Proton-linked Subunit Kinetic Heterogeneity for Carbon Monoxide Binding to Hemoglobin from Chelidonichthys kumu*

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The pH dependence of CO binding kinetics to Chelidonichthys kumu hemoglobin (Hb) and human adult Hb has been investigated between pH 2.0 and 9.0 at 20 °C. For both Hbs, CO binding kinetics is characterized by two proton-linked transitions, with different pKₐ values for α- and β-chains in C. kumu Hb, leading to a relevant functional kinetic heterogeneity at most pH values. On the other hand, in human adult Hb the CO binding does not display a functional heterogeneity. Lowering the pH from 9 to 6 brings about a decrease of the CO binding rate constants, to a different extent for human adult Hb and the two chains of C. kumu Hb. Further lowering the pH from 6 to 2 induces an enhancement of CO binding rate constants, probably related to the protonation of proximal His₈⁻Fe atom and the cleavage (or severe weakening) of the His₈⁻Fe bond. The presence of physiological concentrations of ATP (≈3 mM) affects the pH dependence of CO binding kinetics to C. kumu. Moreover, the effect of temperature (between 8 °C and 38 °C) on CO binding kinetics has been investigated at the absence of ATP at different pH values. These results allow to interpret the functional kinetic heterogeneity of C. kumu Hb on the basis of different regulatory aspects in the α- and β-subunits, as suggested by structural considerations.

In mammalian hemoglobins (Hb), the subunit structural heterogeneity of α- and β-chains usually does not bring about any relevant functional heterogeneity, this being especially true for CO binding both in the T and R quaternary states (1, 2). A different behavior has been observed for O₂ binding to human adult Hb, which displays some kinetic heterogeneity (3–6). This finding clearly indicates that reactivity determinants indeed differ for various ligands and possibly for different quaternary conformations (7, 8).

Previous investigations have shown that in the kinetic pathway of CO binding a crucial role is played by proximal His₈⁻Fe, which modulates the ligand reactivity through the energy barrier for the movement of the ferrous iron to the heme plane (9, 10). Thus, even keeping a T-like quaternary conformation, the cleavage (or the severe weakening) of the proximal His₈⁻Fe bond dramatically changes the reactivity of deoxy human adult Hb for CO at low pH, leading to an increase of the rate constant for ligand binding consistent with an R-like kinetic behavior (10). However, no kinetic functional heterogeneity has been detected for human adult Hb, as indicated by the observation that alteration of the proximal His₈⁻Fe bond may be described as an apparent single protonation process. Therefore, the proximal side of the heme pocket appears to display energy parameters closely similar for the two types of subunits in the T state of human adult Hb.

On the other hand, a marked functional heterogeneity has been observed for CO binding to some fish Hbs (11–13), and it becomes especially evident whenever experimental conditions tend to stabilize the T quaternary state also in the liganded form. However, the structural basis for such a markedly heterogeneous behavior has not been established unequivocally (14, 15).

The functional properties of fish Hb from Chelidonichthys kumu have been reported recently (16). At neutral pH values, this Hb displays a markedly heterogeneous kinetic CO binding behavior, which parallels O₂ equilibrium ligand binding properties typical of a Hb staying in the T quaternary state even in the liganded form (16). In view of these features, and of the very stable tetrameric assembly observed in C. kumu Hb, like other fish Hbs (16, 17), the pH and temperature dependence of the dynamic behavior for CO binding to this fish Hb has been investigated and compared with the same process in human adult Hb. In this respect, C. kumu Hb offers a very interesting opportunity for studying the different proton-linked modulation of ligand reactivity in the two types of subunits within the tetramer. The main purpose of this approach is thus the characterization of some energy parameters, which play major roles in regulating the reactivity of this fish Hb, and, more in general, of the role of different subunits in modulating the dynamic pathway of ligand binding. This aspect has not been investigated for CO binding to mammalian Hbs, and in particular for human adult Hb, since only a fairly small (almost undetectable) kinetic functional heterogeneity has been observed in Fe-Co hybrids in the presence of inositol hexakisphosphate (18). The comparison of the behavior observed in C. kumu Hb with respect to human adult Hb also allows one to envisage some detailed aspect(s) of potential effects of the heme environment on the regulation of the CO binding process, and thus on the origin of the functional heterogeneity in C. kumu Hb.

EXPERIMENTAL PROCEDURES

Human adult Hb and C. kumu Hb were prepared as reported previously (16, 19), and stripped of cations and anions according to Riggs (20).

