Ultrafast Heme−Residue Bond Formation in Six-Coordinate Heme Proteins: Implications for Functional Ligand Exchange

Marten H. Vos,*[,‡,§] Andrea Battistoni,[||,⊥] Christophe Lechauve,[#] Michael C. Marden,[#] Laurent Kiger,[#] Alain Desbois,[▽] Eric Pilet,[○] Eve de Rosny,[○] and Ursula Liebl[‡,§]

Laboratoire d'Optique et Biosciences, CNRS, Ecole Polytechnique, F-91128 Palaiseau, France, INSERM U696, F-91128 Palaiseau, France, Dipartimento di Biologia, Università di Roma Tor Vergata, Via della Ricerca Scientifica, 00133 Roma, Italy, Consorzio Interuniversitario "Istituto Nazionale Biostrutture e Biosistemi", Viale delle Medaglie d'Oro 305-00136 Roma, Italy, INSERM U779, F-94276 Le Kremlin-Bicêtre, France, Institut de Biologie et de Technologie de Saclay, Service de Bioénergétique, Biologie Structurale et Mécanismes, CNRS URA 2096, CEA/Saclay, F-91191 Gif-sur-Yvette Cedex, France, and Laboratoire de Cristallographie et Cristallogenèse des Protéines, Institut de Biologie Structurale Jean-Pierre Ebel, 41 rue Jules Horowitz, F-38027 Grenoble; CEA; CNRS; Université Joseph Fourier, 41 rue Jules Horowitz, 38027 Grenoble, France

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ABSTRACT: A survey is presented of picosecond kinetics of heme−residue bond formation after photolysis of histidine, methionine, or cysteine, in a broad range of ferrous six-coordinate heme proteins. These include human neuroglobin, a bacterial heme-binding superoxide dismutase (SOD), plant cytochrome b_{559}, the insect nuclear receptor E75, horse heart cytochrome *c* and the heme domain of the bacterial sensor protein Dos. We demonstrate that the fastest and dominant phase of binding of amino acid residues to domed heme invariably takes place with a time constant in the narrow range of 5−7 ps. Remarkably, this is also the case in the heme-binding SOD, where the heme is solvent-exposed. We reason that this fast phase corresponds to barrierless formation of the heme−residue bond from a configuration close to the bound state. Only in proteins where functional ligand exchange occurs, additional slower rebinding takes place on the time scale of tens of picoseconds after residue dissociation. We propose that the presence of these slower phases reflects flexibility in the heme environment that allows external ligands (O_2, CO, NO, . . .) to functionally replace the internal residue after thermal dissociation of the heme−residue bond.

Chemical bonds between amino acid residues and cofactors can play important roles in protein stability. In some cases the associated bond breaking and bond formation processes are directly linked with the function of proteins. The structural and energetic characteristics of such bonds are often well determined. However, dynamic properties of intrinsic bond formation are generally difficult to assess, as slow diffusion and folding processes determine overall bond formation rates in mixing experiments. A different approach is possible in heme proteins, where selectively axial bonds with the heme iron can be dissociated by a light pulse, and bond reformation dynamics followed by optical spectroscopy (*1–3*).

Many hemes involved in electron transfer processes are coordinated to two axial residues, like the histidine-methionine linked heme in cytochrome *c* (cyt *c*[1]). During the past decade, a rapidly expanding group of proteins has also been characterized that contain 6-coordinate (6-c) hemes, which can bind either an internal residue or an external ligand (like O_2, NO or CO) to the sixth position. In this group of proteins, the exchange between the ligands is thought to be functional. In several of these proteins, binding of the internal residue has been studied after photolysis of CO from the heme. This process invariably takes place on the tens of microseconds to milliseconds time scale as established in experiments on various 6-c hemoglobins (*4, 5*), neuroglobin (*6–8*), the gas sensors CooA (*9*) and Dos (*10, 11*), as well as modified cyt *c* (*12*). Here, residue binding after CO dissociation is rate-limited by CO migration and protein rearrangement, whereas dissociation of the heme−residue bond in the absence of external ligands leads to at least 6 orders of magnitude faster rebinding, on the picosecond time scale (*2, 10, 13–15*). This latter process, formation of the residue−heme iron bond from a near-binding configuration, is the subject of this paper.

