# Mechanism of Hydrogen Cyanide Binding to Myoglobin†

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ABSTRACT: Cyanide binding to myoglobin is much slower than that of other ferric and ferrous ligands, suggesting rate limitation by bond formation and disruption within the distal pocket. This interpretation is supported by two key experimental observations. First, His64(E7) to Gly and Ala mutations, which open a direct channel from the solvent to the iron atom, and Phe46(CD4) to Leu, Ile, and Val mutations, which increase the mobility of the distal histidine, have little effect on the association rate constant for cyanide binding. In contrast, these mutations cause 100−1000-fold increases in the rate constant for azide binding, showing convincingly that the binding of this ligand is limited by the rate of its movement into the protein. Second, the rate constant for cyanide dissociation is unaffected by changing the size of the residue at position 64(E7) in the series Gly, Val, Leu, Ile, Phe, whereas there is a 2000-fold decrease in the rate of azide dissociation in going from Gly64 to Phe64 metmyoglobin. The major determinants of the cyanide affinity are the ease of water displacement from the ferric iron atom in metmyoglobin, the acid dissociation constant of HCN inside the protein ($K^*_a$), and steric hindrance and electrostatic interactions at the sixth coordination position. Direct hydrogen bonding to the distal histidine does not appear to play an important role in stabilizing bound cyanide. Instead, the general polarity of the distal pocket and its effect on $K^*_a$ are the key factors regulating cyanide affinity under physiological conditions.

The basis of heme protein function is dictated by the structural elements which regulate ligand binding and discrimination. During the last decade site-directed mutagenesis and ultrafast laser photolysis methods have been used to examine the roles of key distal pocket residues in modulating $O_2$, CO, and NO binding to ferrous myoglobins and hemoglobins. Hydrogen-bonding, steric hindrance, overall polarity of the distal pocket, and the presence of internal water molecules all contribute to ligand recognition and discrimination (Springer *et al.*, 1994). Cyanide and azide binding to the ferric forms of these proteins have been examined for over 100 years (Antonini & Brunori, 1971). In addition to general questions of toxicity, the $Fe^{3+}CN^-$ complex is thought to be isoelectronic with the physiologically significant ferrous CO complex, and cyanometmyoglobin has been used as a structural model for CO-myoglobin (Emerson & La Mar, 1990; Rajarathnam *et al.*, 1993).

Brancaccio *et al*. (1994) measured rate and equilibrium constants for azide binding to 46 different mutants of sperm whale, human, and pig metmyoglobins and presented a detailed mechanism for the binding of this ligand. The association rate constant for azide binding is governed by the speed of ligand movement into the protein through a polar channel, the size of which is regulated by the conformation of His64(E7). Although the distal histidine impedes azide movement into the heme pocket, it does form a strong hydrogen bond with the bound ligand. Replacement of His64(E7) with apolar amino acids decreases the equilibrium association constant for azide binding 20−50-fold.

A comprehensive description of cyanide binding to metmyoglobin has not been presented previously because dissociation rate and equilibrium constants for a complete set of heme pocket mutants have not been measured. Mintorovitch and Satterlee (1988) presented a mechanism for cyanide association based on studies with hemoglobins from *Glycera dibranchiata* which contain a leucine residue at the E7 position. Cyanide binding to these hemoglobins is ∼100-fold slower than that to native mammalian myoglobins containing a distal histidine. They interpreted this decrease in terms of a decreased tendency of HCN to deprotonate in the apolar distal pocket of the *Glycera* hemoglobins. Ikeda-Saito *et al.* (1992) and Brancaccio *et al.* (1994) observed a similar pattern: His64(E7) to Val, Thr, Ile, Leu, and Phe substitutions showed marked decreases in $k'_{CN}$. To account for the effects of mutagenesis on the association rate constant for cyanide association, Brancaccio *et al.* (1994) proposed the four-step reaction scheme described in Figure 1, involving: (1) dissociation of coordinated water to form a pentacoordinate ferric intermediate; (2) partitioning of HCN into the distal pocket; (3) deprotonation of HCN to form the much more reactive anion; and (4) bond formation to generate the $Fe^{3+}-CN^-$ complex. The crucial step for cyanide association is formation of $CN^-$ in the distal pocket. Deprotonation of HCN is an unfavorable process at neutral pH because the p$K_a$ of HCN is ∼9. Thus, the rate of cyanide association appears to be determined by the fraction of deprotonated HCN in the distal pocket times the rate of bond formation between internal $CN^-$ and pentacoordinate $Fe^{3+}$ (see eq 8; Brancaccio *et al.*, 1994). If this mechanism is correct, then the overall cyanide dissociation rate constant,

