ELSEVIER

Biophysical Chemistry 72 (1998) 323–335

Biophysical Chemistry

Glycated human hemoglobin (HbA_{1c}): functional characteristics and molecular modeling studies

M. Cristina De Rosa [a], M. Teresa Sanna [b], Irene Messana [a], Massimo Castagnola [b], Antonio Galtieri [c], Ester Tellone [c], Roberto Scatena [a], Bruno Botta [d], Maurizio Botta [e], Bruno Giardina [a,*]

[a] *Istituto di Chimica e Chimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica, Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, CNR, Roma, Italy*
[b] *Istituto di Chimica Biologica, Università di Cagliari, Cagliari, Italy*
[c] *Dipartimento di Chimica Organica e Biologica, Università di Messina, Messina, Italy*
[d] *Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università di Roma, La Sapienza, Roma, Italy*
[e] *Dipartimento Farmaco Chimico Tecnologico, Università di Siena, Siena, Italy*

Received 24 November 1997; revised 9 February 1998; accepted 9 February 1998

Abstract

A minor hemoglobin component of human red cell hemolysate, HbA_{1c}, is the result of the non-enzymatic reaction of glucose with the α-amino groups of the valine residues at the N-terminus of the β-chains of human hemoglobin. In this paper, the effect of protons, chloride and 2,3-diphosphoglycerate (DPG) on the functional properties of HbA_{1c} has been investigated in some details. Moreover, the structural modifications induced on the native molecule by the sugar moieties, studied by computer modeling, do agree with the observed functional alterations. In particular, the functional results indicate that: (a) the low-affinity conformation (or T-state) of HbA_{1c} is destabilized by the chemical modification per se; (b) the Bohr effect is reduced with respect to that of native HbA_0; (c) the affinity of the T-state of HbA_{1c} for 2,3-diphosphoglycerate is about 2.6 $\times$ lower than that of the corresponding conformational state of HbA_0, while the R-state is less affected with, the affinity being 1.7 $\times$ lower. At the structural level, computer modeling studies show that the two sugar moieties are asymmetrically disposed within the 2,3-diphosphoglycerate binding site. In addition, molecular mechanics and dynamics calculations concerning the interaction with 2,3-diphosphoglycerate indicate that while in HbA_0 the effector can assume two different stable orientations, in glycated Hb only one orientation is possible. All together, the results show that glycation of the Val 1 residues of both β-chains does not impair the binding of DPG but imposes a different mode of binding by changing the internal geometry of the complex and the surface distribution of the positive electrostatic potential within the binding pocket.

Keywords: Protein structure; Non-enzymatic glycosylation; Protein-ligand interactions; Molecular dynamics; Electrostatic potential

* Corresponding author. Istituto di Chimica e Chimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica, Largo F. Vito 1, 00168, Roma, Italy. Tel.: +39-6-3058494; fax: +39-6-3053598; e-mail: b.giardina@uniserv.ccr.rm.cnr.it

0301-4622/98/$19.00
PII S0301-4622(98)00117-3

1. Introduction

The oxygen affinity of hemoglobin is greatly influenced in vivo by a complex interplay of interactions involving protons, chloride, 2,3-diphosphoglycerate (DPG), carbon dioxide and temperature. Due to the great physiological importance of this network of interactions, many studies have been devoted to determining how and to what extent these allosteric effectors may modulate the ability of hemoglobin to bind and release oxygen [1]. In this respect, the understanding of the allosteric behaviour of Hb has been greatly helped by naturally occurring mutants, artificially induced point mutations and specific chemical modifications at the level of key amino acid residues. Human red blood cell hemolysate contains several non-enzymatically modified minor hemoglobin components, the most abundant of which is HbA_{1c}. This hemoglobin component, whose percentage (normally about 3–4% of total hemolysate) is related to the blood glucose concentration, provides useful information about the patient glycemic status. HbA_{1c} is the result of the non-enzymatic reaction of glucose with the α-amino groups of the valine residues at the N-terminus of β-chains. The labile glucosamine adduct derived from this reaction is converted, throughout an Amadori rearrangement, into fructosamine [2] that cyclizes in a pyranosidic ring [3]. Isolation of this naturally and specifically modified human hemoglobin creates the possibility of investigating in some detail, the molecular perturbations induced by the chemical modification on the aforementioned network of heterotropic interactions. Hence, although previous studies have clearly shown that glycation of human hemoglobin affects the T quaternary structure (low-affinity conformational state) bringing about a more relaxed conformational state [4], no study has been devoted to the dissection of the induced functional perturbation at the level of the various heterotropic effectors.

In this paper, we have investigated the functional properties of HbA_{1c} with particular attention to the effect of protons, chloride and 2,3-diphosphoglycerate. Moreover, the structural perturbation induced by the chemical modification has been studied by computer modeling based on the crystal structure of deoxy-human hemoglobin (HbA_0).

This computational part of the work has been mainly directed towards: (a) the study of possible conformations of the DPG/β-cleft complex additional to that showed by the crystallographic model of Arnone in the awareness that this latter represents only one aspect of the conformational fluctuations available to the system; (b) the effects of the chemical modification on the interactions of the protein with its main physiological effector, namely 2,3-diphosphoglycerate.

We should recall that, in human HbA_0, the binding of DPG occurs at a specific site, which, at neutral pH, involves a cluster of eight positively charged amino acid residues (Val NA1, His NA2, Lys EF6 and His H21 of each β-chain) located on the dyad axis of the hemoglobin tetramer [5]. Hence, we wished to investigate how and how much the stereochemistry of this important heterotropic site could have been changed by the chemical modification of Val NA1. The results give a consistent picture of the functional alterations linked to the glycation of the N-terminal groups of the β-chains of human hemoglobin and add useful information to our understanding of structure-function relationships in this class of oxygen-transport proteins.

2. Materials and methods

2.1. Purification procedure

HbA_0 and HbA_{1c} were purified from hemolysates of volunteers following published procedures [6]. Briefly, cells were washed $3\times$ with *iso*-osmotic NaCl solution by centrifugation at $1000\times g$ and the packed cells were lysed by adding 2 volumes of cold hypotonic buffer. Stroma were removed by centrifugation at $12\,000\times g$ for 30 min. Hemoglobin purification was performed by preparative cation-exchange chromatography on Bio-Rex 70 (BioRad, Richmond, CA). The purity of Hb preparations, assessed by isoelectrofocusing in 8 M urea of globin chains [7], was always greater than 96%. Removal of organic and inorganic phosphates (i.e., stripped hemoglobin) was obtained by passing the hemolysate first through a Sephadex G-25 column, equilibrated with 0.01 M Tris–HCl buffer (pH = 8.0) containing 0.1 M NaCl, and afterwards through a column of mixed bed ion-exchange resin (BioRad 501 X8).