The photolysis of small *external* ligands from hemes has been exploited for many decades. The first indications of photodissociation of an *internal* protein residue from heme were reported in a subpicosecond resolution transient absorption study of Jongeward and co-workers (*13*) on ferrous cytochrome *c* and soluble cytochrome b_5 (respectively His-Met and His-His coordinated). From the transient spectra obtained after excitation with intense UV pulses they

* Corresponding author. Tel.: +33169085066, Fax: +33169085084, email: marten.vos@polytechnique.edu.
‡ CNRS, Ecole Polytechnique.
§ INSERM U696.
|| Università di Roma Tor Vergata.
⊥ Consorzio Interuniversitario "Istituto Nazionale Biostrutture e Biosistemi".
INSERM U779.
▽ CNRS URA 2096.
○ Laboratoire de Cristallographie et Cristallogenèse des Protéines, Institut de Biologie Structurale Jean-Pierre Ebel; CEA; CNRS; Université Joseph Fourier.

[1] Abbreviations: cyt, cytochrome; DAS, decay associated spectrum; DosH, heme domain of Dos; Ngb, neuroglobin; SOD, superoxide dismutase.

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suggested formation of a 5-c species and rebinding in ~7 ps in both cases, although under these excitation conditions a longer-lived bleach of the absorption band was also observed. Based on the spectral similarity of both transient species it was suggested that in both cases His was dissociated. Later work on cyt *c* using transient absorption (*2*) and subpicosecond resonance Raman spectroscopy (*3*) confirmed photodissociation and fast rebinding of the heme−residue bond, even under excitation with visible excitation pulses with moderate energy, but demonstrated that Met and not His is dissociated. In addition, the transient Raman work showed that, upon Met dissociation, the heme iron moves out of plane leading to a domed heme (*3*). Photodissociation and picosecond rebinding of a residue has been reported for two other heme proteins: the CO sensor CooA (His-Pro coordination, ref *16*) (*14*) and the heme domain DosH of the sensor protein Dos (His-Met coordination), where Met is presumably dissociated (*10, 17*).

Comparing the rebinding kinetics in this small number of proteins studied, it is intriguing to observe that in all of them the fastest phase of rebinding takes 5−7 ps. With the exception of DosH, this phase is also by far the major phase. Here, we set out to determine more systematically general properties of the internal ligand binding process. We present a survey of a broad range of 6-c ferrous heme proteins, varying in heme coordination, heme environment, solvent exposure and function. Other than cyt *c* and DosH, these include human neuroglobin (Ngb), a bacterial heme-binding superoxide dismutase (SOD), plant cytochrome b_{559} and the insect nuclear receptor E75.

Ngb is a mammalian monomeric globin protein of known structure (*18*). Its function is not unambiguously established; local oxygen supply for brain and eye tissue (*19*) as well as a signaling function (*20*) have been proposed. The *b*-type heme is axially coordinated by two near-orthogonally oriented His residues; external ligands can bind in place of distal His64 (His E7 in globin notation). Low-yield long-lived photodissociation of His from the ferrous bis-His protein has been implied from single-wavelength measurements with nanosecond resolution (*6*).

Cyt b_{559} from spinach chloroplasts is a membranous electron transfer protein closely associated with the photosystem II reaction center (*21*). The heme of cyt b_{559} is coordinated by two His residues (*22*) and does not bind external ligands.

Haemophilus ducreyi is a bacterial pathogen that does not synthesize heme, but imports it from a mammalian host. It contains a SOD that is capable of binding *b*-type heme (*23*). The role of this heme is not well established; it appears not to be involved in the superoxide dismutase function of the protein (A.B., unpublished results). Recent studies have suggested that this protein protects *H. ducreyi* from toxicity of exogenous heme (*24*). Possibly the protein might also play a role as heme transport protein. A very peculiar and interesting feature is that the heme is exposed to the solvent. It is coordinated by two His residues from two different subunits (*23*). Therefore, this protein constitutes a model system for studying heme−ligand dynamics in the absence of a well-defined protein heme pocket.

E75 is a nuclear receptor from *Drosophila* that was very recently shown to bind heme and to be gas-responsive (*25*). In the absence of external ligands, the heme is coordinated by a His and a Cys residue (*26*). The published absorption spectra of the CO and NO bound form (*25*) are consistent with CO replacing the Cys residue and NO forming a 5-c NO-bound species.

