Conformational changes induced by polyanions in haemoglobin from *Camelus dromedarius*

Studies on the ferric derivatives

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The effect of inositol hexakisphosphate on the redox equilibria and on the c.d. spectra of ferric derivatives of haemoglobin from *Camelus dromedarius* has shown that: (i) two distinct functionally relevant binding sites for polyanions are present on the protein; (ii) conformational changes promoted by inositol hexakisphosphate are largely dependent on spin state of the iron; (iii) tertiary and quaternary changes are not necessarily linked; (iv) structures induced by polyanions can be mixed forms that are neither T-state nor R-state.

INTRODUCTION

Previous studies (Amiconi *et al.*, 1985) on O₂ binding by haemoglobin from *Camelus dromedarius* [whose amino acid sequence reveals the presence of all the amino acid residues considered relevant for the functional properties of human haemoglobin (Braunitzer *et al.*, 1979)] have shown the existence of two distinct binding sites for polyphosphates, which modulate, as a function of their concentration, the conformation of the tetrameric molecules. In addition, an investigation on the oxidation–reduction equilibrium of this protein has suggested the existence of intermediate conformers (R. Santucci, F. Ascoli, G. Amiconi & M. Brunori, unpublished work), neither R-state nor T-state in the Monod–Wyman–Changeux terminology (Monod *et al.*, 1965), that could affect the functional properties of this haemoglobin. Independent evidence for the existence of mixed forms has also been reported for the oxy derivative (Santucci *et al.*, 1985).

It is clear from available information (Baldwin & Clothia, 1979) that: (i) on ligand binding the subunits of haemoglobin move relative to each other (quaternary change) and more localized atomic displacements occur within each subunit (tertiary change); (ii) the coupling between these two types of structural changes is an essential factor in the co-operative mechanism of haemoglobin. Since the co-operativity in the redox reaction varies with solvent composition (e.g. pH), it was decided to investigate with dromedary haemoglobin both the effect of inositol hexakisphosphate on the Hill coefficient in the redox equilibrium and the role played by polyphosphates in modulating the structure (i.e. the conformational states) of the protein.

The results have indicated that: (i) the conformational changes in ferric haemoglobin promoted by inositol hexakisphosphate are largely dependent on spin state; (ii) the structures acquired in the presence of inositol hexakisphosphate do not coincide completely with the typical T-state or R-state, but are essentially mixed

forms; (iii) the inter-relationships between tertiary and quaternary changes are complex.

MATERIALS AND METHODS

Specimens of dromedary blood were collected from the jugular veins of animals in acid/citrate/glucose. Haemoglobin was purified as previously described (Amiconi *et al.*, 1985).

The ferric derivative was obtained by oxidation of the ferrous protein with K₃Fe(CN)₆ in a protein/oxidant molar ratio of 1:2 in buffered solution at neutral pH. The oxidant in excess and its reduction product were eliminated by extensive dialysis against the appropriate buffer.

The protein concentration was determined from the optical absorption on the basis of the following absorption coefficients: $\epsilon_{577} = 14600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the oxygenated derivative, $\epsilon_{500} = 10000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the aquomet ferric derivative (pH 6.5) and $\epsilon_{540} = 11000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the hydroxymet ferric derivative (pH 9.3).

Toluyene Blue, the mediator used in the potentiometric titrations, was obtained from ICN Pharmaceuticals Co.; inositol hexakisphosphate was purchased from Sigma Chemical Co.; all the other reagents were of analytical grade and used without further purification.

The redox titrations were carried out in a jacketed glass cell, at $25 \pm 0.02^\circ \text{C}$, equipped with a Radiometer K401 calomel reference electrode and a Radiometer P101 platinum measuring electrode according to a procedure previously described (see Santucci *et al.*, 1984). A Radiometer model 62 pH-meter/potentiometer was used for the potential measurements.

The measured electrode potentials were referred to the normal hydrogen electrode after the convention of Clark (1960). The oxidation–reduction potential at 50% of oxidation gave the mid-point potential E_h .

C.d. spectra were recorded with a Jasco J-500 A

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spectropolarimeter equipped with a Jasco DP-500 N processor. The molar ellipticity ($\text{degrees} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$) is expressed as $[\theta]_M$ on a molar haem basis.

