Molecular Dynamics Analysis of a Second Phosphate Site in the Hemoglobins of the Seabird, South Polar Skua. Is There a Site-Site Migratory Mechanism along the Central Cavity?

Antonio Riccio,* Maurizio Tamburrini,* Bruno Giardina,† and Guido di Prisco*
*Institute of Protein Biochemistry and Enzymology, C.N.R., I-80125 Naples, Italy; and †Institute of Chemistry and Clinical Chemistry, Faculty of Medicine, Catholic University "Sacro Cuore," I-00168 Rome, Italy

ABSTRACT Hemoglobin function is modulated by several non-heme ligands; among these effectors, organic phosphates generally bind to heterotropic sites with a one-to-one stoichiometry. The phosphate binding site of human hemoglobin is located at the interface between the two β chains. An additional binding site for polyanions has been studied at the molecular level (Tamburrini, M., A. Riccio, M. Romano, B. Giardina, and G. di Prisco. 2000. *Eur. J. Biochem.* 267:6089–6098) in the hemoglobins of the south polar skua (*Catharacta maccormicki*). It is formed by a cluster of six positive charges of both α chains (Val-1, Lys-99, Arg-141); the two Lys-99α have an essential role in the site structure. The present investigation, carried out on skua deoxyhemoglobins by using a molecular dynamics approach, confirms the structural feasibility of the additional site, possibly having the role of an entry-leaving site, and leads to the proposal of a novel migration pathway for phosphate along the central cavity of hemoglobin from one binding site to the other, occurring according to the hypothesis of a site-site migratory mechanism, which may assign a functional role to the central cavity. The role of Lys-99α was further confirmed by molecular dynamics experiments on the mutant Lys-99$\alpha\rightarrow$Ala in which, at the end of the simulation, the phosphate was external to the additional site.

INTRODUCTION

Hemoglobin (Hb) increases the oxygen-carrying capacity of the blood of vertebrates ~20-fold compared to that due to diffusion of physically dissolved oxygen. Oxygen transport and release into tissues depends on the functional properties of Hb, namely 1) its intrinsic oxygen-binding properties and 2) its interactions with factors that modulate these properties within the erythrocyte. Generally, Hb is functionally modulated by several non-heme ligands, such as organic phosphates, protons, and chloride ions (Weber and Jensen, 1988); the former bind with a one-to-one stoichiometry to heterotropic sites topologically distinct from the heme pocket.

In the past, the heterotropic sites in human Hb have been widely investigated in an attempt to establish a stereochemical rationale for the development of drugs against sickle cell anemia (Perutz et al., 1986). The x-ray structure of the phosphate binding site in human Hb (Hb A) has been elucidated by studying the interaction with the physiological effector 2,3-diphosphoglycerate (2,3-DPG) (Richard et al., 1993) and with inositol hexakisphosphate ($InsP_6$) (Arnone and Perutz, 1974). The structure revealed that this site is located at the interface between the two β chains, mainly involving four residues of each β subunit (Val-1, His-2, Lys-82, His-141) (Richard et al., 1993; Arnone and Perutz, 1974).

The existence of more than one binding site for polyanions has also been hypothesized in several mammalian Hbs on the basis of several experimental approaches (Zuiderweg et al., 1981; Amiconi et al., 1985). In particular, it was observed that the binding curve of phosphate to Hb A is characterized by a biphasic profile, suggesting the presence of at least two sites per tetramer with different affinity constants (Zuiderweg et al., 1981). Furthermore, these authors suggested that the second site is formed by the cluster of the four positive charges of the N- and C-termini of the two α chains. This hypothesis was strongly supported by the observed decrease of phosphate affinity upon carbamylation of the N-terminal residues of the α chains. Moreover, following the observation that the fast exchange between bound and free $InsP_6$ on the ^{31}P-NMR time scale cannot be reconciled with a single-step binding mechanism, they proposed that the additional site may possibly have the role of an entry-leaving site, with migration of phosphate on the Hb surface occurring according to the hypothesis of a site-site migratory mechanism. In this mechanism, the main site and the additional site can bind $InsP_6$ independently, and phosphate can be released into solution either immediately or after migration between the two sites on the same Hb molecule (entry-leaving site).

A recent, first study at the molecular level of an additional phosphate binding site in non-mammalian vertebrate Hbs (Tamburrini et al., 2000) deals with the two Hbs (Hb 1 and Hb 2) of the south polar skua (*Catharacta maccormicki*, Stercorariidae), a seabird breeding in coastal Antarctic regions. It has been proposed by molecular modeling analysis that the additional site is formed by a cluster of six positive charges of the two α chains instead of the four previously reported, evidencing for the first time the fundamental role

Received for publication 17 April 2001 and in final form 9 July 2001.

Address reprint requests to Dr. Guido di Prisco, Institute of Protein Biochemistry and Enzymology, C.N.R., Via Marconi 12, I-80125 Naples, Italy. Tel.: +-39-081-7257242; Fax: +39-081-5936689; E-mail: diprisco@dafne.ibpe.na.cnr.it.

0006-3495/01/10/1938/09 $2.00

of the two Lys-99α in the site structure. Because these charged residues are identical to those found in Hb A at the same sequence positions (see below), it can be assumed that the additional site of skua Hbs has an association constant very similar to that of deoxy-Hb A for $InsP_6$ (1.00×10^4 M^{-1}) (Zuiderweg et al., 1981). Moreover, the observation that Lys-99α is strongly conserved in Hbs suggested that most Hbs possess a potential additional binding site, whose physiological importance may be linked to evolutionary adaptations and life style (Tamburrini et al., 2000).

