pulse radiolysis experiments were performed on all *Mytilus edulis* MeT isoforms [35]. We determined the metals content of MeT by atomic absorption spectroscopy.

In all pulse radiolysis experiments on (300 cm^3) myoglobin and (100 cm^3) metallothioneins solutions, 1×10^{-1} mol dm^{-3} tertiobutanol was added to scavenge the hydroxyl radicals and the hydrogen atoms leaving only hydrated electron as the reactive species. Before the experiments, the samples were centrifuged at $24000\times g$