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Characterization and oxygen binding properties of des-Arg human hemoglobin

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The role of chloride in the stabilization of the deoxy conformation of hemoglobin (Hb), the low oxygen affinity state, has been studied in order to identify the nature of this binding. Previous studies have shown that arginines 141α could be involved in the binding of this ion to the protein. Thus, des-Arg Hb, human hemoglobin modified by removal of the α-chain C-terminal residue Arg141α, is a possible model for studies of these interactions. The loss of Arg141α and all the salt bridges in which it participates is associated with subtle structural perturbations of the α-chains, which include an increase in the conformational flexibility and further shift to the oxy state, increasing oxygen affinity. Thus, this Hb has been the target of many studies of structural and functional behavior along with medical applications. In the present study, we describe the biochemical characterization of des-Arg Hb by electrophoresis, high-performance liquid chromatography and mass spectroscopy. The effects of chloride binding on the oxygen affinity and on the cooperativity to des-Arg Hb and to native human hemoglobin, HbA, were measured and compared. We confirm that des-Arg Hb presents high oxygen affinity and low cooperativity in the presence of bound chloride and show that the binding of chloride to des-Arg does not change its functional characteristics as observed with HbA. These results indicate that Arg141α may be involved in the chloride effect on Hb oxygenation. Moreover, they show that these residues contribute to lower Hb oxygen affinity to a level compatible with its biological function.

Key words: des-Arg hemoglobin; Oxygen affinity

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Introduction

Hemoglobin (Hb) is an allosteric protein found mainly in red cells whose main function is to deliver oxygen from the lungs to the tissues. Hbs are tetrameric and are composed by two α-chains and two β-chains, each containing a heme group to which a single oxygen molecule binds reversibly (1). To perform its function, Hb assumes different structural conformations with distinct O_2 affinities. The shift between these states is regulated by the presence of allosteric effectors such as organic phosphates and monovalent anions. These anions preferentially bind to the deoxy form (T-state), increasing the stability of the low-affinity T-state compared to the high-affinity R-state, thus increasing p_{50}, the O_2 pressure at half saturation (2). In human red cells, 2,3-diphosphoglycerate is the main allosteric effector of hemoglobin A (HbA), whose binding has been very well described in the literature. This phosphate has high affinity for HbA, with binding occurring at a very specific site that consists of eight cationic residues located in the central cavity of the protein (3). However, it has been shown that HbA also binds monovalent anions that also stabilize the deoxy form, reducing its affinity for O_2. The most abundant of these physiological ions is the chloride ion (4).

The nature of the chloride modulation of Hb affinity for

O_2 has been the object of many studies in the past years, but no consensus has been reached about how this binding occurs. First it was suggested that this binding would involve specific sites, i.e., the N-terminal Val 1 β2 and the guanidine group of Arg 141α1, the C-terminal residue (5-7). In other studies, Perutz et al. (8) proposed that the binding of chloride to Hb is not specific but is due to the opening of the central cavity during the T-R transition, in which the positively charged amino acids would attract the anions. In more recent studies, Fronticelli et al. (9), comparing human and bovine Hb, again identified specific chloride binding sites, justifying their findings about the different affinities of the two Hbs for oxygen (9-11).

In 1999, Hui et al. (12) proposed Arg141α1 and Arg141α2 as the chloride binding sites based on the comparison of crystallographic structure maps of deoxy HbA and chemically modified Hbs. Crystallographic studies carried out by our research group have also identified these arginines as chloride sites. This evidence was obtained by analyzing maps of different electronic densities of deoxy Hb crystallized in the total absence of anions and in the presence of high chloride concentrations. In the same study, arginines 92α1 and 92α2 were also identified as these possible sites. These four sites follow the chloride binding pattern identified for other proteins (Seixas FAV, Colombo MF**,** unpublished results).

des-Arg Hb, that loses Arg141α1 and Arg141α2, the presumed sites of chloride binding, is found *in vivo* in the placenta, where the carboxypeptidase-M enzyme, present in the microvillous syncytiotrophoblast, removes the arginine residues (13).

These residues play an important role in stabilizing the T structure by interaction with other amino acids. Arginine 141α forms salt bridges with Lys127α2, Asp126α2, and Valα2 (14,15), with a consequent increase in molecule flexibility that shifts the equilibrium to the high-affinity structure R and reduces the cooperativity value indicated by the Hill coefficient. Despite this structural alteration, there is no modification in the heme group region and the Tyr140α residue is positioned in the same direction as in HbA (16). Because of these functional changes, des-Arg Hb has been used in vasoconstrictive studies and in the development of blood substitutes (17).

