Binding of Imidazole to the Heme of Cytochrome c₁ and Inhibition of the bc₁ Complex from Rhodobacter sphaeroides

II. KINETICS AND MECHANISM OF BINDING

The kinetics of imidazole (Im) and N-methylimidazole (MeIm) binding to oxidized cytochrome (cyt) c₁ of detergent-solubilized bc₁ complex from Rhodobacter sphaeroides are described. The rate of formation of the cyt c₁-Im complex exhibited three separated regions of dependence on the concentration of imidazole: (i) below 8 mM Im, the rate increased with concentration in a parabolic manner; (ii) above 20 mM, the rate leveled off, indicating a rate-limiting conformational step with lifetime ~1 s; and (iii) at Im concentrations above 100 mM, the rate substantially increased again, also parabolically. In contrast, binding of MeIm followed a simple hyperbolic concentration dependence. The temperature dependences of the binding and release kinetics of Im and MeIm were also measured and revealed very large activation parameters for all reactions. The complex concentration dependence of the Im binding rate is not consistent with the popular model for soluble c-type cytochromes in which exogenous ligand binding is preceded by spontaneous opening of the heme cleft, which becomes rate-limiting at high ligand concentrations. Instead, binding of ligand to the heme is explained by a model in which an initial and superficial binding facilitates access to the heme by disruption of hydrogen-bonded structures in the heme domain. For imidazole, two separate pathways of heme access are indicated by the distinct kinetics at low and high concentration. The structural basis for ligand entry to the heme cleft is discussed.

The binding of small molecules to c-type hemes has been widely studied in type I cytochromes c and is known to result in displacement of the native methionine ligand and to cause significant conformational changes in the heme binding domain (1–4). The affinities and kinetics of binding reflect the interactions among the exogenous ligand, the heme, and the protein and provide a window into the static and dynamic properties of the heme domain.

In cytochromes (cyts)² c and c₂, the methionine ligand of the heme is located in the middle of a 12–18-residue linker connecting two α-helices of the conserved cytochrome c fold. Structural studies of cyts c and c₂ with exogenous ligands bound (1, 2, 5) show that part of the linker sequence (termed the “hinge” by Dumortier et al. (3)) is rotated to create a slight opening or loosening of the heme binding cavity. The initial and final states are generally referred to as closed and open. However, there is no direct evidence that ligand binding is preceded by a spontaneous conformation change that closely resembles the open state seen in ligand-bound structures or that the heme-methionine linkage is actually broken in any intermediate state. Indeed, the mechanism of ligand entry and access to the heme is still unknown.

Here we report on the kinetics of binding of imidazole and N-methylimidazole to cytochrome c₁ in isolated cyt bc₁ complex from Rhodobacter sphaeroides. Cytochrome c₁ is an integral component of the bc₁ complex, playing the role of electron carrier between the iron-sulfur protein and water-soluble cyt c (6). Little is known about ligand interactions with the heme of cyt c₁, although mutational studies have shown that changing the endogenous distal axial ligand (methionine) significantly modifies the midpoint potential of cyt c₁ (7–9). Even less has been reported for exogenous ligand interactions with the c₁ heme. Oszycka et al. (10) reported that cyanide partially inhibits its cyt bc₁ from Rhodobacter capsulatus by binding to ferric cyt c₁, with Kᵦ ~ 25 μM. Inhibition of R. sphaeroides His-tagged bc₁ complex, when isolated with imidazole, was described by Guegova-Kuras et al. (11). This was attributed to lowering of the Eₘₗ of cyt c₁ by binding Im on the basis of its known interaction with cyt c.

The very unusual kinetics of imidazole binding to cyt c₁ lead us to propose a novel mechanism for ligand binding to the heme, in which an initial and superficial binding near the heme cleft facilitates the subsequent entry into the heme pocket. The model is developed in the context of known structural and dynamic features of the heme domain, with general applicability to cyt c heme ligand binding.

EXPERIMENTAL PROCEDURES

Growth of R. sphaeroides strains, preparative procedures for the His-tagged cytochrome bc₁ complex, and methods of assaying ligand binding are described in the accompanying paper (43).
Ligand Binding to Cytochrome c1

The imidazole and N-methylimidazole release rates were determined by adding 1 mM sodium ascorbate and 10 μM DAD to bc1 complex fully saturated with ligand and measuring the slow recovery of the α-band of cyt c1, at 552 minus 541 nm. Because of the lability of oxidized c1 at room temperature, saturation with Im involved preincubation of stock bc1 with 10 mM Im for at least 2 h on ice. After dilution into the assay medium (at room temperature), the residual concentration of ligand was low enough (~0.1 mM) that the rate of release of free c1 by ascorbate/DAD was much greater than the rate of ligand binding. Thus, after release, the rebinding of ligand was negligible compared with the reduction of the c1 heme.

Imidazole and N-methylimidazole have essentially identical pHKa = 7.00 ± 0.05 (12), and both are close to 90% deprotonated at pH 8, as used in this work. Concentrations and concentration-dependent parameters (e.g. Kp, Kp, kobs(on), etc.) are given in terms of total concentration of Im or Melm without correction.

Kinetic measurements of reactions with rate constants slower than 0.5 s−1 were performed on a diode array Agilent 8453 spectrophotometer (integration time 100 ms) equipped with a UV-blocking filter (Corning Glass, CS 0–52, cut-off wavelength 320 nm) to prevent a light-induced reduction of cyt c1. All reactions faster than 1 s−1 were studied using an Applied Photophysics SX-17MV stopped-flow spectrophotometer, with mixing time less than 2 ms and integration time 2.5 ms. Reactions with rate constants in the 0.5–1 s−1 range were typically studied using both instruments to provide overlap of the measurement ranges.