Carbon monoxide was obtained by CaracciOssigeno S.p.A. (Rome, Italy). Chemicals were from Merck AG (Darmstadt, Germany) and Sigma. All products were of analytical grade and used without further purification.

Kinetics of CO binding was carried out as previously reported at 419 nm and between 8 °C and 38 °C (9, 10), keeping deoxy Hb at pH 7.0 in
a very low ionic strength buffer (I = 2 mM) and mixing with a higher ionic strength buffer (I = 0.3 M) at the desired pH and with varying concentrations of dissolved CO.

CO binding kinetics was carried out in the absence and presence of 3 mM ATP (only for *C. kumu* Hb) between pH 2.0 and 9.0 (0.15 M phosphate buffer between pH 2.0 and 4.0 and between pH 5.5 and pH 7.5; 0.15 M acetate buffer between pH 4.0 and pH 5.5; 0.15 M MES1 buffer between pH 5.0 and 7.0; 0.15 M HEPES buffer between pH 6.5 and 8.0; 0.15 M Tris/HC1 buffer between pH 7.5 and 9.0). No ion effects were observed for buffers overlapping in pH values.

The transient spectrum of unliganded *C. kumu* Hb at pH 2.3 was obtained as described previously at 21 °C (9, 10), mixing deoxy Hb in a low ionic strength buffer at pH 7.0 with a degassed 0.15 M (final concentration) phosphate buffer at pH 2.0 (final pH = 2.3), and determining the amplitude of the denaturation process between 480 nm and 600 nm.

Kinetic experiments were performed at the Department of Biochemical Sciences "Alessandro Rossi Fanelli" of the University of Roma "La Sapienza," using a Gibson-Durrum stopped-flow apparatus with a 2-cm pathlength observation cell, connected to a desktop computer for fast data acquisition (On Line Systems, Jefferson, GA). The pH values of the reaction mixture were checked at every temperature.

### RESULTS AND DISCUSSION

*C. kumu* Hb has been shown to bind O2 noncooperatively at neutral pH, cooperativity occurring at alkaline pH values; in parallel, kinetic heterogeneity for CO association and O2 dissociation fades out going from pH 6 to pH 9. Such behavior suggests that the lack of cooperativity may be at least partially attributed to the marked functional heterogeneity present in the T state, possibly accompanied by a stabilization of this low affinity quaternary conformation even in the liganded form. This feature likely contributes to impair the appearance of a cooperative ligand binding in *C. kumu* Hb. Furthermore, the physiological allosteric effector ATP has been shown to significantly decrease the ligand affinity of *C. kumu* Hb, impairing the appearance of cooperativity even at alkaline pH values. Such a finding indicates that ATP may play a significant role in modulating the ligand affinity, the quaternary conformational equilibrium, and possibly the functional heterogeneity of *C. kumu* Hb (16).

Fig. 1 shows the pH dependence of CO binding bimolecular rate constants to human adult Hb (panel A) and to *C. kumu* Hb in the absence (panel B) and presence (panel C) of 3 mM ATP. The kinetic functional heterogeneity of *C. kumu* Hb is present essentially over the whole pH range, but it tends to disappear at pH 2.0 and 9.0. The pH dependence of the CO binding kinetic properties of adult human Hb refers to the overall rate constant, since no detectable kinetic heterogeneity is observed (10). Both adult human Hb and *C. kumu* Hb display a pH-dependent CO binding behavior, which requires two apparent protonation events, such that lowering pH from 9.0 to 6.0 brings about a decrease of the second-order rate constant followed by an enhancement of the kinetics upon further lowering of pH from 6.0 to 2.0 (Fig. 1). In all cases, the reverse bell-shaped pattern displays a well at pH values around 6, suggesting that the apparent singly protonated species is characterized by the slowest CO binding rate constant. Parameters reported in Table I have been obtained from the fitting of data according to the following equation.

\[
I'_{\text{obs}} = I'(\Delta T) + I'_1(K\text{H}^+P) + I'_2(K_1K_2\text{H}^+\text{P}) \quad \text{(Eq. 1)}
\]

\(I'_{\text{obs}}\) is the observed second-order rate constant for CO binding; \(I'_{\text{o}}\) and \(I'_1\), and \(I'_2\) are the bimolecular rate constant for CO binding to the unprotonated, singly protonated, and doubly protonated molecule, respectively; \(K_1\) and \(K_2\) are the apparent proton equilibrium association constants to the unliganded Hb for the

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1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
being especially relevant for one type of subunit (see Fig. 1, panels A and B). An additional peculiar aspect of the first protonation event (on the alkaline side) is the fact that the pH of the transition is fairly high in both chains (pK_a = 7.6 and 8.2 for the fast and slow subunit, respectively; see Table I) with respect to human adult HB (pK_a = 6.9; see Table I). Moreover, the pK_a value differs between the two subunits by ~0.6 and ~1.0 pH unit in the absence and presence of 3 mM ATP, respectively (see Fig. 1 and Table I).