Our results show that the ligand rebinding kinetics on the sub-10 ps time scale in these widely varying systems are remarkably homogeneous. The origin of this finding and the implications of more dispersive kinetics on a longer time scale will be discussed.

MATERIALS AND METHODS

Protein Purification. SOD from *H. ducreyi* was overexpressed in *Escherichia coli* and purified in a form lacking the N-terminal protein domain as described in ref *23*. Human neuroglobin (wild type as well as C46S/C55S mutant Ngb, where no intradisulfide bridge can be formed (*27*)) was overexpressed in *E. coli* BL21DE3 by autoinduction (*28*) and purified as described in ref *29*. Cytochrome b_{559} was isolated from spinach following a procedure described in ref *30*. E75 from *Drosophila* was overexpressed in *E. coli* and purified as described in ref *26*. The heme domain DosH of the sensor protein Dos from *E. coli* was expressed and purified as described in ref *17*. Horse heart cytochrome *c* was purchased from Sigma.

Sample Preparation. The proteins were prepared to a sample concentration of 50−70 μM (except for E75, 12 μM) in a gastight cell with an optical path length of 1 mm. The buffer compositions were 10 mM potassium phosphate, pH 7.0 (SOD), 100 mM potassium phosphate, pH 7.0 (Ngb), 50 mM Tris, pH 8.0, 250 mM KCl, 5 mM $MgCl_2$, 0.05% (w/v) Triton X-100 (cyt b_{559}), 50 mM Tris, pH 8.0, 300 mM NaCl (E75) or 50 mM Tris, pH 7.4 (DosH, cyt *c*). The samples were degassed and reduced with sodium dithionite (SOD, Ngb, E75, DosH, cyt *c*) or sodium ascorbate (cyt b_{559}). In some experiments with Ngb in addition 10 mM dithiothreitol was added.

Spectroscopy. Steady-state spectra were recorded using a Shimadzu UV−vis 1601 spectrophotometer. Multicolor femtosecond absorption spectroscopy (*31*) was performed with a 30 fs pump pulse centered at 565 nm and a <30 fs white light continuum probe−pulse, at a repetition rate of 30 Hz. Data were acquired in scans with a double time window, with windows of 4 and 50 ps full scale (and additional time windows up to 300 ps for DosH). The experiments were performed at 20 °C. Data analysis in terms of decay associated spectra (DAS) was performed as described in ref *32*.

RESULTS

The ground-state absorption spectra of the reduced deoxy forms of the investigated proteins are shown in Figure 1. All spectra are qualitatively similar and indicate formation of a 6-c low spin form, in agreement with previous reports on the respective proteins. For all proteins, transient spectra in the Soret region were collected at different time delays after excitation in the α band.

Figure 2 shows selected transient spectra, and a DAS analysis, for SOD as an example. Upon excitation the Soret band red-shifts, as expected for dissociation of an axial ligand. The transient spectra slightly shift to the blue during the first few picoseconds and decay almost to zero within

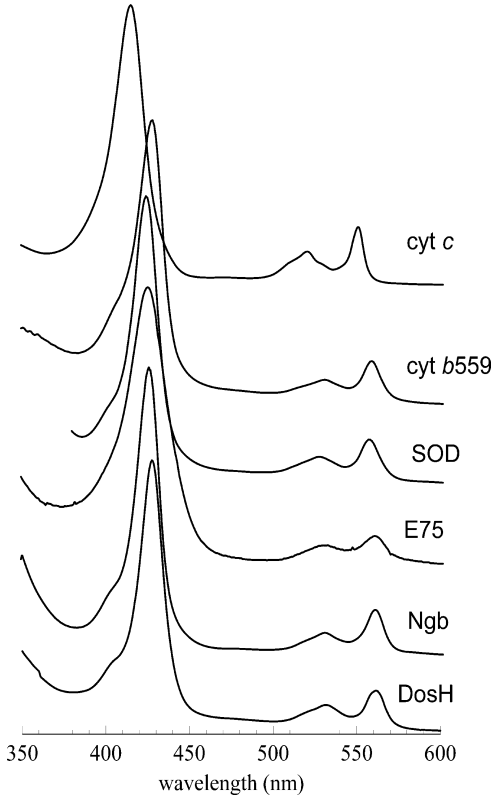

FIGURE 1: Steady-state spectra of the ferrous 6-c heme proteins used in this study. With the exception of cyt *c* all proteins contain *b*-type heme.

