Fast Coordination Changes in Cytochrome c Do Not Necessarily Imply Folding*

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Folding of globular proteins occurs with rates that range from microseconds to minutes; consequently, it has been necessary to develop new strategies to follow the faster processes that exceed stopped-flow capabilities. Rapid photochemical methods have been employed to study the rate of folding of reduced cytochrome c. In this protein, the iron of the covalently bound heme binds a His and a Met, proximal and distal. Unfolding by guanidine or urea weakens the Fe–Met bond and the reduced unfolded cytochrome c easily binds CO and other heme ligands, which would react slowly or not at all with the native protein. Therefore in the presence of CO, reduced cytochrome c unfolds at lower denaturant concentrations than in the absence of this ligand, and rapid photochemical removal of CO from unfolded cytochrome c, is expected to trigger at least an incomplete refolding. This approach is complicated by the breakage of the proximal His–Fe bond that may occur as a consequence of CO photodissociation in the unfolded cytochrome c because of the so-called base elimination mechanism. Rebinding of CO to the four-coordinate heme yields kinetic intermediates unrelated to folding. Our hypothesis is supported by parallel observations carried out with protoheme and microperoxidase.

Cytochrome c (cyt-c)1 has been widely used as a model system to study the kinetics and mechanism of protein folding (1–4) because of several suitable properties, the most relevant being its rapid and reversible unfolding when exposed to denaturants such as urea or guanidine hydrochloride (Gdn) and the presence of a covalently bound heme, which offers a convenient spectroscopic probe (because of its optical spectrum and strong quenching of the Trp fluorescence in the folded state). In native cyt-c, the heme iron is six-coordinate because the polypeptide chain provides two axial ligands, namely a His (His-18, the proximal His; numbering is for the sequence of horse heart cyt-c) and a Met (Met-80) (5, 6). The distal Fe–Met bond contributes to a significant extent to the overall thermodynamic stability of native cyt-c (7, 8) (in the unfolding process this bond is weakened), and the unfolded cyt-c is a mixture of the five-coordinate and several six-coordinate states, the sixth ligand being provided either by Met-80 or by other His or Met residues of the polypeptide chain (a phenomenon called miscoordination) (3, 4, 9, 10). Because of the weakening of the Fe–Met bond, external heme ligands, such as CO, induce unfolding at lower denaturant concentrations by competing with Met-80 for the sixth coordination position.

The native state of reduced cyt-c is more stable than the oxidized one; thus, under appropriate experimental conditions, reduction promotes refolding (Ref. 11 and references therein). Moreover, since CO destabilizes reduced cyt-c, it is possible to select experimental conditions where the protein is mostly unfolded in the presence of CO and mostly folded in its absence (e.g. 40 °C and 4.6 M Gdn (3) for horse heart cyt-c). Under these conditions, rapid photochemical removal of CO from unfolded, reduced cyt-c may promote refolding with transient rebinding of Met-80 to the heme iron. Indeed at selected wavelengths a characteristic “raise and fall” optical density change was observed, and, if complete absorbance spectra are collected as a function of time, several kinetic intermediates may be seen to build up and then decay (3, 12). With horse heart cyt-c the time course is actually more complex than outlined above because of the possible miscoordination events by residues other than Met-80. This experiment, pioneered by Eaton and co-workers (3), has been interpreted according to a coordination/miscoordination kinetic mechanism shown in Scheme 1.

\[
\text{hv} \\
\text{X} - \text{Fe} - \text{CO} \rightarrow \text{X} - \text{Fe} + \text{CO} \leftrightarrow \text{X} - \text{Fe} - \text{Y} + \text{CO}
\]

\text{SCHEME 1}

In Scheme 1, X indicates the proximal His while Y indicates Met-80 or any other residue capable of coordinating to the heme iron (His-26, His-33, and Met-65 for horse heart cyt-c). To investigate the folding mechanism for this class of proteins in the absence of miscoordinations, we studied a simpler cytochrome c, cyt-c551 from \textit{P. aeruginosa}, whose amino acid sequence allows coordination only with the proximal His (His-16) and the distal Met (Met-61); the additional Met (Met-18) is too close to the proximal His to bind to the distal coordination position of the heme iron (13).

