Conformational changes induced by polyanions in haemoglobin from the dromedary (*Camelus dromedarius*)

Role of chloride ions

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The role of chloride ions in modulating polyanion-induced conformational changes in haemoglobin from the dromedary (*Camelus dromedarius*) has been investigated. The results obtained have shown that: (i) in the ferric derivative at pH 6.5 the effect of single polyanion (dextran sulphate and inositol hexakisphosphate) on the conformation is essentially local, thus involving only the tertiary structure of the protein; (ii) the presence of chloride ions at a concentration close to the physiological value (i.e. 150 mM) is essential to induce quaternary conformational changes in the polyanion–ferric protein system; (iii) comparison between structural and functional data correlates polyanion-induced tertiary conformational changes with changes in the value of midpoint potential, E'_0 , and quaternary changes with co-operativity.

INTRODUCTION

The allosteric properties of macromolecules are generally attributed to ligand-promoted conformational changes whereby the oligomeric proteins are converted from a constrained or low-affinity state (the so-called 'T-state') into a relaxed conformation ('R-state') having a higher affinity for substrates. How the effect of ligand binding at one site is propagated throughout the molecule is still the subject of much investigation. In fact, it is difficult to distinguish experimentally between direct (i.e. local) effects and indirect (i.e. propagated) changes at a remote position. Haemoglobin from the dromedary (*Camelus dromedarius*) [an $\alpha_2\beta_2$ tetramer having in its sequence all the amino acids considered relevant for the functional properties of human haemoglobin (Braunitzer *et al.*, 1979)] appears to be a good system for the investigation of the linkage between ligand-induced tertiary structural changes and the relative stability of quaternary conformations. Recent studies have revealed the existence of two distinct binding sites for polyanions, which modulate the structural and functional properties of this protein (Amiconi *et al.*, 1985; Santucci *et al.*, 1985). Moreover, investigations on the ferric derivatives have suggested the existence of mixed forms (neither T-state nor R-state) induced by the binding of polyanions (Santucci *et al.*, 1986a). Therefore, in order to investigate the detailed relationship between the structural flexibility (and in particular the nature of the coupling between tertiary and quaternary conformational changes) and the functional properties of dromedary haemoglobin, c.d. studies of the ferrous and ferric derivatives together with redox equilibrium measurements have been undertaken both in the absence and in the presence of various allosteric effectors, such as inositol hexakisphosphate, dextran sulphate and chloride ions.

The results have indicated that, in ferric dromedary haemoglobin: (i) the effect of single polyanion on the conformation appears to be essentially local, i.e. only involving the tertiary structure of the protein; (ii) the presence of chloride ions is essential to induce quaternary

changes in the polyanion–protein system and hence to affect the equilibrium between the main stable conformations (T-state and R-state).

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 adding $K_3Fe(CN)_6$ to the ferrous protein in a molar ratio of 2:1 (oxidant/protein) in a buffered solution at neutral pH. The oxidant in excess was eliminated by extensive dialysis against the appropriate buffer.

Haemoglobin concentration was determined on the basis of ϵ 14.6 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 577 nm for the oxygenated derivative and ϵ 10.0 $\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 500 nm for the ferric derivative at pH 6.5.

Inositol hexakisphosphate (Sigma) and dextran sulphate (Gallard–Schlesinger Chemical Co., Carle Place, NY, U.S.A.; average M_r 8000; 2.3 mmol of sulphate groups/g; 5% of $\alpha 1 \rightarrow 4$ linkages) were of analytical grade and used without further purification.

Toluyene Blue, the mediator used in the potentiometric titrations, was purchased from ICN Pharmaceuticals Co.

All the experiments were performed in 20 mM-Bistris/HCl, pH 6.5.

C.d. spectra were recorded with a Jasco J-500 A spectropolarimeter equipped with a Jasco DP-500 N processor. The molar ellipticity ($[\theta]_M$ on a haem basis; in the spectral range studied (250–300 nm), the 'noise' level was always lower than 0.3 millidegree.

The redox titrations were carried out in a jacketed glass cell, at $25 \pm 0.02^\circ\text{C}$, equipped with a Radiometer P101 platinum measuring electrode and a Radiometer K401 calomel reference electrode according to a procedure previously described (Santucci *et al.*, 1984).

Fig. 1. Effect of dextran sulphate on the near-u.v. c.d. spectra of dromedary ferric haemoglobin (0.2 mM in haem) at pH 6.5 in the absence (a) and in the presence (b) of 0.15 M-NaCl

The concentrations of dextran sulphate were: in (a) —, none; ----, 10 μ M; in (b) —, none; ----, 10 μ M; ····, 0.6 mM. The measurements were made in 20 mM-Bistris/HCl. The temperature was 25 °C.

A Radiometer model 62 pH-meter/potentiometer was used for the potentiometric measurements.

The measured potentials were referred to the standard hydrogen electrode after the convention of Clark (1960). The oxidation-reduction potential at 50% of oxidation gave the midpoint potential, E'_0 .

