ISOCYANIDE BINDING KINETICS TO MONOMERIC HEMOPROTEINS

A Study on the Ligand Partition between Solvent and Heme Pocket

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ABSTRACT The kinetics of methyl-, ethyl-, *iso*-propyl-, and *ter*-butyl-isocyanide binding to *Aplysia limacina* myoglobin (distal His → Lys) and the isolated β chains from hemoglobin Zurich (distal His → Arg) have been investigated by flash photolysis at various temperatures above 0°C. Sperm whale (*Physter catodon*) myoglobin and the isolated β chains from normal adult hemoglobin have been used as references. In most reaction systems investigated the apparent extent of photolysis increases with temperature. For sperm whale myoglobin and the normal β chains the increase is of the same magnitude and not correlated to the type of ligand used. On the contrary, for the two proteins lacking the distal histidine, the phenomenon is dependent on the size of the alkyl side chain of the ligand. The results, analyzed on the basis of the multibarrier model (Austin, R. H., K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus, 1975, *Biochemistry,* 16:5355–5373), suggest that the partition of the ligand molecules between the solvent and the heme pocket, occurring during the photolysis process, is primarily determined by interactions between the ligand and residues in the heme cavity rather than by diffusion through the protein matrix.

INTRODUCTION

Since the pioneering work of St. George and Pauling (2), alkyl-isocyanides have often been used as molecular probes to investigate phenomena of steric hindrance at the heme cavity of ferrous hemoproteins (3). A comprehensive kinetic investigation on various proteins has more recently been reported by Mims et al. (4). These authors also analyzed the available data for distal effects of the protein on the binding of various members of the homologous series of alkyl-isocyanides. We expected a comparative investigation on the binding of isocyanides to *Aplysia limacina* myoglobin (Al-Mb) and to the isolated β chains from hemoglobin Zurich (β^{ZH}) to shed new light on this topic. Sperm whale (*Physter catodon*) myoglobin (SW-Mb) and isolated β chains from normal adult hemoglobin (β^{A}) served as references. In β^{ZH} chains and Al-Mb the distal histidine is replaced, respectively, by arginine and lysine (5, 6). Both proteins display a large cavity at the distal side of the heme, as shown by x-ray crystallographic analysis (6–9). Therefore, structurally speaking, these two proteins are rather similar. Nevertheless, they differ dramatically in their functional properties: Al-Mb has a CO binding rate of $5 \cdot 10^5\ M^{-1} \cdot s^{-1}$(10), as is also found for SW-Mb (3), while β^{ZH} chains, like many other hemoproteins in which the distal histidine is replaced by another amino acid (11), display a markedly increased CO binding rate (12–16). It has been suggested that the rate of ligand binding to a heme protein at physiological temperatures is determined by the velocity of the ligand combination from inside the heme pocket (14, 15). Proximal side effects play an important role in determining the intrinsic reactivity of the heme iron (11, 17–19). However, information is lacking on how the partition of the ligand between the solvent and the heme pocket is regulated and what is its importance in determining the apparent velocity of ligand binding to hemoproteins. With the comparative study reported here we attempt to contribute to the understanding of this phenomenon.

From the homologous series of alkyl-isocyanides we have chosen those that can be accommodated in the heme pocket, without having the side chain imbedded into the protein matrix (4), i.e., the methyl-, ethyl-, *iso*-propyl-, and *ter*-butyl-isocyanide.

MATERIALS AND METHODS

SW-Mb was obtained from Sigma Chemical Co. (St. Louis, MO). Al-Mb was purified according to Rossi Fanelli and Antonini (20). Isolated β^{A} and β^{ZH} chains where prepared in the carbonyl form as described elsewhere (13, 14) and converted to the oxy form, by illumination under a stream of oxygen (21), immediately before use. Isocyanides were synthesized by the procedure described elsewhere (22) and their quality checked by nuclear magnetic resonance, infrared, and mass spectroscopy. Their densities were found to be all between 0.74 and 0.76, with an error of

~2%. Therefore, a unique value of 0.75 was used for all isocyanides. After the synthesis, the compounds were frozen in liquid nitrogen and stored, in the absence of oxygen, at −70°C. Immediately before use, stock isocyanide solutions were obtained by mixing the pure compound with argon-saturated water and shaking vigorously. The desired concentrations of the ligand were obtained by anaerobic dilution of the appropriate stock solution with oxygen-free buffer.