2.2. Functional studies

Oxygen binding isotherms were determined by the tonometric method [8] in the absence and presence of allosteric effectors. Determination of the oxygen affinity as a function of DPG concentration made it possible to obtain the binding constants of both the oxy and deoxy conformational states of Hb according to the equation:

$$\log P_{50}^{obs} = \log P_{50}^{0} + \frac{1}{4}\log \times \left(\frac{1 + K_{deoxy} \cdot [2,3-DPG]}{1 + K_{oxy} \cdot [2,3-DPG]} \right)$$

where P_{50}^{0} refers to the oxygen affinity in the absence of the effector. The use of P_{50} instead of P_m is justified by the highly symmetric shape of the binding curve.

2.3. Molecular modeling studies

2.3.1. Model building procedure for HbA_0

Since the crystal structure of deoxyhemoglobin A_0 with DPG is not available, the coordinates of the X-ray structure [9] of deoxyhemoglobin A_0 at 1.9 Å resolution, obtained from Brookhaven Protein Data Bank, served to build up the starting molecular model. The structure comprises 574 amino acid residues and 192 crystallographic water molecules. Hydrogen atom coordinates were generated using the INSIGHTII software package from MSI. [1] All of the hydrogen atoms added to the crystal structure were explicitly treated. INSIGHTII was also used for the construction of the DPG molecule and the geometry optimization was performed with the DISCOVER software package from MSI. Molecular graphics and force field calculations were carried out on a INDY 4400 SGI workstation running the 5.2 IRIX operating system. The initial coordinates of the complex were created by reproducing the binding of DPG at the receptor site, as observed by Arnone [5]. In addition, water molecules were added to fill an area of 7.0 Å radius, centered on the DPG molecule, and an area of 3.0 Å radius, centered on each one of the charged residues.

2.3.2. Model building procedure for HbA_{1c}

Glycated hemoglobin HbA_{1c} was built starting from the crystal structure of HbA_0 and replacing one of the hydrogen atoms of the N-terminal group of Val 1, with the ketoamine adduct. The Amadori adduct was designed with a β-pyranose conformation according to ^{13}C nuclear magnetic resonance (NMR) studies on protein glycation [3].

In order to find the structures of relatively low energy, assuming that glycation produces only a small local change in the hemoglobin structure [10], a search of the local conformational space of the modified binding site was performed. The first step required finding the conformations of minimum energy for the glycated *N*-methylvalinamide (Fig. 1). Initially, the search was restricted to varying systematically at 20° the angles β, γ and δ of the glycated amino acid while maintaining all the bond lengths and angles fixed. For each dihedral position, the energy of the modified amino acid was calculated using the AMBER All Atom force field [11] with Homans extension to carbohydrates [12]. The resulting potential maps of energy were created by the ANALYSIS module of INSIGHTII. A search in the presence of the potential energy of the unmodified protein was then performed on the three dihedral angles of the modified amino acid bound to the two β chains of the deoxyhemoglobin structure to reveal any possible perturbation induced in the conformation of the glycated amino acid by the neighbour side chains of the protein moiety. The conformational analysis was performed following the same procedure employed before. Since the coordinates for deoxyhemoglobin were held constant, only residues with at least one atom within a 15 Å distance from the sugars were included in the computation. The study of the potential maps of energy created by the ANALYSIS module of INSIGHTII showed the dihedral-angular values corresponding to conformations of minimum energy for the Amadori adduct. All conformations of minimum energy were taken in consideration and, for each of them, a model of HbA_{1c} was built, following the same procedure as that described for HbA_0.

[1] MSI, 9605 Scranton Rd. Suite 101, San Diego, CA 92121.

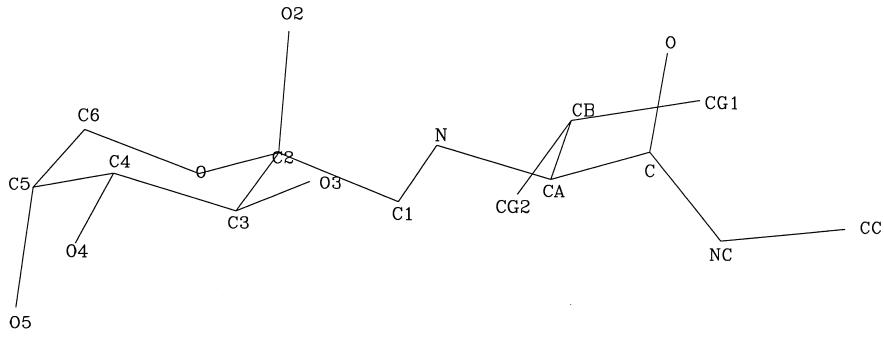


Fig. 1. The glycated *N*-methylvalinamide. The torsional angles varied in the conformational search are: β (CB–CA–N–C1), γ (CA–N–C1–C2) and δ (N–C1–C2–C3). Hydrogen atoms are not displayed in the figure.

