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Trehalose Glass-Facilitated Thermal Reduction of Metmyoglobin and Methemoglobin

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The disaccharide trehalose is believed to play a major role in anhydrobiosis.[1−3] The glass-forming properties of trehalose solutions under drying condition coupled with the ability of trehalose to form strong hydrogen bonds with surface groups of biomolecules are key features that enable trehalose-producing plants and animals to survive extended periods of dehydration.[2,4] These properties have resulted in trehalose being the focus of many efforts to develop new methods of preserving proteins and peptides.[4−8] Proteins embedded within such matrices show a dramatic reduction in the amplitude and nature of their conformational fluctuations.[9−18] In the case of myoglobin (Mb) and hemoglobin (Hb), the damping of conformational fluctuations by the glassy matrix is sufficient to prevent the escape of dissociated heme ligands from the protein.[14−16,19]

As part of an effort to expose the extent to which glassy matrixes can protect proteins from thermal stress, we embedded derivatives of horse Mb and human adult hemoglobin (HbA) in trehalose glasses and followed spectroscopic markers as a function of heating protocols. In these protocols, samples consisting of a thin layer of a protein-containing trehalose glass are heated at a fixed temperature for 15 to 60 min and then examined spectroscopically (visible and near-IR absorption, CD, front face fluorescence, and in some cases UV resonance Raman). The spectroscopic studies which are the focus of a future more detailed account, show that in the glassy state the protein is protected against thermally induced unfolding even at temperatures exceeding 100 °C. Quite unexpectedly, we observe that when the samples are exposed to temperatures approaching 100 °C, the optical spectrum of glassy samples of aquo-metMb irreversibly evolved into spectra characteristic of deoxyMb. Since trehalose is not a reducing sugar but does often contain trace glucose impurities, we reasoned that glucose, a reducing sugar, was the likely reductant.

To test the hypothesis that the thermally induced reduction is mediated by a reducing-sugar impurity in the trehalose, we repeated the protocol on glassy samples made from trehalose doped with glucose (10 wt %). The glucose-doped samples of aquo-met Mb and -Hb samples both underwent thermally induced reduction at lower temperatures (between 70 and 80 °C). The addition of maltose, also a reducing sugar, had a similar impact. Figure 1 shows the visible absorption spectra of the initial glass-embedded aquo-met samples of Mb prior to, during, and after the heating protocol. The initial spectrum, (a), obtained after the formation of the glass, is essentially the same as that of aquo-metMb in solution. The final spectrum (c), obtained after the heating protocol, is characteristic of deoxyMb (with a trace amount of the remaining aquo-met derivative). The intense Soret absorption band in the 380−440 nm spectral region similarly reflects these assignments. The weak near-IR band III, characteristic of ferrous five-coordinate heme, is also

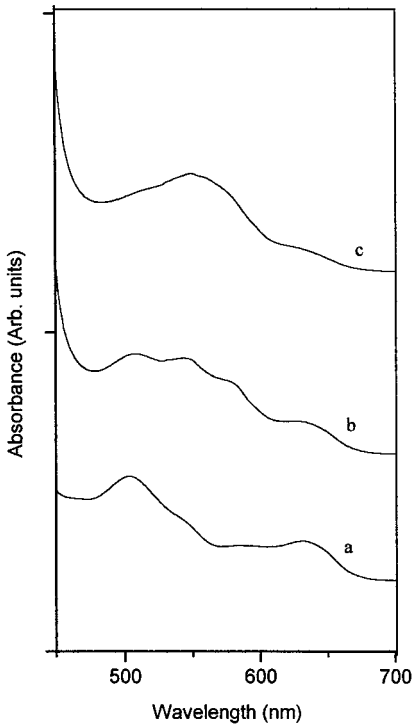

Figure 1. Room-temperature absorption spectrum of aquo-metMb in a glucose-doped trehalose glass: (a) Prior to heating, (b) after 15 min of heating at 75 °C, and (c) after 45 min of heating at 75 °C. Sample preparation and analysis are described in the Supporting Information.