Equilibrium Measurements

Isocyanide binding equilibria to Al-Mb were measured spectrophotometrically on a DU7 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) connected on line to a Olivetti M-20 personal computer (Olivetti & C., S.p.A., Ivrea, Italy). The experiments were performed at different temperatures between 5° and 40°C, using ~3 μM protein solutions, in the presence of trace amounts of sodium dithionite, under strictly anaerobic conditions. Monitoring was done between 425 and 450 nm. The binding curves were computed using the optical density changes, induced by the combination of the protein with the ligand, at 50 different wavelengths. No equilibrium measurement could be performed on the β^{ZH} chains due to the instability of their deoxy derivative.

Kinetic Measurements

Stopped-flow measurements were performed using the apparatus described elswhere (23). For the flash-photolysis measurements a flash-lamp pumped dye-laser was used, with a pulse centered at 590 nm, having a duration of ~4 μs and an energy of ~60 mJ (11, 24). In some cases we used a Nd:YAG laser, capable of producing 12-ns pulses at 532 nm with energies between 60 and 270 mJ. The protein concentration in the reaction mixture was always ~3 μM. All solutions were saturated with argon and complete removal of oxygen was attained by addition of trace amounts of sodium dithionite.

For SW-Mb and Al-Mb, isocyanide binding kinetics were investigated both by rapid mixing and by flash photolysis. Ligand binding velocities were measured at various isocyanide concentrations, ranging between 10- and 1,000-folds the molar equilibrium constants. In all cases the second order rate constants could be estimated by linear regression analysis. The measurements were repeated at several temperatures from 5° up to 40°C and the absence of denaturation phenomena was checked after the experiments at the highest temperature by repeating the measurement at 20°C. Linear Arrhenius plots were obtained from which the activation energies were calculated by least squares fittings. Measurements of the isocyanide dissociation rates were done by the ligand displacement method (25), i.e., mixing, in the stopped-flow apparatus, the isocyanide derivative of the protein with buffer containing 1 mM CO.

RESULTS

Equilibrium Measurements

The affinity constants at 20°C for the binding of the various isocyanides to Al-Mb and β^{ZH} chains are given in Table I and compared with those reported in the literature for SW-Mb and β^{A} chains (4). The values given for Al-Mb are the result of five to ten independent equilibrium measurements. The standard errors on the affinity constants were always within 10% of their values. The equilibrium constants given for the β^{ZH} chains were computed from the kinetic combination and dissociation constants.

The temperature dependence of the equilibrium constants for isocyanides binding to Al-Mb was investigated. This allowed the estimation of the thermodynamic parameters for the various reaction systems, as reported in Fig. 1. A substantial enthalpy/enthropy compensation effect is evident for all ligands used. As already reported for other proteins (4), the highest free energy change upon ligand binding is observed in correspondence to two carbon atoms in the alkyl side chain.

TABLE I

APPARENT EQUILIBRIUM CONSTANTS (K), KINETIC RATES (λ_{on}, λ_{off}), AND ACTIVATION ENERGIES ($E^{\ddagger}_{on}$) FOR THE BINDING OF ISOCYANIDES TO SPERM WHALE MYOGLOBIN, *APLYSIA* MYOGLOBIN, AND THE ISOLATED β CHAINS FROM NORMAL ADULT HUMAN HEMOGLOBIN AND FROM HEMOGLOBIN ZURICH

	K	λ_{on}	$E^{\ddagger}_{on}$	λ_{off}
	$\cdot 10^{-4} M^{-1}$	$\cdot 10^{-4} M^{-1}s^{-1}$	*kcal/mol*	s^{-1}
SW-Mb				
MIC	2.3*	13.0	13.0	5.6§
EIC	17.0*	10.0	9.5	0.6§
*i*PIC	2.2*	1.1	14.0	0.5§
*t*BIC	0.2*	0.3	13.0	1.5§
Al-Mb				
MIC	0.6	60.0	5.0	100.0¶
EIC	3.6	54.0	7.7	15.0§
*i*PIC	1.7	4.6	13.0	2.7§
*t*BIC	0.2	0.8	16.0	4.0§
β^{A}				
MIC	11.0*	82.0	4.4	7.5§
EIC	38.0*	22.0	6.2	0.6§
*i*PIC	15.0*	6.5	9.0	0.4§
β^{ZH}				
MIC	123.0‖	80.0	5.0	0.6§
EIC	371.0‖	104.0	6.4	0.3§

K and λ were measured at 20°C while $E^{\ddagger}$ refers to measurements between 5° and 40°C. In all cases 0.1 M phosphate buffer was used and a protein concentration of ~3 μM. MIC, methyl-isocyanide. EIC, ethyl-isocyanide. *i*PIC, *iso*-propyl-isocyanide. *t*BIC, *ter*-butyl-isocyanide.