2.3.3. Molecular mechanics and dynamics calculations

To verify the reliability of our procedure, we attempted to reproduce the results of Arnone using both AMBER All Atom and CVFF [13] force fields. Initially, the complex HbA_0–DPG was minimized using the steepest descent method with all heavy atoms fixed, until the first derivative was less than 10.00 kcal mol^{-1} $Å^{-1}$. In this way, any artifact introduced by the addition of hydrogen atoms was removed. Manual docking simulations were then performed on deoxyhemoglobin and DPG with the DOCKING program of the module INSIGHTII. In order to optimize the non-bonding (van der Waals and Coulomb) interaction energy between the two molecules, the docking has been repeated several times with different initial orientations of the ligand. Molecular mechanics calculations were used to refine the potential complexes. Because of the large number of atoms in the model, the following constraints have been imposed: (a) a subset, centered on DPG, comprising all the residues with at least one atom within a 14 Å distance from the DPG molecule was created; (b) all the atoms external to the subset remained fixed, even if their non-bonding interactions with all the relaxing atoms were calculated. Using AMBER All Atom force field, the 1–4 van der Waals interactions (van der Waals interactions separated only by three bonds) were reduced by a factor of 0.5 in order to avoid their overestimation [11]. Using CVFF force field, a harmonic potential was used without the inclusion of cross terms. A dielectric constant of 1.0 was used in both AMBER and CVFF minimizations. In addition, because of the charge on the amino acids residues, electrostatic and non-bond cut off were not employed. The complexes were subjected to energy minimizations for 8000 iterations by the method of steepest descent (root mean square gradient 0.1 kcal mol^{-1} $Å^{-1}$). Afterwards, the system was minimized by the conjugate gradient method, which required about 3000 iterations to converge to a RMS gradient of 0.001 kcal mol^{-1} $Å^{-1}$ (corresponding to a maximum first derivative of less than 0.01 kcal mol^{-1} $Å^{-1}$). Docking procedure and minimization of the different complexes of HbA_{1c} were the same as those employed for HbA_0 complex. However, since there is no X-ray structure, the energy minimization of HbA_{1c} and of HbA_{1c}–DPG complex was followed by 170 ps of molecular dynamics. The HbA_{1c} conformation of minimum energy (refined to a maximum first derivative of 0.1 kcal mol^{-1} $Å^{-1}$) was equilibrated for 20 ps at 300 K. Following the equilibration procedure, the molecular dynamics run was continued for 150 ps. The MD integrations were done using the Verlet leapfrog algorithm with a time step of 1.0 fs, and a non-bond cut off of 15 Å was employed. Analogous procedure was applied to the HbA_{1c}–DPG complex.

2.3.4. Electrostatic potentials

Crystallographic studies [5] have shown that the binding site of 2,3-diphosphoglycerate contains eight ionizable amino acid side chains. DPG itself has two phosphate and one carboxylate charged groups. Since electrostatic interactions are believed to be the driving force in the formation of the Hb–DPG complex, the electrostatic field at the binding site was calculated both for HbA_0 and HbA_{1c} using the DelPhi

software package from MSI. DelPhi applies a macroscopic description of either the protein and the solvent, treating them as continuums with different dielectric constants [14–16].

The electrostatic potential was calculated over the solvent accessible surface of the DPG binding site. The solvent-accessible surface was calculated, according to the Connolly algorithm, rolling a sphere of 1.4 Å over a van der Waals surface of 10 Å centered on the DPG molecule. The atomic coordinates of the subset were mapped onto a three-dimensional grid and the 'focusing' technique was used to reduce the errors on the potential values at the grid boundary points. Two 'finite difference' calculations were performed, with a final grid step of 0.6 Å/grid pt. Additional water molecules that would have been treated as part of the solute by the program were not included in the DelPhi study. All calculations were carried out with a dielectric constant of 2, inside the protein and 80 in the solvent region. The ionic strength parameter, which simulates a solution containing charged molecules was fixed at 0.1450 mol l^{-1}; changes in this parameter did not modify the result appreciably.

3. Results

3.1. Functional studies

The oxygen affinity of glycated hemoglobin, under full stripped conditions, i.e., in the absence of both organic phosphates and chloride, is significantly higher than that of native hemoglobin A (see Table 1). The effect of chloride on the oxygen affinity of both HbA_0 and HbA_{1c}, measured at pH 7.4, is reported in Table 1. Upon addition of chloride ions, the oxygen affinity of both proteins decreases significantly, although the effect is slightly less marked for the modified hemoglobin. The lower effect of chloride on glycated hemoglobin increases the difference in oxygen affinity between the two proteins. Hence, the P_{50} (partial pressure of oxygen at 50% oxygen saturation) values are almost 1.8 × higher in the case of native hemoglobin at all pH values within the range 6.0 to 7.5 (Fig. 2). At pH values ≥ 8.0, the oxygen affinities tend to become identical; this, in turn, implies that the amplitude of the Bohr effect of

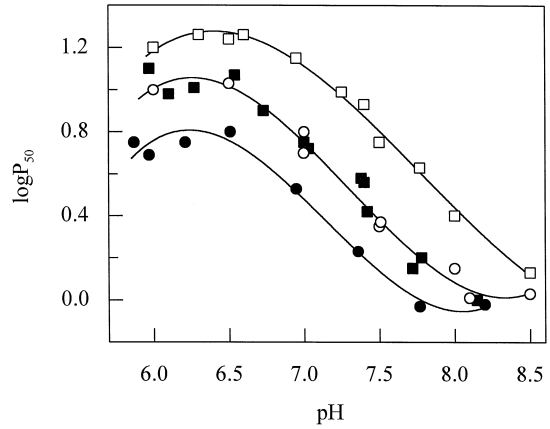


Fig. 2. Variation of the oxygen affinity as a function of pH for HbA_0 and HbA_{1c}. log P_{50} of HbA_0 (open symbols) and HbA_{1c} (closed symbols) as a function of pH at 20°C. Circles refer to measurements performed in MES (2-[*N*-morpholino]ethanesulfonic acid) or HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) or TAPS (*N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid) buffers 0.1 mol l^{-1} + NaCl 0.1 mol l^{-1}. Squares refer to the same buffers + 3 mmol l^{-1} DPG.

Table 1
Some functional properties of HbA_{1c} compared to those of HbA_0

Parameter	Buffer	pH	*T* (°C)	HbA_0	HbA_{1c}
log P_{50}	stripped (HEPES 0.1 M)	7.4	20	0.12 ± 0.01	0.02 ± 0.01
	NaCl 0.1 M (HEPES 0.1 M)	–	–	0.40 ± 0.02	0.20 ± 0.01
	NaCl 0.1 M + DPG 3 mM (HEPES 0.1 M)	–	–	0.87 ± 0.03	0.55 ± 0.02
K_{Deoxy} (M^{-1}) (DPG)	NaCl 0.1 M (HEPES 0.1 M)	7.4	20	$(3.6 \pm 0.4) \cdot 10^4$	$(1.4 \pm 0.1) \cdot 10^4$
K_{Oxy} (M^{-1}) (DPG)	–	–	–	$(3.5 \pm 0.6) \cdot 10^2$	$(2.1 \pm 0.2) \cdot 10^2$
K_{Deoxy}/K_{Oxy} (DPG)	–	–	–	$(1.0 \pm 0.2) \cdot 10^2$	$(0.7 \pm 0.2) \cdot 10^2$
$K_{Overall}$ (M^{-1}) (DPG)	–	–	–	$(5.6 \pm 0.6) \cdot 10^3$	$(1.2 \pm 0.2) \cdot 10^3$

The table reports log P_{50} of HbA_0 and HbA_{1c} measured under different experimental conditions and the binding constants of DPG to the oxygenated and deoxygenated derivatives of the two hemoglobins.
Relative ratios and overall binding constant values are also reported.

