Experimental Report

Proposal: **8-03-755** **Council:** 4/2012

Title: Structural characterization of thetwo T and R conformers of Carcinusaestuarii hemocyanin trapped in sol-gel matrix

This proposal is a new proposal

Researh Area: Biology

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Samples: hemocyanin

Instrument	Req. Days	All. Days	From	To
D11	0	1	12/11/2012	13/11/2012

Abstract:

This proposal is aimed to the structural characterization of hemocyanins, the oxygen transport proteins of mollusks and arthropods, in order to describe the conformational features that define their affinity for oxygen. Under anaerobic condition the protein exists as T-oxy (tense) state in the deoxygenated form, while under oxygen saturation as R-deoxy (relaxed) state. Neither T-oxy nor R-deoxy can be isolated in solution. Our experimental strategy is based on embedding R and T conformations within the pores of a sol-gel matrix where the reduced mobility of water strongly decreases the rate of the conformational shift that occurs during the oxygen saturation process. In previous SANS measurements within a BAG section (BAG-8-27) we have checked the adequacy of the experiment-targeted sample holders, the gel preparation and the optimum matching contrast conditions between solvent and sol-gel matrix. occurring at 72 mol % D2O. SANS data will be analyzed using a newly developed method that will allow the determination of the quaternary structure differences between the R and the T state.

Experimental Report

SANS experiments were performed at D11 from 12/11/2012 to 13/11/2012. Our aim was to characterize the structure of the different *Carcinus aestuarii* hemocyanin conformers entrapped into a sol-gel matrix, in order to validate the cooperativity model proposed in our laboratory: the three-state model. The structural effect of lactate, a positive allosteric effector of hemocyanin, was also evaluated.

According to the results obtained for undoped silica matrix (Ex N°: BAG-8-27), we performed SANS measurements of hemocyanin (~5mg/ml) entrapped into sol-gel matrix at the optimum deuteration grade of 0.720, using cuvettes of 2 mm thickness. A total of 8 samples plus buffers were measured. SANS spectra were collected for hemocyanin entrapped both in presence and absence of oxygen, and in oxygenated and deoxygenated conditions. The scattering signals from the different oxy- and deoxygenated conformers seem to confirm the presence of three distinct conformers (R-state: high affinity state, S-state: intermediate state and T-state: low affinity state) (**Fig. 1**), in agreement with our functional results. Moreover, at the lowest Q-values there is a negative correlation between the intensity of the scatter signals and the oxygen affinity of the conformers. Indeed, higher intensities are observed in the conformers characterized by lower oxygen affinities (S- and T-states) compared to the fully oxygenated R-state conformer.

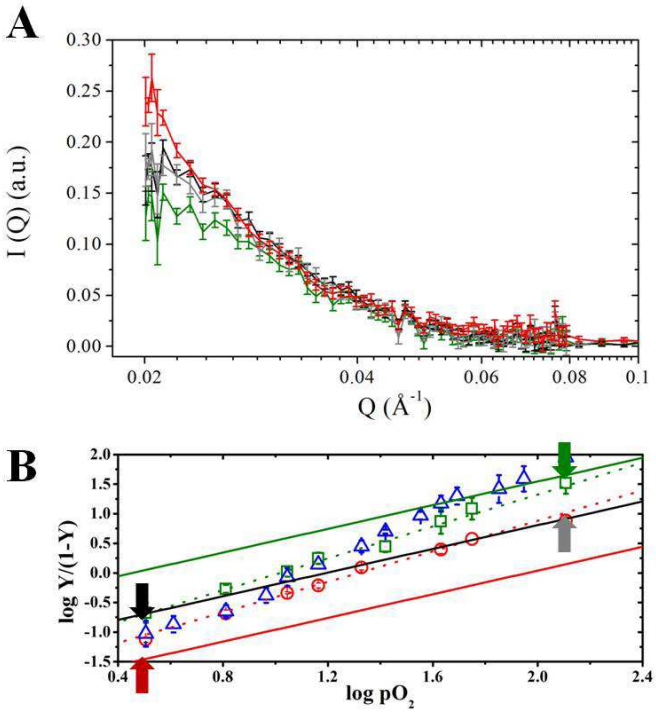

FIGURE 1. Semilogarithmic SANS patterns curves of C. *aestuarii* hemocyanin entrapped in presence or absence of oxygen, in oxygenated and deoxygenated conditions at pH 7.8 (**A**). For sake of clarity, the conditions at which the spectra were collected are indicated by arrows in the "three state" Hill-plot at pH 7.8 (**B**). *Red arrow*: T-state deoxygenated; *black arrow*: S-state deoxygenated; *grey arrow*: S-state oxygenated; *green arrow*: R-state oxygenated.