As the probe of this photochemical experiment is the heme, the picture may be considerably more complex if any of its characteristic reactions were to occur. The heme can be dissolved in organic solvents or dispersed in micelles of detergents, and its reactivity with ligands can be studied in the absence of the protein moiety. It is known that in the presence

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41073
of imidazole (Im) or 2-methyl imidazole (MeIm), the heme binds CO more strongly than in the absence of these bases and readily forms a six-coordinate state of the type (Im or MeIm)–Fe–CO; conversely, when CO is present the organic base in trans is bound with higher affinity (14, 15). If the (Im or MeIm)–Fe–CO complex is submitted to a short and bright flash of light, the photolabile Fe–CO bond is broken and, in the dark, CO recombination proceeds largely via a mechanism called base elimination (16) in Scheme 2.

\[
\begin{align*}
X + Fe - CO & \rightarrow X + Fe + CO \\
X + Fe - CO & \rightarrow X + Fe + CO
\end{align*}
\]

**SCHEME 2**

In Scheme 2, X indicates the proximal base, either Im or MeIm, and is formally analogous to the proximal His of Scheme 1. Because of the lower affinity for the proximal base, the five-coordinate photoproduce X–Fe easily dissociates X and converts to a four-coordinate species, which first rebinds CO and then X. The base elimination kinetic mechanism (16) populates three intermediates, i.e. one four-coordinate and two five-coordinate (Fe–CO and Im–Fe) states; if it operates also in the case of unfolded CO-bound cyt-c, it is expected to complicate considerably the interpretation of the observed time course. The four-coordinate species is less stable than either of the five-coordinate, hence it remains scarcely populated throughout; however, it equilibrates quickly with both of them, because of its high reactivity, and the prevailing path to recover the equilibrium six-coordinate is via the CO-bound intermediate rather than the base-bound one.

In this paper we present evidence that a base elimination mechanism occurs in photochemical experiments on CO-bound unfolded cyt-c. Indeed, at least one of the main kinetic intermediates populated during the time course, which was assigned to a partially folded or collapsed species, is also observed in detergent-dissolved protoheme and in the heme-containing oligopeptide microperoxidase (17). Our results suggest that intermediate species populated after photolysis of CO include those originally postulated by Eaton and co-workers (Scheme 1) as well as those of the base elimination mechanism (Scheme 2); therefore interpretation of photochemical experiments on unfolded, CO-bound cytochromes is considerably more complex than hitherto anticipated, and part of the observed kinetics probably bears no relationship to folding.

**EXPERIMENTAL PROCEDURES**

Horse heart cyt-c, microperoxidase (MP-11) and heme were purchased from Sigma-Aldrich; all other reagents were of analytical grade. *P. aeruginosa* cyt-c_{551} was purified as described (18).

The instrument used for photolysis experiments was described elsewhere (19). Briefly the 5-ns pulse of Nd-YAG solid-state laser (Quanta System HIL 101, second harmonic λ = 532 nm, E = 80 mJ/pulse) was focused onto an optical Thunberg tube containing the desired solution. The transmittance of the sample can be monitored using two separate recording devices, differently oriented with respect to the laser light pulse: a photo multiplier tube and a CCD solid state camera. The photo multiplier monitoring line is orthogonal to the laser beam; it consists of a 50-W lamp, a Spex 1681 monochromator, a Hamamatsu R1398 photo multiplier tube and a digital Tektronix TDS 360 oscilloscope equipped with either an amplifier Comlinear Corporation Model E203 or the preamplifier Tektronix ADA400. The CCD optical line is arranged at 15° from the laser beam; it consists of a 300-W lamp (ILC technology), an Acton-Princeton 320 Pl spectrometer (Princeton Instruments), and a pulsed CCD camera (Princeton Instruments) capable of time resolution down to 3.5 ns. The delay between the laser pulse and the action of recording a transmittance spectrum is controlled either by the Princeton FG100 pulse generator necessary to operate the CCD camera or, for longer time delays, by a Tektronix AFG 310 function generator.