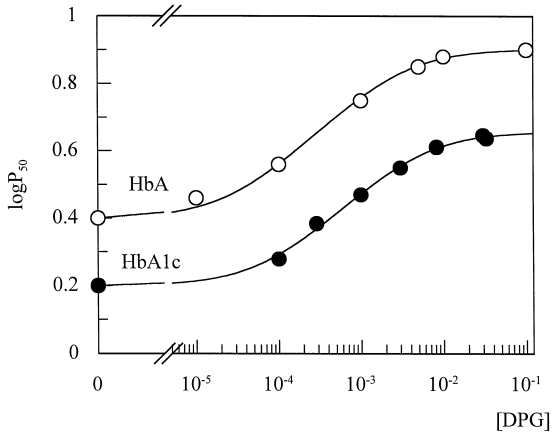


Fig. 3. Variation of the oxygen affinity as a function of DPG concentration for HbA_0 and HbA_{1c}. log P_{50} of HbA_0 (open symbols) and HbA_{1c} (closed symbols) as a function of DPG concentration at 20°C. HEPES 0.1 mol l^{-1} and NaCl 0.1 mol l^{-1}.

glycated hemoglobin is reduced with respect to that of the native hemoglobin.

The oxygen binding properties of naturally glycated hemoglobin have been studied in comparison with those of HbA_0 as a function of pH in the absence and in the presence of 2,3-diphosphoglycerate, under conditions of constant concentration of chloride (100 mM). The difference between the two proteins becomes much more marked in the presence of 2,3-diphosphoglycerate since the effect of this effector is significantly reduced by the chemical modification at the level of the amino terminal of Val NA1. On the whole, the oxygen affinity of glycated hemoglobin in the presence of DPG corresponds to that of the native hemoglobin in the presence of only chloride (Fig. 2). The reduction of the Bohr effect of the naturally glycated Hb remains evident also in the presence of DPG.

The interaction of DPG with native and glycated hemoglobins has been investigated in more detail at

Table 2
RMS deviations between the X-ray structure of HbA_0 and the minimized structures obtained by AMBER All Atom and CVFF force field

RMS Gradient	RMS Backbone	RMS Heavy atoms
AMBER All Atom		
0.001	0.704	0.871
CVFF		
0.001	0.821	0.945

Root mean square (RMS) deviations (Å) for the minimized backbone and heavy atoms of the subset compared with the coordinates from the starting crystal structure are reported in the table. Calculation of RMS deviations at an RMS gradient of 0.001 kcal mol^{-1} $Å^{-1}$.

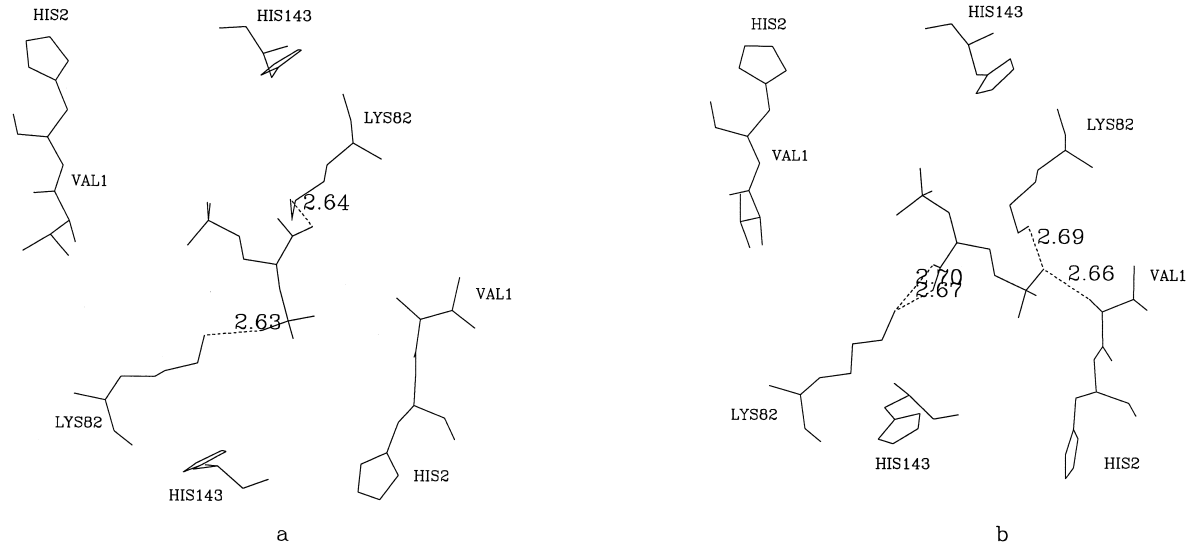


Fig. 4. A comparison between the two structures of minimum energy (a and b) of HbA_0–DPG complex. Hydrogen bonding interactions of DPG with the binding site of HbA_0 are shown (dotted lines). All distances in Angstrom (Å).

Table 3
Dihedral angles of glycated *N*-methylvalinamide

Conformation	β	γ	δ
I	180	−100	180
II	180	−160	180
III	−80	160	−80

The angles correspond to the values of β, γ and δ in the conformations of minimum energy of glycated *N*-methylvalinamide. All values in degrees.

Table 4
Dihedral angles of the Amadori adduct in β_1 and β_2 chains

	β	γ	δ
β_1	120	180	−160
β_2	180	180	−60

The angles correspond to the values of β, γ and δ in the lowest energy conformation of the Amadori adduct of β_1 and β_2 chains. The conformational search was performed in the presence of the potential energy of the unmodified protein.

pH 7.4, as reported in Fig. 3, which shows the change in log P_{50} induced by increasing concentrations of DPG.

Two main overall features appear immediately evident:

a) the total amplitude of the effect is reduced in the case of glycated Hb (see Fig. 3);

b) the overall affinity constant of HbA_{1c} for 2,3-diphosphoglycerate is about 5× lower than that of native human hemoglobin.

