Heme–protein fission under non-denaturing conditions
(protein dynamics/polyptide–water interactions/heme release)

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ABSTRACT Slow heme transfer from horseradish peroxidases C2 and A2, cytochrome c peroxidase, chloroperoxidase, and leghemoglobin to a heme acceptor protein, apomyoglobin, has been studied under mild conditions. The reaction is best described as heme release into water followed by quick engulfment by apomyoglobin. The energetics of the activated process are large and interpreted as connected to both polypeptide motions during release and the ordering of water around the heme during solvation. The free energy required to break the iron(III)–ligand 5 (LS) bond is a minor but crucial portion of the activation free energy. Donor–acceptor protein interactions are not involved in the transfer. Fast heme release from inactive protein has also been observed. Apoprotein recombination with porphyrins and hemes suggest that this lack of activity is a result of Fe–L5 bond breaking.

The coenzyme and the prosthetic group share the role of being executive partners to a directing apoprotein. The former operates with an on/off relation to the protein during the functional cycle, whereas the latter remains attached. Heme, the archetypical prosthetic group, can, in a reversible but nonphysiological manner, be disconnected when the protein is perturbed by an acid, an alcohol, or a detergent (1). There is also a slow release of heme under mild conditions as regards temperature and ionic strength (1–4) and at elevated temperatures (5). Release of heme from hemoglobin under mild conditions responded perceptibly to structural alterations due to single-point mutations (6). Some biological mechanisms seem to operate with a release of heme (7). Heme release from tryptophan 2,3-dioxygenase, a key enzyme in the regulation of tryptophan and serotonin, is critical for the synthesis and activity of this protein (8). In hemoglobininas, the heme group may reside in various modified pockets (9). A migration of heme between sites has been reported e.g., in the in vivo assemblage of hemoglobin from globin and its prosthetic group (7, 10, 11). From a physicochemical point of view, facts about heme release are requisite for the determination of the equilibrium between apoprotein and heme. Here we present the kinetics of heme release under mild conditions and its dependence upon some hemeprotein parameters. The results can be expressed in an energy profile through stages involved in the migration of heme from donor protein to recipient apomyoglobin (apoMb). The free energies of activation (ΔG‡) for the release of heme from native proteins range from 95–105 kJ/mol with Arrhenius activation energies (Ea) of typically 130 kJ/mol. This activation energy matches previous observations (12) or predictions (13), but two anomalous values of Ea for native proteins have also been observed: 92 kJ/mol for leghemoglobin (Lb) below 23°C and 439 kJ/mol for cytochrome c peroxidase (CCP) at all temperatures. The latter energy is much higher than that normally thought to be available to proteins in water under non-denaturing conditions.

MATERIALS AND METHODS

Horseradish peroxidases A2 and C2 were isolated as described (14), and A2 was preliminarily fractioned on DEAE-cellulose (pH 5.8–5.95), yielding subfractions that were homogeneous by discontinuous gel electrophoresis (15). Chloroperoxidase was purchased from Sigma. Lb a, c1, c2, d1, d2 were isolated from soybean nodules.
apoMb has a higher affinity for Fe(III) hemes than other apoproteins, and heme release could be monitored by the formation of holoMb. The reaction was initiated by addition of a small (1%) volume of apoMb concentrate to the hemo-protein, usually in 50 mM phosphate/1 mM EDTA, pH 7.0 (standard buffer). Heme transfer was monitored by the increase in holoMb absorbance at 409 nm or by the decrease in donor protein absorbance at <400 nm. Release was not observed from deoxyLb or carboxyLb.

