THE REPLACEMENT OF CALCIUM BY TERBIUM AS AN ALLOSTERIC EFFECTOR OF HEMOCYANINS

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1. Introduction

The role of ligands, such as H^+, Ca^{2+} and Na^+ on the association–dissociation and oxygen binding properties of hemocyanins is well established [1,2]. In [3] we presented evidence that in the case of *Panulirus interruptus* hemocyanin these ions react with the macromolecule in a linked fashion exerting a control on its biological function. Since Ca^{2+} are silent to a great number of spectroscopic techniques, we have investigated the possibility to employ lanthanides as substitutes for Ca^{2+}. In this study we report:

(i) Fluorimetric measurements with *Panulirus* hemocyanin indicating that terbium (Tb^{3+}) competes with Ca^{2+} for the same sites;
(ii) Oxygen-binding experiments showing that Tb^{3+} has an effect on the oxygen-binding properties of this protein similar to that demonstrated for Ca^{2+} [3].

We conclude that Tb^{3+} may be used as a replacement species for Ca^{2+} in hemocyanin, thus providing direct information on the effects exerted by various ions on the control of the biological function of the macromolecule.

2. Materials and methods

Panulirus interruptus hemocyanin was prepared and stored as in [4]. The protein was dialyzed initially against 0.02 M Tris–HCl (pH 7.2) and 3 mM EDTA and subsequently against 0.05 M bis–Tris (pH 6.5). Apoprotein was prepared by dialysis against 0.1 M Tris–HCl (pH 7.6) and 10 mM KCN for 48 h at room temperature, followed by dialysis against 0.05 M bis–Tris (pH 6.5). Deoxygenated hemocyanin used for fluorescence measurements was prepared in a Thunberg tonometer by evacuation and flushing with argon. $TbCl_3$ was obtained from Koch-Light Labs Ltd and dissolved in 0.05 M bis–Tris (pH 6.5). For titration experiments successive amounts of a degassed stock solution of 10^{-2} M $TbCl_3$ or 10^{-1} M and 1 M $CaCl_2$ were added by means of a microsyringe. Above pH 7.2 $TbCl_3$ is less soluble through formation of hydroxide complexes. All experiments were made under conditions where the protein is in its aggregated state (M_w 450 000). The protein concentration is expressed in terms of oxygen binding sites/litre, taking 1 binding site/75 000 mol. wt [4]. Fluorescence measurements were made with a FICA 55L corrected spectrofluorimeter operated at 25°C. Oxygen-binding experiments at 20°C were performed essentially as in [5]. Stopped-flow experiments were carried out with a Gibson-Durrum stopped-flow apparatus equipped for fluorimetric measurements. The dead time of the apparatus is ~3.5 ms.

3. Results

3.1. *Binding of Tb^{3+} to* Panulirus *hemocyanin at pH 6.5*

The fluorescence spectrum of deoxygenated *Panulirus* hemocyanin excited at 285 nm in the absence and presence of Tb^{3+} is shown in fig.1. In

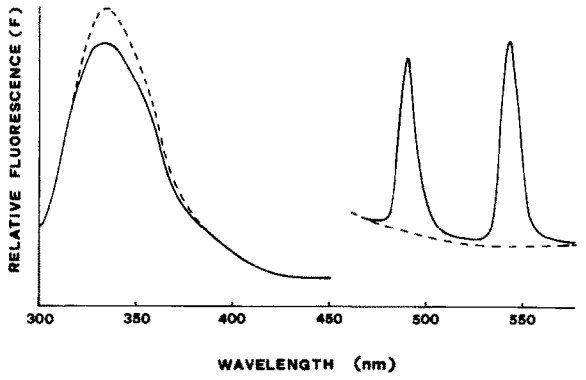

Fig.1. Fluorescence spectra of deoxygenated *Panulirus* hemocyanin in the absence (– – –) and presence (——) of 1.9 mM $TbCl_3$. Excitation wavelength 285 nm. Protein was 7.6×10^{-6} M in 0.05 M bis–Tris (pH 6.5). The sensitivity of emission intensity above 450 nm has been increased by a factor of 50. Upon addition of Tb^{3+} the sample is diluted by 22%.