Analysis of the data in Fig. 3, in terms of the two states allosteric model, resulted in the parameters reported in Table 1. The calculated equilibrium constants for the binding of DPG to the R (representative of the oxygenated derivative) and T (representative of the deoxygenated derivative) state of the two hemoglobins clearly indicate that both conformational states are affected by the chemical modification, although to a different extent. Thus, while the affinity of the T-state of HbA_{1c} for DPG is about 2.6× lower than that of the corresponding conformation of HbA_0, the affinity constant of the R-state is less affected being only 1.7× lower than that of the R-state of native human hemoglobin. On the whole, the ratio K_{deoxy}/K_{oxy} is decreased by about 30% by glycation of the N-terminal of the β chains.

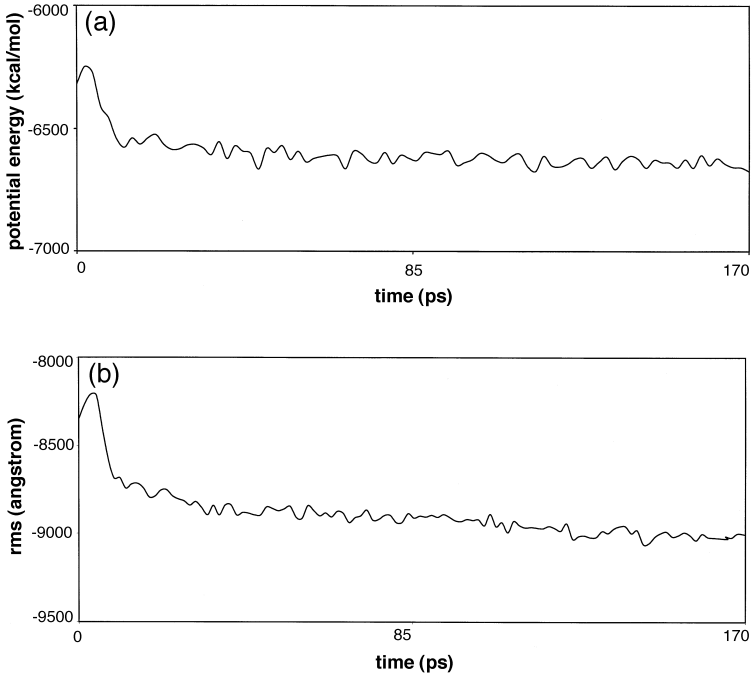


Fig. 5. Time evolution of the potential energy for HbA_{1c} (a) and HbA_{1c}–DPG complex (b), respectively.

Moreover, glycation of the amino terminal residues of the β chains results in an overall destabilization of the DPG binding, since the overall affinity constant decreases by about $5\times$ (see Table 1).

3.2. Molecular modeling studies

During docking simulations of deoxygenated hemoglobin-2,3-diphosphoglycerate interactions, the lowest non-bonding energy corresponds to the structure proposed by Arnone on the basis of X-ray diffraction studies [5]. In particular, AMBER molecular mechanics calculations show two low-energy conformations of the complex which are related by almost a 180° rotation (Fig. 4a and b) and in general agreement with the two different orientations of the asymmetric DPG molecule seen by Arnone within the central cavity between the β chains. On the whole, the anionic groups of DPG interact with the eight cationic groups which form the polyphosphate binding site and precisely with Val 1, His 2 and 143, and the Lys 82. The main difference between the two calculated structures involves the two Lys 82 and the Val 1 of β_1 chain.

In one low energy conformation (Fig. 4a), the carboxylate and one phosphate group of the effector molecule form two hydrogen bonds involving both Lys 82. In the second one (Fig. 4b), the same groups interact with both Lys 82 and also with Val 1 of β_1 chain, forming four hydrogen bonds. This finding is at variance with the structure proposed by Arnone: thus, the DPG molecule does not interact alternatively with the dyad related lysine residues but with both lysine residues at the same time. In this respect, it should be noted that in a more recent study, concerning the X-ray structure of the human deoxyhemoglobin–DPG complex performed on crystals obtained at low-salt concentration [17], the contemporaneous involvement of both lysine residues has been clearly pointed out. However, in that case, the two lysine residues are bridged only by the carboxyl group of the diphosphoglycerate molecule. On the basis of this comparison, the calculated structures may be considered as depicting a somewhat intermediate situation with respect to those represented by the structures proposed by Arnone [5] and by Richard et al. [17]. On the whole, the present computational analysis, emphasizing both the role of the Lys 82 residues and of the whole cationic field contributing to the interactions, outlines once more the existence of different structural subpopulations as consequence of the conformational fluctuations of DPG inside the β-cleft.

The minimized structures of HbA_0 obtained by the molecular mechanics calculations were superimposed onto the initial X-ray structure and the RMS deviations were calculated for both the subsets at the end of the energy refinement. The structural results on the effect of AMBER All Atom and CVFF force fields are reported in Table 2. The results, confirming the reliability of the molecular mechanics methods employed in this study, prompted us to apply molecular mechanics force field to the study of HbA_{1c} and of HbA_{1c}–DPG complex. Table 3 reports the dihedral angles of the three low energy conformations (I, II, III) of glycated *N*-methylvalinamide, while the angles in the lowest energy conformation

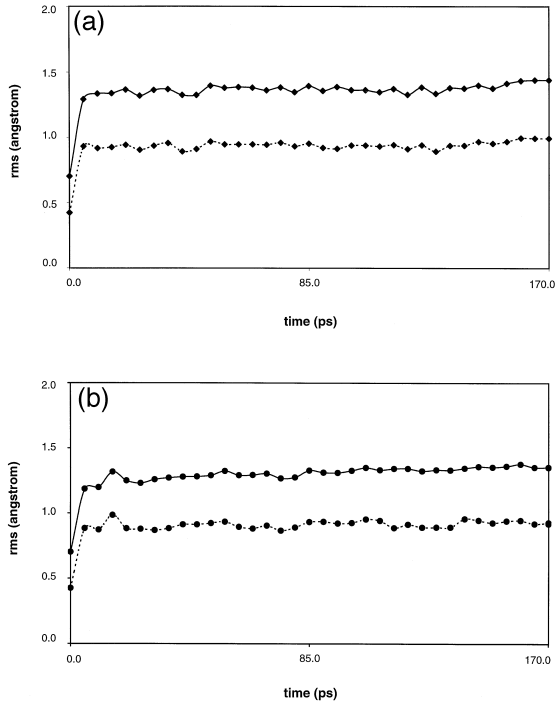


Fig. 6. Root mean square deviation in position away from the crystallographic coordinates for the heavy atoms (—) and for the backbone atoms (---) of HbA_{1c} (a) and HbA_{1c}–DPG complex (b), respectively.