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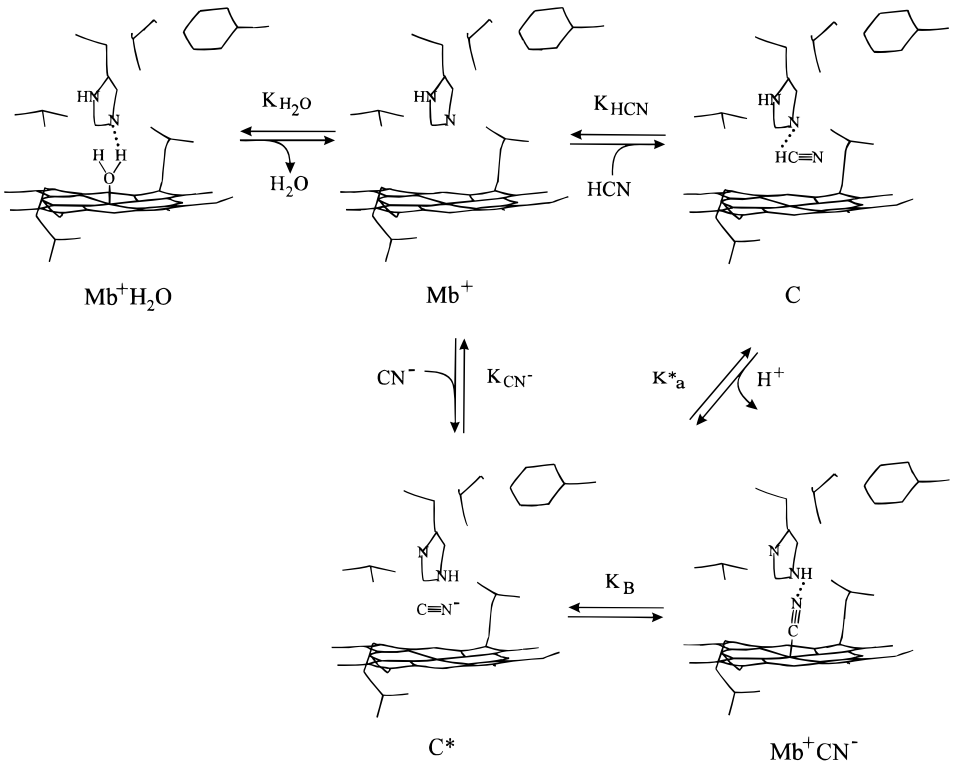


FIGURE 1: Mechanism of cyanide binding to metmyoglobin. Distal amino acid residues shown are from left to right Val68(E11), His64(E7), Leu29(B10), and Phe46(CD4). $Mb^+H_2O$, $Mb^+$, C, C*, and MbCN are the water-coordinated metmyoglobin, unligated metmyoglobin, unligated metmyoglobin containing HCN in the distal pocket, unligated metmyoglobin containing a cyanide anion in the distal pocket, and cyanide-coordinated metmyoglobin, respectively.

$k_{CN}$, should be determined solely by $k_{-b}$, the rate of thermal bond disruption of the $Fe^{3+}-CN^-$ complex.

In order to test the mechanism in Figure 1, we have carried out kinetic measurements of cyanide dissociation from mammalian myoglobins under physiological conditions. Four sets of mutants were examined. Amino acids were varied at the three key positions surrounding the bound ligand: Leu29(B10), His64(E7), and Val68(E11). The effects have been interpreted in terms of (a) steric hindrance; (b) the overall polarity, accessibility, and size of the distal pocket; and (c) direct hydrogen bonding to bound cyanide. The fourth set of proteins involved mutation of Phe46(CD4), which has been shown to regulate the exact conformation and orientation of the distal histidine (Lai *et al.*, 1995). Association rate constants for those mutants not studied by Brancaccio *et al.* (1994) were measured so that a complete set of equilibrium constants could be computed and interpreted in terms of the scheme in Figure 1.

## EXPERIMENTAL PROCEDURES

Construction and expression of the human, sperm whale, and pig myoglobin mutant genes have been described by Ikeda-Saito *et al.* (1991), Springer and Sligar (1987), Carver *et al.* (1992), and Smerdon *et al.* (1991). Purification of recombinant myoglobin and mutant proteins was carried out as described in these references.

Cyanide dissociation rate constants were determined by ligand replacement methods (Antonini & Brunori, 1971; Olson, 1981). We used high concentrations of azide to replace bound cyanide. The reaction mechanism is as shown in eq 1.

$$Mb^+CN^- \underset{k'_{CN}}{\overset{k_{CN}}{\rightleftharpoons}} CN^- + Mb^+ + N_3^- \underset{k_{N_3}}{\overset{k'_{N_3}}{\rightleftharpoons}} Mb^+N_3^- \quad (1)$$

At high concentrations of azide, $d[Mb^+]/dt = 0$, and the observed displacement rate, $r_{obs}$, is given by eq 2 (Olson, 1981)

$$r_{obs} = \frac{k_{N_3}k'_{CN}[CN] + k_{CN}k'_{N_3}[N_3] + k_{N_3}k_{CN}}{k'_{CN}[CN] + k'_{N_3}[N_3] + k_{N_3}} \quad (2)$$