A Cary 219 or a Beckman DU-7 spectrophotometer was used for all optical measurements.

RESULTS AND DISCUSSION

The effect of inositol hexakisphosphate on the oxidation–reduction equilibrium of human haemoglobin (Kilmartin, 1973) is characterized by a reduction to unity or below of the degree of co-operativity (n), which is directly related to the steepness of the redox curves (Antonini *et al.*, 1964; Santucci *et al.*, 1984). According to the explanation advanced by Perutz *et al.* (1974), the diminished co-operativity induced by the polyanion results from the stabilization of the ferric derivative in the T-state (the conformation typically associated with deoxyhaemoglobin). This view is part of a general hypothesis (Perutz *et al.*, 1978) that relates the high spin of certain of ferric haemoglobin with a tendency to assume the T-state. In order to evaluate the validity of this model, i.e. the influence of the equilibrium of the extreme conformations T and R on the degree of co-operativity, the values of the midpoint potential of dromedary haemoglobin were determined at pH 6.5 by potentiometric titrations in the absence and in the presence of inositol hexakisphosphate (Fig. 1). As previously observed for the oxygenation reaction (Amiconi *et al.*, 1985), dromedary haemoglobin clearly reveals in the redox process the existence of two distinct

Fig. 1. Effect of inositol hexakisphosphate concentration on the redox potential (E_h) and co-operativity (n) of dromedary haemoglobin (0.2 mM in haem) at pH 6.5

Measurements were made in 0.1 M-Bistris/HCl buffer containing 0.1 M-NaCl. The temperature was 25 °C. The rightmost points (S) refer to 'stripped' haemoglobin.

functionally relevant binding sites for polyanions. As expected from the picture, identical in all essentials, of redox and oxygenation processes (Wyman & Ingalls, 1941), saturation of the higher-affinity site, which stabilizes the T-state forms and thus the ferrous deoxy state of the protein, increases the value of the midpoint potential; then, the progressive saturation of the lower-affinity site returns the redox potential of the protein (thus stabilizing the R-state forms) to about its starting value. The degree of co-operativity is also affected by inositol hexakisphosphate, but the n value never reaches unity (Fig. 1), contrary to what was observed with human haemoglobin (Kilmartin, 1973).

In order to investigate in more detail the structural basis of such functional effects, the c.d. spectra of dromedary haemoglobin, in solvents of different compositions, were determined. In Fig. 2 the near-u.v. c.d. spectra of the ferric dromedary and human haemoglobins (the latter included for comparison) in the aquomet (Fig. 2a) and hydroxymet (Fig. 2b) forms are shown. The pH values (6.5 and 9.3) were chosen to explore the spectroscopic behaviour of the extreme states, given that the midpoint of transition between the two forms is pH 8.1 (R. Santucci, F. Ascoli, G. Amiconi & M. Brunori, unpublished work). The overall features of the spectra are similar for each derivative of the two proteins, except for the larger intensity of the positive dichroic band of dromedary protein in the 280–290 nm region; this evidence was found previously for the ferrous derivatives and attributed to the lack of Trp- α 14(A12) in dromedary haemoglobin (Santucci *et al.*, 1985).

It is known (Li & Johnson, 1969) that the intensity of the band at 260–270 nm depends strongly on the spin state of the ferric iron in haemoglobin, being higher in the low-spin derivatives; accordingly, the band magnitude of the aquomet derivative (which is a high-spin species) is lower in both proteins (Fig. 2a). However, some differences can be observed between the two haemoglobins, attributable to differences in the tertiary conformation. This is

Fig. 2. Near-u.v. c.d. spectra of dromedary (—) and human (---) ferric haemoglobin at pH 6.5 (a) and pH 9.3 (b)

Measurements were made in 0.1 M-Bistris/HCl or -Tris/HCl buffer containing 0.1 M-NaCl. The temperature was 25 °C.