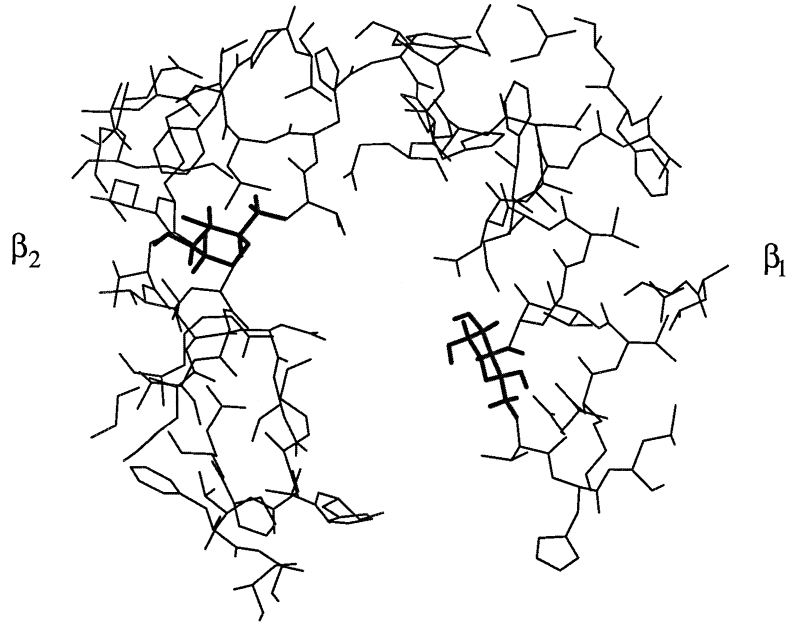


Fig. 7. Central cavity of deoxy-HbA$_{1c}$. The sugar moiety of β_1 chain is directed towards the cavity, while the sugar moiety of β_2 enters the polypeptidic chain.

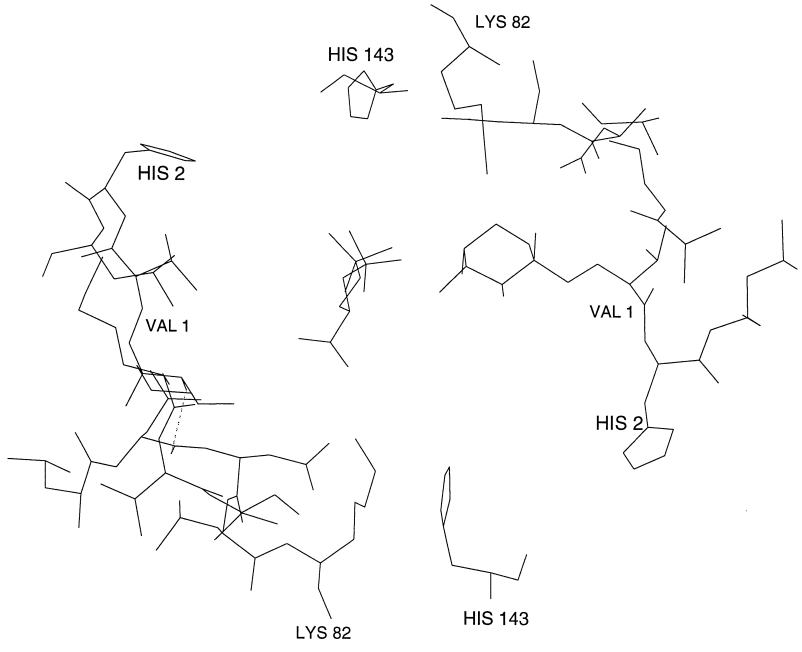
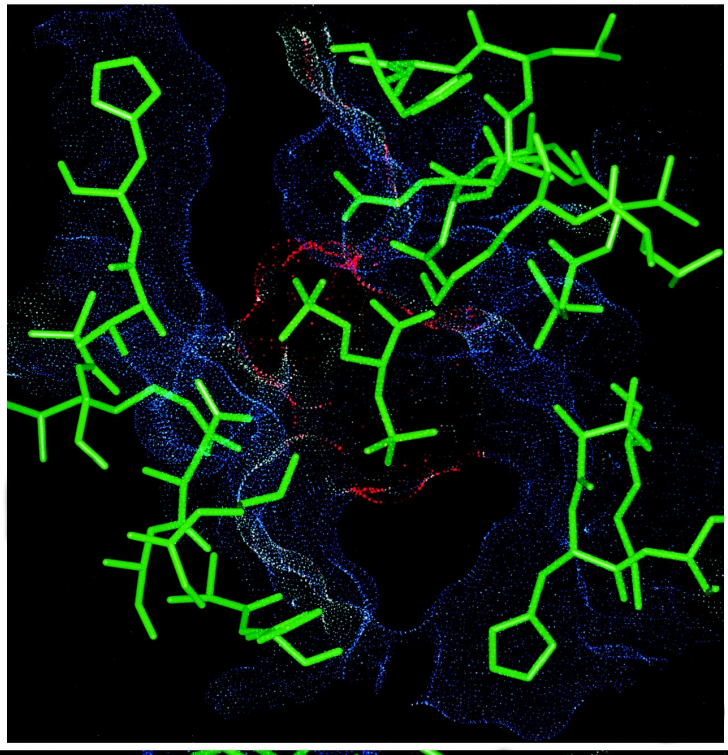
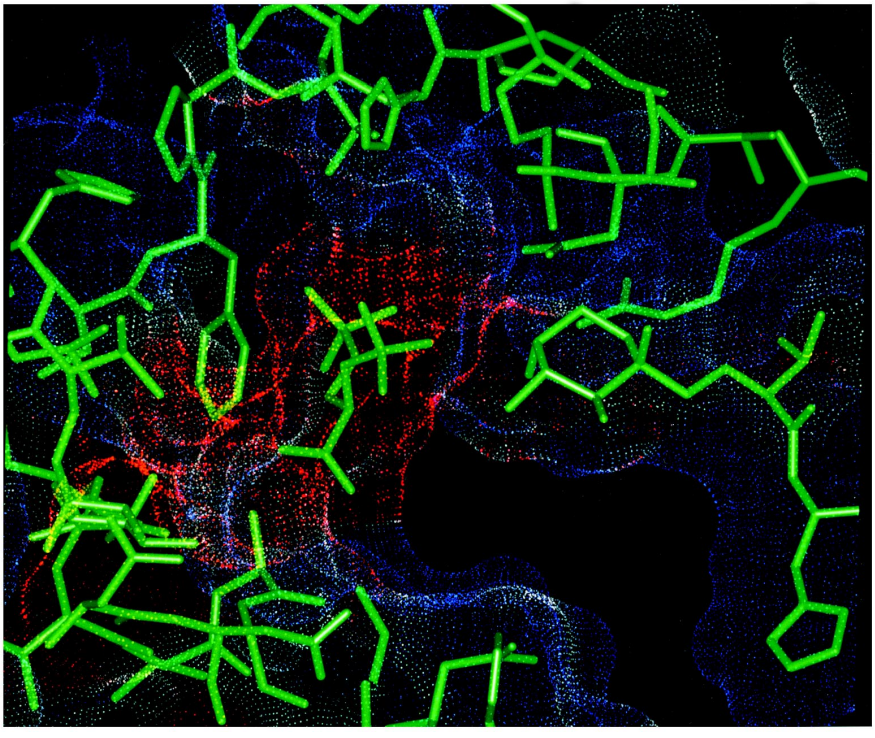