A molecular dynamics (MD) simulation study on the two Hbs of skua and on human Hb A is herewith reported in an attempt to ascertain the role of the additional site and the possible migration pathway of phosphate on the surface of Hb according to the site-site migratory mechanism previously hypothesized (Zuiderweg et al., 1981). We suggest a novel communication pathway between the two phosphate binding sites along the central cavity. Furthermore, to test the role of Lys-99α in the additional site we have carried out MD simulations also on the molecular model of a mutant Hb 1-$(InsP_6)_2$ complex having the substitution Lys-99$\alpha \rightarrow$Ala (αK99A).

All computational studies were carried out using $InsP_6$ because most of the available data on Hbs from other species refer to the interaction with this allosteric effector, which is structurally and functionally very similar to inositol pentakisphosphate, the physiological allosteric cofactor of bird Hbs.

MATERIALS AND METHODS

Molecular modeling

Computer graphics, structural manipulations, energy minimization calculations, and MD simulations were carried out with a Silicon Graphics (Mountain View, CA) O^2 workstation using the INSIGHT II software package (Biosym/Molecular Simulation Incorporated (MSI), San Diego, CA).

Because a detailed analysis of the phosphate binding sites at the molecular level requires a deoxy T-state structure, and crystallographic structures of avian deoxy Hbs are not available, the high-resolution structure (1.74 Å) of human deoxy Hb A (Fermi et al., 1984) (Brookhaven Protein Database, code 2hhb) was used as template to build the molecular model of skua Hbs. The available crystallographic structures of complexes of Hb A with $InsP_6$ (Luisi et al., 1990; Waller and Liddington, 1990) were not used as template because in one of them $InsP_6$ was poorly resolved (Luisi et al., 1990) and in the other, although Hb A was resolved at high resolution, a non-refined $InsP_6$ molecule was described (Waller and Liddington, 1990), and therefore the structure of the phosphate binding site is not fully reliable. Thus, we built the whole complex of skua Hbs with $InsP_6$ ex novo to rely on completely independent final molecular models. Alignment of the amino acid sequences of the α chains of skua Hb 1 and Hb 2 with that of Hb A showed 70% and 60% identity, respectively, whereas the β chains of skua Hbs showed 69% identity with Hb A (Tamburrini et al., 2000). Molecular models of skua Hbs and of Hb A, and of complexes with $InsP_6$, were built as previously described (Tamburrini et al., 2000).

Two $InsP_6$ molecules were added to skua Hb 1 with the AFFINITY program (Biosym/MSI), one in the main binding site, i.e., the central cavity located between the two β chains of the deoxy form (Richard et al., 1993; Arnone and Perutz, 1974), and the other in the cavity formed by Val-1, Lys-99, and Arg-141 of the two α chains, as previously described (Tamburrini et al., 2000). The Hb A-$(InsP_6)_2$ complex was built following the same procedure, and used as a control to verify the validity of the skua Hbs results. In addition, to study the role of Lys-99α in the binding of the additional phosphate, a model of skua Hb 1 with $InsP_6$ bound to both sites was built bearing the mutation αK99A.

To study possible migration of phosphate between the two binding sites, two skua Hb 1-$InsP_6$ complexes were built by automated docking of $InsP_6$ in the central internal cavity that links the two sites. $InsP_6$ was placed by the AFFINITY program (Biosym/MSI) in positions chosen on the basis of MD simulations (see below), i.e., 1) a position along the dyad axis and proximal to the main binding site (Hb1MBS), and 2) a position along the dyad axis and proximal to the additional binding site (Hb1ABS). Two analogous complexes of Hb A (HbAMBS and HbAABS) were built for comparison. To ensure the stability of the models, a 5-Å water shell (Walshaw and Goodfellow, 1993) was subsequently added around all models by using the Soak Assembly function of the INSIGHT II package (Biosym/MSI).

Minimizations and MD simulations

The DISCOVER_3 package (Biosym/MSI) was used for all computational studies. Refinements were performed until an energy minimum was obtained, by the following steps: 1) hydrogen atoms only, 2) water shell, 3) amino acid side chains, 4) Hb main-chain and $InsP_6$ molecules, and 5) Hb-$(InsP_6)_2$ complex.

The first four steps of the refinements, performed with quadratic restraints (tethering restraint), were carried out with 1650 cycles of the steepest descent (SD) algorithm, whereas the last step was carried out with 600 cycles of the conjugate gradient (CG) algorithm until the maximum derivative values were lower than 0.1 kcal/mol/Å. The final models were refined with 100 steps of SD algorithm and with 250 steps of CG algorithm, until the maximum derivative values were lower than 0.01 kcal/mol/Å.

Energy minimizations of the two complexes of skua Hb 1 with only one $InsP_6$ molecule in the central cavity were carried out following a procedure similar to that described above, until an energy minimum was obtained. The minimized complexes were used as initial structures to carry out MD simulations, for further conformational analysis of both sites and to follow the possible movements of both $InsP_6$ molecules on the Hb surface.

MD simulations were carried out with the Verlet method, and the NVE ensemble was used in all calculations. During MD the RATTLE_BOND method (Andersen, 1983) was also used, with a time step of 2 fs. The equilibration phase was 300.0 ps long, whereas the second phase was up to 1.0 ns long; in all calculations a temperature of 300 K was used.