The experimental data thus collected are either time courses at single wavelength or a series of spectra; they are stored as MS-DOS files on an Intel Pentium-based PC and converted to absorbencies by means of the package MATLAB (The Math Works Inc., Natick, MA). Single wavelength time courses are fitted to the desired kinetic model using the non-linear least squares routines provided by MATLAB; spectra are arranged into matrices, submitted to the singular value decomposition (20), and the significant columns of the V matrix are treated as time courses at a single wavelength.

**RESULTS**

Unfolding of c-type cytochromes in Gdn or urea is associated to spectroscopic changes of the covalently bound heme group. Surprisingly, in the reduced unfolded cyt-c it is difficult to obtain the spectrum of a pure five-coordinate heme characterized by a single peak in the visible region. Even when fluorescence and CD spectra are indicative of a fully unfolded protein, the visible spectrum indicates a significant amount of some derivative(s) displaying two peaks. Since in this region both the four- and six-coordinate states have two maxima, all the three coordination states may be populated under these conditions.

The reasons we believe that all coordination states are populated under denaturing conditions are as follows: i) in the unfolded hemoprotein the affinity of the iron for its physiologically ligands is reduced due to loss of the stereochemical fit imposed by the native three-dimensional architecture; ii) the five-coordinate state of unfolded cyt-c may react with the amino acid residues of the polypeptide chain (mostly His) able to form a stable coordination bond with the heme iron; indeed non-physiological bis-His six-coordinate states are described for the ferric unfolded horse heart cyt-c (1, 2, 4); and iii) therefore, in view of the high cooperativity of binding of the axial ligands, the five-coordinate heme iron in unfolded cyt-c is likely to equilibrate with a minor fraction of the four-coordinate state and a significant fraction of the six-coordinate one (14–16).

Even reduced MP-11, the 11-residue heme-binding peptide obtained by proteolytic digestion of horse heart cyt-c (17), has the spectrum of a six-coordinate complex. Since in this case the proximal His is present but other His or Met are absent, it was postulated that the sixth ligand might be provided by the terminal amino groups of the same molecule or via aggregation of different molecules (21, 22). To obtain model spectra for the four-, five- and six-coordinate protoheme in the reduced state, the prosthetic group was dissolved in 5% SDS in the presence of different types of ligands (Fig. 1).

CO is a strong ligand of the reduced heme iron; moreover, it strengthens the bond of the reduced iron with the ligand on the opposite side (trans) of the heme, i.e. the proximal His in hemoproteins (14, 15). Since a significant contribution to the overall stability of the folded state of c-type cytochromes is due to the axial coordination of the heme iron with a Met residue (5, 6), unfolding occurs at lower denaturant concentrations in the presence of CO, which competes with Met. As a consequence, upon rapid photochemical removal of bound CO, cyt-c is expected to populate the very initial folding intermediates by coordinating suitable residues of the polypeptide chain before CO can rebind to restore the initial (dark) state. Indeed it has been shown that, in denatured horse heart cyt-c, photolysis of bound CO is followed by a complex array of reactions involving several intermediates (3, 12). We carried out this experiment not only on horse heart cyt-c, but also on cytochrome c_{551} from *P. aeruginosa* (cyt-c_{551}) that seems interesting since it cannot give miscoordinations (13, 23).