Data were analyzed by using either ENZFITTER (Biosoft) or as/1 (BBN) programs. Kinetic parameters were calculated from the expressions

\[ E_a = -R(\Delta \ln k/\Delta T^{-1}) \]

\[ E_a = \Delta H^{\ddagger} + RT \quad \text{at } 298 \text{ K,} \]

\[ \Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad \text{at } 298 \text{ K,} \]

and

\[ k = RT/Nh \exp(-\Delta G^{\ddagger}/RT) \quad \text{at } 298 \text{ K.} \]

RESULTS

The simplest model for the net migration of heme is a direct route from donor hemoprotein to acceptor apoprotein. However, some samples take a biphasic course, particularly at lower temperature. An initial phase (k1) is 5–100 times faster than the second, quantitatively dominant phase (k2), the rate constants refer to the model in Fig. 1. Here k1 and k2 are the rate constants for the fast and the slow phases, respectively, and k1 is the rate constant of the formation of the fast donor holoprotein from the slow donor holoprotein. The data were analyzed assuming k1 << k2 and k1. Both rates obeyed a first-order course and could be followed for one to five half-lives. With the biphasic mode, two isosbestic points appeared simultaneously in the Soret region, at 390–405 nm and 420–440 nm, positions that depended upon the hemoprotein. If the donor protein was incubated for several hours in the absence of apoMb, the proportion of the fast donor holoprotein was noticeably increased at the expense of slow

Abbreviations: CCP, cytochrome c peroxidase; HRP, horseradish peroxidase; Mb, myoglobin; Lb, leghemoglobin.
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The results are described separately for some hemoproteins.

**Lb**. When monitored at 412 nm, the release of heme from Lb α displayed a single first-order course throughout. The rate constants for the various subfractions of Lb differed significantly. Lb α, after isolation, gave a single first-order rate constant of $7.1 \times 10^{-12} \text{ min}^{-1} (n = 6)$ at 25°C. Two different preparations of Lb c and Lb d gave rate constants of $2.0 \times 10^{-12}$ and $2.5 \times 10^{-12} \text{ min}^{-1}$. These rate constants are covariant with the relative stabilities of the three isoproteins (17).

Three phenomena concerning Lb α occurred at about the same temperature, 23–25°C. The slow phase was cleanly first order over four or five half-lives between 25°C and 45°C; below this temperature the slow phase gradually decreased to one half-life at 8°C, which may hide some fast phase. Second, the change in the entropy sign for Lb α above and below 23°C is striking and only was observed for Lb (Table 1). Finally, there is a discontinuity in the Arrhenius plot (Fig. 2). The data were well fit by two straight lines, intersecting at about 23°C for the native protein as well as for Lb recombined with protoheme or mesohem. For native protoheme-Lb, data for three preparations were analyzed giving $E_a = 132 \text{ kJ/mol}$ above 23°C and 92 kJ/mol below 23°C (Table 1); the difference is significant. The results from repeated experiments support the notion that the upward change in activation energy at higher temperature—rarely reported—is real. Similar results were observed for the heme–protein fission of mesoheme-Lb. The thermal agreement between the three effects can hardly be a chance.

**Horse-radish Peroxidase (HRP)** A2. At 25°C a fast phase and an adjacent slow phase with rate constants of $2.2 \times 10^{-2} \text{ min}^{-1}$ and $4.7 \times 10^{-4} \text{ min}^{-1}$, respectively, were discerned. At temperatures above 23°C, isosbestic points at 393 and 434 nm simultaneously appeared and remained during both phases of the reaction between HRP A2 and apoMb; at lower temperatures these positions changed as the reaction proceeded. Therefore, only two optically active compounds exist in spite of the two-phase course. The fast donor holoprotein fraction of HRP A2 increased by aging in buffer at room temperature or above ($k_f = 1.2 \times 10^{-4} \text{ min}^{-1}$ at 37°C, $r^2 = 0.96$); this means a decrease in $A_{403}/A_{280}$ ("RZ") from 4.2 to 2.9 in 24 hr; the opposite was observed for aging CCP (18). This decrease in absorbivity is consistent with loss of high-spin, hexacoordinated character and with an increase in pentacoordination. A concomitant decrease in enzymatic activity was also observed during preincubation, which roughly corresponded to the fractional increase in fast donor holoprotein. The data in Fig. 2 show that the temperature dependence of the slow phase is a simple function.