On the basis of Equation 1 and of parameters reported in Table I we can state that at the alkaline pH limit l'_obs ~ l'_1 since under these conditions the binding polynomial turns out to be P ~ 1 ([K_a(H^+) + K_aK_p(H^+)^2]), whereas at the acid pH limit l'_obs ~ l'_2 because the binding polynomial is P ~ K_aK_p(H^+)^2 ([1 + K_a(H^+)]). At intermediate pH values, that is at the bottom of the well for the pH dependence (see Fig. 1), we have the predominance (i.e., >90%) of singly protonated species, so that the binding polynomial is P ~ K_a[H^+] ([1 + K_aK_p(H^+)^2]), and l'_obs approximates l'_1. Therefore, the activation energy parameters indeed can be reasonably ascribed to the unprotonated (at pH 8.9 ± 0.2), to the apparent singly protonated (at pH 6.0 ± 0.2), and to the apparent doubly protonated form (at pH 2.5 ± 0.1) (see Fig. 1). The good linearity of the Arrhenius plot at the selected pH values (Fig. 2) confirms the hypothesis that at each of these pH values we are indeed observing the activation energy of only one of the three species mentioned above. This evidence allows us to calculate the transition state parameters (24) of the unprotonated, singly protonated and doubly protonated forms for the CO binding kinetics to human adult HB and to both subunits of C. kumu HB at 20°C, using the molar concentration of the ligand as a reference (see Table II).

A first look at the parameters reported in Table II reveals that lowering pH from 9.0 to 6.0 has a completely different effect for the two chains of C. kumu HB (see Table II). The very marked kinetic heterogeneity significantly decreases as temperature rises (see Fig. 2, panel B), whereas it is enhanced as the temperature is decreased. Therefore, the reaction of the two subunits with CO in the apparent singly protonated species displays a large difference between the two chains in the activation energy E_a, and thus for the activation enthalpy ∆H^* = E_a - RT). This results in a totally different reaction mechanism for the two types of subunits, which is enthalpy-driven for the fast-reacting chain, whereas it is entropy-driven for the slow-reacting subunit (see Table II). The occurrence of a positive entropy change for the formation of the transition state suggests that the very slow binding rate constant of the slow-reacting subunit in the singly protonated form is likely to be related to some proton-linked bond(s), which strongly impair(s) the conformational change of the heme pocket required for the formation of the HbCO adduct.

The behavior observed for human adult HB is more similar to that of the fast-reacting chain of C. kumu. Thus, at alkaline pH human adult HB and C. kumu HB display closely similar values for the activation enthalpy, the lower second-order rate constant of human adult HB being referable only to a more negative activation entropy (see Table II). Lowering the pH from 9 to 6 is characterized by a slight decrease for the activation
enthalpy, the activation entropy becoming significantly more negative in the case of human adult Hb (see Table II). The different trend of energetic parameters in the two Hbs, associated to the difference in the $pK_a$ of the process (see Table I), indeed seems to suggest that, in spite of the similar CO binding rate constant at pH 6.0, a somewhat different mechanism is underlying the alkaline transition in human adult Hb and in the fast-reacting chain of *C. kumu* Hb.

A structural interpretation of this behavior is not straightforward, since there are a large number of substitutions in the heme pocket of *C. kumu* Hb with respect to human adult Hb, and some of them might have closely similar $pK_a$ values, thus belonging to the same class of protonating residues and contributing to the same proton-linked transition. Therefore, in what follows we attempt to discuss their role, also taking into account the effects produced by each substitution in human mutants. In the case of human adult Hb, the alkaline transition observed for CO binding displays a $pK_a$ value, which is consistent with the protonation of a histidyl residue, and it can be tentatively attributed to the distal His. On the other hand, the fairly high $pK_a$ values for the alkaline transition of the two subunits of *C. kumu* (see Fig. 1 and Table I) do not find an obvious explanation, and a more careful analysis of the alterations in the heme pocket of the two subunits is required.