The time evolution observed upon photolysis of CO from unfolded, reduced horse cyt-c (at pH 7, 25–40 °C, [CO] = 1 atm and [Gdn] = 4.6 M) has been described by four main kinetic
that one single second order CO recombination process should, in these cases no folding occurs, and the model predicts being observed at very high Gdn or in free heme and MP; in species. This explanation is inconsistent with these processes, we collected full spectra of the reaction mixture at determined at 25°C leading to the formation of a second order.

The experiment was run as a function of CO concentration, and the results obtained on horse cyt-c and on P. aeruginosa cyt-c were reported in Fig. 2, A and B, respectively. It is clear that lowering CO concentration slows down the formation of 500-μs transient, which is therefore assigned to the bimolecular rebinding of CO with an apparent second order rate constant of \( \sim 2 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \). The same experiments were carried out on mercaptoimidazole (0.1 mM, corresponding to four-coordinate heme), imidazole 0.2 mM (upper panel, dotted line, five-coordinate), imidazole 0.1 mM and CO 1 mM (lower panel, solid line, six-coordinate), CO 1 mM alone (lower panel, dotted line, mostly six-coordinate with CO and H2O). Experimental conditions: 5% SDS and 50 mM Tris, pH 7.4. Spectra in the visible region were multiplied by a factor of 5.

processes with \( \tau = 2, 60, \) and 500 μs, and 7 ms, respectively (3). Since the effect of temperature on the population of the intermediates is small, from now on we refer mostly to the values determined at 25°C. To better characterize the fast kinetic processes, we collected full spectra of the reaction mixture at various time delays after the laser pulse (not shown).

The experiment was run as a function of CO concentration, and the results obtained on horse cyt-c and on P. aeruginosa cyt-c are reported in Fig. 2, A and B, respectively. It is clear that lowering CO concentration slows down the formation of the 500-μs transient, which is therefore assigned to the bimolecular rebinding of CO with an apparent second order rate constant of \( \sim 2 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \). The same experiments were carried out on mercaptoimidazole and on free heme in the presence of Melm, a good model for the proximal His; the results, shown in Fig. 2, C and D, demonstrate that also in these simpler systems rebinding of CO proceeds through an intermediate whose formation is second order.

The coordination/miscoordination model (Scheme 1 and Refs. 3, 11, 12, 24, 25) assigns the slow spectroscopic changes (\( \tau > 1 \) ms) to the CO recombination of partially folded or collapsed species. This explanation is inconsistent with these processes being observed at very high [Gdn] or in free heme and MP; in fact, in these cases no folding occurs, and the model predicts that one single second order CO recombination process should be observed and should return the protein to its pre-flash condition. Therefore these experiments suggest that also the decay of the photoproduct of cyt-c involves species bearing no relationship to folding.

Since in the case of free heme, the complex Melm–Fe–CO populates at least one of the kinetic intermediates observed starting from unfolded cyt-c, we investigated in parallel protoheme varying the concentration of both CO and MeIm. This system had been already described in great detail by White et al. (16), and our only point here is to verify whether the base elimination pathway proposed for the Melm–Fe–CO complex may also apply to the case of unfolded cyt-c. In the absence of a base (Im or MeIm), the heme iron coordinates CO and a water molecule; upon photolysis of the Fe–CO bond, over 95% of the absorbance change recorded corresponds to the rebinding of CO in a simple second order process with a rate constant \( 2.8 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \) (Fig. 3A). This value is in good agreement with that reported for the four-coordinate heme (16); more important in the present context, almost no intermediate is seen in this reaction. Addition of 28 mM Melm, a low concentration \( \text{vs} \) a vs the affinity of this ligand, results in the reaction becoming slower and populating an intermediate (Fig. 3B). At higher Melm concentration (100 mM) less of the intermediate is formed (Fig. 3C), but to make it to disappear altogether, the higher affinity base (Im) is required (Fig. 3D). It is relevant that the apparent second order rate constant for CO combination becomes smaller and smaller as the Melm or Im concentration is raised, till it plateaus (not shown). It is difficult to compare the rate constants of CO recombination in the presence of Im and Melm; the former mimics the “in-plane” heme iron typical of R-state Hb, whereas the latter favors the “out-of-plane” dined configuration of T-state Hb (26). Typically \( k_{CO} \) for Melm–Fe approaches \( 1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \), while for Im–Fe it is one order of magnitude faster (16).