CCP, CCP showed a strong temperature dependence. Neither fast nor slow release could be observed at 20°C, and at 37°C only the slow phase was evident. The two phases may correspond to the two forms of CCP reported (18). The limited number of data for the fast phase prohibits any evaluation of the energetics of this phase, but a remarkable activation energy in 439 kJ/mol is found for the slow phase (Fig. 2 and Table 2). This energy is considerably higher than for any other protein in this study.

**Other Hemoproteins.** Table 2 presents the reaction parameters for the slow phases of six native hemoproteins. The upper temperature limit, 45°C, was set by apoMb precipitation. HRP C2 heme–protein fission could not be observed below 30°C because of the slow rate. Heme release from Hb α chains, which, in agreement with literature data (19), was slower than from β chains, was irreproducible due to apoHb precipitation. Heme release from Hb β chains has been reported for human Aβ and several mutants (6). Even mutations distant from the heme crevice greatly influenced the rate. More surprising is the slow release from Mb, $1.1 \times 10^{-2}$.

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**Table 1.** Kinetic parameters for the slow phase release of the prosthetic group from substituted Lb α and HRP A2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Preexponential factor, $E_a$, kJ/mol</th>
<th>$\Delta H^*$, kJ/mol</th>
<th>$\Delta S^*$, J/mol K$^{-1}$</th>
<th>$\Delta G^*$, kJ/mol</th>
<th>$k(25^\circ C, \text{SB})$, min$^{-1}$ × 10$^3$</th>
<th>Range, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lb</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native (&lt;23°C)</td>
<td>8.9 × 10$^{13}$</td>
<td>92 ± 2</td>
<td>90</td>
<td>$-20$</td>
<td>96</td>
<td>6.30</td>
</tr>
<tr>
<td>PH-rec (&lt;23°C)</td>
<td>2.6 × 10$^{11}$</td>
<td>78 ± 8</td>
<td>76</td>
<td>$-67$</td>
<td>96</td>
<td>5.30</td>
</tr>
<tr>
<td>MH-rec (&lt;22.7°C)</td>
<td>5.7 × 10$^{7}$</td>
<td>58 ± 2</td>
<td>56</td>
<td>$-138$</td>
<td>97</td>
<td>4.00</td>
</tr>
<tr>
<td>Native (&gt;23.2°C)</td>
<td>9.5 × 10$^{20}$</td>
<td>122 ± 6</td>
<td>130</td>
<td>117</td>
<td>95</td>
<td>7.10</td>
</tr>
<tr>
<td>PH-rec (&gt;23.7°C)</td>
<td>1.3 × 10$^{21}$</td>
<td>132 ± 8</td>
<td>132</td>
<td>121</td>
<td>96</td>
<td>6.10</td>
</tr>
<tr>
<td>MH-rec (&gt;22.7°C)</td>
<td>1.1 × 10$^{19}$</td>
<td>111 ± 8</td>
<td>120</td>
<td>80</td>
<td>96</td>
<td>5.10</td>
</tr>
<tr>
<td>PP-rec</td>
<td>1.3 × 10$^{19}$</td>
<td>111 ± 8</td>
<td>120</td>
<td>80</td>
<td>85</td>
<td>440</td>
</tr>
<tr>
<td>MP-rec</td>
<td>1.4 × 10$^{12}$</td>
<td>129 ± 8</td>
<td>127</td>
<td>141</td>
<td>85</td>
<td>420</td>
</tr>
<tr>
<td><strong>HRP A2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>1.1 × 10$^{21}$</td>
<td>139 ± 10</td>
<td>137</td>
<td>117</td>
<td>102</td>
<td>0.47</td>
</tr>
<tr>
<td>PH-rec</td>
<td>2.5 × 10$^{21}$</td>
<td>141 ± 12</td>
<td>139</td>
<td>124</td>
<td>102</td>
<td>0.50</td>
</tr>
<tr>
<td>MH-rec</td>
<td>3.6 × 10$^{19}$</td>
<td>130 ± 8</td>
<td>128</td>
<td>94</td>
<td>100</td>
<td>0.95</td>
</tr>
<tr>
<td>Native (fast)</td>
<td>7.5 × 10$^{19}$</td>
<td>197 ± 11</td>
<td>195</td>
<td>342</td>
<td>93</td>
<td>23</td>
</tr>
</tbody>
</table>