*Data from reference 4.

§Data computed from K and λ_{on}.

‖Data computed from λ_{on} and λ_{off}.

¶The value obtained by direct measurement is 72 s^{-1}.

Kinetic Measurements

Isocyanide binding to all four proteins is exponential in time in the temperature range investigated. The combination rates (λ_{on}) measured by rapid mixing and flash photolysis agree within the experimental error. The values obtained at 20°C for SW-Mb and Al-Mb are given in Table I together with those referring only to flash photolysis measurements on the combination of methyl-, ethyl- and *iso*-propyl-isocyanide to β^{A} chains and of methyl- and ethyl-isocyanide binding to β^{ZH} chains. When reacted with *iso*-propyl- and *ter*-butyl isocyanide, β^{ZH} chains are unstable. Thus no measurements could be performed with these ligands.

Overall Arrhenius activation energies ($E^{\ddagger}$) for the "on" rates, obtained from at least three sets of measurements performed at various ligand concentrations between 5° and

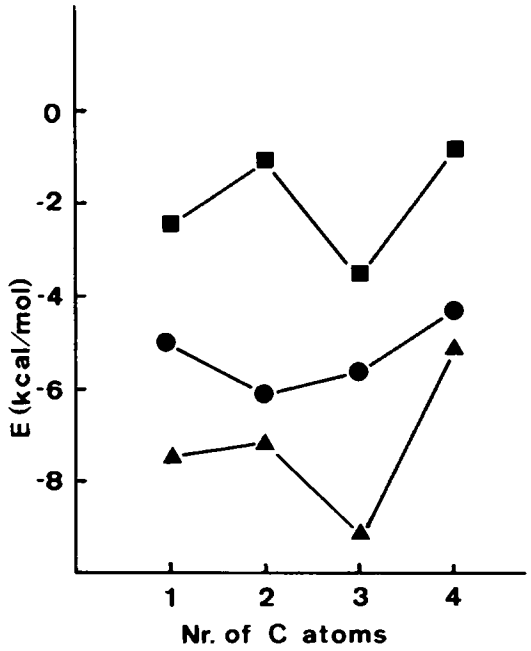

FIGURE 1 Thermodynamic parameters, at 20°C, for the reaction of Al-Mb with isocyanides, as a function of the number of carbon atoms in the alkyl side chain of the ligand. *Triangles*, $\Delta H°$; *circles*, $\Delta G°$; and *squares*, $T \cdot \Delta S°$. Measurements were performed with ~3 μM protein in 0.1 M potassium phosphate buffer, pH 7.4. The standard errors on $\Delta G°$ and $\Delta H°$ are lower than 0.06 and 0.1 kcal/mol, respectively.

TABLE II
EFFECT OF THE DURATION AND INTENSITY OF THE LASER PULSE ON THE APPARENT EXTENT OF PHOTODISSOCIATION θ

	12 ns, 60 mJ	12 ns, 270 mJ	4 μs, 60 mJ
SW-Mb			
MIC	0.02	0.02	0.10
EIC	0.02	0.02	0.14
*i*PIC	0.02	0.02	0.10
*t*BIC	0.02	0.02	0.19
Al-Mb			
MIC	0.24	0.37	0.50
*i*PIC	0.08	0.09	0.09

See Discussion for the definition of θ. The data refer to the derivatives of Sperm whale myoglobin with all four isocyanides and those of *Aplysia* myoglobin with methyl- and *ter*-butyl-isocyanide. Complex intermediate behaviors are found with the ethyl- and *iso*-propyl-isocyanide of *Aplysia* myoglobin. All measurements have been performed at 20°C. Other conditions as reported in Table I.

40°C, are also listed in Table I. The standard errors on the reported $E^{\ddagger}_{on}$ values never exceed 15%.