Since the concentrations of the ligands are kept high, $k_{CN}$, $k_{N_3} \ll k'_{CN}[CN]$, $k'_{N_3}[N_3]$. Under these conditions, eq 2 simplifies to

$$r_{obs} = \frac{k_{N_3}k'_{CN}[CN] + k_{CN}k'_{N_3}[N_3]}{k'_{CN}[CN] + k'_{N_3}[N_3]} \text{ or } \frac{k_{N_3} + k_{CN}\dfrac{k'_{N_3}[N_3]}{k'_{CN}[CN]}}{1 + \dfrac{k'_{N_3}[N_3]}{k'_{CN}[CN]}} \quad (3)$$

In our experiments, [CN] was in the $\mu$M range whereas $[N_3]$ was kept in the mM range. Under these conditions, $k'_{CN}[CN] \ll k'_{N_3}[N_3]$, and eq 3 reduces to

$$r_{obs} = k_{CN} + \frac{k_{N_3}k'_{CN}[CN]}{k'_{N_3}[N_3]} \quad (4)$$

The value of $k_{CN}$ can be calculated from a single measurement if $k'_{N_3}$, $k_{N_3}$, and $k'_{CN}$ have been determined previously in rapid mixing experiments [see Brancaccio *et*

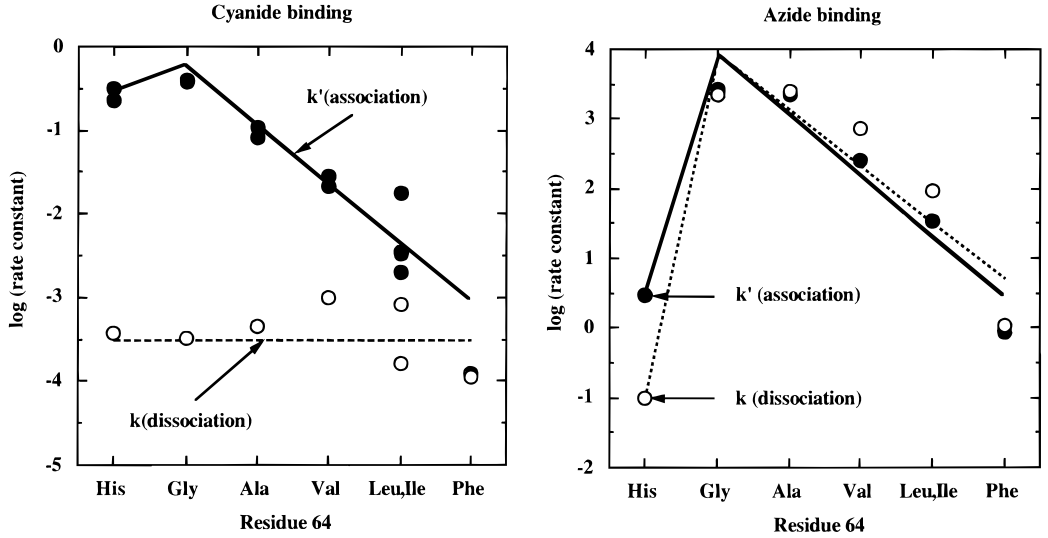


FIGURE 2: Dependence of the rate constants for cyanide and azide binding on the size of residue 64 (E7) in myoglobin.

*al.* (1994)].[1] Alternatively, $k_{CN}$ can be obtained from the *y*-axis intercept of a plot of $r_{obs}$ *versus* [CN]/[$N_3$]. All the replacement reactions were carried out in 0.1 M phosphate buffer at pH 7.0, with [CN] = 10−100 $\mu$M and [$N_3$] = 1−200 mM. The total protein concentration was ∼6 $\mu$M, and absorbance changes were monitored using a Hitachi U-3210 spectrophotometer at 20 °C.

## RESULTS AND DISCUSSION

*Mechanism of Cyanide Binding.* As shown in Figure 2A and Table 1, replacement of His64(E7) by Gly and Ala causes less than 5-fold changes in the kinetic parameters for cyanide binding to mammalian myoglobin. In contrast, these mutations cause dramatic 1000-fold and 20 000-fold increases, respectively, in the association and dissociation rate constants for azide binding (Brancaccio *et al.*, 1994; Figure 2B). The latter observations suggest strongly that the major kinetic barrier for azide binding is ligand movement into and out of the protein through a pathway regulated by the distal histidine. The H64G and H64A mutations create an opening from the solvent to the iron atom which speeds up both azide entry and exit. This idea is supported by the monotonic decreases in both $k'_{N_3}$ and $k_{N_3}$ as the size of the position 64 side chain is increased (Figure 2B). Size and not polarity is the most important factor since $k'_{N_3}$ for Phe64 metmyoglobin is roughly the same as that for wild-type protein containing His64.