The preexponential factor, $E_a$, k (at 25°C in standard buffer), and range were determined from Fig. 2. $\Delta H$ was calculated from $E_a$ by using Eq. 2; $\Delta G$ was calculated from k (at 25°C in standard buffer) by using Eq. 4. $\Delta S$ was calculated from $\Delta H$ and $\Delta G$ by using Eq. 3. SB, standard buffer; PH-rec, recombinated with protoheme; MH-rec, recombinated with mesoheme; PP-rec, recombinated with protoporphyrin; MP-rec, recombinated with mesoporphyrin.

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**Fig. 1.** Energy diagram for heme transfer from two observed forms of a donor holoprotein to an acceptor protein, apoMb. The slow to fast donor holoprotein conversion, $k_s$, is partly irreversible. $k_f$ and $k_s$ are the rate constants for heme transfer from the slow and fast forms, respectively. There is a small activation energy for heme and porphyrin uptake by apoproteins (16).
min⁻¹ at 25°C in the presence of excess apoHRP C2. The rate increased at higher temperatures but could not be measured below 25°C because the equilibrium favors holomB. We can only speculate that the stability of holoMb relates to its secondary structure. Not only does Mb generally have a high α-helix content, but several α-helices in the same domain join to form a tight heme pocket (20). Chloroperoxidase, in a single experiment at 25°C, consisted of 40% fast phase and gave the rate constants \( k_f = 2.5 \times 10^{-2} \) and \( k_s = 1.9 \times 10^{-3} \) min⁻¹. Data for earthworm Hb will be published separately (M.L.S., J.P., and K.G.P., unpublished results).

No heme release could be seen under any mild conditions for catalase, lactoperoxidase, cytochrome c, or cytochrome c oxidase. Considering the deeply buried heme of catalase (21) and probably likewise for lactoperoxidase (22), these were the expected results. The absence of detectable heme release from cytochrome c oxidase is understandable, considering the increased size of heme A, which probably cannot be bound by apoMb and also the possibility that heme A may be sequestered within the detergent micelle.

Energies of Heme–Protein Fission. Two groups of rate constants at 25°C can be discerned in Table 2; the native oxygen carriers are about 20 times faster than the peroxidases. The \( \Delta G^\ddagger \) values between the two groups differ by 8 kJ/mol. Also conspicuous is the \( \Delta H^\ddagger \) for CCP, a value several times higher than those normally seen for reactions of native proteins under mild conditions (12, 23). This \( \Delta H^\ddagger \) value of CCP is balanced by a huge entropy term.

The activation parameters for a reconstituted Lb and HRP A2 are presented in Table 1. The results for protoheme- and mesoheme-substituted Lb a are consistent with those for native Lb a. The \( \Delta G^\ddagger \) are the same, and \( \Delta S^\ddagger \) are negative at 10°C, while \( \Delta H^\ddagger \) is reduced compared to the situation at 25°C. The release of protoporphyrin or mesoporphyrin from Lb a was much faster than the release of heme, with \( \Delta G^\ddagger \) lower by about 10 kJ/mol at 25°C. The two porphyrin-substituted proteins did not exhibit temperature-variable activation energies.