MD simulations were carried out only in the regions located around both $InsP_6$ molecules, keeping all the other residues and water molecules at their initial positions, and using the following subsets: 1) the amino acid residues located within 15 Å around both $InsP_6$ were free from restraints during all simulations; 2) water molecules located at 15 Å around both $InsP_6$ had a quadratic restraint with 5 kcal/Å^2/mol; 3) the amino acid residues and water molecules located in the layer 15–18 Å from both $InsP_6$ had a quadratic restraint (tethering with K = 70 kcal/Å^2/mol); 4) the amino acid residues and water molecules located in the layer 18–20 Å from both $InsP_6$ also had a quadratic restraint, but with a K = 160 kcal/Å^2/mol; 5) all other atoms were kept at their initial positions as determined in the minimized models.

The models obtained after MD simulation were minimized, using the same restraints on the subsets, with 400 steps of SD algorithm and 650 steps of CG algorithm, until the maximum derivative values were lower than 0.01 kcal/mol/Å.

Consistent valence force field (CVFF) parameters were used in all calculations, modified by Biosym/MSI to parametrize the heme (CVFF_HEME), using a 20-Å cutoff distance for the treatment of electrostatic

interactions and a 10-Å cutoff distance for the treatment of all other nonbonded interactions, and using a switching function to smooth the energy function and to remove discontinuities in the force field at the point of truncation, with a distance-dependent dielectric constant $\epsilon = 1 \times R$. The distance-dependent dielectric constant model, used together with a water shell surrounding the protein, has been shown to achieve a trajectory that better agrees with the crystal structure when compared with a constant dielectric model (Guenot and Kollman, 1992).

The complex Hb A-$(InsP_6)_2$ was built, refined, and used in MD simulations with the same procedures described above, and used as a control to verify the validity of the skua Hbs results. A similar procedure was followed with the two Hb A-$InsP_6$ complexes.

RESULTS

Validation of the models

The accuracy of the minimized models of the complexes of skua Hbs and of Hb A was analyzed using the inverse protein folding approach (Bowie et al., 1991) with the PROFILE_3D (Biosym/MSI) program. This algorithm measures the compatibility of an amino acid sequence with a three-dimensional (3D) structure by converting the structure to a 1D representation, known as a 3D profile, which can be aligned with the sequence (Lüthy et al., 1992). The global score of the complexes with two $InsP_6$ molecules (S = 282, 281, and 283 for skua Hb 1, Hb 2, and Hb A, respectively) and of the complexes with one $InsP_6$ molecule (S = 290 and 286 for Hb1MBS and Hb1ABS, respectively; S = 284 and 281 for HbAMBS and HbAABS, respectively) were comparable to the corresponding value found in the crystallographic structure of Hb A (S = 295), and higher than the value (S = 265) expected on the basis of the length of the protein sequences. These data suggested the reliability of all models, which was also confirmed by the comparison between the interactions found in the main binding site and those found in the x-ray structures of complexes of Hbs with organic phosphates (see below).

Fig. 1 reports the root mean square (rms) deviations, as a function of the time of simulation, for the regions of the complexes skua Hb 1-$(InsP_6)_2$ and human Hb A-$(InsP_6)_2$, respectively, maintained free from restraints during simulations. The data clearly suggested the stability of the simulations. Similar results were obtained from MD simulation experiments carried out on the Hb 2-$(InsP_6)_2$ complex and on the mutant αK99A of the complex Hb 1-$(InsP_6)_2$ (not shown).

Molecular modeling and dynamics

The main binding site

The main phosphate binding site of skua Hbs is characterized by a high number of substitutions with respect to Hb A (Table 1), and by a higher total electrostatic charge, a very important feature because phosphate binding to Hb is essentially electrostatic. The minimized model of the complex Hb A-$(InsP_6)_2$ showed very similar interactions of $InsP_6$ with Hb A in the main binding site with respect to those reported in the crystallographic structures of the complexes of Hb A with $InsP_6$ (Arnone and Perutz, 1974) and with 2,3-DPG (Richard et al., 1993). Moreover, the models of skua Hbs were very similar to those suggested for goose (Zhang et al., 1996) and chicken (Knapp et al., 1999) Hbs, which were built by manual addition of $InsP_6$ to the crystallographic structures of those Hbs.

After MD simulations on the skua Hb 1-$(InsP_6)_2$ complex, $InsP_6$ and all residues in contact with it showed the

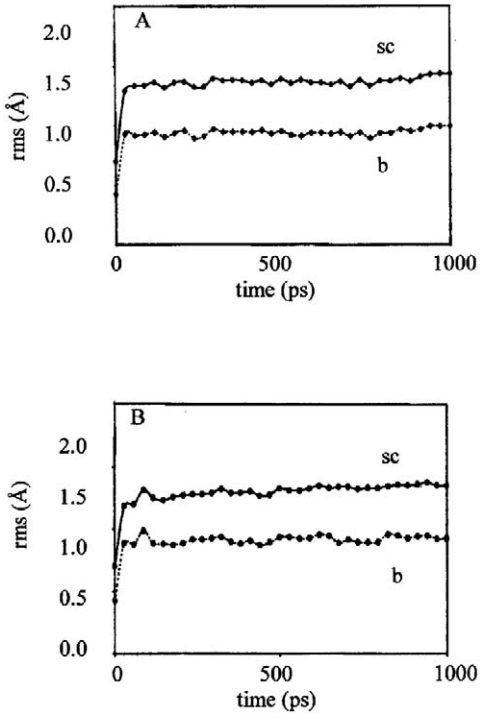

FIGURE 1 Root mean square (rms) deviations, as a function of the time of simulation, of the amino acid side chains (sc) and of the backbone (b) of the protein region located within 15 Å around both $InsP_6$ molecules and free from restraints during MD simulation. Skua Hb 1 (*A*); human Hb A (*B*).