50 ps. A global analysis in terms of decay associated spectra allows description of the entire data set in terms of spectrally distinct exponentially evolving components. DAS analysis of the total data set between 1 and 50 ps reveals two major decay components. The 1.2 ps component reflects the blue shift of an initial red-absorbing band and can be ascribed to heme photophysical effects (*33, 34*). The dominant 6.1 ps red-shifted component is ascribed to rebinding of dissociated histidine to the heme. The final, constant (on the time scale of the experiment), spectrum has a relative amplitude of less than 3%. It has a blue-shift shape and therefore does not reflect dissociated ferrous heme, but can be ascribed to a small fraction of photo-oxidation (cf. ref *10*). We conclude that rebinding of dissociated histidine in SOD is essentially a single exponential process with a time constant of ~6 ps.

A similar analysis was performed for all protein systems studied. Figure 3 shows the DAS associated with decay components with time constants >5 ps. They all display the red shift characteristic of ligand dissociation that can be ascribed to internal residue rebinding. Analysis of the amplitude of the spectra indicates that, as for Met 80 dissociation in cytochrome *c* (*2*), the quantum yield of residue photodissociation is close to 1 for the *b*-type heme containing proteins. The variation in the position of the extrema in the DAS corresponds well to that of the Soret band ground-state absorption maxima (Figure 1). The corresponding time constants (Table 1) of the dominant component are in the remarkably narrow range of 5−7 ps. This point is also illustrated in the comparative rebinding kinetics of Figure 4.

For Ngb and E75, minor longer phases with similarly shaped DAS were also observed (Figures 3, 4). For WT Ngb,

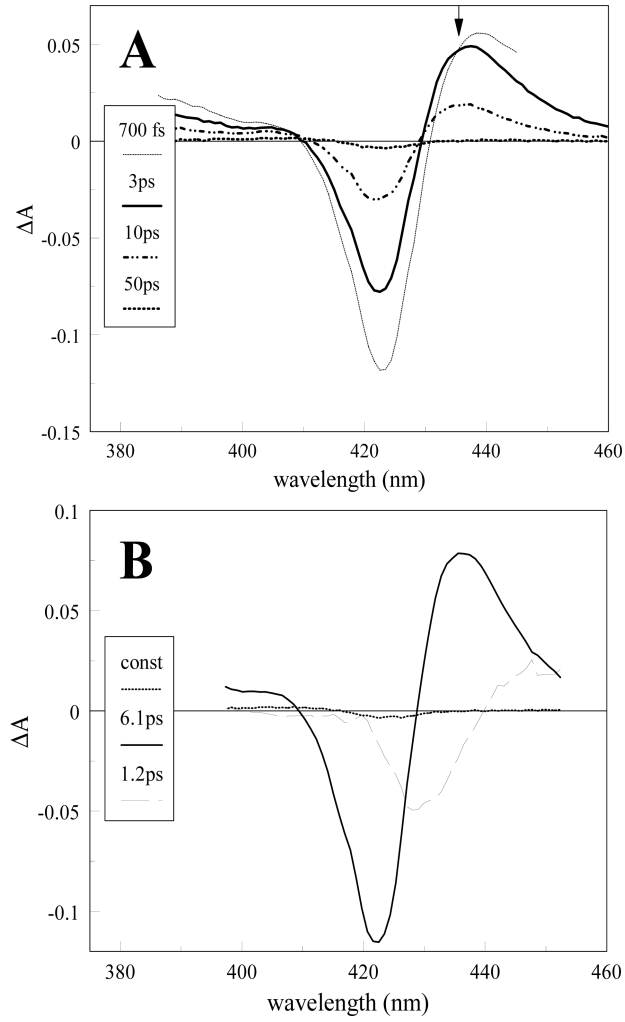

FIGURE 2: Histidine dissociation and rebinding in SOD from *H. ducreyi*. A: Transient spectra at selected delay times. The arrow indicates the wavelength of the kinetics in Figure 4. B: Analysis of the total data set in terms of decay associated spectra. The 1.2 ps component reflects heme photophysics processes. The 6.1 ps component reflects His rebinding to the heme.

the kinetics were identical in the presence and absence of dithiothreitol; also in the C46S/C55S double mutant very similar kinetics were observed (not shown). These observations imply that partial reduction of the disulfide bond in this protein is not responsible for the biphasic kinetics. The only investigated system with substantially slower overall rebinding kinetics is DosH, where rebinding, presumably of Met, occurs, in addition to the "universal" 7 ps phase, with a 35 ps phase with similar amplitude (*10, 17*). The significance of this latter phase as decay of a signaling intermediate has been discussed previously (*17*).