The population of the kinetic intermediate associated to binding of CO is a function of Melm concentration, being maximal at intermediate values (10–50 mM Melm) and negligible or zero both in the absence of the base and at saturating concentrations (Fig. 3). According to White et al. (16), this is explained because at high concentrations of the base the heme remains five-coordinate after photolysis and CO rebinds slowly, whereas in the absence of the base the four-coordinate species is largely populated and CO rebinding is very fast (see “Discussion”). At intermediate base concentrations, photolysis of CO is associated to partial dissociation of either Im or Melm; subsequent CO rebinding involves the quickly reacting four-coordinate species, and a five-coordinate CO intermediate is transiently populated.

Control experiments were carried out to exclude artifacts that may interfere with this interpretation of the photochemical intermediate. We checked the extent of photolysis of the Im–Fe bond in the six-coordinate derivative (Im–Fe–Im). A laser pulse dissociates Im with lower quantum yield than CO, but even for this complex recombination is dominated by the base elimination mechanism since it populates distinguishable kinetic intermediates (data not shown); the rate constant for the rebinding of Im to the five-coordinate heme iron was estimated at \( 3.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) under our experimental conditions. Melm does not form the six-coordinate complex Melm–Fe–Melm, but only the five-coordinate Melm–Fe–Melm, one when a solution of Melm (20–100 mM) and heme reduced with dithionite in the absence of CO was submitted to flash photolysis, only a very small absorbance change was observed; the recovery of the pre-flash absorbance occurred in a pseudo first order process with rate constant \( 2 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \). We take this as the rebinding of Melm to the four-coordinate heme iron. The model system of proteoheme dissolved in detergent in the presence of Melm and CO is a good candidate to mimic the intrinsic heme properties of reduced unfolded cyt-c bound to CO. By changing the Melm concentration from 0 to 100 mM while keeping constant that of CO at 1 mM, we have been able to assign the kind of spectroscopic changes associated to the different steps of the base elimination mechanism; this experiment is reported in Fig. 4. It will be observed that during the time course two distinct intermediates, with time constants and fractional pop-
ulations varying with [MeIm], are populated. At 20 mM MeIm both of them are evident and their $\tau$ are 300 ns and 6 $\mu$s, respectively. The faster process is attributed to the equilibration of the four- and five-coordinate derivatives, whereas the slower one is second order with respect to CO and is thus assigned to the formation of (H$_2$O)-Fe–CO complex (Scheme 2).

In view of the above results, we tested the effect of MeIm on unfolded, CO-bound horse heart cyt-c. Consistently with the results obtained on free heme, the intermediate, which in the absence of MeIm is formed with $\tau = 500 \mu$s, disappears if photolysis is carried out in the presence of 5 mM Gdn and 20 mM or more MeIm (Fig. 5). Lower concentrations of MeIm allow the formation of the 500- $\mu$s intermediate, and its maximal population was obtained in the absence of MeIm; thus a clear titration can be obtained. As observed with free heme, addition of MeIm not only reduces the population of the kinetic intermediate but also slows down the rate constant of CO recombination. Moreover, high concentration of MeIm (250 mM) interferes also with the population of the fast intermediates.

**DISCUSSION**

For the purposes of the present discussion we first outline the interpretation of the slow kinetic processes defined, for any given set of conditions, as those corresponding to, or slower than, the bimolecular rebinding of CO (i.e. $\tau \geq 500 \mu$s for horse cyt-c at pH 7, 25 °C, [Gdn] = 4–5 M, and CO = 1 atm).