Fig. 8. The proposed model for HbA$_{1c}$–DPG complex. The ligand shifted its orientation in order to reduce the interaction with the Amadori adduct of β_1 chain.





of the Amadori adduct of β_1 and β_2 chains are reported in Table 4.

The analysis of the potential maps showed that the conformational search on the Amadori adduct β_2 chain is affected by the orientation of the adduct towards the polypeptidic chain. Only a small angular range is allowed to the dihedral angles, because all the other angles provide high steric hindrance. This result was confirmed by the molecular dynamics calculations. Based on the analysis of the time course of the trajectory vs. the potential energy (Fig. 5a and b) and vs. the RMS deviation (Fig. 6a and b) the simulations were judged to be stable. The averaged structural properties of HbA_{1c} and HbA_{1c}–DPG complex were evaluated from the final 80 ps of simulation.

The model of DPG binding site of HbA_{1c} is shown in Fig. 7. The binding site of DPG is not symmetrically affected by glycation of valine residues: the Amadori adduct of the β_1 chain enters the accessible region of the binding site, whereas the adduct of β_2 is directed away from the site and towards the polypeptide chain. With respect to the interaction with DPG, the model of the complex is shown in Fig. 8. The calculations indicate that: (i) the effector molecule binds to the modified hemoglobin in only one orientation, the structure of the complex being different from both the structures identified for the native protein; (ii) the DPG molecule has shifted its orientation because of the steric hindrance of the deoxy-D-fructopyranose ring linked to the Val 1 residue of β_1.

Finally, Fig. 9a and b show the solvent accessible surface, color-coded according to the electrostatic potentials, respectively for HbA_0 and HbA_{1c}, calculated by using the DelPhi software package. As shown by the figures, the electrostatic potential is uniformly positive within the HbA_0 binding site while, in the case of glycated hemoglobin, a surface of negative potential facing the negatively charged groups of DPG is present on the Amadori adduct of Val 1. Hence, the decrease of the electrostatic field at the level of the glycation site, i.e., of Val 1, contributes to give a major role to both Lys 82 residues which appear to be the most positively charged residues of the site.

4. Discussion

Oxygen equilibria of glycated hemoglobin (HbA_{1c}) under full stripped conditions, i.e., in chloride and DPG free medium, show a small but significant increase in oxygen affinity compared to HbA_0 (see Table 1). This implies that the two glucose moieties covalently attached to the NH_2-termini of the β chains interfere with the allosteric equilibrium of the T and R conformational states which characterize human hemoglobin A_0. The perturbation is not dramatic, but still significant and indicative of a slight stabilization of the high-affinity conformation of the protein induced by the chemical modification 'per se'.

Upon addition of chloride at physiological concentration (100 mM), the oxygen affinity of both HbA_0 and HbA_{1c} decreases significantly, the effect being more marked for the unmodified human hemoglobin (see Table 1). Regarding the structural perturbation at the basis of the observed functional alteration, we like to consider a recent proposal concerning the allosteric properties of bovine hemoglobin [18–20]. In the light of those results, no one of the cationic groups lining the central cavity between the β chains may be considered as a specific chloride binding site. In other words, the binding site is the whole cavity and what is important is the ratio positive/negative charges. Hence, the lower effect of chloride seen in HbA_{1c} should be attributed to the neutralization of the positive charge of the Val 1 residues brought about by glycation.

Moreover, if one considers that Val 1 α and Lys 82 β are known to contribute to the chloride dependent part of the Bohr effect of human hemoglobin

Fig. 9. Electrostatic potential along the solvent-accessible surface of HbA_0–DPG (a) and HbA_{1c}–DPG (b) complexes. Color code: red $V \leq -1\ KT/e$; white $-1\ KT/e < 1\ KT/e$; blue $V \geq 1\ KT/e$. The electrostatic potential is uniformly positive at the N-terminal of the β chains in HbA_0 (a). On the contrary, the Amadori adduct decreases the electrostatic potential at the binding site of HbA_{1c} (b). The figure also reveals the effect of α_2 36 (C1) Phe which is very close to the ligand in the HbA_{1c} binding site.

A_0, it appears evident as glycation of Val 1 residues could be at the basis of a small but significant reduction of the pH dependence of the oxygen affinity.

As far as the DPG is concerned, the decreased effect on the affinity of HbA_{1c} has been reported several years ago [21] and attributed generically to the covalent modification of the amino terminal by glucose. In a more recent study, the small effect of DPG has been confirmed and attributed to a slight decrease of the T-state binding constant [4]. However, a more detailed structural and functional characterization was still lacking. From the data reported here, it appears that the ratio K_{deoxy}/K_{oxy} is decreased by about 30% by glycation of the N-terminal of the β chains resulting in a decrease of the overall affinity constant for DPG of about 5 × (see Table 1).