TABLE 1 Amino acid residues forming the $InsP_6$ binding sites of skua Hbs

β-Chain Residues Forming the Main Binding Site	α-Chain Residues Forming the Additional Binding Site	
Skua Hbs	Skua Hb 1	Skua Hb 2
Val-1	Val-1 (Val)	Met-1
His-2	Pro-95	Pro-95
Lys-82	Lys-99	Lys-99
Arg-135 (Ala)	Asn-134 (Thr)	Ala-134
Val-136 (Gly)	Thr-137 (Thr)	Ser-137
His-139 (Asn)	Ala-138 (Ser)	Glu-138
Arg-143 (His)	Arg-141	Arg-141

The residues of human Hb A different from those of skua Hbs are reported in parentheses.

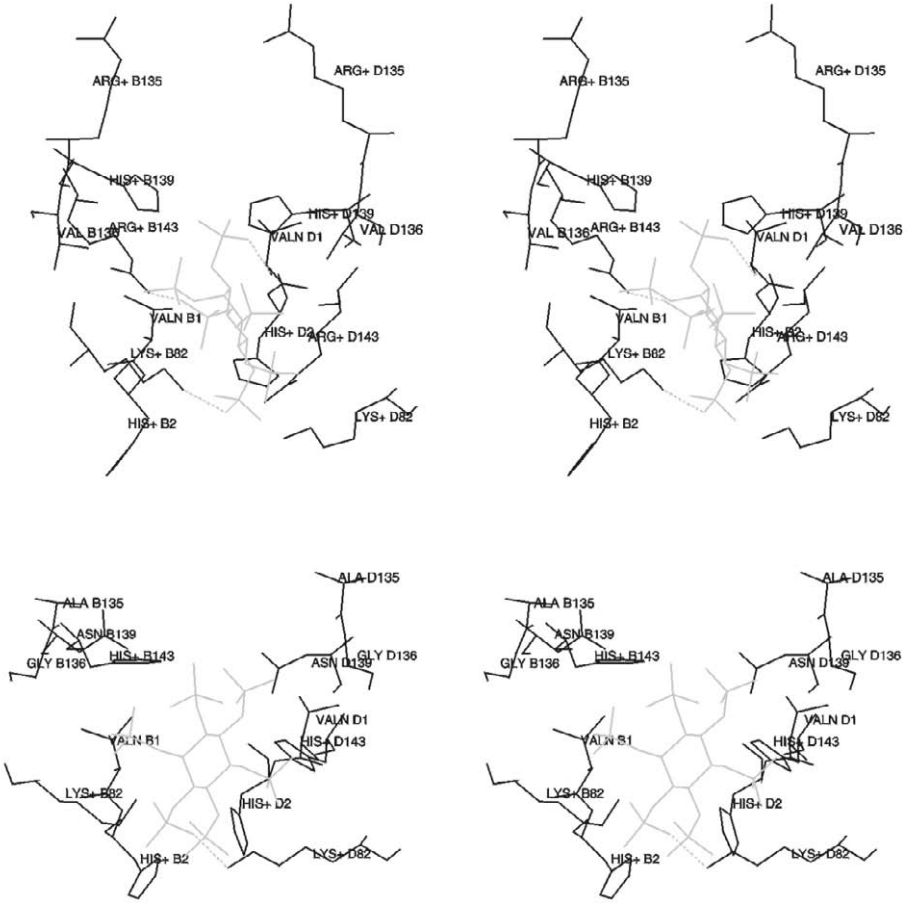

FIGURE 2 Stereo view of the main phosphate binding sites of skua Hb 1 (*top*) and human Hb A (*bottom*), after MD simulation. $InsP_6$ is shown in gray. The H-bonds established between $InsP_6$ and Hb are indicated by dashed lines. The residues indicated with B and D belong to the β_1 and β_2 chains, respectively.

greatest conformational differences with respect to the initial positions (Fig. 2). The side chains of Arg-135β_2 and His-2β_2 were "opened" around $InsP_6$, whereas the same residues of the β_1 chain were "tightened" around phosphate. $InsP_6$ rotated around its initial position, and its center of mass moved ~2.5 Å along the central cavity, characterized by a high number of positively charged residues (Table 2), toward the center of Hb. The binding site showed tightening in its lower part, with the Nϵ atoms of the two Lys-82β at a distance of 6.0 Å, instead of 8.6 Å found in the minimized model. Similar results were found in the Hb 2-$(InsP_6)_2$ complex; in fact, the two skua Hbs have the β chain in common.

MD simulations on the human Hb A-$(InsP_6)_2$ complex revealed some differences in comparison with skua Hbs (Fig. 2). In fact, $InsP_6$ did not show any rotation of the plane of cycloexane, and its migration along the central cavity was not so significant as in skua Hbs. These results might be explained on the basis of the different amino acid composition and, therefore, of the total positive charge, of this site.

The additional binding site

As reported (Tamburrini et al., 2000), the additional phosphate binding site in skua Hbs is made of seven residues of each α chain (sequence positions 1, 95, 99, 134, 137, 138, 141), which are in direct contact with $InsP_6$; in particular,

TABLE 2 Polar residues lining the central cavity of skua Hb 1

α Chain	β Chain
Val-1+	Val-1+
Lys-99+	His-2+
Gln-103 (His+)	Lys-82+
Asp-126−	Glu-101−
Arg-141+	Arg-104+
	Arg-135+ (Ala)
	His-139+ (Asn)
	Arg-143+ (His+)

The residues of human Hb A different from those of skua Hb 1 are reported in parentheses.