According to the coordination/miscoordination model (Scheme 1), the formation of the slow intermediates is due to the transient binding of an amino acid side chain to the heme iron in a partially folded or collapsed protein (3), and their decay to the replacement of this internal ligand by CO. The more “folded” the protein, the slower the replacement of the amino acid acting as distal ligand. Our experiments demonstrate that recombination of CO yields an intermediate rather
than the equilibrium species also under experimental conditions in which the protein is completely unfolded even in the absence of CO (e.g. 8 M Gdn, data not shown); moreover, a similar intermediate was seen also in the simpler systems microperoxidase and Melm–heme. We suggest that the intermediate which forms with $\tau = 500$ ms has CO, but not the proximal His, bound to the heme iron and is populated via a base elimination mechanism as described by White et al. (16); therefore, the other kinetic intermediates postulated by that mechanism may also be present. This interpretation is consistent with the known and as yet unexplained discrepancy between absorbance data obtained on cyt-c, which may suggest a substantial population of a putative folding intermediate, and the failure to detect a signal from unequivocal markers of collapse and initial folding, such as quenching of Trp fluorescence (24) and far UV circular dichroism (12). Moreover, the higher population of the slow intermediate seen at lower pH values (e.g. 5), may be due to the enhanced rupture of the proximal bond due to the protonation of the His (as suggested to us by Prof. Steve Hagen). The decay of this intermediate, with $\tau = 7$ ms, is attributed to rebinding of the proximal His and is still second order with respect to CO being coupled to the binding reaction step.

An extended base elimination kinetic scheme, Scheme 3, is shown as follows.

**Scheme 3**

The starting equilibrium species (A) is a six-coordinate derivative having the reduced heme iron coordinated with CO and with the proximal His or Melm or Im (X), depending on the sample (cyt-c, microperoxidase, or heme). The laser pulse promotes the dissociation of bound CO and populates the poorly reactive five-coordinate state X–Fe (B), which would rebind CO slowly (16). Since removal of CO weakens the bond between X and the Fe in state B, a four-coordinate intermediate (C) is populated; the equilibrium between the four- and five-coordinate species (B $\leftrightarrow$ C) is achieved rapidly, due to the high reactivity of the in-plane four-coordinate iron (27). State C, even if present to low levels and therefore difficult to detect, rebinds CO much faster than its five-coordinate partner; for hemes in organic solvents the ratio between the two rate constants is 50–500, depending on the nature of the proximal base (Im or Melm) (16). Therefore, the photolyzed species are rapidly drained to the species D, which we postulate to have an H$_2$O molecule coordinated in *trans*; this may be displaced by His, Melm, or Im restoring the dark equilibrium species (A). The original model proposed by Traylor and co-workers (16) does not allow for water coordination in *trans* since their experiments were carried out in organic solvents, but it seems plausible that a water molecule may bind under our experimental conditions. This assumption, however, is not crucial to the proposed kinetic scheme.

The base elimination mechanism neatly accounts for the slow kinetic intermediate (forming with $\tau = 500$ ms in cyt-c and cyt-c$_{553}$), which we assume to be mostly species D, and also for its decay (with $\tau = 7$ ms) due to coordination of the proximal His in *trans* and displacement of the putative H$_2$O (D $\rightarrow$ A). Moreover it explains why Melm prevents the appearance of the slow intermediate in horse cyt-c by maintaining the heme iron five-coordinate after photodissociation of CO (Fig. 4). It is relevant to recall that Melm does not bind to the sixth coordination position of a His five-coordinate derivative (16); hence, whatever its effect, it has to be already bound before the flash. The base elimination hypothesis here presented assigns the 500-μs and 7-ms transients to the build-up and decay of the slow intermediate (our species D) and provides a better explanation for the nature of the slow intermediate than that proposed by the coordination/miscoordination mechanism, namely that CO recombination occurs to a collapsed molecule having a His or a Met coordinated on the distal side since the latter hypothesis does not explain why the intermediate is formed at
very high [Gdn] or in MP and the heme-MeIm model systems. However there are good reasons to suggest that some coordination (by His or Met residues) may also occur populating species E since in horse heart cyt-c the slowest process is too complex to be fitted to a single exponential.