The connection of this structural and functional behavior in Hbs, possibly modulated by chloride binding, has led us to study the biochemical and functional properties of des-Arg Hb. One approach to the elucidation of this matter is to perform comparative studies of human Hb and chemically modified des-Arg Hb, obtained by the carboxypeptidase-B (CPB) hydrolysis, which specifically removes the 141α1 and 141α2 arginine residues (18).

Material and Methods

A blood sample was collected from a healthy non-smoking adult male by venipuncture using a heparinized syringe, after obtaining written informed consent. The purification procedure followed the method previously described by Colombo and Bonilla-Rodriguez (19), based on the protocol of Williams and Tsay (20) and was monitored by non-denaturating PAGE. The purified HbA was extensively deionized by several passages through an Amberlite MB-1 column.

des-Arg Hb was obtained from HbA by enzymatic cleavage, as previously described in the literature (21-24). C-terminal residues were removed by the digesting chains with CPB (grade COBC, Worthington, USA) in 20 mM Tris-HCl buffer, pH 8.0, at an enzyme to substrate weight ratio of 1:100 at room temperature for approximately 2 h. The modified Hb was purified by gel filtration through a Sephadex G-25 (PD-10 column, GE Healthcare, USA) equilibrated with 10 mM HEPES-NaOH buffer, pH 6.9, for the removal of free arginine residues.

Once the arginine residues were removed from the solution, the sample was purified chromatographically on an ion-exchange column using DEAE A50 Sephadex, equilibrated with 10 mM HEPES-NaOH containing 0.1 M NaCl buffer, pH 7, for separation of des-Arg Hb from the CPB, on the basis of their isoelectric points: (pI = 7.1 for Hb *vs* pI = 6.0 for CPB) (25,26). HbA and des-Arg Hb were concentrated using Amycon concentrators and stored in liquid nitrogen until use.

The efficiency of the CPB hydrolysis was checked by ESI-Q-TOF mass spectrometry using a Q-TOF Micro apparatus equipped with an electrospray ionization source operated in the positive or negative ion mode. Samples were diluted in 50% acetonitrile, 0.1% trifluoroacetic acid in ultrapure water and applied to the equipment using a syringe pump at a flow rate of 5 µL/min. This procedure permits comparison of the molecular mass of Hb and the products. The difference in mass can be used to identify amino acids released by CPB treatment.

des-Arg Hb was characterized by electrophoretic methods and chromatography.

Isoelectric focusing

HbA and des-Arg Hb samples were analyzed by isoelectric focusing on agarose gel in the presence of the ampholytes, forming a linear pH gradient. The gel was positioned in a horizontal direction and placed on a cool platform. Electrolyte strips saturated with 1 M phosphoric acid and 1 M sodium hydroxide were placed at the anode and cathode extremes of the gel, respectively. The plati-

num electrode connected to the gel maintains the gel in contact with the electronic apparatus. The samples were applied to the center of the gel and subjected to an electrical field of 8 A, with a cathode and an anode pole at each extreme of the gel.

Electrophoretic methods

The HbA and des-Arg Hb samples were characterized by electrophoresis at alkaline pH on cellulose acetate with Tris-EDTA-boric acid (TEB) buffer at pH 8.6, and at acidic pH on agar gels (Oxoid, UK) with phosphate buffer at pH 6.2, as described by Bonini-Domingos (27).

Globin chain analysis

The α- and β-globin chains were separated by the urea-2 mercaptoethanol method and analyzed by electrophoresis in TEB at alkaline pH 8.6 on acetate cellulose according to the method described by Schneider (28). Acidic pH electrophoresis was carried out on cellulose acetate at pH 8.7 in TEB-urea-2 mercaptoethanol buffer and on a 12% polyacrylamide gel with 5% acetic acid buffer. When the electrophoresis was completed, amido black on Coomassie blue staining was used to detect protein, followed by destaining with 7% acetic acid and 30% methanol solution (29).

High-performance liquid chromatography

Hb and des-Arg Hb were separated on the basis of their ionic interactions with the cationic column by elution with two sodium phosphate buffers to form a pH gradient of increasing ionic strength. The apparatus contains 3 x 0.46-cm nonporous cation exchange resin, which was eluted at 2 mL/min (30). The elution profile of the column was standardized for quantitative screening of Hb variants using the heterozygote β thalassemia kit (BioRad, USA), containing the Hbs A2/F patterns, which allows the quantification of Hb A2, Hb F, Hb A, Hb S, and Hb C. Protein elution was monitored inline by absorbance measurements at 415 and 690 nm as a function of time.