For the release of 2,4-disubstituted hemes from HRP C2, fast and slow phases were always seen (J.P., M.L.S., and K.G.P., unpublished data). The rate constants for the fast phases correlate to the Van der Waals volumes of the substituents, but there is little response in the slow phase to either Van der Waals volume or Fe(III)/Fe(II) reduction potential, \( E_{n7} \) (24). LaMar et al. (25) derive a much stronger correlation between 2,4-substituents and heme–Mb fission, the most stable form being the native protein.

### DISCUSSION

There are two principal steps in the transfer of heme or porphyrin from one protein to another: migration out of one pocket into water and from water into another pocket. The lack of rate dependence upon acceptor protein concentration and the concentration-independent gel-filtration results strongly suggest that donor–acceptor protein interactions are not involved in the heme transfer process. These results do suggest that heme and porphyrin are freely water soluble. Aggregation is not observed because an excess of acceptor protein rapidly removes free heme or porphyrin, preventing

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**Table 2. Kinetic parameters for the slow phase of protoheme release from native heme proteins at 25°C**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rate constant, min⁻¹ ( \times 10^5 )</th>
<th>Preexponential factor, min⁻¹</th>
<th>( \Delta H^\ddagger ), kJ/mol</th>
<th>( \Delta S^\ddagger ), J mol⁻¹ K⁻¹</th>
<th>( \Delta G^\ddagger ), kJ/mol</th>
<th>Range, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lb (&gt;23°C)</td>
<td>7.10</td>
<td>9.5 ( \times 10^{20} )</td>
<td>130</td>
<td>117</td>
<td>95</td>
<td>23–37</td>
</tr>
<tr>
<td>Lb (&lt;23°C)</td>
<td>6.30</td>
<td>8.9 ( \times 10^{23} )</td>
<td>90</td>
<td>20</td>
<td>96</td>
<td>7–23</td>
</tr>
<tr>
<td>HRP A2</td>
<td>0.47</td>
<td>1.1 ( \times 10^{21} )</td>
<td>137</td>
<td>117</td>
<td>102</td>
<td>14–45</td>
</tr>
<tr>
<td>CCP</td>
<td>0.17</td>
<td>1.5 ( \times 10^{23} )</td>
<td>437</td>
<td>1110</td>
<td>103</td>
<td>23–37</td>
</tr>
<tr>
<td>HRP C2</td>
<td>0.24</td>
<td>5.2 ( \times 10^{20} )</td>
<td>131</td>
<td>91</td>
<td>104</td>
<td>30–45</td>
</tr>
<tr>
<td>Hb A</td>
<td>7.73</td>
<td>7.6 ( \times 10^{10} )</td>
<td>120</td>
<td>83</td>
<td>95</td>
<td>25–42</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>1.90</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>99</td>
</tr>
</tbody>
</table>

NA, not available
polimerization and precipitation. The on-reaction into the pocket of an acceptor apoprotein requires a $\Delta G^\ddagger$ of $\approx 1$ kJ/mol, which is no barrier at room temperature (16). Apparently heme--donor protein separation is the rate-limiting step in heme transfer, and it is justified to concentrate on this step in the following discussion.

The kinetics of heme release were determined to be a convolution of two rate constants, depicted as a slow and a fast phase, respectively. The slow release of heme from a slow donor solvent wall is the dominant path for all freshly prepared proteins. Below we argue that the slow donor holoprotein represents the active protein and that the fast form reveals an inactive but informative fraction of the donor protein. Furthermore, we estimate the relative importance of different contributions to the barrier for heme release. The widely varying rates between various donor proteins imply that equilibrium between holoprotein and apoprotein--heme is determined by the differences in polypeptide–heme interactions of these holoproteins. Finally, we discuss the rare change in the slope in the Arrhenius plot for the slow phase of Lb.

