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ARTICLE

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Saccharose solid matrix embedded proteins: a new method for sample preparation for X-ray absorption spectroscopy

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Abstract In this study, solid samples of hemoglobin and hemocyanin have been prepared by embedding the proteins into a saccharose-based matrix. These materials have been developed specifically for specimens for X-ray absorption spectroscopy (XAS). The preservation of protein conformation and active site organization was tested, making comparisons between the solid and the corresponding liquid samples, using resonance Raman, infra red, fluorescence and XAS. The XAS spectra of irradiated solid and liquid samples were then compared, and the preservation of biological activity of the proteins during both preparation procedure and X-ray irradiation was assessed. In all cases, the measurements clearly demonstrate that protein solid samples are both structurally and functionally quite well preserved, much better than those in the liquid state. The saccharose matrix provides an excellent protection against X-ray damages, allowing for longer exposure to the X-ray beam. Moreover, the demonstrated long-term stability of samples permits their preparation and storage in optimal conditions, allowing for the repetition of data collection with the same sample in several experimental sessions. The very high protein concentration that can be reached results in a significantly better signal-to-noise ratio, particularly useful for high molecular weight proteins with a low metal-to-protein ratio. On the bases of the above-mentioned results, we propose the new method as a standard procedure for the preparation of biological samples to be used for XAS spectroscopy.

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Introduction

X-ray absorption spectroscopy (XAS) is a very powerful method to study the active site geometry of metalloproteins with a high degree of accuracy. This technique can be considered complementary to X-ray diffraction, with the further advantage of providing detailed structural information on the environment of the metal site for solution samples under physiological conditions. Furthermore, it allows one to obtain relevant information even on proteins not suitable for crystallization. A recent improvement in the use of XAS for the investigation of the active site structure of metalloproteins can be seen in the development of the third-generation storage rings. These new machines present significant advantages, such as: the availability of an intense and well-focused highly monochromatic beam, allowing a reduction of both the protein concentration and the volume of the sample, as a consequence of the increased photon density on the sample. In fact, second-generation machines have a brilliance on the order of 10^{13}–10^{15} photons s^{-1} mm^{-2} $mrad^{-2}$ 0.1% BW, whereas third-generation machines have a brilliance 5–6 orders of magnitude higher. However, damage to biological materials has been reported even with first- and second-generation machines (Halpern 1982). This mainly consists of the degradation of the protein backbone (Peisach et al. 1982) or in photo-reduction of the metal centre (Chance et al. 1980), related to the generation of a free radicals (mainly produced from the breakdown of water molecules).

The harmful effects of free radicals can be reduced by carrying out the XAS experiments at low temperatures (liquid nitrogen temperature or lower), working with buffered protein solutions in solid phase, where the free

diffusion of the damaging species is actually hindered. However, the electronic transfer of the radical centre along hydrogen bond chains of the structured water remains essentially unaffected or it is even improved. Furthermore, it has been demonstrated that freezing induces conformational modifications and often denaturation of proteins in solution (Franks 1985; Chang et al. 1996). Freeze-denaturation of chymotrypsinogen is an example of the complexity of this process. Denaturation does not occur even at −70 °C in the pH range 2.55–10.00, but the denaturation of the protein has a non-linear dependence on temperature at pH 1.78. Furthermore, a considerable protective effect against denaturation is observed by increasing the protein concentration and in the presence of 0.1 M NaCl or 0.1 M sucrose. In such conditions, no denaturation is observed even at pH 1.78 (Franks 1982). In line with the last observations, it has been further demonstrated that protein denaturation can be prevented by addition to the protein solution of specific compounds like some inorganic salts, polyhydroxylic alcohols (glycerol, polyethylene glycols), amino acids (betaine, proline), or disaccharides (saccharose, threalose). All of the mentioned compounds are known to exert a protective action against protein conformation damage caused by freezing or dehydration (Gekko and Timasheff 1981a, b; Lee and Timasheff 1981; Carpenter and Crowe 1988; Crowe et al. 1992).