The two models of DPG binding that emerged from our computational approach (Fig. 4a,b and Fig. 8) seem to agree with the functional alterations observed in the present study. Hence, the decrease of the DPG binding constant has mainly an entropic origin because of the limitations imposed by the stereochemistry of the glucose moiety on the conformational fluctuations of DPG inside the β-cleft. Moreover, the decreased amplitude of the effect could well be explained by the specific structure of the complex. Thus, based on the work of Arnone, one of the most conspicuous effect of DPG on the structure of each β subunit is a movement of the A helix, that is pulled along moving closer to helix E and the EF corner (Table 5). This perturbation arises, upon DPG binding, because it attracts the charged amino groups of Val 1 and the side chain of His 2. As pointed out by Richard et al. [17], this attraction, if not direct, could be mediated by two water molecules. The peculiar stereochemistry of the glycated Hb–DPG complex together with the chemical modification of the Val 1 residues greatly lowers the possibility of such an effect, inhibiting the above mentioned movement of the A helix. This is a point of great importance since this helix seems to play a major role in the modulation of the hemoglobin function. In fact, during the course of a careful investigation on the structural and functional properties of bovine hemoglobin [18–22], which is known to be characterized by an intrinsic low oxygen affinity, it has been pointed out that the structure of this molecule reveals a significant shift of the helix A closer to the molecular dyad even in the absence of the organic phosphate effector.

Table 5
Distances between the helix A backbone atoms of HbA_0 and HbA_{1c}. Distances (Å) were measured in the absence (−) and in the presence (+) of DPG

Amino acid	Atoms	HbA_0		HbA_{1c}	
		−DPG	+DPG	−DPG	+DPG
Val 1 (NA1)	N–N	17.73	14.87	17.52	17.42
	CA–CA	17.83	16.54	18.55	18.52
	C–C	18.83	17.90	20.34	20.39
His 2 (NA2)	N–N	18.21	18.57	20.75	20.39
	CA–CA	19.91	20.57	22.77	22.52
	C–C	22.68	23.16	24.31	24.72
Leu 3 (NA3)	N–N	24.18	23.80	24.67	24.59
	CA–CA	26.96	26.53	26.66	26.62
	C–C	28.73	28.34	29.12	28.90
Thr 4 (A1)	N–N	30.68	30.17	31.12	31.15
	CA–CA	32.79	32.28	33.09	33.12
	C–C	34.30	34.15	34.70	34.65
Pro 5 (A2)	N–N	35.53	35.44	35.80	35.74
	CA–CA	37.56	37.48	37.46	37.43
	C–C	39.63	39.61	39.64	39.67
Glu 7 (A4)	N–N	39.25	39.25	39.29	39.29
	CA–CA	37.97	37.97	38.02	38.13
	C–C	37.84	37.86	37.70	37.90
Lys 8 (A5)	N–N	36.85	36.83	37.26	37.21
	CA–CA	37.17	37.15	37.27	37.23
	C–C	40.14	40.13	40.13	40.13

Glu 6 (A3) is not reported since it does not belong to the subset which has been minimized.

Finally, it appears more and more evident from the available data, that the charge cluster of the major anion binding site of hemoglobin represents a key region of the molecule. The extent to which this cluster may operate as a structural determinant of functional modulation will depend on the charge density and on the stereochemistry of the residues involved. Hence, glycation of the Val 1 residues of both β chains does not impair the binding of DPG but imposes a different mode of binding by changing the internal geometry of the complex and the surface distribution of the positive electrostatic potential of the binding pocket. These structural alterations have important reflections on the interplay of the various heterotropic ligands and therefore, on the functional properties of the hemoglobin molecule.

References

[1] M.F. Perutz, Annu. Rev. Physiol. 52 (1990) 1.
[2] R.J. Koenig, S.H. Blobstein, J. Biol. Chem. 252 (1997) 2992.
[3] C.I. Neglia, H.J. Cohen, A.R. Garber, P.D. Ellis, S.R. Thorpe, J.W. Baynes, J. Biol. Chem. 257 (1983) 14279.
[4] M. Coletta, G. Amiconi, A. Bellelli, A. Bertollini, J. Carsky, M. Castagnola, S.G. Condò, M. Brunori, J. Mol. Biol. 203 (1988) 233.
[5] A. Arnone, Nature 237 (1972) 146.
[6] M. Castagnola, P. Caradonna, M.L. Salvi, D.V. Rossetti, J. Chromatogr. 272 (1983) 51.
[7] M. Castagnola, P. Caradonna, L. Cassiano, C. Degen, F. Lorenzin, D.V. Rossetti, M.L. Salvi, J. Chromatogr. 307 (1984) 91.
[8] B. Giardina, G. Amiconi, Methods Enzymol. 76 (1981) 417.
[9] J.S. Kavanaugh, P.H. Rogers, D.A. Case, A. Arnone, Biochemistry 31 (1992) 4111.
[10] H.H.-L. Brady, J. Brady, M. Karplus, Proc. Natl. Acad. Sci. U.S.A. 82 (1985) 1697.
[11] S.J. Weiner, P.A. Kollman, D.T. Nguyen, D.A. Case, J. Comp. Chem. 7 (1986) 230.
[12] S.W. Homans, Biochemistry 29 (1990) 9110.
[13] P. Dauber-Osguthorpe, V.A. Roberts, D.J. Osghuthorpe, J. Wolff, M. Genest, A.T. Hagler, Proteins 4 (1988) 31.
[14] I. Klapper, R. Hagstrom, R. Fine, K. Sharp, B.H. Honig, Proteins 1 (1986) 47.
[15] M.K. Gilson, B.H. Honig, Proteins 4 (1988) 7.
[16] A. Nicholls, B.H. Honig, J. Comp. Chem. 12 (1991) 435.
[17] V. Richard, G.G. Dodson, Y. Mauguen, J. Mol. Biol. 223 (1993) 270.
[18] M.F. Perutz, G. Fermi, C. Poyart, J. Paglier, J.A. Kister, J. Mol. Biol. 232 (1993) 536.
[19] A.M. Nigen, J.M. Manning, J.O. Alben, J. Biol. Chem. 255 (1980) 5525.
[20] M.F. Perutz, J.V. Kilmartin, K. Nishikura, J.H. Fogg, P.J.G. Butler, H.S. Rollema, J. Mol. Biol. 138 (1980) 649.
[21] M.J. McDonald, M. Bleichman, H.F. Bunn, J. Biol. Chem. 254 (1976) 702.
[22] C. Fronticelli, I. Pechik, W.S. Brinigar, J. Kowalczyk, G. Gilliland, J. Biol. Chem. 269 (1994) 23965.