THEORY

The binding of small molecules to c-type hemes has generally been described by minimal two-step mechanisms. Two previously presented versions differ according to whether the second order process of ligand binding precedes or follows the rate-limiting step (3, 4, 13–16). Most authors have adopted a scheme in which cyt c undergoes a rate-limiting but spontaneous structural change that ruptures the relatively weak FeIII–Met bond, forming a five-coordinate heme in an “open” conformation of the protein that permits second order binding of ligand, L.

\[
\begin{align*}
\text{cyt c} & \rightleftharpoons \text{cyt c} + L \\
\text{cyt c} & \rightleftharpoons \text{cyt c} - L \\
\text{cyt c} & \rightleftharpoons \text{cyt c} + L \\
\text{cyt c} & \rightleftharpoons \text{cyt c} - L
\end{align*}
\]

SCHEME 1

The “open” conformation is considered to have a short lifetime at neutral pH, but it allows a number of small molecules (imidazole, cyanide, nitric oxide, azide, pyridine, etc.) to penetrate the heme binding pocket and occupy the position of the sixth ligand of the heme, effectively locking the cytochrome in the open conformation. Applying the steady state approximation \((k_{close} \gg k_{open} + k_{off})\), Scheme 1 yields the following,

\[
k_{obs} = \frac{k_{open} K_{p} [L] + k_{close} [L]}{k_{off} + k_{on}[L]} \quad \text{(Eq. 1)}
\]

where \(k_{obs}\) is the observed, pseudo-first order rate constant for ligand binding to heme.

At low ligand concentration, \(k_{obs} \approx K_{open} k_{on}[L] + k_{off}\) where \(K_{open} = k_{open}/k_{close}\). The apparent second order rate constant is \(k_{obs}(on) = K_{open} k_{on}\) and \(K_{open}\) is expected to be very small at neutral or mildly alkaline pH. At high ligand concentration, a limiting rate is reached, \(k_{obs}(lim) = k_{open}\), which is taken to indicate the frequency of spontaneous rupture of the Fe–Met bond. The measured dissociation constant is \(K_{d} = (k_{open} K_{bind})^{-1}\), where \(K_{bind} = k_{on}/k_{off}\) and the kinetic “Michaelis constant” is given by \(K_{m} = k_{obs}/k_{on}\).

An alternative scheme has the ligand binding prior to breakage of the FeIII–Met bond, followed by a first order rearrangement (ligand exchange) to yield the final product (16, 17).

\[
k_{obs} = \frac{k_{p} K_{b}[L]}{1 + K_{b}[L]} + k_{off}\quad \text{(Eq. 2)}
\]

If the initial binding establishes a fast equilibrium \((K_{B} = k_{p}/k_{-p}\) and \(k_{-p} \gg k_{p}\)), Scheme 2 gives the following.

At low ligand concentration, \(k_{obs} = k_{p} K_{b}[L] + k_{-p}\) and the apparent second order rate constant is \(k_{obs}(on) = k_{p} K_{b}\). At high ligand concentration, the limiting rate is \(k_{obs}(lim) = k_{p} + k_{-p} = k_{p}\). The measured dissociation constant is \(K_{d} = (K_{p} K_{b})^{-1}\), whereas \(K_{p}^{-1}\) is the effective Michaelis constant.

Both mechanisms predict similar behaviors for the rate of product (cyt c – L) formation: first order in ligand at low ligand concentration (second order overall) and zeroth order at high concentration of ligand, as is observed. In either case, the rate-limiting event, characterized by either \(k_{open}\) or \(k_{p}\), corresponds to a substantial structural rearrangement of the heme environment.

As originally proposed (14, 15), the initial binding in Scheme 2 was in the heme pocket, at the Fe–S bond, and the ligand exchange process was by an S_{N}2 mechanism. Formally, however, it describes any mechanism in which the ligand preassociates with the protein in some way that facilitates the subsequent displacement of the Fe–S bond. This view will be developed further below.

RESULTS

Kinetics of Imidazole Binding to Cyt c1—We previously found equilibrium binding of imidazole to cyt c1 to be quite tight, with \(K_{d} \approx 0.33 \text{ mM at } 25^\circ\text{C}\), pH 8 (43). The rate of binding shows a complex dependence on Im concentration and is very temperature-dependent. The kinetic parameters for Im binding are summarized in Table 1, with error estimates.

The initial rate of binding was proportional to the concentration of Im over the range 0.1–2 mM (Fig. 1), with an apparent second order rate constant, \(k_{obs}(on) \approx 27 \text{ M}^{-1} \text{s}^{-1}\), at 25°C. However, at Im concentrations between 2 and 10 mM, the rate increased faster than expected for a second order reaction, indicating a roughly quadratic or parabolic dependence. The apparent second order rate constant increased to...
Ligand Binding to Cytochrome c₁

TABLE 1
Temperature dependence of the kinetics of ligand binding to cyts c
Measurements were at 298 K, except where noted (values in parentheses).