Oxygen binding curves

Oxygen binding experiments were performed with 60 µM (heme) Hb in 10 mM HEPES-NaOH buffer, pH 7.2, at room temperature by the tonometric-spectrophotometric method (31). The protein concentrations were estimated using the extinction coefficients reported by Benesch et al. (32). Oxygen binding curves were measured in the presence and in the absence of 100 mM NaCl, and the functional parameters p_{50} and Hill's coefficient (n_{50}), a measure of cooperativity, were calculated from the Hill Plot by linear regression near half saturation.

Results and Discussion

HbA and des-Arg Hb (prepared from the same Hb sample) were submitted to mass spectrometry in order to assess the efficiency of arginine release and to determine if the Hb and its products were homogeneous (Figure 1). The masses of the human Hb chains are 15126.38 Da for the α-chain and 15867.24 Da for the β-chain. The masses of the des-Arg Hb are 14970.74 ± 0.16 Da for the α-chain and 15867.36 ± 0.21 Da for the β-chain. When the mass of the des-Arg α-chain is substracted from that of the HbA α-chain, the difference is 155.99. This corresponds to the mass of one arginine residue, 156.17 per α-chain. No significant quantities of other proteins or α-chains were detected.

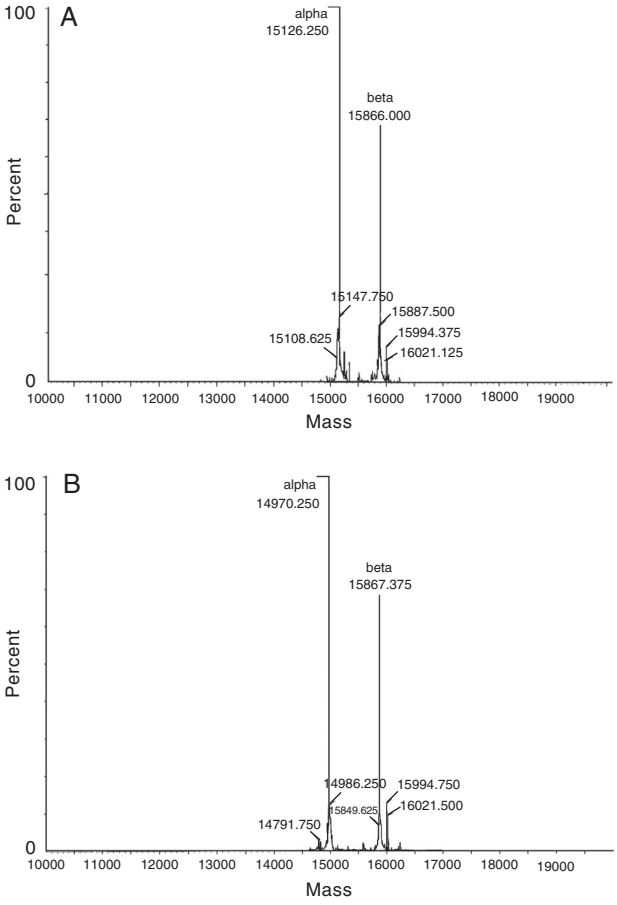

Figure 1. Mass spectrometric analysis of hemoglobin A (HbA), and des-Arg Hb. *A*, The masses of the HbA chains are 15126.38 Da for the α-chain and 15867.24 Da for the β-chain. *B*, The masses of the des-Arg Hb chains are 14970.74 ± 0.16 for the α-chain and 15867.36 ± 0.21 for the β-chain. The difference between the α-chains is ~156 Da, the exact mass of the arginine residue.

The methods used for the electrophoretic characterization of des-Arg Hb were those widely used in studies of Hb variants. The electrophoretic pattern of des-Arg Hb migration differs from that found for intact Hb. Figure 2 shows the results of the isoelectric focusing experiment, which reveal a difference in the migration of the two Hbs. des-Arg Hb (lanes 1 and 3) migrates more towards the cathode than intact Hb (lane 2) due to the loss of two positively charged arginine residues.