**Fast Heme Release.** The relative concentration of fast phase varied between different donor proteins and also with the incubation time and temperature. These changes could be followed as changes in the Soret band region and in the concentration of active enzyme. The difference between $k_i$ and $k_o$ parallels the shift from hemes to porphyrins (Fig. 2), and it was concluded that a broken Fe--ligand 5 (L5) bond is a necessary prerequisite for the fast release of heme (J.P., M.L.S., and K.G.P., unpublished results). The large activation energy of the fast phase shows that Fe--L5 bond rupture is not simply the first step in a two-step model for heme release. The situation is summarized in the model shown in Fig. 1.

**Heme Release.** The above discussion and a separate communication concentrated on the irregular fast separation of heme and protein (J.P., M.L.S., and K.G.P., unpublished results). Below we discuss the normal slow phase and, in particular, discern different contributions to the energy barrier for heme–protein fission.

A free energy profile for heme release is presented in Fig. 1. The barrier to release is reasoned to be a simple potential with the difference in free energies between heme and porphyrin release being the breaking of the Fe--L5 bond. The N--Fe--S bonds in cytochrome c yield an estimated joint free energy of stabilization of about 25 kJ/mol, with the major share for the Fe--N bond (26). Our experimentally determined values of $\Delta G^\ddagger$ for the transfer of heme and porphyrin differ by 10 kJ/mol. We attribute this energy to the breaking of the L5 N--Fe bond. This is shown as a destabilization of the porphyrin–protein interaction relative to the hemoprotein. Although perfectly satisfactory to explain the difference between heme and porphyrin, Table 1 shows that the breaking of the Fe--L5 bond only constitutes a minor portion of the total barrier for heme release. The remaining $\approx 90$ kJ/mol comes from aquisition of the porphyrin moiety and lining the pocket with water. We cannot kinetically discern these contributions, however, from the dissociation of the Fe--L5 bond.

The inseparability is well illustrated by a comparison of the above Fe--L5 free energy and the increased energy of solvation during elongation of this bond. A 30% increase of the equilibrium Fe--L5 bond length at breakpoint is a reasonable estimate and corresponds to a 1.5 Å sliding of the heme plane (J.P., unpublished data). This, in turn, translates to aquisition of 20% of the heme surface. Stellwagen empirically related the altered polarizability of water vs. protein to a change in the reduction potential by means of the expression (27)

$$\Delta E'(mV) = 345 - (15 \times \% \text{ exposure}),$$

and

$$\Delta G^\ddagger = -n\Delta E',$$

yielding $\Delta E' = 45$ mV and $\Delta G^\ddagger = -4.3$ kJ/mol. The above exercise, although crude, confirms that several infeasible forms of comparable strength operate simultaneously.

The continued aquation involves Fe--L5 bond dissociation raises an even higher barrier for heme or porphyrin release. This barrier is the net result of attraction between water and the ionic FeN$_4$ (or H$_2$N$_4$) chelate and repulsion between the hydrophobic porphyrin disc and the new polar environment. The repulsion obviously dominates, and an analysis of the free energy of heme transfer by summation of the free energies of solvation of the heme parts is enlightening (28).

We approximate protoporphyrin as $4(CH_2=CHCH_2) + 4(CH_3H) + 2(CH_2H_2) + 2(CH_2H_3)$, disregarding already aquated carboxyl groups. We next postulate that the protein internal surface can be modeled as a mixture of a liquid hydrocarbon and an alcohol. With these assumptions, the solvation of protoporphyrin from the heme pocket will require an energy in the range 68--110 kJ/mol, which is comparable to our 90 kJ/mol found previously. The glaring exception is CCP, where the heme is deeply buried and the high $\Delta H^\ddagger$ indicates gross polypeptide rearrangements (Table 2).