Quite different behavior is observed for cyanide binding. Replacing the distal histidine with smaller residues does not cause an increase in $k'_{CN}$. Instead, there is a monotonic decrease in the association rate constant as the apolar character of the position 64 side chain is increased in the order Gly, Ala, Val, Ile/Leu, to Phe. Proof that size is not the key determinant comes from comparisons of the rate constants for His64 and Gln64 with those for Phe64 and Leu64 metmyoglobins. The values of $k'_{CN}$ for proteins containing the large polar residues are 0.1−0.3 $mM^{-1}$ $s^{-1}$, whereas those for mutants containing large aliphatic or aromatic residues are 0.0001−0.003 $mM^{-1}$ $s^{-1}$. In contrast, the rate constant for cyanide dissociation shows little systematic dependence on either the size or the polarity of the residue at position 64 (Figure 2A). Taken together these results suggest strongly that cyanide binding is governed by "inner" kinetic barriers involving $Fe^{3+}-CN^-$ bond formation and disruption and not by ligand movement into and out of the protein. This situation is analogous to that observed for CO binding to the ferrous form of myoglobin [see Carver *et al.* (1990)] and led Brancaccio *et al.* (1994) to propose the mechanism shown in Figure 1.

Table 1: Rate and Equilibrium Constants for Cyanide Binding to Position 64 (E7) Mutants of Mammalian Myoglobins in 0.1 M Phosphate, pH 7.0, 20 °C[a]

| residue at position 64 | $k'_{CN}$ [a] ($mM^{-1}$ $s^{-1}$) | $k_{CN}$ ($s^{-1}$) | $K_{CN}$ ($mM^{-1}$) |
|---|---|---|---|
| His (human WT) | 0.23 | 0.00038 | 600 |
| His (sperm whale WT) | 0.32 | 0.00040 | 800 |
| His (pig WT) | 0.34 | 0.00039 | 870 |
| Gln (human) | 0.18 | 0.000010 | 18 000 |
| Gln (sperm whale) | 0.083 | | |
| Tyr (sperm whale) | 0.0010 | 0.0017 | 0.60 |
| Trp (sperm whale) | 0.0040 | 0.000023 | 170 |
| Gly (human) | 0.40 | 0.00032 | 1200 |
| Gly (sperm whale) | 0.37 | | |
| Ala (human) | 0.11 | 0.00046 | 240 |
| Ala (sperm whale) | 0.08 | | |
| Val (human) | 0.028 | 0.0010 | 28 |
| Val (sperm whale) | 0.021 | | |
| Val (pig) | 0.025 | | |
| Thr (sperm whale) | 0.054 | 0.0013 | 41 |
| Ile (human) | 0.0086 | 0.00083 | 10 |
| Ile (sperm whale) | 0.018 | | |
| Leu (human) | 0.0034 | 0.00016 | 21 |
| Leu (sperm whale) | 0.0020 | | |
| Phe (sperm whale) | 0.00012 | 0.00011 | 1.1 |

[a] Association rate constants, $k'_{CN}$, were measured by stopped-flow rapid mixing techniques [see Brancaccio *et al.* (1994)]. The association equilibrium constants were computed as $K_{CN} = k'_{CN}/k_{CN}$.

The pseudo-first-order association rate constant at high cyanide concentrations is given by the product of the rate of bond formation, $k_b$, and the fraction of unligated metmyoglobin molecules containing the cyanide anion in the distal pocket, $f_{Mb\cdots CN^-}$ or $f_{C*}$. Assuming that ligand movement

[1] The values of $k'_{N_3}$, $k_{N_3}$, and $k'_{CN}$ of H64W, V68N, V68Q, and F46I, which were not included in our previous paper (Brancaccio *et al.*, 1994), have been determined in this study in rapid mixing experiments. The $k'_{N_3}$ values for H64W, V68N, V68Q, and F46I are 13, 7.3, 10.1, and 3400 ($mM^{-1}$ $s^{-1}$), respectively. The $k_{N_3}$ values of these mutants are 4.0, 4.6, 56, and 390 ($s^{-1}$), respectively. The $k'_{CN}$ values are listed in Tables 1, 2 and 3.

into and out of the protein and all protonation steps are in a rapid equilibrium, the fraction of reactive molecules is given by

$$f_{C^*} = \frac{[C^*]}{[Mb^+H_2O] + [Mb^+] + C + C^*} \quad (5)$$

where $Mb^+H_2O$, $Mb^+$, and C are water-coordinated metmyoglobin, unligated metmyoglobin, and unligated metmyoglobin containing HCN in the distal pocket, respectively. The relative concentrations of the $Mb^+H_2O$, $Mb^+$, C, and C* species can be obtained from the following equilibrium expressions:

$$K_{H_2O} = \frac{[Mb^+H_2O]}{[Mb^+][H_2O]}; \quad K_{HCN} = \frac{[Mb^+\cdots HCN \text{ or } C]}{[Mb^+][HCN]};$$

$$K^*_a = \frac{[H^+][Mb^+\cdots CN^- \text{ or } C^*]}{[Mb^+\cdots HCN \text{ or } C]}; \quad K_a = \frac{[H^+][CN^-]}{[HCN]}; \quad (6)$$

$$[HCN] = \frac{[H^+]C_o}{K_a + [H^+]}$$

where $C_o$ is the total concentration of cyanide free in solution (*i.e.*, [HCN] + [$CN^-$]). The final expression for $f_{C^*}$ is

$$f_{C^*} = \frac{K_{HCN}\left(\frac{K^*_a}{K_a + [H^+]}\right)C_o}{1 + K_{H_2O}[H_2O] + K_{HCN}\left(\frac{K^*_a + [H^+]}{K_a + [H^+]}\right)C_o} \quad (7)$$