| Ligand          | kₜₐₜₐ  | Eₜₐₐ | log₄ | Source/Reference |
|-----------------|--------|------|------|------------------|
| Imidazole       |        |      |      |                  |
| Cyt c           |        |      |      |                  |
| kₜₐₜₐ(on) (s⁻¹) | 27 ± 3 | 110 ± 6 | 206 ± 1.1 | This work⁴⁻       |
| kₜₐₜₐ(off) (s⁻¹) | 0.012 ± 0.002 | 161 ± 5 | 26.4 ± 0.7 | This work⁴⁻       |
| kₜₐₜₐ(lim1) (s⁻¹) | 1.2 ± 0.2 | 80 ± 20 | 14.3 ± 3 | This work⁴⁻       |
| kₜₐₜₐ(lim2) (s⁻¹) | -      | -    | -    | This work⁴⁻       |
| Cyt c           |        |      |      |                  |
| kₜₐₜₐ(on) (s⁻¹) | 0.063 | 143  | 24   | Ref. 4           |
| kₜₐₜₐ(off) (s⁻¹) | 27    | 93   | 18   | Ref. 4           |
| N'-Methylimidazole |      |      |      |                  |
| Cyt c           |        |      |      |                  |
| kₜₐₜₐ(on) (s⁻¹) | 71 ± 3 | 160 ± 7 | 30.0 ± 1.3 | This work⁴⁻       |
| kₜₐₜₐ(off) (s⁻¹) | 0.79 ± 0.02 | 110 ± 7 | 19.2 ± 12 | This work⁴⁻       |
| kₜₐₜₐ(lim) (s⁻¹) | 72.7  | 7    | -    | This work⁴⁻       |
| Cyt c           |        |      |      |                  |
| kₜₐₜₐ(on) (s⁻¹) | 0.063 | 143  | 24   | Ref. 4           |
| N-Methylimidazole binding to oxidized cytochrome c₁. Top, MeIm in 100 mM NaCl. Solid line, hyperbolic fit to all data for [MeIm] > 800 mM, with Vₜₐₜₐ = 70 s⁻¹, Kₜₐₜ = 1000 mM; kₜₐₜₐ = 1.4 s⁻¹. Bottom, closed circles, Im in 100 mM NaCl; open circles, Im in 900 mM NaCl. Solid line, fit according to Scheme 4 with parameters as given in Table 3. Conditions were as follows. 1 μM oxidized bc₁ complex in 50 mM Tris, pH 8.0, 100 or 900 mM NaCl, 20 mM cholate was mixed with various amounts of Im or MeIm at 25 °C.

At Im concentrations above 100 mM, it is clear that a novel process appears, and the rate increases steeply (Fig. 2). The slope of the increase in log(Rate) versus log[Im] indicates a parabolic dependence (i.e. an n value of about 2). The apparent second order rate constant recovers to a value (50–70 M⁻¹ s⁻¹) similar to that observed at low concentrations, and the rate continues to increase at 1 M imidazole. Almost identical behavior was seen in the presence of 900 mM NaCl. The slope of the increase in log(Rate) versus log[Im] at high concentration is slightly less steep, but it also corresponds to an n value near 2 with some indication of saturation at 1 M (Fig. 2, open circles).

At lower temperatures, the kinetics of Im binding showed a qualitatively similar concentration dependence (initially secondorder, with partial saturation at intermediate concentrations, followed by further acceleration at high concentrations).

At 15 °C, the rate of binding in the low concentration (linear) range gave a second order rate constant of kₜₐₜₐ(on) = 5.5 ± 0.3 M⁻¹ s⁻¹. The plateau was indistinct, but the rate clearly accelerated again at Im concentrations above 200 mM, and the data were reasonably well fit by a simple model (see “Discussion”) with a plateau rate of kₜₐₜₐ(lim) = 0.35 ± 0.1 s⁻¹.

At 5 °C, Im binding was so slow that the kinetics could not be directly measured at concentrations less than 5 mM. The rate was linear up to 25 mM imidazole, with an apparent second order rate constant kₜₐₜₐ(on) = 1.2 M⁻¹ s⁻¹, decreasing to 0.5 M⁻¹ s⁻¹ over the range 50–350 mM imidazole. At high concentrations (>400 mM), the apparent binding rate constant increased again, to at least 1.6 M⁻¹ s⁻¹, and the rate accelerated with no sign of saturation up to 800 mM. The data could be crudely fit with a plateau rate of 0.09 ± 0.03 s⁻¹.

The temperature dependence of the second order rate constants in the low concentration range indicates a very substan-
Equivalence of activation and equilibrium parameters for ligand binding to cyt c1.

| Ligand  | $k_{obs(on)}/k_{obs(off)}$ | $(K_M)^{-1}$ | $\Delta H^\circ$ | $A/s$ |
|---------|--------------------------|-------------|-----------------|-------|
| Cyt c1-Im | -51 ± 3 | 33 ± 7 | 56 ± 6 | 36 ± 5 |
| Cyt c1-Melm | 50 ± 10 | 61 ± 10 | 47 ± 6 | 59 ± 5 |


tional activation energy for Im binding, $E_a \approx 110$ kJ/mol, and a large preexponential factor, log $A = 20.6$. Comparison of the fitted plateau rates yields $E_a \approx 80$ kJ/mol and log $A \approx 14$ for this limiting process.

Kinetics of N-Methylimidazole Binding to Cytochrome c1—In contrast to Im, the rate of Melm binding increased monotonically with concentration, with the exception of a slight decline at the highest concentrations (Fig. 2). A simple hyperbola fits the data well up to 600 mM, with $K_m \approx 1$ mM and $V_{max} \approx 72$ s$^{-1}$. This is much faster than the plateau value for Im but comparable with the extrapolated maximum rate for Im (see below). The kinetic parameters for Melm binding are summarized in Table 1, with error estimates.

The second order rate constant for N-methylimidazole binding ($k_{obs(on)} = 71$ M$^{-1}$ s$^{-1}$ at 25°C, pH 8) was similar to that for imidazole binding calculated from the hyperbolic fit to the 10–50 mM data range. The apparent rate constant as [Melm] → 0 yields the unbinding rate constant, $k_{obs(off)} \approx 1.4$ s$^{-1}$.

At 15°C, the Melm binding rate was linear with concentration, with a second order rate constant of $7.5 \pm 0.2$ M$^{-1}$ s$^{-1}$. From the binding rate constants at 15 and 25°C, we estimate an activation energy for Melm binding at 160 kJ/mol with a preexponential factor of log $A = 30.0$ (see Table 2).