To deduce the structures of the entrapped conformers in terms of the most probable structure best fitted with SANS data, the spectra were analyzed by the QUAFIT method (Spinozzi & Beltramini, 2012), which has been already tested by studying the structure of the molluscan hemocyanin of *Octopus vulgaris* (Spinozzi et al., 2012). Each SANS spectra was analyzed by the program twenty times through a Montecarlo approach, resulting in twenty independent fits. The fitting procedure resulted in 2 x 6-meric hemocyanin structures characterized by different arrangements of the two hexamers, in terms of rotational orientation (α, β, γ) and distance between them (*d*). A

decrease of the interhexameric distance *d* was observed during the allosteric transition towards higher oxygen affinity-states.
To determine the structural effect of lactate on the quaternary structure of the different conformers, SANS spectra of R-state in presence of oxygen, S-state both in presence and absence of oxygen and T-state fully deoxygenated were collected in presence of 40mM of lactate (**Fig. 2**). S-state deoxygenated in presence of 40mM of lactate shows a scattering profile very different from the other spectra at Q <0.04 $Å^{-1}$. S- and R-state oxygenated spectra are almost superimposable, while small differences between these two conformers and the T-state deoxygenated are evidenced. The key result of the QUAFIT analysis is the lower interhexameric distance *d* of about 2-3Å of all the conformers in presence of lactate, although at the present degree of refinement of SANS data fitting the differences (no lactate versus lactate) are not statistically significant.

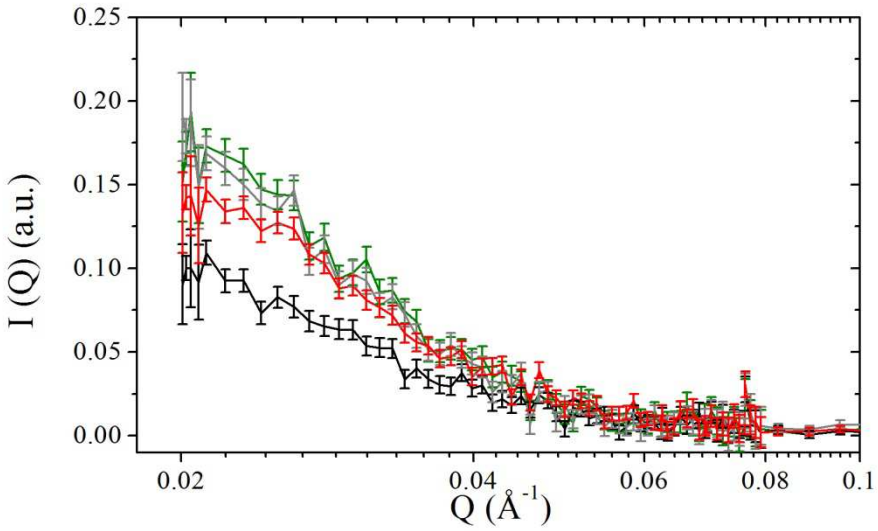

FIGURE 2. SANS patterns of different hemocyanin conformers in the presence of 40 mM lactate at pH 7.8. R-conformer in presence of oxygen (in *green*), S-conformer both in presence (in *gray*) and absence (in *black*) of oxygen and T- conformer fully deoxygenated (in *red*).

In conclusion, we correlated the functional properties of the distinct conformers of hemocyanin to their structural properties obtained through SANS measurements, allowing the definition of a structural model of cooperativity for the *Carcinus aestuarii* hemocyanin. Smaller interhexameric distances were observed either after the allosteric transition towards higher oxygen affinity conformational states, and after the binding of lactate.

1) F. Spinozzi, P. Mariani, I. Mičetić, C. Ferrero, D. Pontoni, and M. Beltramini. Quaternary structure heterogeneity of oligomeric proteins: a saxs and sans study of the dissociation products of octopus vulgaris hemocyanin. PLOS one, 7:e49644, 2012.

2) F. Spinozzi and M. Beltramini. Quafit: A novel method for the quaternary structure determination from small-angle scattering data. Biophys. J., 103:511–521, 2012.