particularly evident in the spectra of the hydroxymet derivative (Fig. 2b, i.e. at alkaline pH); thus, whereas in dromedary haemoglobin one main band located at 264 nm is present, the human protein shows two well distinct c.d. bands at 259 and 264 nm, with about the same intensity. On the basis of previous results (Santucci *et al.*, 1985), these optical properties can be assigned to the presence, at alkaline pH, of a higher percentage of a T-like tertiary conformation for the same derivative of human haemoglobin. Such conclusion is in line with the lower degree of co-operativity observed in the redox process of dromedary haemoglobin ($n = 1.7$) with respect to the human protein ($n = 2.5$) at high pH values, which has been interpreted as being due to the presence of an intermediate conformation (neither R-state nor T-state) in the ferric protein (R. Santucci, F. Ascoli, G. Amiconi & M. Brunori, unpublished work). Thus it appears that the T-like structure of the subunits, present at alkaline pH in the ferric dromedary protein, plays a role in the regulation of the functional properties of the protein that is not secondary.

The spectral changes induced by inositol hexakisphosphate on the c.d. bands of ferric dromedary haemoglobin in the aromatic region (250–300 nm), at both acid and alkaline pH, are shown in Figs. 3(a) and 3(b). At pH 6.5, the presence of the organic phosphate induces conformational changes in the quaternary structure of aquomet derivative, as revealed by changes in the dichroic band at 287 nm, associated by Perutz *et al.* (1974) to changes at Trp- β 37(C3) located at the α_1 - β_2 interface. Low concentrations of the salt (up to 1:1 molar ratio salt/tetramer) decrease the intensity of this band, thus pushing the tetramer towards its quaternary conformational T-state; in contrast, higher concentrations of the salt induce c.d. changes in the opposite direction, and the

protein re-acquires its initial conformation almost completely. On the other hand, the tertiary structure of the protein appears to be slightly affected (towards the intrachain T-like form) by inositol hexakisphosphate; this is evident by comparing the intensity of the 250–270 nm c.d. spectrum of the ferric protein at pH 6.5 (Fig. 3a) and those of cyanomet and fluoromet derivatives in 1 mM-inositol hexakisphosphate (Fig. 4), taken to be in R-state and T-state respectively (see also Perutz *et al.*, 1974). These results provide independent evidence, in addition to the functional properties (see Fig. 1), of the existence on the ferric derivative of two distinct binding sites for polyanions, as already demonstrated for the reduced forms (Santucci *et al.*, 1985); however, whereas the stronger binding site present in the protein reveals an affinity for polyanion comparable in both the reduced and oxidized forms, the weaker site has a much lower affinity for inositol hexakisphosphate when the protein is in ferric state. A lack of protein-salt interaction is in fact observable for the ferric derivative in the inositol hexakisphosphate concentration range between 400 μ M and 50 μ M, just where dromedary oxyhaemoglobin strongly binds the salt (Santucci *et al.*, 1985). Under the experimental conditions used, the dependence of the intensity of the dichroic band on inositol hexakisphosphate concentration may be fitted to a simple titration curve having a mid-point equal to 5 mM in terms of salt concentration (see Fig. 3a inset).

The effect of inositol hexakisphosphate on the hydroxymet derivative of dromedary haemoglobin (pH 9.3) is shown in Fig. 3(b). As expected, no influence of the salt on the c.d. spectrum is observed up to a concentration of 1 mM, in that the protein-salt affinity is very low at alkaline pH. However, in the presence of higher concentration of polyanion (≥ 1 mM) a lowering

Fig. 3. Effect of inositol hexakisphosphate concentration on the near-u.v. c.d. spectra of dromedary ferric haemoglobin (0.2 mM in haem) at pH 6.5 (a) and pH 9.3 (b)

The concentrations of inositol hexakisphosphate were: in (a) —, none; ----, 50 μ M and 400 μ M; ····, 1.5 mM; - - - - - , 8 mM; in (b) —, none; ····, 1 mM; ----, 10 mM. Measurements were made in 0.1 M-Bistris/HCl or -Tris/HCl buffer containing 0.1 M-NaCl. The temperature was 25 °C. In the inset to (a) the change in intensity of the ferric dromedary haemoglobin dichroic band located at 284 nm (●) is plotted as a function of inositol hexakisphosphate (IHP) concentration at pH 6.5. The experimental values may be fitted with a simple conformational transition having mid-point at 50 mM-salt concentration (continuous line) induced by the titration of the weaker polyanion-binding site on the protein.