Since a hyperbolic dependence on the cyanide concentration is not observed experimentally, the third term in the denominator can be neglected and the final expression for the bimolecular association rate constant is

$$k'_{CN} \approx \frac{K_{HCN}}{1 + K_{H_2O}[H_2O]}\left(\frac{K^*_a}{K_a + [H^+]}\right)k_b \quad (8)$$

The overall dissociation rate constant is given by $k_{CN} = k_{-b}$, and the equilibrium association constant is

$$K_{CN} = \frac{k'_{CN}}{k_{CN}} \approx \frac{K_{HCN}}{1 + K_{H_2O}[H_2O]}\left(\frac{K^*_a}{K_a + [H^+]}\right)K_b \quad (9)$$

The pronounced pH dependence of cyanide binding to native myoglobins and hemoglobins is well-characterized and has been analyzed using expressions similar in form to those shown in eqs 8 and 9 (Anusiem *et al.*, 1968; Antonini & Brunori, 1971; Mintorovitch & Satterlee, 1988). The low rate of cyanide binding to myoglobin relative to that of azide is due in part to the fact that only a small fraction of the cyanide present in the distal pocket is ionized at neutral pH (*i.e.*, $K^*_a/(K_a + [H^+])$) whereas azide is fully ionized under these conditions.

*Effects of 64 Replacements on $k'_{CN}$.* Equation 8 provides a useful framework for interpreting the effects of amino acid replacements on the association rate constant for cyanide binding. The small changes in $k'_{CN}$ produced by the H64G and H64A mutations indicate that the polarity of the distal pocket is little affected by these replacements, and as a result, $K^*_a$ is not changed substantially. This view is supported by two independent observations. (1) Water is still coordinated to the iron atom in the ferric forms of these mutants (Quillin *et al.*, 1993; Brancaccio *et al.*, 1994). Thus, the $K_{H_2O}[H_2O]$ term still occurs in eq 8. (2) The major peaks in the IR spectra of Gly64 and Ala64 CO myoglobins are located around 1960 $cm^{-1}$ and are indicative of a loss of positive electrostatic field adjacent to the bound ligand (Li *et al.*, 1994). However, the CO forms of both of these mutants also show substantial absorbance in the 1940−1950 $cm^{-1}$ region, which is indicative of a highly solvated, polar distal pocket.

The linear decrease in log($k'_{CN}$) for the series Ala64, Val64, Leu64, and Phe64 is readily explained in terms of an increase in the p$K^*_a$ for ionization of HCN as the distal pocket becomes more apolar and less accessible to solvent (Figure 2A and Table 1). Since water is no longer coordinated in Val64, Leu64, and Phe64 methemoglobins, the decrease in $K^*_a$ must be substantially greater than the favorable effect of removing the $K_{H_2O}[H_2O]$ term from eq 8. This logic also explains the higher rates of cyanide binding to the Tyr64 and Trp64 mutants when compared to the Phe64 mutant. Both of these residues have negative hydropathy indices and are considered polar compared to Phe which has a positive index (Kyte & Doolittle, 1982). Thus, it is reasonable to assume that $K^*_a$ is larger for these proteins, and as a result, $k'_{CN}$ should be greater than the corresponding parameters for Phe64 metmyoglobin. These increases in polarity are able to compensate for the unfavorable effects of direct Tyr64 coordination to the iron atom and the large size of the indole side chain of Trp64.

*Effects of 64 Mutants on Cyanide Affinity.* The cyanide dissociation rate constant shows little systematic change with mutation of the distal histidine (Figure 2A, Table 1). Replacement of His64 with Gly and Ala causes no change in $k_{CN}$; replacement with Val, Thr, and Ile causes 2−3-fold increases whereas replacement with Leu or Phe causes 2−3-fold decreases. Taken together, these results suggest that bound cyanide is not stabilized significantly by hydrogen bonding to the distal histidine in native myoglobin and that ligand exit from the distal pocket is not limiting the overall dissociation rate constant.

A comparison of the effects of mutagenesis at His64 on azide and cyanide affinities is shown in Figure 3. The H64G mutation has little effect on $K_{CN}$ but causes a 30-fold decrease in $K_{N_3}$ (Table 1; Brancaccio *et al.*, 1994). There is an almost linear decrease in log($K_{CN}$) for the series Gly64, Ala64, Val64, Leu64, to Phe64, whereas all five of these mutants show roughly the same low affinity for azide, which is 30−50-fold less than that of wild-type myoglobin (Figure 3). The latter result shows that specific hydrogen bonding to His64 is a key factor in stabilizing bound azide. The progressive decrease in cyanide affinity is most readily interpreted as due to a systematic decrease in $K^*_a$ with increasing apolar character of the side chain at position 64 (eqs 8 and 9).