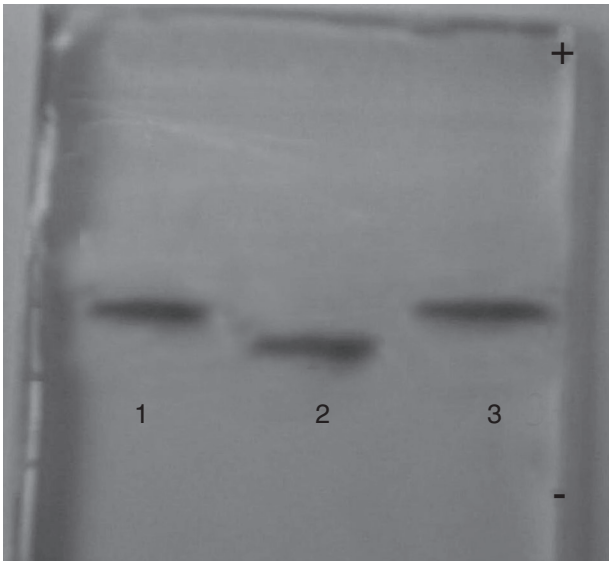

Figure 2. Isoelectric focusing of hemoglobin A (HbA) and des-Arg Hb. The volume applied for each sample was 10 µL of the stock concentrations 7 mM/heme for des-Arg Hb and 11 mM/heme for HbA. *Lanes 1* and *3*, des-Arg Hb; *lane 2*, HbA. (+) = positive pole and (-) = negative pole. des-Arg Hb migrated more to the positive pole than HbA because of the loss of two positively charged arginine residues in each tetramer.

Hb electrophoresis on cellulose acetate, using intact HbA (lane 1) as control and des-Arg Hb (lane 2) as the analyte, was performed both at acidic pH (6.2) and alkaline pH (8.6), as illustrated in Figure 3. The des-Arg Hb sample migrated towards the positive pole under both pH conditions, reaching a position closer to this cathodic region than the HbA sample. This fact can be explained as a consequence of the loss of two arginine residues with positive charges, leaving des-Arg Hb with a more negative charge than HbA.

Figure 4 shows the results of chain electrophoresis at acidic and alkaline pH. The α- and β-globin chains migrate towards the positive pole. The β-chains of HbA and des-Arg Hb are identical and therefore their migration profile was the same, whereas the α-chains had different patterns. The α-chain of des-Arg Hb migrated closer to the positive polar region than the α-chain of HbA due to the loss of the arginine residues, characterizing specific modification of the charge of the α-chain.

The HPLC chromatograms of Hb and des-Arg Hb are shown in Figure 5. des-Arg Hb is eluted at the position of the acetylated subfraction of HbA (P3). Acetylation of Hb eliminates to positive charges of lysines, residues at neutral pH, which makes the overall charge of the protein more

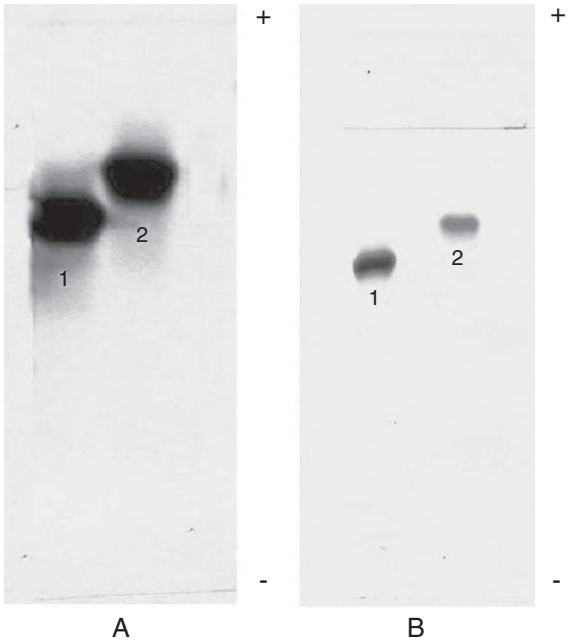

Figure 3. Electrophoresis of hemoglobin on cellulose acetate. *Lane 1*, HbA; *lane 2*, des-Arg Hb. *A*, Phosphate buffer, pH 6.2, stained with Coomassie blue. For each sample, 20 µL of stock concentration of 11 mM/heme for HbA and 7 mM/heme for des-Arg Hb were applied. *B*, Tris-EDTA-boric acid buffer, pH 8.6, and Ponceau stain. For each sample, 15 µL of stock concentration of 11 mM/heme for HbA and 7 mM/heme for des-Arg Hb were applied. The proteins migrate from the negative (-) to the positive pole (+) and in both cases des-Arg Hb migrated closer to the positive pole.

negative (33). The fact that des-Arg Hb presents the same chromatographic behavior as acetylated HbA is consistent with the loss of the positively charged residues by des-Arg Hb.