Given the great interest in XAS application to biological materials, a systematic approach is required to overcome the difficulties of XAS outlined above and to consider the peculiar needs of handling biological samples. This provided us with both the motivation and the rationale for the development and experimentation of a new method for XAS protein sample preparation, based on the inclusion of the protein in cryo- and anhydro-protectant based solid matrices. Saccharose was chosen as the main component of the matrix since it has been already proved to stabilize protein structures in solution (Salvato et al. 1974; Lee and Timasheff 1981; Arakawa and Timasheff 1982), and it may function as a cryo- and anhydro-protectant for biomolecules. A further advantage provided by protein solid solutions, i.e. matrix-included proteins, is that it allows one to reach much higher protein concentrations than in liquid solution. This is particularly useful for those large proteins where the X-ray absorption centre (i.e. the metal ions) represents a minute fraction of the molecular weight.

The aim of this paper is to present a new method for biological sample preparation, specific for XAS. The proteins chosen to test the sample preparation method are hemoglobin (Hb) and hemocyanin (Hc). Both proteins are respiratory pigments able to bind molecular dioxygen reversibly at a metal active site. However, they differ in the nature of the metal, the degree of steric hindrance and chemical characteristics. Both proteins have very informative spectroscopic properties, providing very sensitive probes of the active site structure. In the case of Hb, the X-ray absorption centre is an Fe ion bound in a hydrophobic planar heme group that occupies a large volume inside each sub-unit. It has only one direct bond with the proximal histidine residue. In the case of Hc, the protein from the two investigated phyla are oligomers that have a very different molecular weight, topology and Cu/protein ratio, though for both proteins the active site contains two copper ions each directly bound to the protein matrix by three imidazole nitrogens. Conceivably, the interactions of this dinuclear Cu metal site with the polypeptide chain could be more sensitive to conformational changes caused by X-ray damage.

Materials and methods

Human Hb was obtained from healthy volunteers and purified as reported elsewhere (Antonini and Brunori 1971). One aliquot was used immediately after purification and another was frozen after addition of 20% w/v saccharose. Hc was purified from hemolymph collected from living animals; the general procedure has been described elsewhere (Preaux and Gielens 1984). The purified proteins, dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 20 mM $CaCl_2$, were frozen after addition of 20% w/v of saccharose and stored at −20 °C until used. Hc was obtained from two mollusc species, *Rapana venosa* (gastropod) and *Octopus vulgaris* (cephalopod), and from one arthropod species, *Homarus americanus*.

Powdered solid solutions of the proteins in saccharose were obtained starting from proteins dialysed against the appropriate buffer for 24 h at 4 °C. Buffers were prepared, at a final concentration of 100 mM, using secondary and tertiary ammonium bases such as piperazine and triethanolamine, together with glycine and aliphatic carboxylic acids. The protein solution, at concentration of about 30 mg/ml and containing saccharose in a sucrose/protein ratio of 3:1 w/w (corresponding to a concentration of about 0.250 g of protein per gram of final solid-solution), was then rapidly frozen in liquid nitrogen and lyophilized. The samples of the deoxy form of the proteins were obtained by addition of a concentrated solution (100 mM) of sodium sulfite and sodium dithionite in buffer up to a final concentration of 2.5 mM each. The specimens for XAS measurements were prepared by pressing about 200 mg of lyophilized powdered protein solid solution under 140 atm in a home-made small press having a chamber with lateral dimensions of 2.2×0.3 cm; the slides obtained were about 0.2 cm thick. Solutions of irradiated proteins were obtained by dissolving the corresponding slide in milliQ water and dialysing against the appropriate buffer for 48 h at 4 °C. The clear solution, when necessary, was further concentrated by an Amicon Centricon 30 K.

The X-ray absorption near edge structure (XANES) measurements were performed at the LURE synchrotron facility, in the D21 (EXAFS II) beamline. The Cu and Fe K_α signals were collected in fluorescence configuration by a seven-element CANBERRA detector. A Si (311) crystal was used as a monochromator. For the Hc samples, the energy range was from 8930 to 9090 eV (calibration of energy performed by means of a copper foil reference); for the Hb the energy range was from 7080 to 7200 eV, the resolution was 0.3 eV, with a counting time of 6 s/point. The absorption spectra were collected using a Perkin-Elmer Lambda 16 double beam spectrophotometer equipped with a thermostated cell holder. The spectra were collected on samples of saccharose included Hc, dissolved in buffer, before and after X-ray irradiation. Fluorescence spectra were collected on a L 50 Perkin-Elmer spectrofluorimeter equipped with a thermostated cell holder. Fluorescence emission spectra were collected from solution samples as well as on solid samples prepared as described for the XAS experiments.