in intensity of the dichroic band at 264 nm is evident, although the 280–290 nm region is not affected. This finding indicates that under these conditions the T-like state of subunits of the haemoglobin is stabilized. The intensity of the band at 264 nm of the hydroxymet derivative in the absence of inositol hexakisphosphate is in fact higher than that of molecules in typical T-state [such as the deoxy derivative (Santucci *et al.*, 1985) and fluoromethaemoglobin at pH 6.5 in the presence of 1 mM-salt, shown in Fig. 4(b)], but lower than that of the protein in the R-state [such as the oxy derivative and cyanomethaemoglobin, shown in Fig. 4(a)]. Moreover, the shape of the c.d. spectrum of the hydroxymet derivative resembles that of molecules characterized by a T-state conformation. On the basis of these considerations, it becomes plausible that in the hydroxymet derivative the lowering observed in the intensity of the dichroic band at 264 nm upon addition of inositol hexakisphosphate is strictly related to a conformational transition of the protein towards a T-like state of subunits, whereas the quaternary structure does not seem to be involved. The absence of tertiary and quaternary effects by addition of inositol hexakisphosphate (50 μ M to 8 mM) to the cyanomet derivative of dromedary haemoglobin has already been observed with the human protein (Perutz *et al.*, 1974); this behaviour may find its explanation in a greater rigidity of the protein moiety of the molecule, or in the absence of interaction with inositol hexakisphosphate at the concentrations used. As far as the fluoromet derivative is concerned, the different influences of polyanion on dromedary and human haemoglobins at the level of tertiary structures are relevant. Thus the peak at 264 nm increases in the human protein on the addition of inositol hexakisphosphate, suggesting a stabilization of

the intrachain R-like form, or possibly a rupture of the bond of iron to histidine (Fermi & Perutz, 1977). On the other hand, dromedary haemoglobin shows a decrease in the same band in the presence of polyanion, indicating a tendency towards a T-like form of subunits; at 1 mM concentration of inositol hexakisphosphate, the c.d. spectrum of the fluoromet derivative of dromedary haemoglobin in fact appears to be superimposable on that of the deoxy derivative (see Santucci *et al.*, 1985).

CONCLUSIONS

The results reported suggest that there is a multiple number of ways in which conformational changes can occur in dromedary haemoglobin. In other words, addition of a high-affinity ligand to the protein, such as inositol hexakisphosphate, always produces changes in the protein moiety of haemoglobin, which, however, cannot be described as a globule of 'plastic material' capable of flowing under an applied stress.

In particular: (i) the inter-relationships between tertiary changes and quaternary transitions are complex; on the basis of the c.d. data alone, it is possible to observe tertiary changes in the almost complete absence of quaternary modifications [e.g., by interaction of inositol hexakisphosphate with the oxy derivative (Santucci *et al.*, 1985)] and quaternary changes associated with small tertiary variations (e.g., by binding of inositol hexakisphosphate to aquomethaemoglobin); (ii) the electronic state of iron is in some way transmitted to the protein; thus, in the case of low-spin compounds (such as the oxy derivative), inositol hexakisphosphate is not able to shift sufficiently the molecular population towards the T-state, whereas inter-subunit surfaces appear to be very sensitive in the high-spin species (such as the fluoromet and aquomet derivatives); (iii) the change in co-operativity in the electron-transfer reaction of dromedary haemoglobin appears to be associated with both stabilization of the T-state and the existence of intermediate tertiary conformations (neither R-like nor T-like); this view agrees with the hypothesis advanced by Perutz *et al.* (1978) in that the variation of n can be adequately accounted for in terms of essentially two alternative quaternary structures, whose constituent subunits can assume different conformations depending on the experimental conditions.

Fig. 4. Near-u.v. c.d. spectra of cyanomet (a) and fluoromet (b) derivatives of dromedary haemoglobin at pH 6.5, in the absence (—) and in the presence (---) of 1 mM-inositol hexakisphosphate

Measurements were made in 0.1 M-Bistris/HCl buffer containing 0.1 M-NaCl (a) or -NaF (b). The temperature was 25 °C.

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