In Ngb, a very small (1−2% of the initial amplitude) signal remained after decay of the 6.8 ps (83%) and 21 ps (15%) components, indicating that a small fraction of dissociated His adopts a configuration from which picosecond rebinding cannot occur. Time-resolved measurements on the microsecond and millisecond time range (not shown) revealed a kinetic component with similar (5-c minus 6-c) spectral characteristics and indicate that this fraction rebinds in a multiphasic manner, with a fastest phase of ~3 ms. This value is similar to that previously reported by Kriegl and co-workers (*6*). However the amplitude of this phase (~2%, in agreement with the picosecond measurements) is signifi-

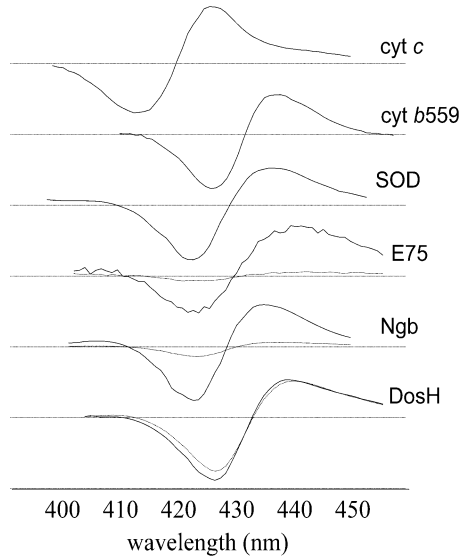

FIGURE 3: Spectra associated with decay components attributed to axial residue rebinding with heme. The solid lines represent the fastest (5−7 ps) decay component. For proteins where a significant slower component was found, this component is indicated with a dashed line.

Table 1: Time Constants Associated with DAS Assigned to Residue Rebinding to Ferrous Heme Proteins[a]

protein	coordination	τ_i in ps (rel ampl)	ref
cytochrome *c* (horse heart)	His-Met	5.6	this work, refs *2, 13*
cytochrome b_{559} (spinach)	His-His	5.9	this work
SOD (*H. ducreyi*)	His-His	5.1	this work
E75 (*Drosophila*)	His-Cys	7.3 (0.92) 70 (0.08)	this work
neuroglobin (human)	His-His	6.8 (0.83) 21 (0.15)	this work
DosH (*E. coli*)	His-Met	7.2 (0.53) 35 (0.47)	this work, ref *10*
CooA (*Rhodospirillum rubrum*)	His-Pro	6.5	ref *14*

[a] For systems with two constants relative amplitudes are given in parentheses.

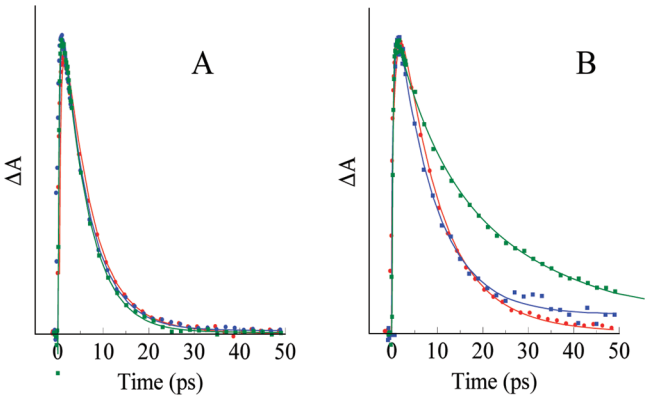

FIGURE 4: Kinetics of axial residue rebinding as measured in the peak of the absorption increase of the DAS. Panel A: cyt *c* (green, 428 nm), cyt b_{559} (blue, 442 nm) and SOD (red, 435 nm). Panel B: E75 (blue, 442 nm), Ngb (red, 442 nm) and DosH (green, 440 nm).

cantly lower than in ref *6*. (5%). This difference may be due to multiple excitations of the 6-c protein by an intense nanosecond flash, which can raise the effective dissociation yield per flash above the photochemical quantum yield of dissociation. Such an effect is not possible when using femtosecond pulses, which are much shorter than the time scale of the picosecond rebinding processes.