We now discuss briefly the faster kinetic processes, which are independent of CO concentration and were assigned to the transient coordination of amino acid side chains Met or His (Y, species E in Scheme 3). Chen et al. (12) explored the effect of CO concentration on the overall time course and recognized that only the two fastest processes ($\tau = 2 \mu s$ and $50-70 \mu s$) are CO independent; therefore they restricted their analysis to these species. Moreover they recognized that most of the CD change is observed before any coordination event, thus pointing to proximal effects. Fast absorbance changes are observed not only in cyt-c–CO but also in MP–CO and in the model system Melm–Fe–CO; in cyt-c their amplitude grows upon increasing denaturant concentration and is affected by addition of MeIm (Figs. 4 and 5). We suggest that the faster transients may be contributed by two distinct processes, i.e. equilibration between the five- and four-coordinate species (B ↔ C) and distal coordination of amino acid side chains (B ↔ E and C ↔ F). Perhaps equilibration of five- and four-coordinate overlaps with binding of Met in the 2-μs process (25), and binding of a distal His corresponds to the 40- to 70-μs one (3); alternatively it might be suggested that the B ↔ C equilibration is responsible for the fast CD changes observed by Chen et al. (12). Addition of MeIm results in partial substitution for the proximal His, and the higher MeIm concentration the lower the population of four-coordinate iron attainable in the 2-μs process; this is why MeIm affects both the amplitude of the fast process and the rate of CO recombination. The difference in reactivity between the five- and four-coordinate species (B and C, respectively) is so large that binding of CO would occur preferentially to C even if this was the minority species (by two order of magnitude); thus C never accumulates to a significant extent contrary to D. The interpretation of the fast processes is difficult because of the superimposition with intermediate C of the base elimination pathway; nevertheless the 40-μs transient was observed also by Gray and co-workers (9, 11) using a different approach, namely the photochemical reduction of unfolded oxidized cyt-c. Since the photochemical reduction method is carried out in the absence of CO and exploits the different stability of the oxidizing and reduced cyt-c, one would expect that the four-, five-, and six-coordinate species remain at equilibrium throughout the reaction time course; this lends support to the 40- to 70-μs intermediate representing a true folding species, also consistent with ultra-rapid mixing experiments (28). It is also consistent with our hypothesis that the experiments carried out by photochemical reduction display a simpler time course (2). Finally we may speculate that the four-coordinate species, present together with the five-coordinate one but very reactive toward CO, may play some role in the folding process by coordinating rapidly Met or His on the distal site before the proximal His (species F in Scheme 3). In this respect it is relevant that the five-coordinate heme reacts quite slowly with Im ($k = 3.9 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$), thus suggesting that the rapid coordination events ascribed to distal residues in cyt-c (Scheme 1) may take advantage of the presence of even a small amount of the highly reactive four-coordinate species. Indeed if it were not so, the reactivity of the imidazole residue of His in the unfolded protein would have to be incredibly high.

We conclude that a very significant fraction of the intermediates observed upon photolysis of cyt-c–CO under unfolding conditions (in Gdn or urea), may be accounted for by a six-coordinate species postulated to be: (H$_2$O–Fe–CO and populated via a four-coordinate species. This explains why so little fluorescence or CD evidence for the collapse of the polypeptide chain was recorded after photolysis of CO (12, 24) despite the large absorbance changes and warns against the use of this approach in the absence of accurate controls that take into account the contribution of the intermediates linked to the base elimination CO rebinding pathway.