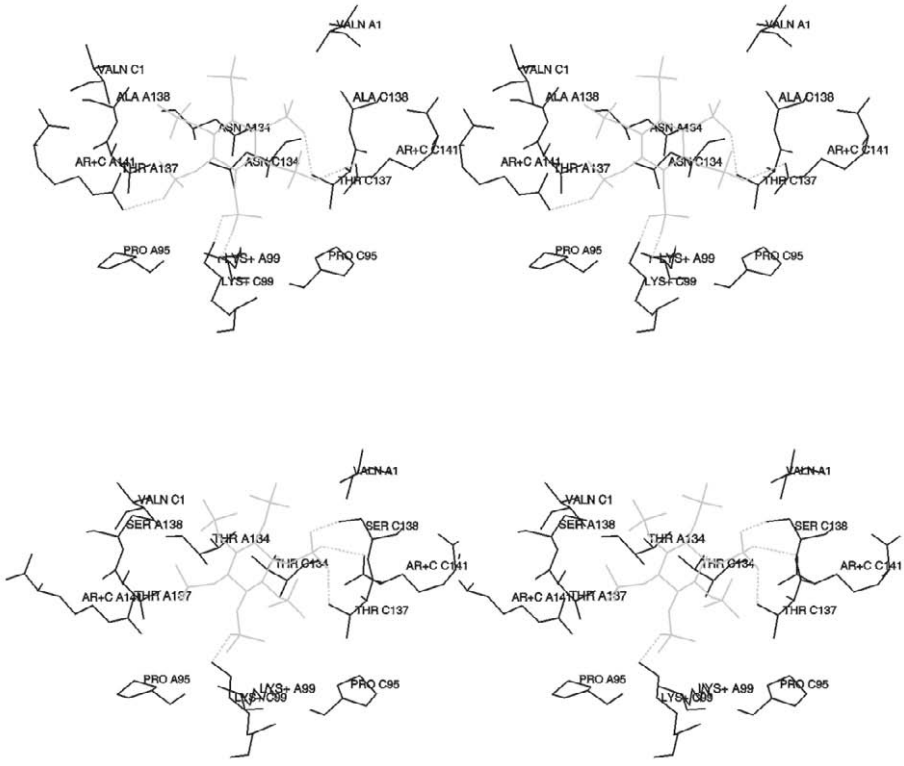

FIGURE 3 Stereo view of the additional phosphate binding sites of skua Hb 1 (*top*) and human Hb A (*bottom*), after minimization. $InsP_6$ is shown in gray. The H-bonds established between $InsP_6$ and Hb are indicated by dashed lines. The residues indicated with A and C belong to the α_1 and α_2 chains, respectively.

the N- and C-termini and Lys-99 establish six salt bridges with $InsP_6$.

Skua Hb 1 has only two conservative substitutions in this site with respect to Hb A, whereas skua Hb 2 has four substitutions, two of which are nonconservative (Table 1). The additional sites of skua Hbs and human Hb A have identical total electrostatic charges; therefore, their binding energy for $InsP_6$ should be similar.

After minimization, $InsP_6$ had the same conformation in skua Hb 1 and Hb A, with phosphate groups having similar interactions within the site and establishing salt bridges with Lys-99 (Fig. 3). It is noteworthy that, although the two skua Hbs have different α chains, Lys-99 is conserved in Hb 2. Moreover, the pK_a value of Glu-138 in both α chains shifts to higher values, i.e., 6.5 and 7.1 for α_1 and α_2, respectively, due to the strongly negative environment produced by $InsP_6$ (Tamburrini et al., 2000).

After MD simulations, the changes of all side chains and of $InsP_6$ were less marked in the additional site of all Hb models as compared with those observed in the main site, indicating higher structural rigidity. In skua Hb 1, $InsP_6$ showed a shift toward the central cavity of Hb, where a high number of positively charged residues are located (Table 2), and a small shift toward an edge of the site (Fig. 4). During all MD simulations the salt bridges between Lys-99α and $InsP_6$ were conserved. Unlike the main site, this one did not show tightening around $InsP_6$, with the Nϵ atoms of the two Lys-99α at a distance of 6.8 Å instead of 6.0 Å found in the minimized model.

Similar results were found after MD simulations carried out on the Hb A-$(InsP_6)_2$ complex. This similarity can be explained on the basis of the high identity in amino acid composition of the additional binding site between the two Hbs. In skua Hb 2, $InsP_6$ showed higher mobility than in Hb 1; in fact, phosphate shifted toward the upper part of the site, which appeared slightly opened, with only Lys-99α_2 bound to $InsP_6$.

The role of Lys-99α in the binding of $InsP_6$ was confirmed by MD simulations carried out on the mutant αK99A of the complex Hb 1-$(InsP_6)_2$. In fact, at the end of the simulations $InsP_6$ was in a position external to the additional site, and not bound to the residues forming the site (Fig. 4), whereas no difference was found in the main binding site with respect to the same complex built with normal Hb 1 (Fig. 2). Therefore, the substitution Lys-99→Ala involved loss of binding between Hb and $InsP_6$.