We determined the oxygen affinity of HbA and des-Arg Hb in the presence and in the absence of chloride ions (Figure 6). The lines through the experimental points represent the best non-linear fit of the parameters of the integrated Hill plot (which takes the functional form of the logistic equation) to the data. These parameters are shown in Table 1.

The results show the different affinities for oxygen in each condition, represented by the sigmoidal adjustments of the curves. The oxygen binding curves are shifted to the left for des-Arg Hb in the absence and in the presence of chloride compared to the curves for HbA under the same conditions. This behavior shows that des-Arg Hb has a higher affinity for oxygen than HbA since the saturation of the protein requires a lower amount of oxygen than that needed for HbA, in both cases. Therefore, a lower p_{50}

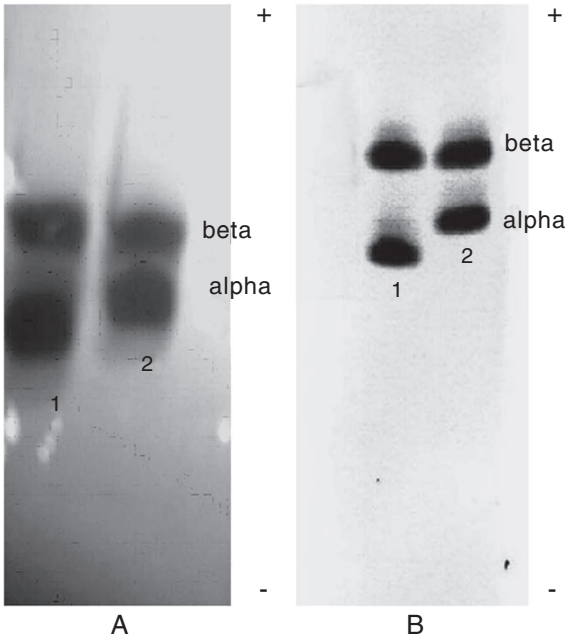

Figure 4. Globin chain electrophoresis. *Lane 1* indicates HbA and *lane 2* indicates des-Arg Hb. *A*, Tris-EDTA-boric acid (TEB) buffer, pH 5.0, on 12% polyacrylamide gel with 5% acetic acid buffer. For each sample, 3 µL of stock concentration of 11 mM/heme for HbA and 7 mM/heme for des-Arg Hb were applied. The gel was stained with Comassie blue for protein detection. *B*, TEB buffer, pH 8.6, in cellulose acetate. For each sample, 50 µL of stock concentration of 11 mM/heme for HbA and 7 mM/heme for des-Arg Hb were applied. The gel was stained with amido black for protein detection. Both electrophoretic runs were carried out in the presence of 8 M urea and 1 M mercaptoethanol. The chains migrate from the negative (-) to the positive (+) pole. The β chains of HbA and des-Arg Hb are identical; however, the α-chains present different patterns of migration, with des-Arg Hb being closer to the positive pole compared to the α-chains of HbA.