Unbinding from the Cyt c1-Ligand Complex—The Im and Melm release rates from cytochrome c1 were determined from the recovery of ascorbate reducibility (Fig. 3). The observed reduction is the net result of at least three reactions in sequence: release of ligand from cyt c1, rebinding of methionine to the heme to yield the high redox potential form, and reduction by ascorbate/DAD. From the plateau rate, we estimated the limiting step in methionine displacement and Im binding to the heme, $k_{obs(im)} \approx 1$ s$^{-1}$ at 25°C. Because the cyt c1 heme is predominantly ligated to Met in the native state, the rate of conformational recovery and Met rebinding must be much faster than 1 s$^{-1}$. Also, the rate of reduction of uninhibited cyt c1 with ascorbate/DAD exceeds 10 s$^{-1}$ at the concentrations used. Finally, the residual concentration of Im after dilution was 0.1 mM, which, together with the above estimate of $k_{obs(on)} = 27$ M$^{-1}$ s$^{-1}$, gives a rate of $3 \times 10^{-3}$ s$^{-1}$ for rebinding of ligand to cyt c1 at 25°C, at least 3 orders of magnitude slower than the rate of native cyt c1 reduction by ascorbate/DAD. Therefore, the net rate of reduction, which was less than 0.1 s$^{-1}$ at all temperatures, is equal to the rate of Im unbinding from the cyt c1 heme, including any associated conformational changes.

For N-methylimidazole, the same argument applies. The observed rate of reduction by ascorbate/DAD, which was less than 1 s$^{-1}$ at all temperatures, is clearly indicative of Melm release rather than the conformational recovery.

The observed rates of reduction at 25°C were 0.012 and 0.79 s$^{-1}$ for Im and Melm, respectively, indicating release rates, $k_{obs(off)}$, that differ by about 65-fold. The off rate for Im agrees well with the estimate obtained by extrapolating the binding rate to zero ligand concentration (0.014 s$^{-1}$; see Fig. 2). However, for Melm, the off rate measured in this fashion is roughly half the value (1.4 s$^{-1}$) determined by extrapolation of the binding rate (Fig. 2). The release rates for both Im and Melm show very strong temperature dependence (Fig. 3), with apparent activation energies of $E_a = 161$ kJ/mol for Im and 110 kJ/mol for Melm. The preexponential factors, determined by extrapolation to 1/T = 0, were also very large: log $A = 26.4$ and 19.2 for Im and Melm, respectively. The measured kinetic parameters for Im and Melm release are summarized in Tables 1 and 2.

**DISCUSSION**

Ligand Binding to c-type Hemes—For cyt c and c$_p$, the equilibrium binding properties are well known for many exogenous ligands, but the mechanism of binding is still under debate. Many ligands to the ferric heme exhibit saturation kinetics, indicating an internal step that is rate-limiting at high concentrations (4, 16–18). Two versions of a minimal two-step model are described by Schemes 1 and 2 (see “Theory”), which, with steady state and rapid equilibrium constraints applied, yield formally identical kinetic behavior-hyperbolic dependence on ligand concentration, reaching a maximum rate determined by an internal event (Equations 1 and 2). Without additional information, the two models cannot be distinguished, although previous reports have generally favored Scheme 1 (3, 4, 13, 16). We observed similar behavior for cyt c$_1$ with many ligands, including Melm described here. However, for Im, the concentration dependence of the rate of binding is complex and necessitates the introduction of an initial (“superficial”) binding interaction.
prior to the conformational and bond formation events that yield the heme liganded state. We first compare the kinetics of Melm and Im binding to cys c1, c2, and c3 before developing a new model of ligand binding that accommodates the unusual behavior of Im binding to cty c1.

N-Methylimidazole Binding and Release—The kinetics of binding N-methylimidazole to cty c1 are well fit by a hyperbolic concentration dependence (up to 0.6 mM). The associated parameters (Table 1) are similar in value to those reported for binding of many nitrogenous ligands to cys c and c2 (kobs(on) = 30–130 M−1 s−1 (4, 16, 19), kobs(lim) = 25–60 s−1 (3, 4, 16, 18)).

The value of kobs(off) = 1.4 s−1 obtained by extrapolating kobs to zero concentration is almost twice as large as the value (0.79 s−1) measured by assaying heme reducibility after preincubation with Melm. No such discrepancy was seen for kobs(off) for Im. The smaller value yielded kobs(off)/kobs(on) = 11.1 mM for Melm, in good agreement with the measured Kd = 9.3 mM (43). It is possible that the difference between the two assays of kobs(off) for Melm indicates a slow conformational or configurational change in the heme-bound state of Melm that is not complete on the time scale (≤1 s) of the initial rate of binding.

Imidazole Binding and Release—The concentration dependence of the kinetics of Im binding to cty c1 is markedly different from that of Melm or that reported for any previously described ligand or cty c combination. Two regimes were apparent, at 0–2.5 mM and >100 mM imidazole. In both regions, the rate increased with concentration in an approximately parabolic manner before approaching a saturation level. Preliminary studies show qualitatively similar dependences for 2-methyl- and 4-methylimidazole (not shown), and so far it is unique to imidazoles with both nitrogens free.

Because of the sigmoidicity of the concentration dependence, estimates of the second order rate constants for Im binding varied from 25 to 70 M−1 s−1. This range is in good agreement with values for horse heart cty c and R. sphaeroides and R. capsulatus cty c2 (50, 125, and 90 M−1 s−1, respectively) (4, 16).

Two limiting rates are evident in the concentration dependence. Above 30 mM, the rate of Im binding approached a limiting value, kobs(lim1), of about 1 s−1, indicating an implicit conformational step as seen in cty c and c2 but with a much longer transition time (1 s versus 30 ms). After reaching an intermediate plateau at 50–100 mM, the rate of binding increased again at higher concentrations, with a parabolic dependence (n = 2 in the log(Rate) versus log[Im] plot). Saturation was not reached at 1 mM imidazole, but the negative curvature indicated a limiting rate (i.e., kobs(lim2)) of about 100 s−1. This is similar to the limiting rates seen for cys c and c2 (3, 4, 16, 18).

The steep acceleration at high concentrations is not an ionic strength effect. The contribution to ionic strength from the imidazole is small because only 10% is charged at pH 8, and raising the ionic strength from 100 to 900 mM NaCl had only minor effects on the kinetics and concentration dependence. Furthermore, in high salt, the increase in rate at high Im concentration was still indicative of n ≈ 2.