In earlier work, it was shown that excitation of 6-c ferric cyt *c* does not lead to sizable dissociation of axial residues (*15*). On the other hand, we previously suggested a small fraction of photolysis of ferric 6-c DosH, and rebinding in ~20 ps (*10*), but it was later shown that in this case one of the axial ligands is a water molecule (*35*). Transient absorption experiments on the ferric forms of other proteins of this study gave similar results as cyt *c* (not shown). We conclude that the bonds of axial residues with ferric hemes are more photostable than those of ferrous hemes.

DISCUSSION

The characteristics of the transient spectra of all proteins studied here indicate the formation of a 5-c domed species upon absorption of a photon by the 6-c ferrous heme, and thus photodissociation of an internal residue. Heme doming upon residue photolysis is also consistent with molecular dynamics simulations of Met dissociation in DosH (Figure 7 in ref *17*). Thus the process appears similar to the way small ligands like CO can be photodissociated from ferrous heme−His−CO complexes (*1*). As, in contrast to small gaseous ligands, the internal ligands are restrained to heme proximity, full rebinding is observed on the picosecond time scale for all proteins.

In principle, the dissociated ligand can be either of the axial residues. For the His-Met coordinated cyt *c*, it has been demonstrated that the Met residue is dissociated (*2, 3*) with near-unity quantum yield (*2*). For DosH, which contains a His-Met coordinated *b*-type heme, from spectral arguments it was also inferred that the Met residue is dissociated with high quantum yield (*10*); this was recently confirmed by a mutational study (*17*). For the His-His coordinated systems, it cannot be spectroscopically determined which of the two His residues is dissociated. However, in Ngb one can distinguish a proximal and a distal histidine (His64). The latter can be replaced by external ligands. Upon excitation of the complexes with external distal ligands, it is the bond with the distal ligand that is broken, and not the one with the proximal His. Therefore, and by analogy with the situation in DosH, it appears likely that in Ngb the distal His is dissociated. In support of this proposal, the induced absorption parts of the transient spectra associated with CO dissociation and His dissociation in Ngb are very similar (not shown). In addition, dissociation of proximal His from a 5-c heme protein (like deoxymyoglobin) has not been observed, whereas dissociation of NO from a 5-c heme is possible (*36*). We cannot exclude, however, that in Ngb heme excitation leads to dissociation of proximal His rather than distal His in a fraction of the hemes. Following an analogous reasoning as for DosH and Ngb, we suggest that in E75 it is the distal Cys that is dissociated. We note that SOD also can form heme−His−CO complexes (A.B., M.H.V., L.K. and G. Smulevich, unpublished results), but it has not been determined which of the coordinating His residues plays the role of the proximal His in this case.

The ensemble of our data on a variety of 6-c heme proteins shows that the initial, and in most cases dominant, rebinding

phase invariantly takes place with a time constant of 5−7 ps (Table 1). The variations include heme type (*b* and *c*-type hemes), dissociated residue (His, Met, Cys; in CooA possibly also Pro is dissociated *14, 16*), protein environment (soluble proteins as well as a membrane protein) and in particular heme environment. With one exception (see below), the hemes are shielded within the protein moiety. However, whereas the protein matrix in cyt *c* is relatively rigid (*37, 38*) and devoid of ligand-exchange pathways, the globin environment of Ngb is more flexible and allows exchange with external ligands. The same presumably holds true for the gas-responsive protein E75.