In this respect, *C. kumu* Hb displays some potentially important substitutions with respect to human adult Hb, namely (i) on the distal portion of the heme pocket residue E11 of both chains is Ile instead of Val; (ii) Lys$^E_8$ and Lys$^{E_{10}}_8$ of the $\beta$-subunit, close to distal His$^E_8$, are replaced by Glu and Thr, respectively; (iii) Lys replaces Ser in CD3$\beta$; (iv) Lys is present in F7$\alpha$ (i.e. immediately next to proximal His$^F_8$), a position occupied by Leu in human adult Hb; and (v) Lys is observed in F6$\beta$, where Glu is present in human adult Hb (16, 19).

Even though the substitution of Val$^{E_11}_8$ by Ile has been found to be relevant for the reactivity of CO toward the R state of $\beta$-mutants of human adult Hb (25), a major contribution from this residue to the alkaline protonation process reported in Fig. 1 appears unlikely. It seems more convincing to identify a potentially responsible factor for the effect, at least in the $\beta$-subunits of *C. kumu* Hb, in the large electrostatic variation related to the substitution of Lys by Glu in E3$\beta$ and of Lys by Thr in E10$\gamma$ in the distal portion of the heme pocket. This is likely to bring about a large raising in the proton affinity of distal His$^F_8$, which could attain a value of $pK_a > 7$, as observed in the fast-reacting subunit of *C. kumu* Hb. It should be noticed that the apparent $pK_a$ of Glu$^{E_{3}}_8$ is likely to be further decreased by closely positioned Lys$^{CD3}_8$.

On the other hand, the occurrence of Lys$^{F_{7\alpha}}_8$ in the proximal portion of the heme pocket is unique in *C. kumu* Hb, and a positively charged residue (i.e. Arg$^{F_{7\alpha}}_8$) has been previously observed in this position only in the low affinity human adult mutant Hb Moabit (26). The role played by the residue in F7 on the regulation of functional properties has been clearly demonstrated in the case of mutants of pig myoglobin, where in the native molecule Ser$^{F_7}$ forms an H-bond with N$_6$ of proximal His (27). In *C. kumu* Hb the occurrence of a positively charged lysyl residue at F7$\alpha$ in place of Leu (present in human adult Hb) might be responsible for a relevant alteration of the conformation of the proximal portion of the heme pocket, dramatically reducing the reactivity toward CO. Therefore, the protonation of Lys$^{F_{7\alpha}}_8$ might be responsible for the dramatic reduction of CO binding second-order rate constant of $\alpha$-chains in *C. kumu* Hb, possibly attributing the $pK_a > 8.0$ observed in the slow-reacting subunit of *C. kumu* Hb to this process and to the $\alpha$-chain (see Fig. 1 and Table I). Although the observed $pK_a$ value (= 8.2, see Table I) is ~2 pH unit lower than that of free lysine, such a decrease indeed can be related to the burying of Lys$^{F_{7\alpha}}_8$ inside the proximal portion of the heme pocket, and to the consequent separation from the bulk solvent. The two additional substitutions with respect to human adult Hb, which are observed in the distal portions of the heme pocket in $\alpha$-chains of *C. kumu* (where Asn$^{E_{10}}_8$ replaces Lys at a heme contact position and Ser$^{E_{3\beta}}_8$ replaces Gln next to distal His), should not bring about electrostatic alterations. Furthermore, in the $\beta$-subunits of *C. kumu* Hb the presence of Lys$^{F_{6\beta}}_8$, which substitutes Glu (present in human adult Hb) near proximal His$^{F_8}$. 

Below pH 6, an acidic protonation process can be observed, which brings about a marked enhancement of the CO binding rate constant to both *C. kumu* Hb and human adult Hb (see Fig. 1). This second protonation is reminiscent of that described previously in human adult Hb (10), as well as in other monomeric and dimeric hemoproteins (9, 29, 30), and it may be referred to the protonation of the N$_6$ atom of the His$^{F_8}$ imidazole ring, and the consequent cleavage (or severe weakening) of the proximal His$^{F_8}$-Fe bond. This interpretation seems supported by the observation that the transient absorption spectrum of unliganded *C. kumu* Hb in the visible region displays features characteristic of a tetracoordinated heme (see Fig. 3, and Ref. 9). In C. kumu Hb the $pK_a$ value is drastically different for the two subunits both in the absence and presence of 3 mM ATP (see Fig. 1, and Table I). Such a behavior clearly indicates that in the singly protonated species the conformation of the proximal side of the heme pocket is markedly different in the two chains, this being reflected in a much lower energy for the