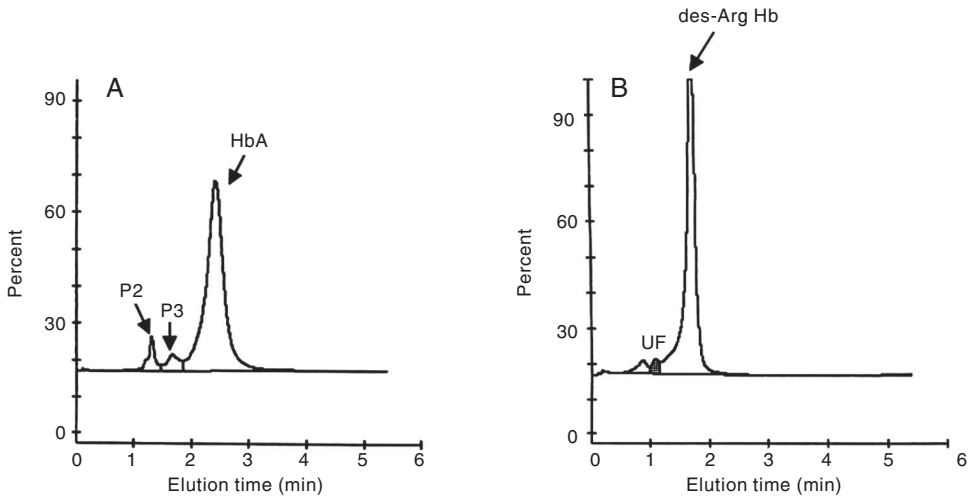

Figure 5. Cation exchange high-performance liquid chromatography of human Hb and the elution times (t). *A*, Chromatogram of HbA: t = 2.4. The glycosylated (P3): t = 1.66 and acetylated (P2): t = 1.3 forms of Hb are also shown. *B*, Chromatogram of des-Arg Hb, with the peak shifted to the acetylated fraction P3: t = 1.71. Also an unknown fraction (U): t = 0.88, and fetal (F): t = 1.81 Hb are present in small amounts.

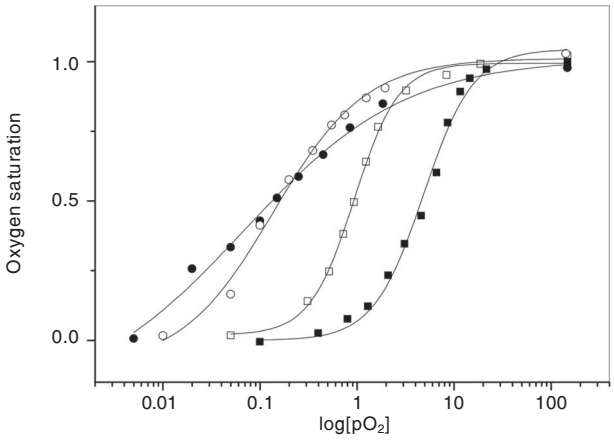

Figure 6. Oxygen binding curves of des-Arg Hb in 10 mM HEPES-NaOH, pH 7.2, in the absence (open circles) and in the presence (filled circles) of 100 mM NaCl. HbA in 10 mM HEPES-NaOH, pH 7.2, in the absence (open squares), and in the presence (filled squares) of 100 mM NaCl. All experiments were carried out with Hb concentration of 60 µM/heme, at room temperature. All data curves were fitted with the best non-linear fit of the parameters of the integrated Hill plot, and the shift to the right of HbA (open and filled squares) indicates its lower affinity for oxygen compared to des-Arg Hb.

Table 1. Oxygen affinities and cooperativities of HbA and des-Arg Hb.

	HbA	des-Arg Hb
p_{50} (mmHg)		
– chloride	1.0 ± 0.4	0.17 ± 0.01
+ chloride	5.6 ± 0.5	0.14 ± 0.01
n_{50}		
– chloride	2.2 ± 0.20	1.0 ± 0.07
+ chloride	2.3 ± 0.20	0.6 ± 0.01

Data are reported as means ± SD. p_{50} = oxygen pressure at half saturation; n_{50} = Hill's coefficient indicating the cooperativity; Hb = hemoglobin. The buffers used were 10 mM HEPES in the absence or in the presence of 100 mM NaCl, at pH 7.2 and 25°C. The hemoglobin concentration was 60 µM/heme.

value for des-Arg Hb in all cases compared to the value for HbA indicates its high affinity for oxygen, which was confirmed for different concentrations of chloride (Tosqui P, Colombo MF, unpublished results).

Table 1 shows the p_{50} and the cooperativity (n_{50}) values for both hemoglobins in the presence and in the absence of chloride. Both Hbs show a higher affinity for oxygen in the absence of chloride, in agreement with the fact that chloride stabilizes the deoxy state. However, when we consider only the curves for des-Arg Hb, we notice that the results for the presence and absence of chloride are similar, a fact that could indicate the loss of chloride binding sites.

Taken together, the results show the characterization of des-Arg Hb, and reflect the structural change in the functional behavior of this Hb. The increased oxygen affinity is a consequence of the loss of stability of the deoxygenated quaternary structure, in which the arginines play an important role. Furthermore, this shift of the equilibrium for the oxygenated state, in the absence and presence of chloride, could act together with the loss of possible binding sites of this anion.

The physiological mechanism of this interaction has not been fully elucidated, but the biochemical analysis of des-Arg Hb can be used to identify a pattern of high-affinity hemoglobins in clinical studies.

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