The Gln64 mutant shows an anomalously low cyanide dissociation rate constant. Since $k'_{CN}$ is only slightly smaller than that of wild-type metmyoglobin, the net effect of the His64 to Gln substitution is a 30-fold increase in cyanide affinity. The structural cause of this dramatic increase is unclear. Increased hydrogen bonding to the bound ligand

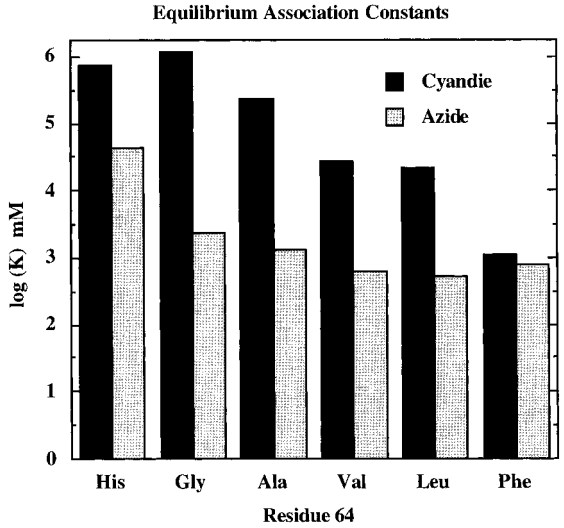


FIGURE 3: Dependence of cyanide and azide affinity on the size of residue 64 (E7) in myoglobin.

is unlikely for two reasons. First, this interaction does not appear to play a key role in cyanide binding as judged by the lack of effect of the H64G and H64A mutations. Second, the His64 to Gln substitution causes a 5-fold decrease in oxygen affinity which is known to be enhanced markedly by hydrogen bonding to residue 64(E7) (Springer *et al.*, 1994). The glutamine side chain does have a slightly more negative hydropathy index and is more flexible than that of histidine. Thus a combination of increased polarity and/or solvent accessibility and less steric hindrance could account for the large value of $K_{CN}$ for Gln64 myoglobin. This appears to be a general phenomenon for ferric ligands since the H64Q mutation also causes a 5-fold increase in azide affinity (Brancaccio *et al.*, 1994).

Replacement of His64 with Tyr causes a ~1000-fold decrease in cyanide affinity, presumably because the phenol oxygen of the tyrosine side chain is coordinated directly to the iron atom and must be displaced by cyanide (Hargrove *et al.*, 1994). This decrease in cyanide affinity is manifested as a ~200-fold decrease in $k'_{CN}$ and a ~5-fold increase in $k_{CN}$. A much smaller, ~4-fold decrease in $K_{CN}$ is produced by the H64W mutation. In this case, both $k'_{CN}$ and $k_{CN}$ decrease markedly, suggesting that the large indole side chain may be limiting the rate of ligand entry and exit.

The 4-fold decrease in cyanide affinity produced by the Trp64 replacement is much less than the 800-fold decrease produced by the Phe64 substitution. Again, this difference appears to be related to the polarity and not the size of the two side chains. The indole side chain is much more polar as indicated both by its negative hydropathy index (Kyte & Doolittle, 1984) and by the presence of a strong absorbance band around 1940 $cm^{-1}$ in the IR spectrum of Trp64 CO-myoglobin (Li *et al.*, 1994). Thus, ionization of HCN in the Trp64 mutant should be greater than that in the more apolar pocket of Phe64 metmyoglobin.

*Phe46 Mutants.* The mechanism proposed in Figure 1 and eqs 8 and 9 was tested further by replacing Phe46(CD4) with a series of smaller, apolar amino acids: Val, Ile, Leu (Table 2). These substitutions do not affect $k'_{CN}$, increase $k_{CN}$ 2−4-fold, and decrease $K_{CN}$ ~4-fold. In contrast, the same mutations cause 200−1000-fold increases in the both association and dissociation rate constants for azide binding (Figure 4; Brancaccio *et al.*, 1994).

Table 2: Rate and Equilibrium Constants for Cyanide Binding to Position 46 (CD4) Mutants of Sperm Whale Myoglobin at pH 7.0, 20 °C

| residue at position 46 | $k'_{CN}$ ($mM^{-1}$ $s^{-1}$) | $k_{CN}$ ($s^{-1}$) | $K_{CN}$ ($mM^{-1}$) |
|---|---|---|---|
| Phe (sperm whale WT) | 0.32 | 0.00040 | 800 |
| Leu (sperm whale) | 0.13 | 0.00080 | 160 |
| Ile (sperm whale) | 0.20 | 0.0016 | 130 |
| Val (sperm whale) | 0.27 | 0.0011 | 240 |

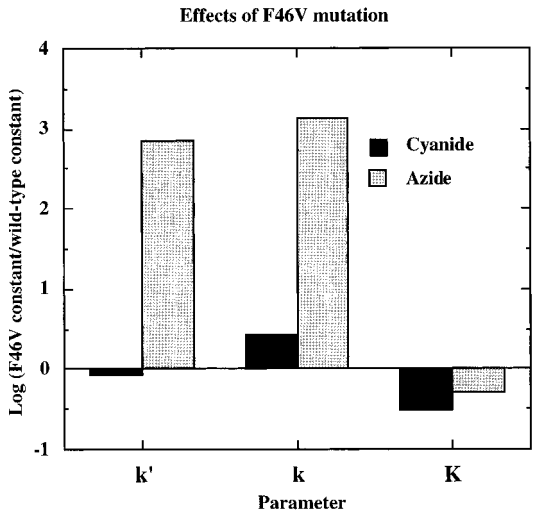


FIGURE 4: Effects of the F46V mutation on the rate and equilibrium constants for cyanide and azide binding to myoglobin.

