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Analysis of Fe(III) Heme Binding to Cysteine-Containing Heme-Regulatory Motifs in Proteins

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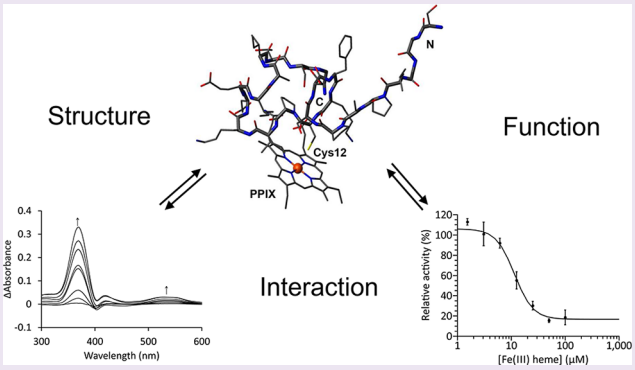
Supporting Information

ABSTRACT: Regulatory heme binds to specific motifs in proteins and controls a variety of biochemical processes. Several of these proteins were recently shown to form complexes with ferric and/or ferrous heme *via* a cysteine residue as axial ligand. The objective of this study was to examine the heme-binding properties of a series of cysteine-containing peptides with focus on CP motif sequences. The peptides displayed different binding behavior upon Fe(III) heme application with characteristic wavelength shifts of the Soret band to 370 nm or 420−430 nm and in some cases to both wavelengths. Whereas for most of the peptides containing a cysteine only a shift to 420−430 nm was observed, CP-containing peptides exhibited a preference for a shift to 370 nm. Detailed structural investigation using Raman and NMR spectroscopy on selected representatives revealed different binding modes with respect to iron ion coordination, which reflected the results of the UV−vis studies. A predicted short sequence stretch derived from dipeptidyl peptidase 8 was additionally examined with respect to CP motif binding to heme on the peptide as well as on the protein level. The heme association was confirmed with the first solution structure of a CP-peptide-heme complex and, moreover, an inhibitory effect of Fe(III) heme on the enzyme's activity. The relevance of both the use of model compounds to elucidate the molecular mechanism underlying regulatory heme binding and its potential for the investigation of regulatory heme control is discussed.



Heme binding to proteins involved in important physiological processes such as oxygen transport, electron transfer, and substrate oxidation has been investigated intensively.[1−4] For hemoproteins with the heme-iron complex as the functional center, structural aspects concerning the orientation of the protein to heme and distinct sequence features of the protein required for binding are well known, mostly because of the large variety of crystal structures for heme-protein complexes.[5−8] In contrast, the recently suggested regulation of proteins through binding to heme[9−13] is not well understood.

The iron-coordinating amino acid is one characteristic feature of heme-binding motifs (HBM) or heme-regulatory motifs (HRM). Both motifs were discussed to play an important role in heme recruitment and heme sensing.[5,6,9,12−15]

Indeed, association and dissociation of the heme-iron complex to and from heme sensor proteins was proposed to be associated with a switch on/switch off function in processes such as catalysis and transcription.[12] A fast dissociation of the heme-protein complex as a consequence of a rather weak heme binding compared to hemoproteins excludes a strong coordination or covalent binding of heme to these proteins. An analysis of the HRM proteins described up to now revealed that primarily cysteine is involved in these temporary heme-binding events.[9] In addition, such cysteine residues were often shown to be accompanied by a C-terminal proline residue, thus

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Table 1. Characterization of Fe(III) Heme Binding to Peptides[a]