A very remarkable result is that the rebinding of dissociated His in SOD is also characterized by single-exponential, extremely fast (5 ps) kinetics. In this protein, the heme is coordinated by two solvent-exposed His residues, and is thought to be "suspended" between two protein subunits (*23*). Thus, dissociation of one heme−His bond would give the heme in principle considerable motional freedom. Yet, rebinding occurs on the same time scale as in the other systems, where both the dissociated residue and the heme are presumably confined in the heme pocket. Together, it appears that the fast rebinding phase is not limited by movements of the dissociated residue and the heme. Rather, the $(5-7\ \text{ps})^{-1}$ rate reflects the intrinsic speed of formation of the heme−residue bond, starting from the 5-c domed heme and the residue in a configuration close to the bound state. The finding that the rate is independent of the nature of the dissociated residue strongly suggests that motions along residue coordinates are not rate-determining. One might therefore suggest that rearrangement of the heme moiety (in particular heme doming) limits the binding rate. At this point a comparison with heme−NO binding characteristics is of interest. The fastest phases of heme−NO rebinding in many (but not all) heme proteins take place in the very similar range of 5−10 ps (*1*). For MbNO, this phase is essentially barrierless (*39*), and it has therefore been proposed that NO can bind to a domed heme configuration (*39*); relaxation to a planar heme would follow immediately. In view of the similarity of the rates, and consistent with the temperature independence in the physiological range (15−37 °C) found for heme−Met rebinding in DosH (*17*), it is reasonable to propose a similar mechanism for the ensemble of fast heme−residue bond formation processes we have studied. In this view, the ∼6 ps rate corresponds to the barrierless formation of the Fe−N (His) or Fe−S (Met, Cys) bond. The intrinsic parameters determining this rate (as well as that for NO binding) remain to be determined; possibly barrierless migration toward the transition state plays a role.

In some systems (DosH, Ngb, E75) we also observed an additional slower heme−residue recombination phase. In principle such phases can arise from two origins: (i) a heterogeneous distribution of configurations populated directly upon dissociation and (ii) competition between fast rebinding and residue rearrangement to a configuration from which binding is slower. For the case of DosH, biophysical studies demonstrated that the first possibility applies (*17*). Simulations indicated that the two phases can be associated with two different configurations of Met after dissociation (corresponding to rotations along the dihedral angles of the flexible Met side chain), with one yielding a strained configuration with the S atom rotated away from the Fe. For the case of Ngb, the dissociated residue is His. The aromatic His side chain cannot easily adopt different configurations in the heme-bound state. In addition, we observed that the presence or absence of a disulfide bond in this protein, a parameter strongly influencing the subnanosecond rebinding kinetics of the external ligand CO (*40*) (M.H.V. and L.K., unpublished results) and the thermal dissociation of the distal His ligand (*27*), did not significantly influence the His rebinding kinetics. Therefore it is unlikely that for Ngb a similar mechanism as for the Met residue in DosH applies or that the two phases reflect rebinding of proximal and distal His (see above), and we suggest that possibility ii may explain the appearance of the slower rebinding phase. The same reasoning may be true for the short side chain of the Cys residue in E75. It is also possible, however, that a structural heterogeneity in the heme pocket plays a role, due to the fact that two different Cys residues (C396 and C468) can act as a heme ligand (*26*).

It is noteworthy that the proteins in this study displaying a slower second phase correspond to those where a functional exchange of the internal residue with an external gaseous ligand takes place. This observation suggests a specific role for the slow rebinding phase. Indeed, in Ngb, after decay of the slow picosecond phase, a small fraction of the dissociated residue could be reconfigured to a position from which rebinding occurs only on the millisecond time scale (this work and ref *6*). We propose that this position, that clearly facilitates access to the heme for small ligands in the protein environment, is reached from the position associated with the slow (in the case of Ngb 21-ps) rebinding phase. Furthermore, if an external ligand is present near the heme upon dissociation of the heme−residue bond, its access to the heme will be facilitated if the dissociated residue is moved away from the closest-binding position. As discussed in the previous paragraph, we believe that this situation is reflected by the presence of a slower binding phase. Thus we suggest that the inferred possibility of the dissociated residue to adopt a different configuration enables external ligands to bind. The presence of such external ligands in the heme pocket may even cause additional strain on the dissociated residue and thus favor motion away from the heme. Unfortunately, at present such a situation is experimentally not directly accessible.

We note that Kumazaki and co-workers (*14*) reported for the CO sensor CooA, apart from the major 6.5 ps decay phase of residue rebinding, also a minor slower (170 ps) phase in single wavelength kinetics. This phase was ascribed to the effect of protein relaxation on the spectrum of the 6-c heme. However, the spectral characteristics of this phase were not determined and, in the context of the above discussion, we suggest that it might reflect an additional slow residue binding phase.

In conclusion, we have demonstrated that binding of amino acid residues to domed heme invariably takes ∼6 ps in 6-c heme proteins. Only in proteins where functional ligand exchange occurs, additional slower rebinding after residue dissociation takes plays. We propose that these slower phases reflect flexibility in the heme environment that allows external ligands to replace the internal residue after thermal dissociation of the heme−residue bond.

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