Lai *et al.* (1995) have shown that Phe46 restricts the mobility of the imidazole side chain of the distal histidine in wild-type metmyoglobin. When this residue is replaced with valine, there is a marked increase in the conformational freedom of the distal histidine. The imidazole side chain can rotate into the space adjacent to the smaller Val46 residue, creating an open and solvated pathway from the outside of the protein into the iron atom. The large increases in both $k'_{N_3}$ and $k_{N_3}$ caused by the F46V mutation show that the major kinetic barrier to azide binding is movement through this channel which is "gated" by the position of the His64(E7) side chain. In the case of cyanide binding, the F46V, like the H64G, mutation has little effect on the observed rate constants, showing that ligand movement into the protein is not rate limiting (Figure 4, Table 2).

*Valine 68 Mutants.* Most of the position 68 substitutions listed in Table 3 cause only moderate (≤2−3-fold) changes in the rate and equilibrium constants for cyanide binding. The major exception is the V68T mutation which decreases $k'_{CN}$ ~30-fold, increases $k_{CN}$ ~5-fold, and decreases $K_{CN}$ 150-fold. Smerdon *et al.* (1991) have shown that the non-bonded electrons of the Thr68 hydroxyl accept a proton from coordinated $H_2O$. Thus, in Thr68 metmyoglobin there are two strong hydrogen bonds holding the bound water in place, and a marked increase in $K_{H_2O}$ explains the large decreases in both $k'_{N_3}$ and $k'_{CN}$ observed for the V68T replacement (Table 3; eq 8; Smerdon *et al.*, 1991; Brancaccio *et al.*, 1994). The same structural feature of the mutant side chain accounts for the increase in $k_{CN}$. The negative portion of the $\beta$-hydroxyl dipole of Thr68 points directly toward all bound ligands. Since water can donate a partial positive charge in the form of a proton, it is stabilized. Anionic or neutral ligands are destabilized, and their dissociation rate constants increased. For example, $k_{N_3}$, $k_{O_2}$, and $k_{CO}$ increase 8-, 3-,

Table 3: Rate and Equilibrium Constants for Cyanide Binding to Position 68 (E11) Mutants of Mammalian Myoglobins in 0.1 M Phosphate pH 7.0, 20 °C

| residue at position 68 | $k'_{CN}$ ($mM^{-1}$ $s^{-1}$) | $k_{CN}$ ($s^{-1}$) | $K_{CN}$ ($mM^{-1}$) |
|---|---|---|---|
| Gly (human) | 2.0 | 0.0030 | 670 |
| Ala (human) | 1.0 | 0.00086 | 1200 |
| Ala (sperm whale) | 1.4 | 0.0012 | 1200 |
| Val (human WT) | 0.23 | 0.00038 | 600 |
| Val (sperm whale WT) | 0.32 | 0.00040 | 800 |
| Val (pig WT) | 0.34 | 0.00039 | 870 |
| Leu (sperm whale) | 0.32 | 0.00027 | 1200 |
| Ile (sperm whale) | 0.15 | 0.00043 | 349 |
| Phe (sperm whale) | 0.37 | 0.00023 | 1600 |
| Ser (pig) | 0.52 | 0.0058 | 90 |
| Thr (pig) | 0.012 | 0.0021 | 5.8 |
| Asn (pig) | 0.3 | 0.0010 | 300 |
| Gln (sperm whale) | 0.19 | 0.0018 | 110 |

and 4-fold, respectively, when Val68 is replaced with Thr in either pig or sperm whale myoglobin (Smerdon *et al.*, 1991; Springer *et al.*, 1994).

The V68S mutation causes a pronounced increase in $k_{CN}$ with only a small change in $k'_{CN}$. An explanation of this effect is difficult. The increase in the dissociation rate constant can be attributed to unfavorable polar interactions between the Ser hydroxyl group and bound cyanide. This mutation produced a similar 20-fold increase in $k_{N_3}$; however, as in the case of cyanide binding, the association rate constant for azide binding was little affected (Brancaccio *et al.*, 1994). The Ser68 side chain should be able to stabilize bound water and thus increase $K_{H_2O}$. However, this unfavorable effect may be compensated by a favorable reduction in steric hindrance due to the flexibility of the methoxy side chain (*i.e.*, an increase in $k_b$ in eq 8).