MD simulations did not show any direct interaction between the two binding sites, probably because of the restraints imposed on structural movements beyond the binding site region and because the distance between the two sites (22 Å in the final model of the complex Hb 1-$(InsP_6)_2$) was higher

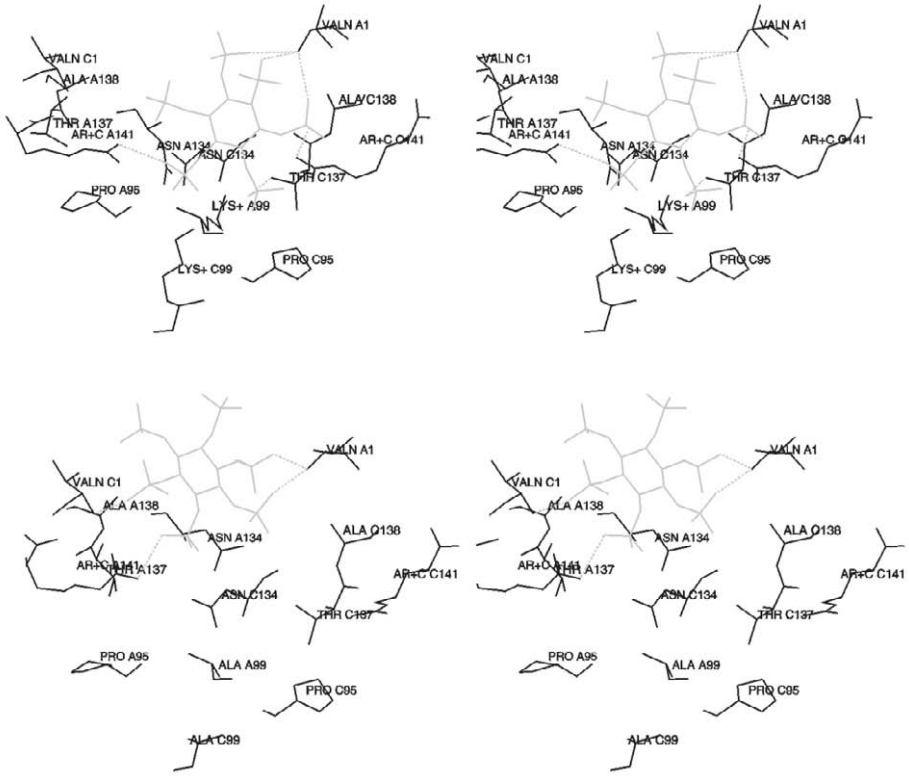

FIGURE 4 Stereo view of the additional phosphate binding sites of skua Hb 1 (*top*) and mutant αK99A Hb 1 (*bottom*) after MD simulation. $InsP_6$ is shown in gray. The H-bonds established between $InsP_6$ and Hb are indicated by dashed lines. The residues indicated with A and C belong to the α_1 and α_2 chains, respectively.

than the cutoff distance for electrostatic interactions (20 Å). These results were confirmed by MD simulations carried out on the complexes of all Hbs with only one $InsP_6$ molecule in either site (not shown). After all MD simulations, carried out on both the main and the additional site, $InsP_6$ did not change its chair conformation, preserving all the stereochemical parameters of the ring and of the phosphate groups.

Hb-$InsP_6$ complexes

After minimization of the Hb1MBS model, $InsP_6$ showed several H-bonds with charged residues of the central cavity (Fig. 5). The phosphate binding region in the central cavity contained residues of both α and β chains, some of which (Lys-99$\alpha_{1,2}$ and Arg-135$\beta_{1,2}$) belong to both phosphate binding sites. Six of 18 residues located within 5 Å around $InsP_6$ had a positive charge (Fig. 5); this was not unexpected because the central cavity of Hb is characterized by many charged residues (Table 2).

In the minimized HbAMBS model there were only four positively charged residues in direct contact with $InsP_6$, namely Lys-99$\alpha_{1,2}$ and Arg-104$\beta_{1,2}$, establishing salt bridges with the phosphate (Fig. 5). There were several substitutions in Hb A with respect to skua Hb 1, decreasing the total positive charge of this region of the central cavity. In fact, three of the changes were Arg-135$\beta\rightarrow$Ala, His-139$\beta\rightarrow$Asn, and Asp-108$\beta\rightarrow$Asn.

The minimized model of Hb1ABS showed several H-bonds between $InsP_6$ and residues belonging to both α chains. This model was also characterized by H-bonds between $InsP_6$ and uncharged residues. Moreover, Lys-99$\alpha_{1,2}$ were still bound to phosphate (Fig. 6). Similar results were found in the minimized model of HbAABS (Fig. 6). It has to be emphasized that no unfavorable contact was found in any of the minimized models examined.

DISCUSSION

Organic phosphates are the most important Hb heterotropic effectors in vertebrates; they generally bind to Hb with a one-to-one stoichiometry. Besides the main site, the presence of an additional binding site for polyphosphates has previously been hypothesized in several mammalian Hbs (Zuiderweg et al., 1981; Amiconi et al., 1985). The first study at the molecular level of this site suggested the involvement of Lys-99α and the N- and C-termini of the two α chains (Tamburrini et al., 2000).

In this study, the role of Lys-99α in the binding of phosphate has been confirmed by MD simulations carried out on the complexes of skua Hbs and human Hb A with