### Table II

**Activation parameters for CO binding to *C. kumu* Hb at 20°C, normalized for 1 M ligand concentration**

| C. kumu Hb | pH 8.9 | pH 6 (fast) | pH 6 (slow) | pH 2.5 |
|------------|--------|------------|------------|--------|
| $E_a$      | 28.9 $\pm$ 1.3 | 31.0 $\pm$ 1.1 | 86.2 $\pm$ 2.8 | 33.3 $\pm$ 1.2 |
| $\delta G_f^t$ | 39.2 $\pm$ 1.2 | 44.3 $\pm$ 1.2 | 50.0 $\pm$ 1.4 | 37.2 $\pm$ 1.0 |
| $\delta H_f^t$ | 26.4 $\pm$ 1.2 | 28.5 $\pm$ 1.0 | 83.8 $\pm$ 2.5 | 30.9 $\pm$ 1.1 |
| $T\Delta S_f^t$ | $-12.8 \pm 1.2$ | $-15.8 \pm 1.8$ | $+33.8 \pm 3.2$ | $-6.3 \pm 0.5$ |

| Human adult Hb | pH 8.9 | pH 6.0 | pH 2.5 |
|----------------|--------|--------|--------|
| $E_f$ | 28.7 $\pm$ 1.2 | 24.8 $\pm$ 1.1 | 26.6 $\pm$ 1.4 |
| $\delta G_f^t$ | 41.5 $\pm$ 1.3 | 44.3 $\pm$ 1.2 | 33.0 $\pm$ 1.0 |
| $\delta H_f^t$ | 26.3 $\pm$ 1.2 | 22.4 $\pm$ 1.1 | 24.2 $\pm$ 1.4 |
| $T\Delta S_f^t$ | $-15.2 \pm 1.3$ | $-21.9 \pm 1.5$ | $-8.8 \pm 0.4$ |
HisF8-Fe bond in human adult Hb and in the fast-reacting shows that the cleavage (or severe weakening) of the proximal in which no heterogeneous kinetic behavior is observed in

$$pK_a$$

than that of human adult Hb (see Fig. 1), which (together with the p



$$pH$$

of the proximal HisF8-Fe bond, the ligand kinetic path-
difference indicates that, even in the absence (or severe weak-



$$pK_a$$

Hb at pH 2.3, 1 min after mixing. The experimental points (•) represent the initial optical density changes at every wavelength after rapid mixing of ferrous deoxygenated C. kumu Hb with degassed 0.3 M phosphate buffer (pH = 2.0) to bring the pH to 2.3. The concentration of C. kumu Hb was 8 μM heme after mixing. The absorption spectrum in the visible region of C. kumu Hb at pH 9.0 was superimposable to that at pH 7.0. For further details, see text.

cleavage of the proximal HisF8-Fe bond in the fast-reacting chain than in the slow-reacting subunit (by ~10 and 6 kJ/mol in the absence and presence of ATP, respectively).

The cleavage (or severe weakening) of the proximal HisF8-Fe bond essentially abolishes the kinetic functional heterogeneity (see Fig. 1), which (together with the p



$$pH$$

of the protonation process) suggests that the structural basis of the chain kinetic difference mostly resides in the conformation of the proximal side of the heme pocket in the singly protonated species. However, it must be remarked that the asymptotic value of C. kumu Hb ($$$I_2 = 2.1 \times 10^6 \text{ m}^{-1} \text{ s}^{-1}$$$) is much lower than that of human adult Hb ($$$I_2 = 3.0 \times 107 \text{ m}^{-1} \text{ s}^{-1}$$$). Such a difference indicates that, even in the absence (or severe week-



$$pH$$

pathway for the interaction with the heme iron displays a larger activation free energy in C. kumu Hb than in human adult Hb (see Table II). In the absence of a proximal control, this effect can only be attributed to differences in the distal portion of the heme pocket between human adult Hb (showing Val211 in both subunits) and C. kumu Hb, where both chains display Ile211 (16), which is known to represent a larger barrier for ligand binding (25).