The ligand dissociation rates (λ_{off}), obtained by direct measurement on β^{ZH} chains and computed from the equilibrium and combination rate constants for the other proteins, are reported in Table I.

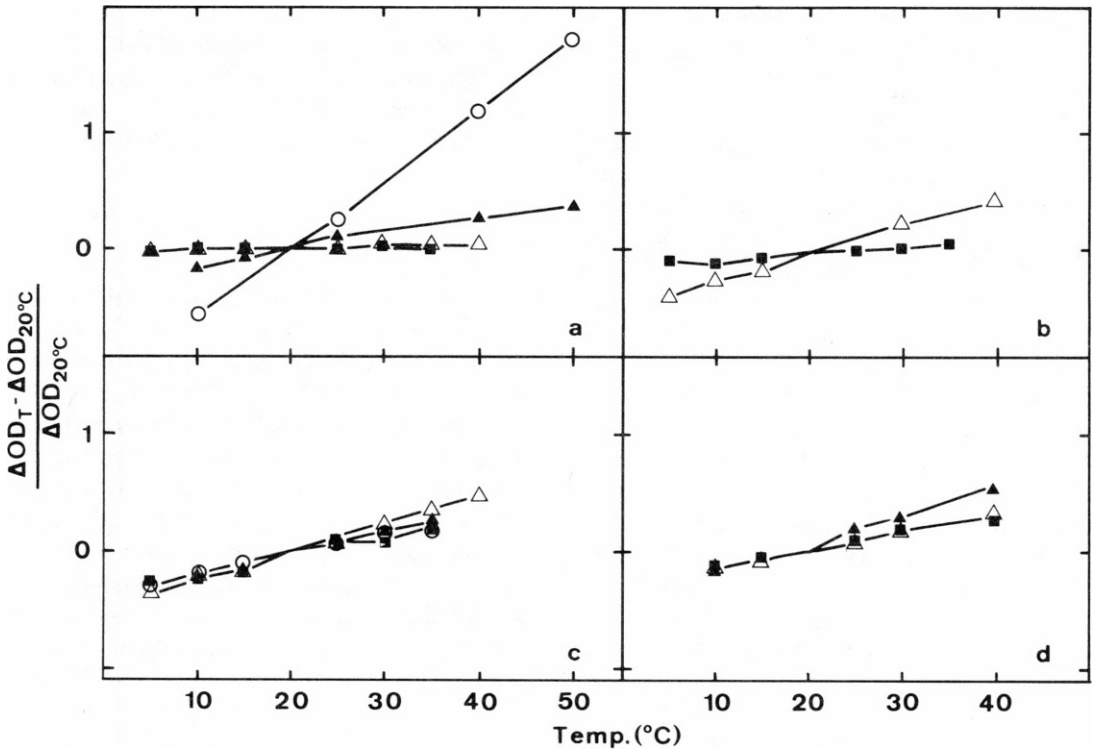

FIGURE 2 Temperature dependence of the absorption change, induced by the photolysing laser pulse, in flash photolysis measurements. The data refer to the reaction of Al-Mb (*a*), β^{ZH} chains (*b*), SW-Mb (*c*), and β^{A} chains (*d*) with methyl- (*squares*) ethyl- (*open triangles*), *iso*-propyl- (*solid triangles*), and *ter*-butyl-isocyanide (*circles*). All experiments were done using 0.1 M potassium phosphate buffer pH 7.4 and a protein concentration of ~3 μM.

Fig. 2 shows how the time zero optical density changes, induced by the laser pulse, vary with temperature. The data refer to all four proteins and isocyanides studied. No data are quoted for the β^A-*ter*-butyl isocyanide complex as its apparent degree of ligand photodissociation is found to be very close to zero at all temperatures between 5° and 40°C.

In rapid mixing experiments no temperature-induced increase of the time zero amplitude of the signal is observed.

For SW-Mb and Al-Mb we have investigated the effect of the duration and energy of the photolysing pulse on the apparent extent of ligand photodissociation θ. The results are reported in Table II.

DISCUSSION

As pointed out in the introduction our investigation involves two myoglobins and two β hemoglobin chains. Each protein pair is representative for a different situation at the distal site of the heme, i.e., the presence or absence of a histidinyl residue pointing towards the heme iron.

The data reported in Table I allow an evaluation of the effect of the distal side in determining the ligand properties of the two pairs of related proteins.