The measured rate constants for Im binding and release yield kobs(off)/kobs(on) = 0.012/27 = 0.44 × 10−3 M. This is reason-ably consistent with the measured dissociation constant, Kd = 0.33 × 10−3 M (43).

Temperature Dependence of Ligand Binding and Release Kinetics—The temperature dependences of the observed rates for Im and Melm binding and release by cty c1 were studied in the low concentration region (i.e., kobs(on), kobs(off), and kobs(lim1)), and the activation parameters, Ea and logA, are summarized in Table 1. The temperature dependences of the high concentration limit rate for Im, kobs(lim2), and Melm were not accessible due to lack of saturation at the lower temperatures.

The activation parameters for the on and off rates are consistent with the independently determined thermodynamic factors of equilibrium binding (43). Applying a simple transition state analysis to the temperature dependence of the kinetics, we can compare Ea and RlnA for the on and off rate constants with the enthalpy (ΔH°) and entropy (ΔS°) of the equilibrium binding constant. The non-enthalpic components, the intercept at 1/T = 0 in transition state theory is given by the equation, lnA = lnkTSST + ΔS°TSST/R + lnk. Assuming that kTSST and the adiabaticity factor, κ, are the same for on and off reactions, R(lnAon − lnAoff) = ΔS°TSST, which equates with ΔS°. These comparisons are summarized in Table 2 and demonstrate the equivalence of the kinetic activation and equilibrium terms, within reasonable expectations of the data.

The magnitudes of the activation parameters associated with all of the rate processes are very large but are comparable with those reported for Im and Melm binding to cty c (20–22) and cty c2 (4) (Table 1). In particular, Dumortier et al. (4) analyzed Im binding to cty c2 according to the mechanism of Scheme 1. They decomposed the overall reaction into two steps, the closed-open transition and Im binding to the open state, and reported separate parameters for kopen and koff. Their temperature data yield values of Ea and logA for koff which agree well with those for the unbinding rate constant, kobs(off), for cty c1-Im. Their results for the rate-limiting step in Scheme 1, which is identified with the closed-open transition, kopen, are comparable with our estimate of Ea for the plateau rate, kobs(lim1), for Im binding to cty c1. However, a more suitable comparison might be with the maximum rate, kobs(lim2), for which we have no temperature dependence data.

All net binding mechanisms under consideration involve significant structural reorganization to make room for the exogenous ligand and to stow the released methionine side chain. The very large Arrhenius preexponentials and enthalpies are consistent with the kinetic barrier arising from highly cooperative events akin to protein folding or, more generally, structural changes in a complex, fluctuating matrix (23).

A Four-site Binding Model for Imidazole—The dependence of imidazole binding in both low and high concentration regions is parabolic, suggestive of a substrate activation process (24). Within each concentration region, this can be accounted for by an initial or “superficial” binding of two Im's that precedes

3 The correction to the activation energy to yield the activation enthalpy, ΔH°TSST = Ea − RT, cancels in the difference between on and off rate values and in any case is well within the error of the data (RT ≈ 2.5 kJ/mol).
4 Calculated from Table III of Dumortier et al. (4).
Ligand Binding to Cytochrome c₁

TABLE 3
Fit parameters for imidazole binding (Scheme 4)

| Parameter | Value |
|-----------|-------|
| \( k_{B1} \) | 0.3 ± 0.1 s⁻¹ |
| \( k_{B2} \) | 0.85 ± 0.15 s⁻¹ |
| \( k_{R3} \) | ≤ 5 s⁻¹ |
| \( k_{R4} \) | 110 ± 20 s⁻¹ |
| \( k_{-R1} \) | 0.014 ± 0.003 s⁻¹ |
| \( k_{-R3} \) | ≤ 0.1 s⁻¹ |
| \( k_{-R4} \) | ≤ 0.001 s⁻¹ |
| \( k_{-R} \) | ≤ 1 s⁻¹ |

The non-zero value of the second order rate constant for Im binding at low concentrations indicates that the substrate activation is “non-essential” (i.e. the single ligand state, \((L)c\), has finite reactivity, but the double ligand state, \((L)c(L)\), is more active (24)). This yields Scheme 4, with \( k_{R1} \) < \( k_{R2} \). The inclusion of the binding equilibria after reaction, \( K_{B1} \) and \( K_{B2} \), is necessary for a complete thermodynamic description that meets the requirements of microscopic reversibility. However, it has little impact on the concentration dependence of the kinetics unless the pre- and postreaction equilibria are dramatically different.

The effective second order rate constant for Im binding at low concentrations is given by \( k_{R2} \), and the dissociation constant is \( K_{B2} \), which is also the dissociation constant for Im binding at low concentrations, \( K_{B1} \).

Scheme 4 leads to an observed rate of ligand binding (see supplemental material) as follows,

\[
k_{obs} = \frac{k_{R1}K_{B1}[L] + k_{R2}K_{B2}[L]^2}{1 + K_{B1}[L] + K_{B2}[L]^2} + k_{-R}
\]

where \( k_{-R} \) includes \( k_{-R1} \) and \( k_{-R2} \) and is weakly concentration-dependent due to \( K_{B1} \) and \( K_{B2} \). However, at \([L] \to 0\), it resolves to \( k_{-R1} \). Analytical expressions are obtained for the second order binding rate constant, \( k_{obs}(on) = k_{R1}K_{B1} + k_{-R1}K_{B2} \), the off rate constant, \( k_{obs}(off) = k_{-R2} \), and the dissociation constant, \( K_a = (K_{B1}K_{R1})^{-1} \), all at low Im concentration, and a limiting rate constant, \( k_{obs}(lim1) = k_{R2} \), at intermediate concentrations. This describes very well the kinetics of Im binding in the 0–50 mM concentration range. However, the rate of Im binding increased again at higher concentrations, also with a parabolic dependence as seen at low concentration.