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REFERENCES
1. Rumbley, J., Hoang L., Mayne, L., and Englander, S. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 105–112
2. Telford, J. R., Wittung-Stafshede P., Gray H. B., and Winkler J. R. (1998) Acc. Chem. Res. 31, 755–760
3. Jones, C. M., Henry, E. R., Chan, C. K., Luck, S. D., Bhuayan, A., Roder, H., Hofrichter, J., and Eaton, W. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11860–11864
4. Yeh, S. R., Takahashi, S., Fan, B., and Rousseau, D. L. (1997) Nature Struct. Biol. 4, 51–56
5. Takano, T., Kallai, O. B., Swanson, R., and Dickerson R. E. (1973) J. Biol. Chem. 248, 5234–5255
6. Bushnell, G. W., Louie G. V., and Brayer G. D. (1990) J. Mol. Biol. 214, 585–595
7. Taler, G., Schejter, A., Navon, G., Vig, I., and Margoliash, E. (1995) Biochemistry 34, 14209–14212
8. Stoll, M. M. M., and Wilson, M. T. (1988) Inorg. Chim. Acta 153, 99–104
9. Telford, J. R., Torkan, F. A., Gray, H. B., and Winkler, J. R. (1999) Biochemistry 38, 1944–1949
10. Colon, W., Walzem, L. P., Sherman, F., and Roder, H. (1997) Biochemistry 36, 12535–12541
11. Pascher, T., Chesick, J. P., Winkler, J. R., and Gray, H. B. (1998) Science 271, 1558–1560
12. Chen, E., Wood, M. J., Fink, A. L., and Kliger, D. S. (1996) Biochemistry 35, 5589–5598
13. Detlefsen, D. J., Thanabal, V., Pecoraro, V. L., and Wagner, G. (1991) Biochemistry 30, 9040–9046
14. Rouege, M., and Brunet, D. (1975) Biochemistry 14, 4100–4105
15. Momentau, M., Rouege, M., and Loox, B. (1976) Eur. J. Biochem. 71, 63–76
16. White, D. K., Cannon, J. B., and Traylor, T. G. (1979) J. Am. Chem. Soc. 101, 2443–2454
17. Harbury, H. A., and Lorch, P. A. (1960) J. Biol. Chem. 235, 3640–3645
18. Cutruzzola, F., Ciabatti, I., Rolli, G., Falcinelli, S., Arrese, M., Ranghino, G., Anselmino, A., Zennaro, E., and Silvestrini, M. C. (1997) Biochemistry 36, 35–42
19. Wilson, E. K., Bellelli, A., Liberti, S., Arrese, M., Grasso, S., Cutruzzola, F., Brunori, M., and Brzezinski, P. (1999) Biochemistry 38, 7556–7564
20. Henry, E. R., and Hofrichter, J. (1992) Methods Enzymol. 210, 129–192
21. Jehanty, A. M., Stutter, D. A., and Wilson, M. T. (1970) Eur. J. Biochem. 71, 613–616
22. Laberge, M., Vreugdenhil, A. J., Vankor, M. J., and Butler, I. S. (1998) J. Biol. Chem. 273, 1039–1050
23. Travaglini-Alpetto, C., Cutruzzola, F., Bigotti, M. G., Staniford, R. A., and Brunori, M. (1999) J. Mol. Biol. 289, 1459–1467
24. Chan, C. K., Hofrichter, J., and Eaton W. A. (1996) Science 274, 628–629
25. Hagen S. J., Hofrichter J., and Eaton W. A. (1997) J. Phys. Chem. B 101, 2352–2365
26. Perutz, M. F. (1979) Annu. Rev. Biochem. 48, 327–338
27. Giacometti, G. M., Traylor, T. G., Ascenzi, P., Brunori, M., and Antonini, E. (1977) J. Biol. Chem. 252, 7447–7448
28. Chan, C. K., Hu, Y., Takahashi, S., Rousseau, D. L., Hofrichter J., and Eaton W. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1779–1784
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