The resonance Raman spectra of saccharose-embedded samples of Hb in deoxy and in oxy form were obtained with 442 nm excitation from a HeCd laser (Liconix) on an instrumental set-up

previously described elsewhere (Smulevich et al. 1999). FT-IR spectra were recorded at room temperature with a Nicolet 5SxC FT-IR spectrometer equipped with a triglycine-sulfate (TGS) detector. Spectra were recorded for liquid solutions of Hc, saccharose-embedded Hc (lyophilized powder) and for Hc lyophilized without saccharose.

Results

In Fig. 1A the XANES spectra of human oxy Hb in buffer solution and in the solid matrix are shown. The comparison does not highlight any substantial differences between the spectra in solution and in the matrix. In the following experiment the temperature dependence of the active site structure of Hb in solid solution was investigated. In Fig. 1B is shown a series of XANES spectra of an oxy Hb at increasing temperatures, from 50 to 250 K with steps of 50 K. The same sample was then re-measured at 200 K (Fig. 1B) and the comparison of XANES spectra of Hb obtained at the various temperatures of the cycle shows no differences. After measurements, the X-ray-exposed sample used in the temperature dependence study was stored for two weeks at −20 °C and then re-exposed to the X-ray beam. The XANES spectrum of the sample after storage (shown in Fig. 1C) does not show significant differences when compared with that obtained for a new sample.

The identity of the Hb structure in solid state and in solution is further confirmed by the resonance Raman results. The spectra of solid oxy and deoxy Hb in the Soret scattering regions (Fig. 2A and B) perfectly correspond with the spectra obtained for the solution samples reported by Spiro and Strekas (1974). In Table 1 are compared the positions of the absorption peaks both for the solid and for the solution samples. Furthermore, the resonance Raman spectra of the solid solution of oxy Hb before and after irradiation are the same (Fig. 3).

In Fig. 4A the XANES absorption spectra relative to the liquid solution and solid matrix samples of them native oxy Hc form of *R. venosa* are shown. Also in this case, the spectra of the samples prepared following the

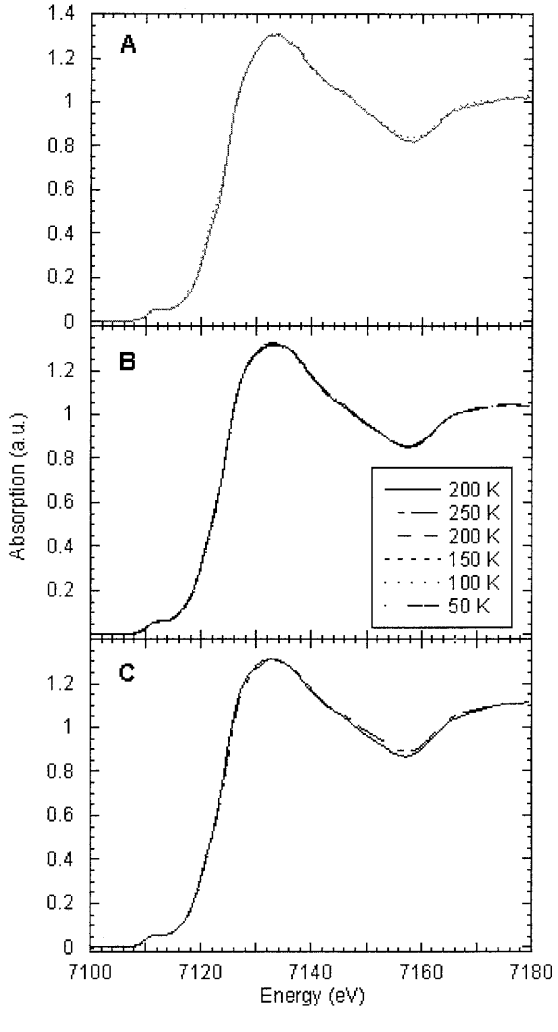

Fig. 1 **A** Comparison of the XANES spectra of human Hb in 50 mM phosphate buffer, pH 7.0. *Solid line*: liquid form; *dotted line*: solid sample. The latter has maintained its structure even under thermal cycle (**B**). Moreover, after 15 days storage at −20 °C, no substantial changes can be detected (**C**); *dotted line*: before storage; *solid line*: after storage