Because of the clear separation between the low and high concentration regimes, the full range of the Im binding kinetic data can be well accounted for as the sum of two independent reaction paths, both exhibiting substrate activation at a pair of binding sites, sites 1 and 2, with relatively high affinity \( (K_{B1} \) and \( K_{B2} \)), as described above, and sites 3 and 4 with much weaker affinity \( (K_{B3} \) and \( K_{B4} \)). Each path is described by Equation 3, with distinct parameter sets for the low and high concentration regions (subscripts 1 and 2 and subscripts 3 and 4, respectively). This full description provides an additional limiting case of \( k_{obs}(lim2) = k_{R2} + k_{R4} \equiv k_{R4} \) at \( K_{B4}K_{B4}[L]^2 \to \infty \).

Implications of the Four-site Model for Imidazole Binding—The resulting fit to the imidazole data, using two Equations 3, is shown in Fig. 2. Both sets of Im data at 25 °C, at low and high ionic strength, are well fit by similar parameters, and the compensation between rates and affinities does not warrant refinement beyond the range of values given in Table 3.

The effective second order rate constant for each state is approximately given by \( k_{R1}K_{B1} \), \( k_{R2}K_{B2} \) (\( n = 1-4 \)). In the lowest concentration range, the inverse relation provided an initial estimate of \( k_{R1} \) using the measured value of \( k_{obs}(on) = 27 M^{-1} s^{-1} \). In the intermediate plateau region, we can still largely ignore the low affinity sites, and there is one dominant state with both high affinity sites occupied and \( k_{R2} \) = 0.85 s⁻¹. The apparent association constant is approximately \( K_{B2}^{pp} = 75 M^{-1} \), giving an on-rate constant of \( \sim 65 M^{-1} s^{-1} \). This is in good agreement with the maximum slope of 55 M⁻¹ s⁻¹ observed in the range of 3–10 mM imidazole. For occupation of low affinity site 3, which appears with \( K_{B3} \approx 0.5 M^{-1} \), a significant reaction rate would obliterate the plateau. The observed dependence requires that each single occupancy of low affinity site 3 exhibits only low reactivity, with \( k_{R3} < 5 s^{-1} \). However, with both low affinity sites occupied, the reactivity is greatly enhanced, with \( k_{R4} = 110 s^{-1} \). Estimating \( K_{B3}^{pp} \approx 0.65 M^{-1} \), this corresponds to \( k_{obs}(on) \approx 70 M^{-1} s^{-1} \).

When high affinity binding sites 1 and 2 are both occupied, the maximum rate \( k_{R2} = 0.85 s^{-1} \) is 2–3 times faster than for...
the singly bound state \( (k_{R1} = 0.3 \text{ s}^{-1}) \). For the low affinity sites, comparison of \( k_{R4} \) and \( k_{R3} \) shows a substantially greater enhancement (at least 20-fold) for double versus single occupancy of sites 3 and 4. Comparison of \( k_{R4} \) with \( k_{R2} \) and \( k_{R1} \) shows the fully occupied state to be more than 100-fold more reactive than when only the high affinity sites are filled and 300 times faster than for singly occupied high affinity site 1.

The reaction equilibrium constants for each state are given by \( K_{Rn} = k_{Rn}/k_{Cn} \) or \( K_{Rn} = (K_{Rn}K_{C})^{-1} \). The measured and fitted kinetic data sets are incomplete for this purpose, but we can obtain a likely range of values by assuming either that \( k_{C} \approx k_{R1} \) (except for \( k_{R3} \) which must be less) or that \( K_{R} \) is roughly constant. Although \( K_{R} \) can vary, microscopic reversibility requires it to be the same for both binding pathways with single occupancy (i.e. \( (K_{R1}K_{R3})^{-1} = (K_{R3}K_{R3})^{-1} \approx 0.4 \text{ mM} \)).

The resulting reaction equilibrium constants are summarized in Table 4. Despite the range of values obtained for each, using the two relationships, it is clear that there are two distinct classes of postbinding reaction equilibrium: for the high affinity binding sites \( K_{Rn} < 100 \) and for the low affinity binding sites \( K_{Rn} > 1000 \). Also, for \( K_{R3} \approx 5000 \), the limit value of \( k_{R3} < 5 \text{ s}^{-1} \) requires \( k_{R3} \approx 0.001 \text{ s}^{-1} \). This is substantially smaller than \( k_{R1} \). These differences in kinetic and equilibrium constants between the high and low affinity binding sites probably originate in the open/close and ligand exchange contributions to the “reaction step,” which we discuss next.

The Constituent Events of the Reaction Steps—Although the underlying events comprising the “reaction step” are unknown in detail, they must include conformational and liganding components. To provide insight into the nature of the initial binding events and their influence on heme ligand formation, we consider a two-step expansion of the reaction step in Scheme 3,

\[
K_{R1}[L] \rightleftharpoons (L) c \rightleftharpoons (L)c^* (L) \rightleftharpoons (L)c - L
\]

SCHEME 5

where \( c^* \) represents a state of cyt c, with the heme accessible to the exogenous ligand. Scheme 5 identifies open/close and ligand exchange processes, such that \( K_{R} = K_{C}K_{c} \). Assuming that \( k_{C} \gg k_{O} \) (i.e. \( K_{O} \ll 1 \)), we have the following.

\[
k_{R} = \frac{k_{O}k_{L} + k_{R}k_{c}}{k_{O} + k_{c}} \quad \text{(Eq. 4)}
\]

\[
k_{R} = \frac{k_{L}k_{c}}{k_{R} + k_{c}} \quad \text{(Eq. 5)}
\]

These reduce to distinct limits, depending on whether \( k_{C} \gg k_{L} \) or \( k_{C} \ll k_{L} \).