The affinity of Al-Mb for methyl- and ethyl-isocyanide is about four times lower than that of SW-Mb. On the other hand, a comparison of the affinity of the isolated β^A and β^{ZH} chains for the same ligands clearly shows that, in this case, the presence of the distal histidine is producing the opposite effect than in the myoglobins. The β^{ZH} chains have a 10-fold higher affinity for the same ligands than do the β^A subunits.

The replacement of the distal histidine with arginine is known to produce large structural modifications in the β^{ZH} chains (8, 9, 26, 27). On the other hand, SW-Mb and Al-Mb differ in many residues besides the distal histidine. It is therefore impossible to evaluate the specific effects of the replacement of the distal residue on the ligand binding properties of the molecule. However, the data in Table I for SW-Mb and β^A clearly indicate that the distal residue is not playing a dominant role in the regulation of their affinity for methyl- and ethyl-isocyanide.

It is worth noticing that the differences in affinity for ligands of increasing size between SW-Mb and Al-Mb do not change considerably in going from methyl- to ethyl- and from *iso*-proply- to *ter*-butyl-isocyanide, but drastically increase in going from the ethyl-to the *iso*-propyl-derivative. This finding gives an idea on the differences in the size of the heme pocket at the distal region in the two myoglobins.

Based on the isocyanide binding rates reported in Table I, we could group Al-Mb, β^A, and β^{ZH} as fast reacting molecules, as compared with SW-Mb. Steric effects at the distal side, combined with a different flexibility of the protein structure around the heme, might explain such differences. This interpretation is in keeping with the finding that as the size of the ligand side chain increases and hydrophobic interactions within the heme cavity become dominant, such differences tend to level off. On the other hand looking at all the combination rates given in Table I, minor differences are found between β^{ZH}, β^A, and Al-Mb. Only in the case of the ethyl-isocyanide the interaction with the distal residue seems to play a significant role both in SW-Mb and in β^A. This picture possibly reflects a higher structural rigidity of myoglobin with respect to the isolated hemoglobin chains, leading to an enhancement of steric phenomena at the distal region.

An interesting aspect of the present investigation deals with the temperature dependence of the apparent extent of photodissociation for the various protein–ligand systems.

According to the multibarrier model proposed by Frauenfelder and co-workers, the binding of a ligand to a hemoprotein takes place through a series of sequential processes involving first the penetration of the protein matrix from the solvent to the active site and then the formation of the bond with the heme iron (1). Thus, in a flash photolysis experiment, an intermediate state is populated by the light pulse where the photolysed ligand, still inside the protein, can either rebind to the heme iron or escape from the pocket into the solvent through the protein matrix.

Multiphasic time courses for ligand binding to monomeric hemoproteins have been reported (15, 28). However, for reasons of simplicity, an approximation to a two barrier model will be used to explain the results presented here. This implies that all processes related to the migration of the ligand from the solvent to the inside of the heme cavity, and vice versa, will be included in a single reaction step. The model is illustrated by the following reaction scheme:

$$P + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P \cdot X \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PX, \qquad (1)$$

where P + X represents the situation in which the ligand is present only in the solvent phase. P · X is the intermediate situation where the ligand is in the pocket but not bound to the iron, and PX is the liganded protein.

In the following discussion the assumption is implicit that, within our approximation, the geminate recombination step (step 2) does not directly contribute to the recorded traces. In fact, rebinding from the pocket cannot be observed in our experimental set-up for it is too fast (14). Therefore, the observed amplitude of the optical density change at time zero is proportional to the fraction $\mathcal{F} = k_{-1}/(k_{-1} + k_2)$ of ligand molecules that have escaped from the heme pocket after the photolysing pulse.

We will refer to the apparent extent of photodissociation θ as to the ratio between the observed amplitude of the absorption change, extrapolated to time zero, and the static absorption difference between the ligated and unligated protein. Within our simple scheme 1 this is proportional to the product of the photochemical quantum yield ϕ by the