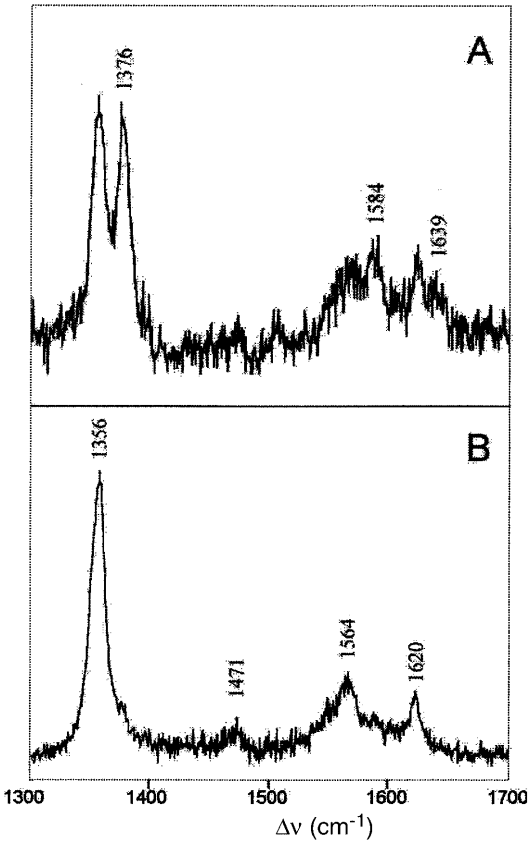

Fig. 2 Resonance Raman spectra of solid solution samples of human Hb in 50 mM phosphate buffer, pH 7.0, in oxy (**A**) and deoxy forms (**B**). The deoxy form was obtained by addition of sodium sulfite and sodium dithionite in buffer up to a final concentration of 2.5 mM each

Table 1 Comparison of resonance Raman peaks of deoxy and oxy human Hb in solid solution and liquid solution

Sample	$\Delta\nu$ (cm^{-1})	$\Delta\nu$ (cm^{-1})	$\Delta\nu$ (cm^{-1})	$\Delta\nu$ (cm^{-1})
Deoxy				
Solid solution[a]	1356	1471	1564	1620
Liquid solution[b]	1358	1473	1565	–
Oxy				
Solid solution[a]	1376	–	1584	1639
Liquid solution[b]	1374	1506	1582	–

[a] Soret region (λ_0 442 nm). Incident power: 5 mW, cylindric lens
[b] Spiro and Strekas (1974)

two different methods are superposable. The same results have been obtained with the other molluscan Hc (*O. vulgaris*) and with the arthropodan *H. americanus* Hc (data not shown). The deoxy forms of Hc show an analogous structural identity between the solid and solution samples: in Fig. 4B the data relative to *H. americanus* deoxy Hc are displayed. Figure 5 shows the effects of X-ray exposure on the oxy form of *R. venosa* Hc up to 5 h.

To verify if the matrix embedding process induces permanent functional and/or structural modifications in the samples, UV and fluorescence spectra were collected on solutions obtained from solid samples dissolved in buffer before and after exposure to the X-ray beam. The comparison of the UV absorption spectra strongly suggests that Hcs do not lose their functionality, i.e. competence to reversibly bind oxygen, as a consequence of the embedding process in the saccharose matrix (Fig. 6). The fluorescence emission spectra of the same sample before and after the X-ray exposure are again identical, indicating that no permanent structural modifications were induced by the presence of saccharose in the protein sample. The fluorescence spectrum measured directly on a slide of embedded *R. venosa* Hc, however, shows a blue shift of 14 nm of the emission maximum (Fig. 7). The IR data obtained on *R. venosa* Hc in buffer solution and prepared according to our procedure in the presence of 300% w/w saccharose agree well with the identity of the protein conformations in the two conditions, as indicated by the same shape and intensity of the amide (II) band (data not shown). The comparison of these data with those obtained on lyophilized samples of *R. venosa* Hc in absence of saccharose highlights a loss of ordered structure in the latter case of about 30% (Di Noto et al. 1998).