The slow reaction rates \( (k_{R1}, k_{R2}) \) and relatively small reaction equilibria \( (k_{R1}, k_{R2}) \) associated with the high affinity binding sites suggest the influence of a very small value of the open/closed equilibrium, \( K_{O} \). We suggest that this is due, in part, to a fast rate of closure, such that \( k_{C} > k_{L} \). This yields \( k_{R} \approx K_{O}k_{L} \) from Equations 4 and 5. Thus, \( k_{R} \) (Melm) and \( k_{R} \) (Im) (i.e. \( k_{Melm}(off) \)) provide measures of the rate of ligand unbinding from the heme, \( k_{L} \). The modest 2–3-fold activation of \( k_{R} \) that occurs upon binding a second imidazole at site 2 can act through an increase in \( K_{O} \) while maintaining the same rate relationship \( (k_{C} > k_{L}) \).

In the high concentration/low affinity regime, the standout features are the small values of \( k_{R3} \) and \( k_{R4} \) and the substantial acceleration upon binding a second Im \( (k_{R4}) \). We suggest that for single occupation of site 3, the conformational change is slow, and in particular, the rate of closure is slow so that \( k_{C} < k_{L} \). This leads to \( k_{R} \approx K_{O} \) and \( k_{R} \approx K_{C}K_{L} \). Thus, as surmised above, the values of \( k_{R3} \) and \( k_{R4} \) reflect quite different processes from \( k_{R1} \) and \( k_{R2} \), etc. Activation of the low affinity path upon binding a second Im to site 4 would probably occur through acceleration of both \( K_{O} \) and \( K_{C} \) possibly changing the rate-determining condition to \( k_{C} > k_{L} \), so that \( k_{R} \approx K_{C}K_{L} \) and \( k_{R} \approx K_{L} \) at high concentrations.

This description implies that singly bound Im at site 3 leads to a kinetically sluggish response of the heme cleft. As discussed below, we propose that this is due to its ability to form two hydrogen bonds that limit the conformational flexibility of the protein. Binding the second Im releases the kinetic constraint, greatly enhancing \( k_{O} \) and \( k_{C} \) and possibly revealing an increased value of \( K_{O} \).

Comparison of Imidazole and N-Methylimidazole Binding—The simple hyperbolic concentration dependence seen for Melm appears to be quite distinct from the complex behavior of Im. However, the adequacy of a hyperbolic fit for Melm is not evidence for the absence of multiple binding sites, whereas the behavior seen for Im requires the minimal assumption of two pairs of sites. Thus, Melm could exhibit a multiplicity of binding sites, but it is possible to eliminate the kinetic significance of high affinity sites. The measured on and off rate constants are fully consistent with the measured dissociation constant, \( K_{d} \approx 10 \text{ mM} \). Because \( K_{d} = (K_{O}K_{C})^{-1} \), if \( K_{O} \gg 1 \), as reasonably expected, then \( (K_{C})^{-1} \gg 10 \text{ mM} \) for all sites. If sites existed with \( (K_{Melm})^{-1} < 10 \text{ mM} \), which is even tighter than Im, then \( K_{R} \) would be \( \approx 1 \), and the maximum extent of heme ligation would be small, contrary to observation (43). Here we compare the characteristics of Im and Melm binding at the low and high concentration limits, assuming a single binding site for Melm.

The determinations of \( k_{Melm}(on) \) and \( k_{Melm}(off) \) at low concentration yield dissociation constants for Im (0.44 mM) and Melm (11.1 mM) that differ by a factor of 25, in excellent agreement with our equilibrium measurements (43). The kinetic affinity constant \( (K_{d}) \) for Im (in the low concentration range) and Melm exhibit even greater disparity (\( \approx 100 \text{ versus } 1 \text{ M}^{-1} \)). Because \( K_{d} = (K_{O}K_{C})^{-1} \), these evaluations yield the net reaction equilibrium \( K_{R} \approx 90 \) for Melm. Alternatively, the kinetic fit yields \( K_{R} = k_{R}/k_{C} = 70/1.4 = 50 \). The equivalent parameter for Im is \( K_{R} \approx 25–50 \) (Table 4).

The relative similarity of these values for Im (at low concentration) and Melm is coincidental because \( K_{R} \) is composite, including both conformational and heme bonding changes (i.e. Methylimidazole...
**Ligand Binding to Cytochrome c₁**

...amino acid two residues from the methionine (βXM motif), and, unique to *Rhodobacter*, a disulfide bond.

The site of ligand entry is not known for any cytochrome *c*, but it is noteworthy that the heme of cyt *c₁* is markedly solvent-exposed and is exposed on two sides, in contrast to cyts *c* and *c₂*. It is particularly striking that the two propionate groups (on rings A and D) of the *c₁* heme are fully exposed because these are completely concealed in cyts *c* and *c₂* (see supplemental material). We suggest that the propionates are key elements of the high affinity sites of Im binding in cyt *c₁*. This face of the cyt *c₁* protein, which is quite polar, also constitutes the docking site for the iron sulfur protein (ISP) subunit of the *bc₁* complex (28, 29), so the heme is alternately exposed and occluded during the turnover cycle of the *bc₁* complex.

The apparently unique ability of Im and methyl derivatives with both nitrogens free to access a high affinity path in cyt *c₁* implicates the capacity to form two hydrogen bonds. Access and binding to the heme from these sites is slow, with a maximum rate of about 1 s⁻¹, reflecting a likely limitation in the structural dynamics along this edge of the heme cleft. The propionates are engaged in electrostatic interactions with residues on both sides of the cleft, which may restrict its movement. Interference with these interactions may be necessary to allow sufficient flexibility for ligand entry.

The parabolic dependence in the low concentration range implicates an activated process, as illustrated by Scheme 3. We suggest that a single Im binds at or near the propionates, forming two hydrogen bonds that either restrict its own mobility or may bridge the heme cleft, thereby restraining its movement. A second Im then substitutes as a hydrogen bond partner of the first, inducing greater flexibility and allowing Im to progress more readily toward the heme. This would be consistent with the superficial binding modes modifying *kₐ* and/or *Kₒp*.