apparent at its characteristic position (∼760 nm) for the end-point heat-treated species. The intermediate spectrum (b) obtained during the heating protocol occurs prior to the appearance of the deoxy spectrum. The shown spectrum is assigned as a mixture derived from the initial metMb and a bis-histidine ferric derivative (hemichrome). The inclusion of the bis-histidine derivative is based both on the two peaks in the absorption spectrum (∼535 nm and ∼563 nm), (Figure 1b) which agree with the reported spectrum. Figure 2 shows a similar series of spectra for glassy samples of HbA. As with Mb, the initial and final spectra (a and c, respectively) of the glucose-containing sample are clearly attributable, respectively, to an initial aquo-met derivative and to a final sample that is predominantly the five-coordinate ferrous form (with some remaining aquo-met). In the absence of added glucose, the heat-treated (75 °C) sample of aquo-metHbA yields a spectrum (b) that is clearly assignable to the hemichrome without any contribution from the five-coordinate ferrous derivative.

This heat-induced reduction clearly depends on the amount of added glucose. The time required to reduce the glassy sample at 70 °C decreases with increasing concentration of added glucose.

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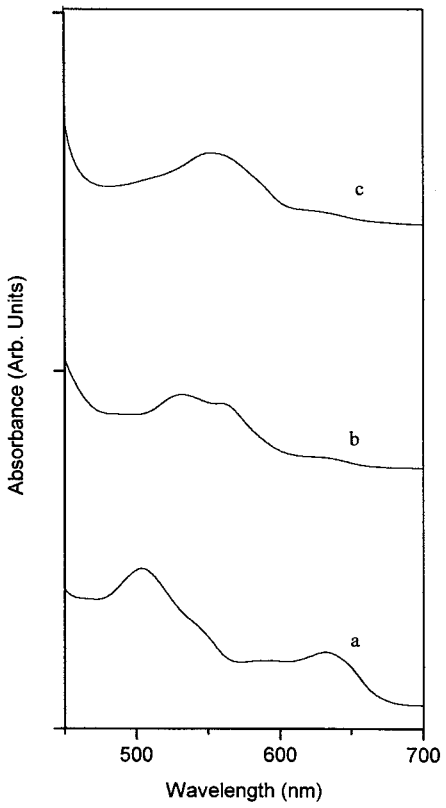

Figure 2. Room-temperature absorption spectrum of aquo-metHb in a trehalose glass: (a) Prior to heating, (b) after 15 min of heating at 75 °C (sample with no added glucose), and (c) after 45 min of heating at 75 °C (sample with added glucose).

Samples containing 12, 10, and 7% glucose by weight (representing approximately 50-fold excess of glucose over Mb) required, respectively, 30, 45, and 60 min to become reduced, whereas samples containing 5 and 2% glucose were only partially reduced at 90 min.

Extended heating of a *solution* of aquo-metMb or -Hb at 75 °C in the presence of trehalose or glucose or both at the levels used in making the glassy samples does not result in the formation of the ferrous derivative as reported in earlier studies.[20] Heating *solution* samples with just glucose or mixtures of glucose plus trehalose (but not just trehalose) at near 100 °C does result in the appearance of reduced protein as well as clear signs of protein denaturation (turbidity and precipitation). However, in the trehalose glass, denaturation is prevented. Thus, the glassy state is a key component in the mechanism.

The spectra of the Mb/Hb intermediate species suggest that the hemichrome may be a necessary intermediate in the thermal reduction pathway. The hemichrome was invoked as the facilitating intermediate for the rapid electrochemical reduction of metMb in a thin film deposited on an electrode.[21] The glassy matrix allows for the thermal generation of a hemichrome population while maintaining a compact tertiary conformation for the globin. In solution, thermal generation of the hemichrome typically leads to protein unfolding. The glassy matrix likely prevents the unfolding process from proceeding. Together these data indicate that trehalose glass facilitates the reduction process by providing a more efficient electron-transfer pathway than is available in solution both by facilitating the formation of a stable hemichrome at a temperature where glucose becomes a more potent reducing agent.

This reduction process has many potential biophysical and biomedical applications. Autoxidation is a serious problem that limits the storage time for acellular hemoglobin-based blood substitutes.[20] The present findings not only support the use of trehalose to slow autoxidation[23] and to limit oxidative damage in general[24] but also indicate that it can be used in a process to reverse autoxidation that has occurred following long periods of storage.

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Supporting Information Available: The description of the preparation of the glass (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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