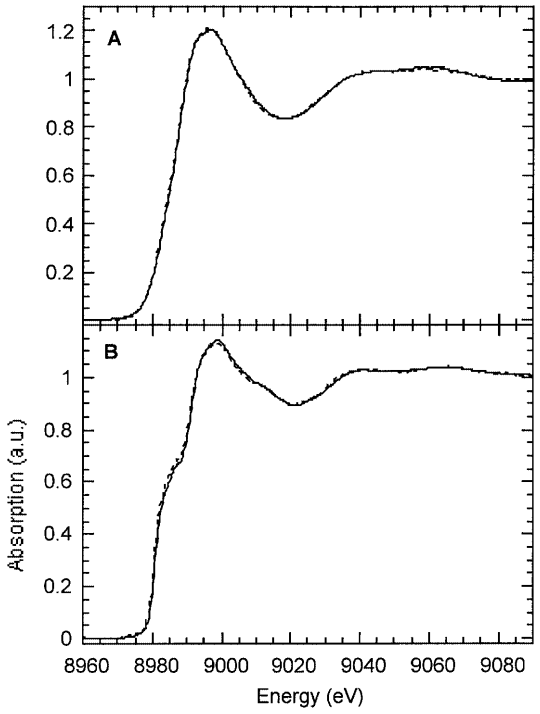

Fig. 4 Comparison of the XANES spectra of Hcs in solid solution (*solid line*) and in liquid form (*dotted line*) in 50 mM phosphate buffer, pH 7.5: **A** *R. venosa* in oxy form; **B** *H. americanus* in deoxy form. The deoxy form was obtained by addition of sodium sulfite and sodium dithionite in buffer up to a final concentration of 2.5 mM each

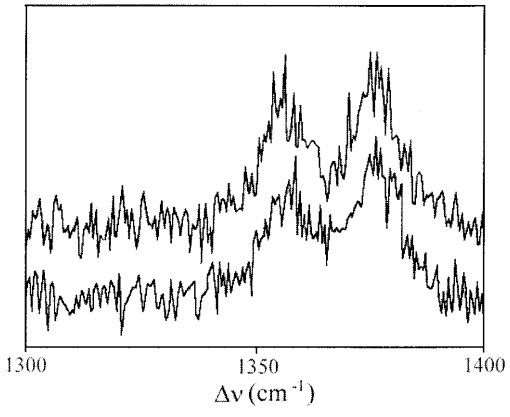

Fig. 3 Comparison of the resonance Raman spectra of a solid solution sample of oxy human Hb in 50 mM phosphate buffer, pH 7.0, before (*lower line*) and after (*upper line*) X-ray exposure

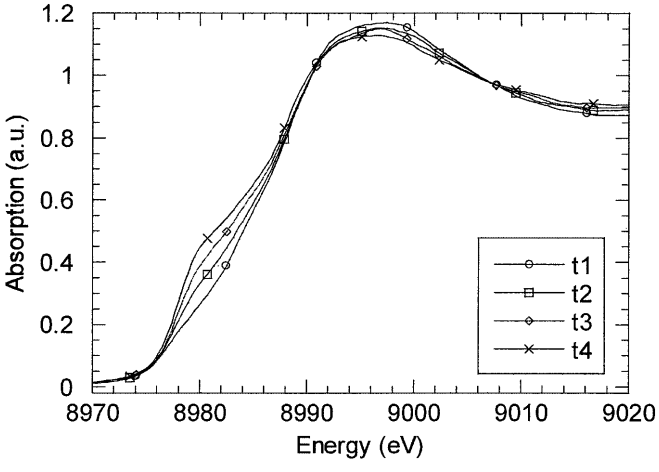

Fig. 5 Observed variation of the XANES spectrum of liquid *R. venosa* Hc in oxy form as a function of the exposure time to the X-ray beam. Measurements have been performed starting from 1 h exposure (*t*1), then after 2, 3 and 4 h (*t*2, *t*3 and *t*4)

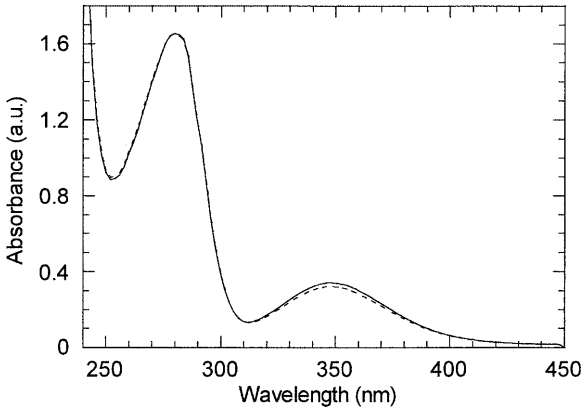

Fig. 6 UV absorption spectra of *O. vulgaris* Hc in 50 mM phosphate buffer, pH 7.0. *Solid line*: before XANES measurement; *dotted line*: the same sample after irradiation