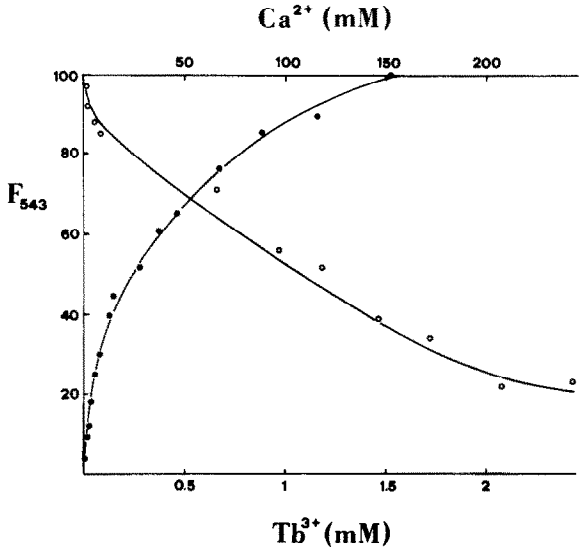

Fig.2. Tb^{3+} titration (•) and displacement by Ca^{2+} of Tb^{3+} bound to deoxygenated *Panulirus* hemocyanin (○). Protein was 7.6×10^{-6} M in 0.05 M bis–Tris (pH 6.5). Total amounts of added $CaCl_2$ and $TbCl_3$ are plotted versus the change in emission intensity as measured at 543 nm.

the absence of Tb^{3+} the tryptophan emission maximum is centered at 335 nm. Upon addition of Tb^{3+} three emission bands, due to bound Tb^{3+}, are observed at 490, 543 and 590 nm. The intensity of the emission band at the highest wavelength is very low and therefore not recorded in fig.1. The appearance of these emission bands is associated with an apparent decrease in the tryptophan emission at 335 nm; however after proper correction for dilution it becomes clear that the bound Tb^{3+} increases the fluorescence yield of tryptophan by ~8%.

Results of a titration experiment with progressive additions of Tb^{3+} to deoxygenated hemocyanin are shown in fig.2. The titration curve is somewhat heterogeneous with an overall Tb^{3+} K_d of 0.1–0.3 mM. for hemocyanin.

3.2. *Displacement by Ca^{2+} of Tb^{3+} bound to deoxygenated* Panulirus *hemocyanin at pH 6.5*

Addition of $CaCl_2$ at pH 6.5 to a solution of deoxygenated hemocyanin saturated with Tb^{3+} (1.9 mM) causes a decrease in fluorescence at 490 nm and 543 nm. The results of a Ca^{2+} titration experiment are also shown in fig.2. About 25% of the original Tb^{3+} signal, after proper correction for dilution, is still present when saturating amounts of Ca^{2+} were added.

3.3. *Effect of oxygen on the fluorescence of* Panulirus *hemocyanin in the presence of Tb^{3+} at pH 6.5*

Addition of oxygen to deoxygenated hemocyanin saturated with Tb^{3+} at pH 6.5 causes an ~80% decrease in fluorescence at 335 nm and a 65–70% decrease at 490 nm and 543 nm. It is well established that the formation of the copper–oxygen complex is associated with a large quenching on the tryptophan emission, through an energy-transfer mechanism [6]. However, we found that a similar decrease in fluorescence at 490 nm and 543 nm is observed when oxygen is added to a solution of apohemocyanin saturated with Tb^{3+}; in this case of course no change at 335 nm is measured. These experiments indicate that oxygen is an effective quencher of the emission of Tb^{3+} bound to *Panulirus* apo- or holo-hemocyanin.

3.4. *Kinetics of Tb^{3+} binding to deoxygenated* Panulirus *hemocyanin at pH 6.5*

Stopped-flow experiments in which deoxygenated hemocyanin was mixed with a deoxygenated $TbCl_3$

solution at pH 6.5 under saturating conditions, indicate that the reaction is completed within the dead time of the stopped-flow apparatus. A 4-fold dilution of the Tb^{3+} solution yields a decrease in the total fluorescence signal observable as expected from the fact that only partial saturation of the protein with Tb^{3+} was achieved. Moreover an additional decrease of the fluorescence signal was observed upon mixing a Tb^{3+} solution equilibrated with air with the protein. These experiments indicate that the combination of Tb^{3+} with hemocyanin is a fast process, with a time constant $>500\ s^{-1}$.

3.5. *Oxygen equilibrium of* Panulirus *hemocyanin in the absence and presence of terbium at pH 6.5*

Figure 3 shows Hill plots of oxygen binding curves of *Panulirus* hemocyanin in the absence and presence of Tb^{3+}. In the absence of Tb^{3+} the protein binds oxygen cooperatively with an Hill coefficient at 50% saturation of 1.5 and a $\log p_{½} = 1.0$. Addition of Tb^{3+} causes an increase in cooperativity ($n_H = 1.7$) and a decrease in oxygen affinity ($\log p_{½} = 1.2$). Tb^{3+} affects primarily the position of the lower (T-state) asymptote, while the upper (R-state) asymptote does not seem to be affected. Similar behaviour was observed [3] when small amounts of Ca^{2+} ($\leqslant 0.1$ mM) were added to the protein.