fraction $\mathcal{F}$. This definition holds for ideally short photolysing pulses. In the real case, the finite duration of the light pulse can induce multiple photodissociations of the recombined ligand with the ultimate effect of pumping it out of the heme pocket (29). In the attempt to evaluate the incidence of such a "pumping effect" on our measurements, we have compared the apparent extent of phododissociation produced by photolysing pulses of different durations and intensities on the various isocyanide derivatives of SW-Mb and Al-Mb (Table I). For SW-Mb, no matter which isocyanide is used as ligand, increasing the energy of the 12-ns laser pulse does not alter the apparent extent of photolysis. However, a drastic rise of θ is observed upon increasing the duration of the low energy pulse from 12 ns to 4 μs. This shows that the intensity of the photolysing light is always enough to fully dissociate all ligands and that a quite pronounced "pumping effect" is present. In contrast, the behavior of Al-Mb changes with the size of the alkyl chain of the ligand. With methyl-isocyanide both the energy and the duration of the photolysing pulse influence the apparent extent of photolysis. This is indicative for a rather low quantum yield and for the presence of a significant "pumping effect." On the other hand, with the *ter*-butyl-isocyanide derivative of Al-Mb, neither the energy nor the duration of the laser pulse alters the apparent extent of photolysis, implying full photodissociation and absence of "pumping effect."

The increase with temperature of the apparent extent of photodissociation, found in some of the investigated reaction systems, cannot be attributed to a trivial change in the equilibrium saturation since the measured negative enthalpy of binding (Fig. 1) would be effective in the opposite direction. Therefore, based on the reasonable assumption that, in the temperature range in which the measurements have been performed, no change occurs of the true quantum yield ϕ, the temperature dependence of θ can be ascribed to a change of the fraction $\mathcal{F}$. This implies, within our simple scheme 1, a higher temperature dependence for k_{-1} than for k_2. As stated above the situation could be complicated by a possible temperature dependence of the "pumping effect." However, this can be excluded in the case of the Al-Mb complex with *ter*-butyl-isocyanide where the "pumping effect" is absent and the temperature dependence of θ is maximal. This indeed seems to be an ideal situation in which a low extent of photodissociation is accompanied by a high quantum yield (photodissociation of the ligand from the heme iron is complete at the laser energies used) in the absence of "pumping effect." For this situation to be possible some bounds to the possible value of k_{-1} must hold: it has to be small compared with the reciprocal of the duration of the longer laser pulse ($2.5 \cdot 10^5\ s^{-1}$) so that the ligand has no time to recombine during illumination. Moreover, to account for the observed low value of the apparent extent of photodissociation ($\theta = 0.09$ at 20°C), k_{-1} has to be 11-fold larger than k_2. Since the overall recombination appears as a strictly bimolecular single exponential in the milliseconds range (at millimolar ligand concentrations), one can fix the order of magnitude for $1/k_{-1}$ in the microseconds time range with a value for the constant of $\sim 3 \cdot 10^4$. In the other cases the extent to which θ depends on temperature cannot be easily rationalized and quantitative information can only be obtained by resolving the elementary kinetic steps of the system. Even though possible complications related to the temperature dependence of the "pumping effect" cannot be excluded, the influence of the bulk of the alkyl side chain is evidently playing an important role in Al-Mb and β^{ZH} chains, where the distal histidine is lacking (5–9). In this case the dominating interaction is that of the hydrophobic side chain of the ligand with the inner surface of the heme pocket. On the contrary, in SW-Mb and in β^A chains a specific interaction between the isocyanide group of the bound ligand and the distal histidine is dominating.

The above concept fits the expectations on the overall rate of ligand dissociation: the presence or absence of a specific interaction between the bound ligand and the distal histidine must influence the ligand dissociation rates. The data of Table I are consistent with this view. It is interesting to note that, as the size of the alkyl group increases, the difference in the "off" rates between SW-Mb and β^A on one side and Al-Mb on the other tends to level off. This shows that the hydrophobic interactions governing the overall ligand binding to Al-Mb, as postulated above, become also increasingly important for Sw-Mb and β^A chains as the bulk of the ligand increases.

For the β^{ZH} chains the isocyanides dissociation rates approach those reported for heme model compounds (30). This finding was expected since the structural modifications induced by the replacement of the distal histidine with arginine (8, 9, 26, 27) lead to an opening of the heme pocket to the solvent (15, 28).

It is clear from Fig. 2 that the effect of temperature on the fraction $\mathcal{F}$ for the β^A chains is not significantly different from the one observed for SW-Mb and, on the contrary, it differs from that of the β^{ZH} chains and Al-Mb. This finding implies that the partition of the ligand molecules between the solvent and the heme pocket is determined by specific interactions between the ligand and the distal residue and is not drastically influenced by overall changes in the protein matrix structure.

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