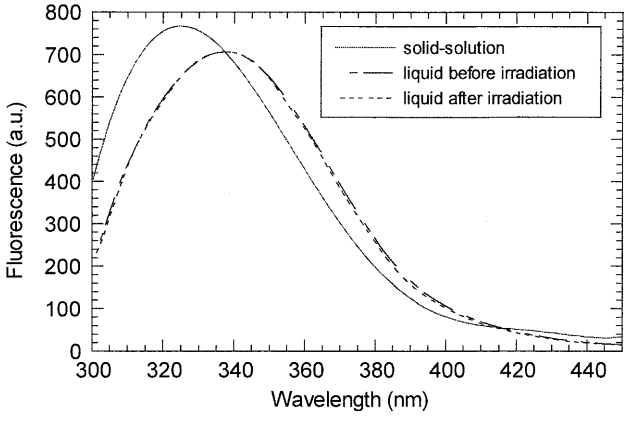

Fig. 7 Fluorescence spectra of oxy *O. vulgaris* Hc in 50 mM phosphate buffer, pH 8.0. Excitation wavelength: 280 nm. The solid solution sample (*solid line*) shows a blue shift of about 14 nm

In order to evaluate the amount of water in the sample as a function of the sugar concentration, dehydration tests were performed on three different solid samples of *R. venosa* Hc, containing respectively 0%, 20% and 300% sucrose w/w, prepared following the protocol described in Materials and methods. The solid samples were dried over phosphoric anhydride under vacuum for about 24 h. The weight loss after dehydration was 5.8%, 5.6% and 1.1%, respectively. In a different experiment the 300% sucrose samples was also dehydrated using an oven at 40 °C for 24 h, showing a comparatively higher weight loss of about 2.6%.

Discussion

Biological structures as DNA, RNA, proteins and membranes undergo extensive and largely irreversible damage when the water clathrates structured around their surfaces are physically disturbed or removed (Franks 1982). Dehydration and freezing are typical processes that can cause irreversible changes at the level of the tertiary and quaternary structures of biological macromolecules. The molecular mechanism responsible for this damage is the same in both cases. In fact, the freezing process involves the removal of water from the surrounding volume around the macromolecular surface, which in turn is immobilized in growing ice crystals. Severe damage to the macromolecular structure arises from these needle-shaped growing ice crystals (Strambini and Gabellieri 1996). Following this mechanism, even cells are severely dehydrated: more than 70% of cytoplasmic water can be lost upon freezing (Franks 1982; Carpenter and Crowe 1988).

Several compounds are known to be efficient in preventing damage to biological structures which can be caused by dehydration or freezing. In fact these compounds can protect biological structures against damage in solution even at relatively high temperature (Timasheff and Arakawa 1989), as well as during freeze-thawing and lyophilization (Carpenter and Crowe 1988). The natural compounds most widely found as preservatives in biological systems are the unreducing disaccharides saccharose (α-glucopyranose-β-fructofuranose) and trehalose (α-glucopyranose-α-glucopranose). Interestingly, monosaccharides have no comparable effects on the biological structures (Crowe et al. 1992). The preservative effects of the former compounds have been usually measured by comparing biochemical and biophysical properties of the biological samples in solution, before and after dehydration or freezing; there are few indications whether the sample maintains the same conformation even in the dehydrated or frozen state. The mechanism involved in the preservation process is mainly based on the exclusion of those compounds from the surrounding volume of the macromolecular system which, in turn, remains preferentially hydrated (Carpenter et al. 1991). The water in the surrounding volume represents ~50% of that of the macromolecule and is structured quite similarly to ice (I). During freeze-thawing and lyophilization these water molecules are removed from the surrounding volume and this causes the damage to the biological structure. Thus conformation-protecting compounds do not interact directly with macromolecules (nucleic acids and proteins) and membranes, but actually they strengthen and stabilize the water structure in their surrounding volume, preventing the removal of hydration water during freezing or dehydration. Damage to biological structures can be also caused by crystallization of solutes during freeze-thawing and lyophilization. Protecting compounds usually give a rapid glass transition at relatively high temperature and concentration, and this process competes with crystallization. The glassy phase does not induce damaging effects on biological structures. Saccharose has been widely used to prevent denaturation of biological samples during lyophilization. However, no further analysis of its role in the prevention of X-ray damage has been performed (Salvato et al. 1974;

Brown et al. 1980; Arakawa and Timasheff 1982; Strambini and Gabellieri 1996). Saccharose is not the only component to have a role in protein stabilization. Divalent cations, organic solutes (Carpenter et al. 1991) and buffers (Izutsu et al. 1993) have a synergistic action. We have previously demonstrated that Hcs and Hbs can be stored for a very long time (Salvato and Beltramini 1990) without appreciable changes in their biochemical and biophysical properties when stored in a frozen state or lyophilized in the presence of saccharose, 20% w/v at −20 °C. The latter functions as a protecting agent against damage which can be induced by freezing and lyophilization (Salvato et al. 1974).