The temperature dependence of the CO binding kinetics has been investigated also at pH 2.5 (see Fig. 2, panel C, a condition in which no heterogeneous kinetic behavior is observed in C. kumu Hb (see Fig. 1). The analysis of the activation parameters for the single dynamic process observed at this pH clearly shows that the cleavage (or severe weakening) of the proximal HisF8-Fe bond in human adult Hb and in the fast-reacting chain of C. kumu Hb brings about an increase of the CO binding rate constant, which is merely due to a decrease of the negative activation entropy (see Table II). This indicates that the modulation of the heme iron reactivity (and thus of the movement in the heme plane upon ligand binding) by the proximal bond is regulated through the activation entropy of the process without significantly influencing the activation en-



$$pK_a$$

In the slow-reacting subunit of C. kumu Hb, the rate en-



$$pH$$

hancement at acidic pH values is regulated by two opposite effects, taking place upon cleavage of the proximal bond,

namely (i) a drastic reduction of the activation enthalpy, and (ii) a negativization of the activation entropy, which was posi-
tive for the singly protonated form of this chain at pH 6.0 (see Table II). This different contribution must be certainly attributed to the peculiar features of the singly protonated form of the slow-reacting subunit of C. kumu Hb, in which the cleavage (or severe weakening) of the proximal HisF8-Fe bond brings about a conformational transition, which is different from that observed for the same phenomenon in human adult Hb and in the fast-reacting subunit of C. kumu Hb. Therefore, it seems that in the singly protonated species the slow-reacting subunits are held in a very unreactive conformation, possibly involving mainly the proximal side of the heme pocket, such as to de-
crease the mobility of the proximal HisF8-Fe bond for ligand binding. The cleavage (or severe weakening) of the proximal HisF8-Fe bond at low pH then releases such a strong constraint in the slow-reacting subunit, which is the cause of the marked functional kinetic heterogeneity at neutral pH, and the reactive toward CO recovers a value essentially undistinguish-
able from that of the partner subunit. The unusually low p



$$pK_a$$

value for this acid protonation process in the slow-reacting subunit (p



$$pK_a$$

< 3.0), as compared with that of human adult Hb (~3.4; see Fig. 1, panel A, and Ref. 10), is in keeping with this interpretation, indicating a very rigid proximal side in the singly protonated form at pH ~ 6.0, or in any event an environ-



$$pH$$

ment unfavorable to the stereochemical changes of the prox-



$$pH$$

imal side that take place upon ligand binding (7, 31).

Furthermore, a comparison in C. kumu Hb between the unprotonated tetramer at pH 8.9 and the doubly protonated species at pH 2.5 shows that the observed similarity for the values of CO binding bimolecular rate constants (see Tables I and II) is only fortuitous, since it stems from meaningfully different contributions of the activation enthalpy and entropy. Thus, in the unprotonated molecule the positive value of $\Delta H^T$ is lower than in the doubly protonated tetramer, whereas the negative contribution of the activation entropy is smaller in the doubly protonated tetramer. Such an observation seems in line with the possibility that the cleavage (or severe weakening) of the proximal HisF8-Fe bond regulates the CO binding kinetics mostly reducing the entropy loss required for the formation of the activated complex, this being true also for human adult Hb (see Table II).

In conclusion, the reported behavior suggests that the dra-
matic functional kinetic heterogeneity in CO binding to C. kumu Hb is likely to be referable to structural variations of the heme pocket with respect to human adult Hb, mostly located in the distal portion of the β-chains and on the proximal side of the α-subunits. Therefore, in the two chains of C. kumu Hb CO binding reactivity is regulated through a different mechanism, which becomes evident only in the singly protonated species, since different protonating groups are altering to a different extent the energetic parameters along the first protonation process on the alkaline side.

Furthermore, this investigation also brings to evidence that the cleavage (or severe weakening) of the proximal HisF8-Fe bond, which is induced by the second protonation event, brings about a relevant enhancement of the binding second-order rate constant mostly through a reduction of the entropy increase required for the formation of the transition state. This observ-



$$pK_a$$

clearly indicates that the degrees of freedom for the movement of the unliganded iron in the heme plane (and thus the entropy changes accompanying this stereochemical varia-



$$pH$$

are an important factor along the reaction pathway of CO binding. In addition, the effect of ATP can be detected only for the p



$$pK_a$$

of the transitions in the slow-reacting chains, suggesting that this heterotropic ligand contributes to an alteration of
the conformation mostly on these subunits, with a marked weakening (by \(\sim 4\) kJ/mol) of the proximal bond.

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