Hydration energies of the large, but water coordination to Fe(III) on the distal side (L6) will essentially not change during the course of heme–protein fission. Coordination on the proximal side (L5) will change from histidine ligation for the slow donor holoprotein to water hydration for free heme (J.P. and O. Edholm, unpublished data). We suggest that water will replace histidine on this side, if sterically unhindered, and that hydration around the ionic FeN$_4$ chelate is fully established for the fast donor holoprotein prior to heme release. We have previously concluded that the prosthetic group of the fast donor holoprotein is separated from the fifth ligand. As a result, the hydrophobic repulsion will not be countered by any hydration attraction, and the barrier for heme–protein fission will rise. This suggestion could partly explain the apparent difference in activation energy for the slow and fast phases (Table 1). In conclusion, heme/porphyrin solvation consumes huge energies, which must be supplied by either polypeptide rearrangements or by concerted collisions of water with protein or both. These conditions are not easily met, making heme–protein fission a slow process.

**Anomalous Conformational Changes.** The kind of change in slope in the Arrhenius plot shown in Fig. 2 is only rarely reported. Nevertheless, the narrow distribution of data points makes it statistically justified to confirm the existence of an upward “knee” for the slow phase of Lb. The breakpoint, 23.2 $\pm$ 0.5°C, is the same for the native protein and the two recombined forms. Two reported examples are the enzymatic activities of pig heart fumerase (22--27°C) (29) and β-lactate dehydrogenase (17°C) (30). This was attributed to protein disaggregation at higher temperature with higher activity for the monomer. In our case an aggregation of Lb and apoMb could offer an explanation for this phenomenon but is ruled out by the gel-filtration experiments. Table 1 shows that the activation energy as well as the preexponential factor are exceptionally small for the slow phase of Lb below 23°C. This observation suggests that heme is abnormally coordinated below the knee temperature. Our data positively rule out the existence of a knee for porphyrin-substituted Lb, but broader distributions prevent any definite answer for other proteins. Clearly the knee is a motivation for further studies, particularly if it relates to a discontinuity in catalytic activity. One likely explanation is that the polypeptide motion is restrained below 23°C. This is consistent with a negative entropy term, the relatively small preexponential
factor in this temperature range (Table 1), and the absence of any effect on the porphyrin-reconstituted protein. Above 23°C, the released polypeptide motions increase the entropy term.

We have previously shown that the Fe(III)/Fe(II) reduction potential correlates well with the vibrational frequency of heme-ligated CO (31, 32). The slow phase reaction rates observed with 2,4-disubstituted HRP C reveal an almost complete lack of any such cis effect (J.P., M.L.S., and K.G.P., unpublished results). Instead, hexacoordinated ferrous hemoproteins constitute a particularly stable form of the active phase, thus demonstrating a pronounced trans effect. The Fe(II)–L5 bond cannot be isolated from the colinear Fe(II)–L6 bond, and the "initial" barrier for heme release involves not only elongation of the former bond but also a simultaneous destabilization of the latter one. This is consistent with the observed diminished rate constants for dioxygen and carbonyl-heme release. A detailed mapping of the vibrational bands of ligated CO and CN could possibly reveal images of the Fe(II)– and Fe(III)–L5 bonds and thus enlighten the mechanistics behind the change of slope and related enthalpy change around 23°C for Lb.

A wide range of dynamic protein motions have been reported for proteins; the slowest changes were interpreted as being due to large polypeptide motions (6, 12, 33). Studies of heme disorientation in Mb and Lb also indicated large protein motions (18, 25, 34). The reaction rates reported in the present study are comparable to reports for heme disorientation within this pocket. The molecular motions involved in heme disorientation and heme–protein fission must be among the most drastic reported for mild conditions.

Samples of CCP were gifts from Drs. T. Yonetani and E. Margoliash. Cytochrome c oxidase was a gift from Dr. B. G. Malmström. Dr. G. Sievers supplied a sample of soybean Lb a for initial studies. Dr. K. Huss-Danell kindly arranged for growing soybeans. This work was supported by grants from Norrlands Gasaktiebolag, Magn. Bergvalls Stiftelse, the Swedish Medical Research Council (3X-6522,7130), and the Faculty of Medicine, University of Umeå.