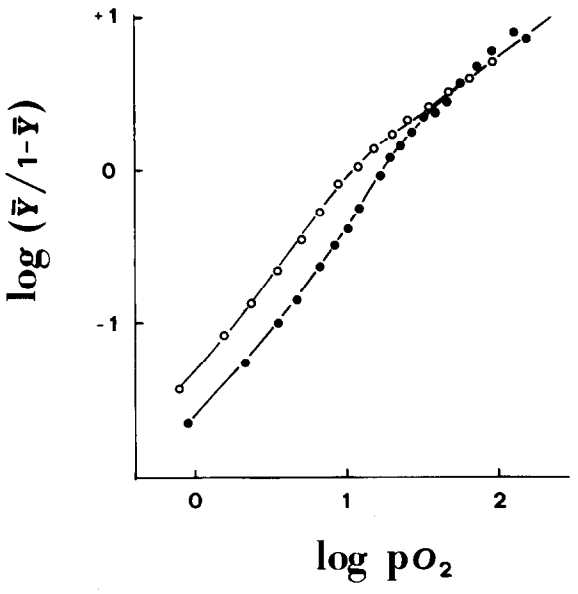

Fig.3. Hill plots of oxygen binding curves of *Panulirus* hemocyanin in the absence (○) and presence (●) of 2 mM $TbCl_3$. Protein was 58×10^{-6} M in 0.05 M bis–Tris (pH 6.5).

4. Discussion

The results presented here suggest strongly that Tb^{3+} may be used as a proper replacement species for Ca^{2+} in *Panulirus* hemocyanin; this may enable us to get valuable information on the nature of the binding site(s) and the relationships with other ligand-binding sites which are involved in the control of the biological function of the protein.

Three arguments support the statement that Ca^{2+} and Tb^{3+} bind to the same allosteric site(s) of the protein and thus are interchangeable:

(i) Fluorescence measurements indicate that Tb^{3+} has an overall affinity for deoxy *Panulirus* hemocyanin which is comparable with that found for Ca^{2+} by measurements with a Ca^{2+}-selective electrode [3]. Precaution must be taken in so far as Tb^{3+} emission only monitors those ions which have the correct stereochemistry to accept energy from the protein aromatic side chains. Therefore no conclusion can be drawn about the homogeneity of these sites.

(ii) The displacement of Tb^{3+} by Ca^{2+} is a direct proof of competition between these ions for at least a certain set of binding sites. However since addition of Ca^{2+} in saturating amounts, leaves ~25% of the original Tb^{3+} signal, it may be concluded that Tb^{3+} binds also to sites which are unaccessible to Ca^{2+}. Furthermore, calculation of the Ca^{2+} K_d, assuming competition with Tb^{3+} for the same site(s), yields a value which is much higher than that found with the Ca^{2+}-selective electrode (11 mM calculated from the fluorescence experiments, 0.05 mM calculated from Ca^{2+}-binding experiments [3]). This indicates that competition of these ions may not be a simple mechanism.

(iii) A third indication that Tb^{3+} can replace Ca^{2+} in *Panulirus* hemocyanin is the fact that the oxygen binding properties are affected in a way similar to that observed for Ca^{2+} [3]. Both the $p_{½}$ and the n_H are altered through a specific effect of Tb^{3+} on the lower (T-state) asymptote.

The linkage between Tb^{3+} and oxygen-binding sites is not accessible to direct fluorescence experiments, since oxygen appears to be an effective quencher of the Tb^{3+} emission. In the holoprotein oxygen quenches to a great extent both the tryptophan emission and the Tb^{3+} emission. Since an effect of oxygen was observed also with the apoprotein saturated with Tb^{3+}, the quenching of Tb^{3+} emission should not be attributed to the oxygenation of the copper site. Reports on oxygen sensitivity of lanthanide ion emission are scarce. An oxygen-sensitive emission of Eu^{3+} in the presence of tryptophan has been reported [7].

Since similar effects are observed in *Helix pomatia* α-hemocyanin, we may conclude that for both mollusc and arthropod hemocyanins Tb^{3+} may be used as a proper replacement ion for Ca^{2+}. The use of these lanthanides may enable us to study in greater detail the nature of the allosteric binding sites in hemocyanins and especially the interactions with other binding sites which control the biological function of these large macromolecules.

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