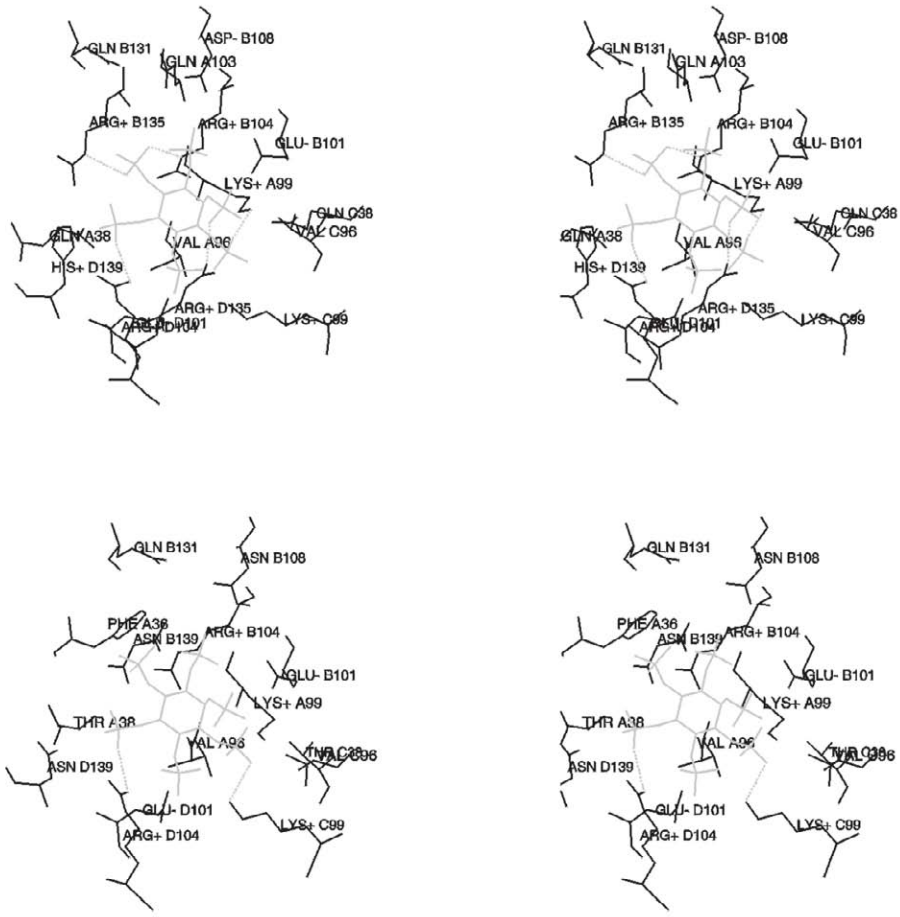

FIGURE 5 Stereo view of the complexes Hb1MBS (*top*) and HbAMBS (*bottom*), after minimization. $InsP_6$ is shown in gray. The H-bonds established between $InsP_6$ and Hb are indicated by dashed lines. The residues indicated with A and C, and with B and D, belong to the α_1 and α_2, and to the β_1 and β_2 chains, respectively.

$InsP_6$. Moreover, MD simulations on the mutant model αK99A of skua Hb 1-$(InsP_6)_2$ showed that in this model $InsP_6$ was external to the additional site (Fig. 4), stressing the essential role of Lys-99α in phosphate binding. This evidence is in agreement with the data available for the binding of $InsP_6$ to human Hb after carbamylation of the N-termini of the α chains (Zuiderweg et al., 1981). In fact, the latter experiment brings about a decrease of the affinity of the additional site for $InsP_6$ of only 40%, which may be explained on the grounds that Lys-99α and the C-termini of the α chains are still able to bind phosphate.

The results of MD simulations on all models of skua and human Hb complexes showed a movement of $InsP_6$ from both sites toward the central cavity, although the simulation time (0.7 ns, plus 0.3 ns to equilibrate the system) might perhaps be too short. These translation movements may be ascribed to migration from one site to the other, although reorganization of $InsP_6$ within the binding sites cannot be excluded (caution is obviously recommended when interpreting reality on the basis of a theoretical approach). Minimization of Hb-$InsP_6$ complexes suggested that the central cavity could be a region of possible phosphate binding, due to the presence of positively charged residues and absence of unfavorable contacts between $InsP_6$ and Hb. Moreover, some of the binding residues of the central cavity also belong to both phosphate binding sites, and could therefore represent intermediate binding regions between the additional and the main sites (Fig. 7). Furthermore, the side chain of Lys-99α might change its conformation to follow $InsP_6$ along the cavity, thus being a direct link between the two binding sites. The overall gradient of positive charge along the central cavity may represent the driving force needed for phosphate migration between the two sites. This mechanism has also been proposed for the binding of chloride because the functional chloride binding sites are contiguous to each other along the central cavity (Ueno and Manning, 1992).

We propose a novel migration pathway for phosphate along the central cavity of Hb from one binding site to the other (Fig. 8). In particular, the orientation of the gradient of positive charge suggests that phosphate may migrate from the additional site to the main site, from which it could be released to return into solution. In fact, $InsP_6$ can bind to the