In this study, it is proposed that a systematic use of co-solutes contributes to the formation of the glassy phase, avoiding damage to biological structures due to crystallization. This study was therefore focused on saccharose as the main protectant, and the effectiveness of the proposed sample preparation method was tested on these two well-characterized proteins. An extensive series of controls was carried out to verify if the room temperature solution structure of these proteins is conserved in the solid saccharose included samples. Also the minimization of the damaging effects of X-rays in the solid samples was evaluated.

The data reported here demonstrate, for both Hb and Hc, the effectiveness in preserving the native conformation when the protein is embedded into the saccharose matrix, following the procedure described in this study. The equivalence of the conformations of the proteins in solution and embedded into saccharose solid matrix is indicated by the superposition of the corresponding XAS profiles for both proteins, Raman spectra for Hb, and absorption, IR and fluorescence spectra in the case of Hcs.

Fluorescence emission is very sensitive to changes of the local dynamics around the fluorophor, being a spectroscopy of electronically and vibrationally excited states. The observed blue shift in the fluorescence emission spectrum of the solid matrix embedded *R. venosa* Hc can be assigned to the greater rigidity of the protein structure in the solid state in comparison with the solution state. XAS, being a structure technique local to the metal centre is unaffected by these more remote effects as demonstrated by the data (Fig. 1A, Fig. 4A and B).

The protective action against damage caused by freezing and lyophilization, exerted by saccharose and the other cryoprotectants, can be assigned to their ability to stabilize the structure of hydration water of the surrounding volume of the protein in solution and to their competence to drive the glass transition of the solution during freezing. Lyophilization can then eliminate only the bulk water, preserving the hydration shell of the protein as indicated by IR spectroscopy and by the dehydration experiments. The preservation of the protein conformation in the solid saccharose matrix depends on the maintenance of this small water volume around the protein, comprising about 5–8% of the total weight of the protein solid solution. The protein itself contributes to the high rates of glass transition. Upon lowering of the protein concentration in the absence of co-solutes, the sugar crystallization rate can largely overcome the rate of the glass transition.

It is important to emphasize that the embedded oxy forms of Hcs and Hbs are highly stable, both conformationally and functionally, over a long time span, even at room temperature, and can be further utilized directly or dissolved in buffer. The deoxy forms are stable in air atmosphere during several months if stored at low temperatures. This long-term stability allows preparation and storage in the best conditions in advance of their use, and this is especially useful for low stability specimens. Furthermore, it is possible to repeat data collection with the same sample in several experimental sessions. The embedding process actually selects and fixes the conformational distribution, which is the most probable for the protein in solution at the conditions of freezing. This conformational distribution is maintained independently from the temperature of observation, owing to the the stabilization by the solid matrix.[1]

With the proposed sample preparation, a very high protein concentration can be reached without precipitation or aggregation processes. This aspect is very important in XAS studies of biological macromolecules, especially for high molecular weight proteins with a low metal-to-protein ratio. The reliability of the new method has been tested in preliminary experiments performed with the third-generation machine (ESRF), by some of the authors (L. Bubacco, personal communication).

In conclusion, this paper presents a new method for biological samples preparation, specific for X-ray absorption spectroscopy.

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[1] The protein structure is also preserved against X-ray damage**.** By contrast, dramatic effects of X-ray can be observed during long exposure (up to 4 h) of samples in liquid solution, even for first generation machines as shown in Fig. 5. These effects are due to the water solution. In fact, the solid *L. polyphemus* oxy-Hc sample, specifically prepared to contain water ~30% w/w, is seen to be bleached in the area exposed to the beam. This indicates damages in the active site; XANES spectra have the same behaviour shown in Fig. 5 except that changes occur over a longer time (9 h). Thus protein preservation in solid solution samples appears to be linked to the reduced availability of water molecules in the matrix, and therefore a reduced production of radicals.

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