Similar compensation between the $K_{H_2O}$ and $k_{-b}$ terms may explain the small effects of the V68I, V68L, V68F, V68N, and V68Q mutations. Replacement of Val68 with larger aliphatic or aromatic residues should create a more apolar binding site. However, these larger residues also tend to hinder sterically both coordinated and non-covalently bound water (Quillin *et al.*, 1995). As a result, $K_{H_2O}$ appears to decrease to roughly to the same extent as $K^*_a$, and little net change in $k'_{CN}$ is observed (eq 8). The opposite situation occurs for the V68N and V68Q mutations. In these cases, $K_{H_2O}$ and $K^*_a$ increase to roughly the same extent with the increasing polarity and hydrogen bonding potential of amide groups. The Asn68 and Gln68 side chains also appear to hinder bound cyanide as judged by the increases in $k_{CN}$.

The V68G mutation shows 10-fold increases in both $k'_{CN}$ and $k_{CN}$ with little change in overall affinity. This replacement causes even larger, 100-fold increases in $k'_{N_3}$ and $k_{N_3}$ (Brancaccio *et al.*, 1994). The increase in azide binding rates is readily explained by a widening of the channel for ligand movement into and out of the distal pocket. The increase in both cyanide rate constants is less readily interpreted since ligand diffusion into the protein is not rate limiting for this ligand. Smaller increases in both $k'_{CN}$ and $k_{CN}$ are observed when either Val68(E11) or Leu29(B10) is replaced with Ala, suggesting that these effects are related to the volume and/or solvent accessibility of the distal pocket (Tables 3 and 4).

*Leu29(B10) Mutants.* The rate and equilibrium constants for cyanide binding show little systematic variation with the size of residue 29. None of the aliphatic substitutions

Table 4: Rate and Equilibrium Constants for Cyanide Binding to Position 29 (B10) Mutants of Mammalian Myoglobins in 0.1 M Phosphate pH 7.0, 20 °C

| residue at position 29 | $k'_{CN}$ ($mM^{-1}$ $s^{-1}$) | $k_{CN}$ ($s^{-1}$) | $K_{CN}$ ($mM^{-1}$) |
|---|---|---|---|
| Ala (sperm whale) | 0.7 | 0.0012 | 580 |
| Val (sperm whale) | 0.3 | 0.00020 | 1500 |
| Leu (human WT) | 0.23 | 0.00038 | 600 |
| Leu (sperm whale WT) | 0.32 | 0.00040 | 800 |
| Ile (sperm whale) | 0.36 | 0.00023 | 1600 |
| Ile (human) | 0.20 | 0.00011 | 1800 |
| Phe (sperm whale) | 0.83 | 0.000072 | 12 000 |
| Trp (sperm whale) | 0.25 | 0.000021 | 12 000 |

produces large effects. In contrast, the L29F and L29W substitutions cause 15-fold increases in cyanide affinity, due primarily to marked decreases in the cyanide dissociation rate constant. The structural origin of this stabilization is likely to be favorable electrostatic interactions between the bound cyanide anion and the positive edges of the multipoles of the benzyl and indole rings. The Phe29(B10) multipole has been shown to stabilize bound oxygen and carbon monoxide, causing 15- and 3-fold reductions in $k_{O_2}$ and $k_{CO}$, respectively (Carver *et al.*, 1992; Li *et al.*, 1994).

The origin of the favorable effect of the L29W replacement on cyanide binding is less clear. This mutation markedly inhibits the rate and extent CO and $O_2$ binding to ferrous myoglobin (*e.g.*, 70-fold decreases in both $k'_{O_2}$ and $k'_{CO}$; Springer *et al.*, 1994). In the case of cyanide binding, hindrance by the large indole ring appears to be compensated by destabilization of coordinated water and favorable multipole interactions. The favorable electrostatic interactions would decrease $k_{-b}$ whereas steric hindrance would decrease both $k_b$ and $K_{H_2O}$. The latter two effects must cancel since $k'_{CN}$ is little affected by the presence of Trp29 (eq 8; Table 4).

*Conclusions.* Hydrogen cyanide binding is a slow process and limited by the internal rate of bond formation and disruption within the distal pocket of myoglobin. As a result, the association and dissociation rate constants are little affected by mutations that open a channel from the solvent into the iron atom (*e.g.*, H64G, H64A, and F46V mutations, Tables 1 and 2). The key determinants of the cyanide association rate and equilibrium constants are (1) the strength of water coordination in the aquometmyoglobin; (2) the acid dissociation constant of HCN inside the protein; and (3) steric hindrance and electrostatic interactions at the sixth coordination position. These three factors correspond to the $K_{H_2O}$, $K^*_a$, and $K_b$ terms in eqs 8 and 9. At neutral pH, the extent of ionization of HCN is small in solution and even smaller within the protein. Thus, the rate of cyanide association is directly proportional to $K^*_a$ which is governed by the general polarity of the distal pocket. In contrast, the rate of cyanide dissociation is relatively invariant and governed primarily by the strength of the $Fe^{3+}-CN^-$ bond. Direct hydrogen bonding to the distal histidine does not appear to play an important role in stabilizing bound cyanide. Instead the general polarity of the distal pocket and its effect on $K^*_a$ is the key factor in regulating cyanide affinity under physiological conditions.

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