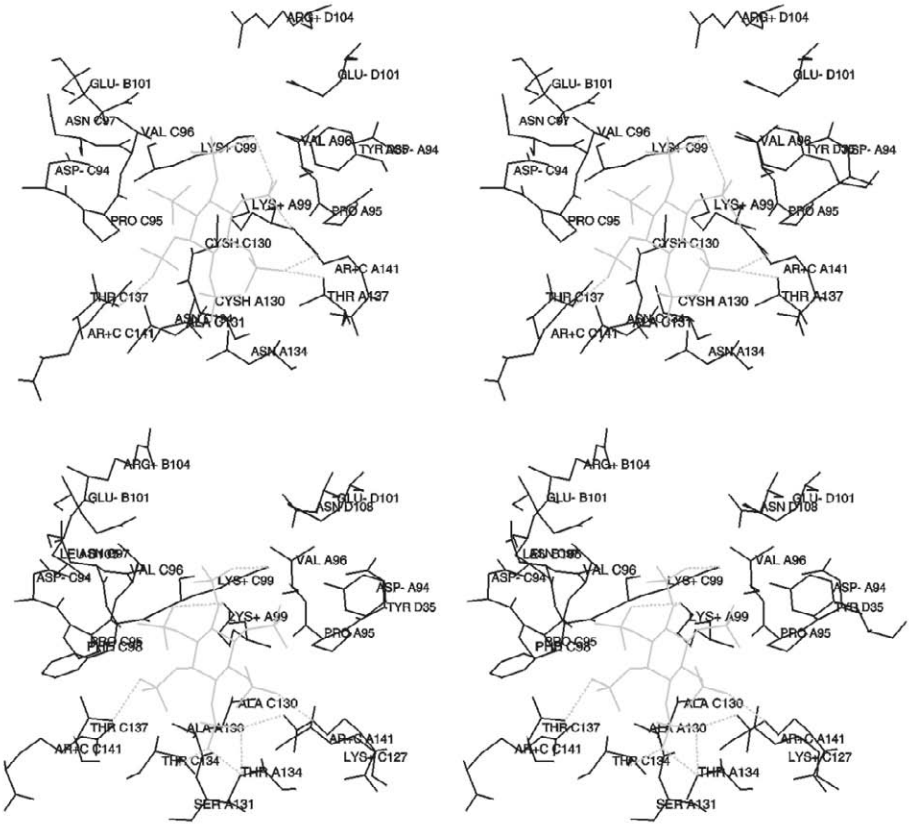

FIGURE 6 Stereo view of the complexes Hb1ABS (*top*) and HbAABS (*bottom*), after minimization. $InsP_6$ is shown in gray. The H-bonds established between $InsP_6$ and Hb are indicated by dashed lines. The residues indicated with A and C, and with B and D, belong to the α_1 and α_2, and to the β_1 and β_2 chains, respectively.

additional site at concentrations close to the physiological ones found in erythrocytes (Tamburrini et al., 2000). This mechanism may assign a functional role to the central cavity, so far considered devoid of a specific function, and also reconciles with the different dimensions of the central cavity found in the T and R structures, which are respectively opened with high affinity for $InsP_6$, and tightened with low affinity for $InsP_6$ (Dickerson and Geis, 1983). The additional site would also enhance the probability of phosphate binding from the solution and prompt transfer to the main site, thus acting essentially as an entry-leaving site. This may contribute to overstabilize the T structure and favor the release of controlled amounts of oxygen under conditions of physiological stress that birds may have to face, for instance during prolonged flights.

The observation that Lys-99α is strongly conserved suggests that most Hbs possess a potential additional binding site that may provide a site-site migration mechanism along the central cavity. The present study prompts further investigations into the role of Lys-99α in the binding of phosphate and on the structure and function of the central cavity of Hb as a possible channel for phosphate migration. To this aim, site-directed mutagenesis would be a most effective tool.

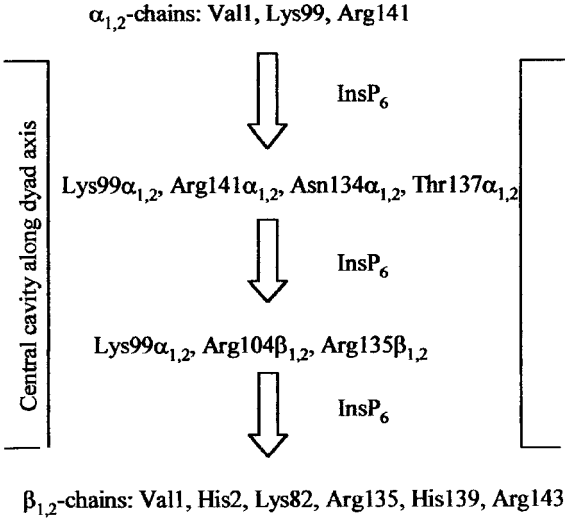

FIGURE 7 Schematic representation of phosphate migration from the additional to the main binding sites of skua Hb 1 along the central cavity. The arrows indicate the overall gradient of positive charge at the basis of phosphate migration between the two sites. The residues in direct contact with $InsP_6$ are reported.

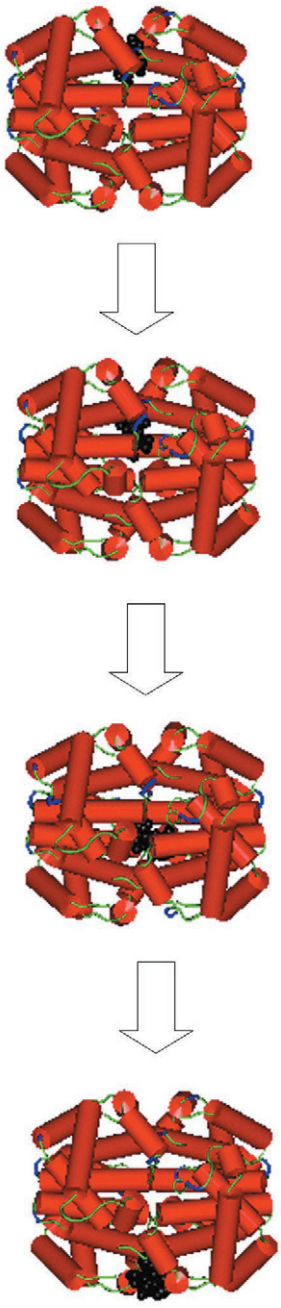

FIGURE 8 Migration pathway of phosphate from the additional to the main binding sites along the central cavity. Hb is shown as a Kabsch-Sander representation of the secondary structure. The van der Waals surfaces of $InsP_6$ are reported in black.

This study is in the framework of the Italian National Program for Antarctic Research.

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