The heme is also exposed along the ring C-D edge. This face of the cyt *c₁* protein is quite apolar, and backbone amides provide the only potential for hydrogen bond interactions around the cleft opening. Only this heme edge is exposed in cyts *c* and *c₂*, which exhibit kinetically simple binding of Im and Melm at high concentrations. It seems likely, therefore, that this edge provides a common site of entry for ligands of these cytochromes and for the low affinity pathway of Im entry to cyt *c₁*. The parabolic dependence of Im binding to cyt *c₁* in the high concentration regime indicates that the surface binding of a single imidazole is also not sufficient to facilitate the entry of imidazole into the heme pocket via the low affinity sites and that binding of a second imidazole is necessary for facile entry. The restriction of this behavior to Im again suggests that the formation of two hydrogen bonds may underlie this effect.

In contrast to Im, monodentate Melm binds more weakly but can still break up a hydrogen-bonded pair. However, it would be unable to restrain the heme cleft dynamics by forming a second hydrogen bond. Binding a single Melm would therefore suffice to facilitate the first steps of entry into the heme pocket.

X-ray structures of cyt *c₂* and its Im adduct show that there is remarkably little perturbation of the heme cleft at the propionate (ring A-D) edge, and the major change in the final structure occurs along the ring C-D and B-C edges of the heme cleft (1). In particular, the native ligand methionine (Met¹⁰⁰ in...
R. sphaeroides cyt c2 is displaced to the heme edge and becomes almost fully solvent-exposed, and Phe102 lifts up from the ring B-C edge to allow a clear view of the bound imidazole (see supplemental material).

The protein structural options for initial binding events in cyt c2 are largely limited to backbone amides. In R. capsulatus cyt c2, mutation of Gly95 causes substantial enhancement of the rate of ligand binding, although it does not move much itself (30). This residue, which is adjacent to the ligand methionine, is not at all conserved (it is Lys99 in R. sphaeroides cyt c2), but the peptide NH is in strong interaction with the D propionate of the heme in both the native and Im-bound states (1). Thus, despite its very similar placement in the initial and final states, the kinetic behavior of the Gly95 mutants suggests a role in the transition.

In simulations of the R. sphaeroides cyt c1-Im adduct (43), the results were quite similar to cyt c2, despite the very different sequences. The conformational change displaces the methionine (Met185) to the heme edge, and the major movement raises the first two of three consecutive prolines (Pro186-Pro188) along the heme C-D edge (Fig. 4). In cyt c1, the residue immediately before the methionine (equivalent to Gly95 in R. capsulatus cyt c2), is Ala184 in R. sphaeroides and Arg182 in R. capsulatus. As for cyt c2, the backbone amide of these unconserved residues is well placed to hydrogen-bond to the D propionate. However, in contrast to cyt c2, the cyt c1-Im simulations showed a substantial displacement of this residue. On the other hand, there is much less perturbation along the B-C edge of the heme. Perhaps because of the rigidity of the triple proline sequence, the backbone movement necessary to accommodate the displaced methionine is exerted more on the preceding residues.

Stability of the Heme Binding Domain of Cyt c1—The significance of two of the features of the cyt c1 “hinge” (the disulfide bond and the βXM motif, which is almost universal but notably absent in R. capsulatus) is substantiated by mutant studies in R. capsulatus and R. sphaeroides. In R. capsulatus, the disulfide bond is required to maintain cyt c1 in the native high potential form. However, second site revertants were recovered in which an alanine located two residues away from the Met-ligand was replaced by a β-branched amino acid, defining the βXM motif (31, 32).

In contrast, cyt c1 of R. sphaeroides contains the disulfide bond but has isoleucine in the βXM motif. Mutants of R. sphaeroides lacking the disulfide bond between Cys145 and Cys169 were still able to grow phototrophically. Although the E m of cyt c1 was substantially lowered, it was at least 100 mV more positive than in the equivalent R. capsulatus mutants (33). Thus, the βXM motif in R. sphaeroides appears to restrain the amplitude of the midpoint potential change associated with the removal of the S–S bond.

We suggest that hydrogen bond networks in and around the heme cleft are involved in stabilizing cyt c1 in its compact, high potential form, in a manner qualitatively similar to the effect of the disulfide bond. The superficial binding of ligand initiates the disruption of these interactions, destabilizing the native form of cyt c1 and lowering the energetic barrier for the conformational transition that opens the heme cleft for direct binding to the heme. The multiplicity of potential ligands in vivo emphasizes the need for stabilizing structures like the disulfide bond and the variety of secondary structures seen in the hinge regions of different c-type cytochromes.

For soluble cyt c, the stability of the heme distal pocket is affected by bulk phase influences, including the solution environment (34) and dielectric constant (35). Destabilization results in lowering of the apparent pK a of the so-called alkaline transition, a major conformational change that occurs at high pH and is accompanied by the displacement of the native methionine ligand by a variety of internal nitrogenous ligands, notably lysine amines (36–38). The alkaline transition, itself, is too slow at pH < 9 (37, 39) to contribute to the spontaneous conformational change required by Scheme 1, for example, but some exogenous ligands are known to promote the alkaline transition. However, the ability of small molecules to do this is not well correlated with their heme-liganding propensity (40, 41). Furthermore, the necessary concentrations are high, in the molar range, and it has not been clear that this action is any more specific than a general chaotropic effect.

Here we have found that even at a few mM, the kinetics of Im binding imply rather specific surface interactions that can affect the structure and dynamics of the protein. The behavior at concentrations above 100 mM suggests similar influences upon specific binding at other sites. Additional, nonspecific interactions at high concentration cannot be ruled out and may be responsible for the fall off in activity at Melm concentrations approaching 1 M, but they do not seem to play a significant role in the main range of this study.

We have proposed that the rate-limiting events in binding imidazole and N-methylimidazole to the heme of cyt c1 include a conformational change that is facilitated by interactions with ligand superficially bound, in some cases at multiple sites. We also suggest that this picture may be extended to c-type cytochromes, generally, whereby exogenous ligand binds first to the protein in a superficial manner, disrupting existing hydrogen-
bonded structures and allowing the ligand to work its way into the cleft and access the heme.

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