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

RESULTS AND DISCUSSION

In a previous paper (Santucci *et al.*, 1986a) it was reported that indices of conformation showed some change upon addition of inositol hexakisphosphate to dromedary ferric haemoglobin but not to its deoxy derivative. In order to shed more light on these features, the structural and functional effect of chain-like flexible polyanions such as dextran sulphate has been determined. The charged polysaccharide was shown (Amiconi *et al.*, 1977; Sacco & Dellacherie, 1986) to interact strongly with human deoxy- and oxy-haemoglobin, affecting both structural and functional properties.

The c.d. study on the ferrous derivatives of the protein at pH 6.5 in the wavelength range 250–300 nm (results not shown) has shown that the spectrum of the deoxy derivative is not affected by the presence of dextran sulphate, whereas the dichroic bands of the oxy derivative are altered similarly to what has been found with inositol hexakisphosphate (Santucci *et al.*, 1985).

These results indicate a phenomenological behaviour induced by polysulphates that is identical with that produced by inositol hexakisphosphate. However, since

combination of inositol hexakisphosphate with ferric dromedary haemoglobin at acid pH (i.e. pH 6.5) produces important quaternary changes in the protein (as revealed by the c.d. spectra) under physiological conditions of chloride ion concentration (i.e. 0.1 M), parallel experiments with dextran sulphate appeared to be relevant; in fact, the length and flexibility of the chain of polysulphate as well as the regular distribution of the negative charges along its structure could offer other binding possibilities with the ferric derivative. Fig. 1(a) shows the c.d. spectrum of the dromedary ferric derivative at pH 6.5 (20 mM-Bistris/HCl buffer, i.e. 20 mM in terms of chloride ions) in the absence and in the presence of dextran sulphate. The chain-like polyanion significantly affects the dichroic bands located at 250–270 nm, whereas no relevant changes are observable in the 280–300 nm region. On the basis of previous studies (Perutz *et al.*, 1974), such a negligible effect in the 280–300 nm region on the c.d. spectrum indicates that the contact region $\alpha_2\beta_2$ of the tetramer is only slightly affected by the presence of the polysulphate; in other words, the overall quaternary structure of the protein appears to be maintained. In contrast, the c.d. changes at 250–275 nm indicate that the binding of dextran sulphate to the ferric derivative produces local (tertiary) changes in the protein (Santucci *et al.*, 1986a). C.d. spectra were obtained in the presence of dextran sulphate concentrations up to 10 mM; at higher concentrations of salt, measurements could not be made because of precipitation phenomena. In order to avoid these, the chloride ion concentration was increased. Fig. 1(b) shows the c.d. spectrum of the same system reported

Fig. 2. Effect of inositol hexakisphosphate on the near-u.v. c.d. spectra of dromedary ferric haemoglobin (0.2 mM in haem) at pH 6.5 in the absence (a) and in the presence of 0.15 M-NaCl (b)

The concentrations of the salt were: —, none; ----, 0.2 mM. The measurements were made in 20 mM-Bistris/HCl. The temperature was 25 °C.

in Fig. 1(a), but in the presence of 0.15 M-chloride ions, which is close to the physiological concentration. Under these experimental conditions, changes in the c.d. bands induced by dextran sulphate are observable in the whole region between 250 and 300 nm, and, in particular, the lowering of the dichroic band at 287 nm clearly reveals a quaternary conformational change occurring in the protein (Perutz *et al.*, 1974). Thus this phenomenon, i.e.

the coupling between tertiary and quaternary conformational changes, appears to be associated with the simultaneous presence of both polysulphate and chloride ions (the latter at a physiological concentration). The effect of dextran sulphate on dromedary ferric haemoglobin strictly resembles that already observed with inositol hexakisphosphate under the same experimental conditions (Santucci *et al.*, 1986a). In fact, a low concentration of polysulphate (salt/tetramer, 1:5 molar ratio) decreases the intensity of the band at 287 nm [associated with Trp- β 37 (C_3) located at the $\alpha_1\beta_2$ interface (Perutz *et al.*, 1974; Santucci *et al.*, 1985)], thus pushing the tetramer towards the T-state quaternary conformation. The maximal c.d. change in this direction is produced by an apparent stoichiometry much less than 1:1 (in terms of molar species); this means that each molecule of dextran sulphate (which has about ten sulphated extremities) interacts with more than two haemoglobin tetramers, as already observed by Sacco & Dellacherie (1986). On the other hand, when the concentration of dextran sulphate is increased, dichroic changes in the opposite direction are observed and the protein appears to reacquire its initial quaternary conformation almost completely. Therefore, in view of the double character of the effect of dextran sulphate, in dromedary haemoglobin there must be at least two binding sites with different affinity constants for polysulphate: one that stabilizes the T-state conformations and the other stabilizing preferentially the R-state forms (see Santucci *et al.*, 1986a). The similarity of structural effects (as measured by c.d. spectra) induced in ferric dromedary haemoglobin by polysulphate and polyphosphate suggests that the main sites of interaction on the protein are probably the same for both polyanions, a finding similar to that observed for human haemoglobin (Sacco & Dellacherie, 1986).

Because of these results, a c.d. study of the interaction between the ferric dromedary haemoglobin and inositol hexakisphosphate at low chloride concentration and acid

Fig. 3. Oxidation-reduction equilibrium curves of dromedary haemoglobin in the absence and in the presence of inositol hexakisphosphate (a) and dextran sulphate (b), at pH 6.5

(a): ●, No inositol hexakisphosphate; △, 0.2 mM-polyphosphate and 0.15 M-NaCl; ■, 0.2 mM-polyphosphate. (b): ●, No dextran sulphate; △, 10 μM-dextran sulphate and 0.15 M-NaCl; ■, 10 μM-dextran sulphate. Measurements were made in 20 mM-Bistris/HCl. The temperature was 25 °C.

Table 1. Influence of different allosteric effectors on the redox parameters of haemoglobin from *Camelus dromedarius*

The protein concentration was 50 μ M in tetramer; the buffer was 20 mM-Bistris/HCl, pH 6.5. The temperature was 25 °C. The value of E'_0 (midpoint potential) are referred to a standard hydrogen electrode.

Effector	E'_0 (mV)	n
—	+175	1.3
0.15 M-Chloride	+175	1.3
10 μ M-Dextran sulphate	+206	1.3
0.2 mM-Inositol hexakisphosphate	+194	1.3
0.15 M-Chloride + 10 μ M-dextran sulphate	+190	1.0
0.15 M-Chloride + 0.2 mM-inositol hexakisphosphate	+185	1.1

pH (i.e. pH 6.5) has been performed in order to clarify the possible role played by chloride ions in modulating the quaternary changes occurring in this protein. The results are shown in Fig. 2. At low concentration of chloride ions (Fig. 2a), inositol hexakisphosphate slightly affects the spectral region 250–300 nm. On the other hand, in the presence of 0.15 M-chloride (Fig. 2b), dichroic changes are mainly observable in the 280–300 nm range, similar to those found with dextran sulphate. Thus chloride ions appear to be essential for quaternary conformational changes in ferric dromedary haemoglobin, in contrast with what is observed in human haemoglobin, where inositol hexakisphosphate produces quaternary transitions also in the absence of appreciable amounts of chloride ions (R. Santucci, unpublished work). As a whole, the data reported above give strong evidence for the importance of a simultaneous presence of polyanions and chloride ions (the latter in a concentration close to physiological, namely 0.1–0.2 M) to obtain propagation of the tertiary conformational changes promoted by polyanions to the other subunits in ferric dromedary haemoglobin.

The structural results reported above indicate that it is possible to discriminate between local intrasubunit conformational modifications and quaternary changes. This fact offers a means of investigating the relative role of these two aspects of protein dynamics in determining the co-operative effects in the haemoglobin–ligand equilibrium. Thus a potentiometric study of the redox properties of dromedary haemoglobin has been performed to correlate the observed structural features with functional properties of the protein. Fig. 3 shows the effect of inositol hexakisphosphate (Fig. 3a) and dextran sulphate (Fig. 3b) on potentiometric titration curves of the redox process of the protein in the presence of 20 mM- or 150 mM-chloride ions. In 20 mM-Bistris/HCl buffer, polyanions shift the redox titration curve of the protein towards higher values of potential without affecting the slope. On the other hand, the simultaneous presence in solution of polyanion and a high concentration (150 mM) of chloride gives rise to a redox titration curve intermediate between that in buffer alone and that in

polyanion at low chloride concentration; this is to be expected, since this curve has to approach that obtained in buffer alone (lower value of E'_0) and that in polyanion (higher value of E'_0). Therefore the slope of the actual experimental curve in both chloride and polyanion will be the lower the larger is the distance between the extreme curves (i.e. in buffer and in polyanion alone). It must be recalled that the redox curve in the presence of 150 mM-chloride ions is superimposable on that obtained in 20 mM-Bistris/HCl buffer (Santucci *et al.*, 1986b). The values of midpoint potential, E'_0 , and degree of co-operativity, n , obtained in the different experimental conditions are reported in Table 1. Evidence here reported also supports the proposal (Kilmartin, 1973) of the relevant role played by quaternary conformation transition from T-state to R-state in controlling the shape (i.e. the slope) of the redox equilibrium curves.

On the basis of these functional effects, it is evident that dextran sulphate globally forms a stronger interaction with ferric dromedary haemoglobin if compared with inositol hexakisphosphate. Thus these results are in line with those obtained by Sacco & Dellacherie (1986) and suggest that, in addition to the main binding sites (common with inositol hexakisphosphate), polysulphate forms additional interactions with the protein, due to its molecular nature (i.e. flexibility, length, regular distribution of negative charges along the chain).

In conclusion, for dromedary haemoglobin, comparison between structural and functional data correlates tertiary conformational changes induced by polyanions with changes in the value of the midpoint potential, E'_0 , and quaternary changes to co-operativity. Moreover, polyanions do not significantly affect the occupancy of T-state by methaemoglobin, but their influence is localized in the subunits. Lastly, the accessibility to the T-state depends on chloride concentration, and therefore the free-energy gain provided to the ferric form by binding chloride is such to bring the two main quaternary conformations (T-state and R-state) closer in stability.

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