no.	peptide sequence[b]	protein (ref)	origin	nUV (nm)	Soret (nm)	K_d for nUV (μM)[c]	K_d for Soret (μM)[c]	Slo1 competition[d]
			C-Containing Motifs					
			Known					
1	APSRCTWQL	CBS (42)	*H. sapiens*	364	419	4.41 ± 0.80	nsat	nd
2	SSIPCLHYK	DGCR8 (43)	*H. sapiens*	366	410	0.81 ± 0.51	3.99 ± 2.35	++
			Predicted					
3	AAAACAAAA	(9)	negative control				nb	nd
4	QFSQCRIBN	(9)	screening		424		2.78 ± 0.78	nd
5	YVSRCIBBA	(9)	screening		430		3.44 ± 0.64	nd
6	FYWDCNHYW	(9)	screening		420		0.67 ± 0.12	nd
7	RDQYCSPTK	(9)	screening		425		nsat	+
8	DICVHLNRK	hEAG1	*H. sapiens*	368		nsat		nd
9	RADICVHLN	hEAG1 (9)	*H. sapiens*		430		0.40 ± 0.17	+ (9)
10	SSIPCLFYK	mutant of **2**	*H. sapiens*	369		0.28 ± 0.19		nd
			CP-Containing Motifs					
			Known					
11	ALTGCPWHD	Irr (24, 35)	*B. japonicum*	368		5.32 ± 0.55		nd
12	SQSSCPAVP	hPer2 (25)	*H. sapiens*	364		6.43 ± 0.53		+
13	DESACPYVM	HRI (18)	*H. sapiens*	364		3.27 ± 1.44		+
14	TPILCPFHL	IRP2 (36)	*H. sapiens*	368	415	0.60 ± 0.41	2.03 ± 1.38	+
15	RPDGCPDEI	Jak2 (11)	*H. sapiens*	398			0.50 ± 0.23	nd
			Predicted					
16	AAAACPAAA		negative control	364		4.73 ± 1.83		nd
17	IGVVCPFVR		screening	368		0.87 ± 0.41		++
18	QFSSCPHYW		screening	368	422	1.94 ± 0.42	10.37 ± 1.19	++
19	SEGGCPLIL	IL-36 α	*H. sapiens*	366		3.75 ± 0.77		++
20	SGGLPAPSDFKCPIKEEIAITSG	DP8 (9)	*H. sapiens*	369		1.42 ± 0.24		+ (9)
21	ARLGCPVIP	feoB	*E. coli*	367		1.37 ± 0.33		nd
22	EDKDCPIKE	PTP-1B	*H. sapiens*	368		48.40 ± 2.73		nd
23	DESACPVYM	mutant of **13**	*H. sapiens*	367		13.26 ± 1.44		+

[a]nUV, near-UV band; nsat, not saturated; nd, not determined; nb, no binding. [b]B = norleucine, as used in an earlier study.[9] [c]Data are mean ± SEM. [d]Evaluation according to the competitive binding assay as follows: +, moderate competition; ++, strong competition.

forming a "CP motif". Such a CP motif has first been proposed to be important for the regulation of pre-5-aminolevulinate synthase (ALAS-E).[16] Since then, a role of proline with respect to the regulation process has been discussed in several reports.[5,10,12,17−19] Although proline in $C^{264}P^{265}$ was not required for heme binding to heme oxygenase-2 (HO-2),[17] the motif proved to be essential for heme-regulated inhibitor/eukaryotic translation initiation factor 2-α kinase 1 (HRI) within HRM $C^{409}P^{410}$.[18] Impairment of heme-dependent function was also observed for other proteins such as Hap1 upon substitution of the proline residue.[10] Subsequently, it was suggested that proline supports the coordination of the cysteine in the thiolate form to the Fe(III) heme complex.[5,12]

Concerning proteins that contain several CP motifs, however, heme binding could not be proven for all of these sequences. Two representatives are heme activator protein 1 (Hap1)[10] and transcription regulator protein Bach1,[20] for which the existence of nonresponsive CP motifs was demonstrated.[10,14] There is strong evidence that the Cys environment plays a crucial role for the association with Fe(II)/Fe(III) heme. The example of HO-2 already indicated that, besides CP motifs, individual cysteine residues may be responsible for heme binding in an appropriate sequence context as well. Further examples are arginyl-tRNA-protein transferase 1 (ATE1),[21] STC1,[19] and PpsR protein.[22] Thus, numerous reports indicate that the CP motif is a preferred amino acid arrangement for heme binding, but not absolutely essential.[18]

As a consequence of an earlier study,[9] we focused our attention on cysteine-containing sequences and, in particular, on the characteristics required for heme binding of CP motifs. In addition, we and others[12] fully subscribe to the view that further studies are needed to validate the proposed critical role of protein flexibility and/or global structural changes for heme binding. The current state of knowledge does not allow for an overall assessment of the process leading to temporarily existing heme-protein complexes, in particular of the conformational differences between the heme-bound and heme-unbound state. A survey of X-ray crystallography and/or NMR reports on CP-motif-containing proteins reveals that six out of 18 heme *b* proteins containing the GX[HR]XC[PLAV]G motif indeed possess proline C-terminal to the coordinating cysteine as shown by Guo *et al.*[5] While the cysteine serves as the contact ligand to the heme-iron ion, the proline residue was found to introduce a bend for the downstream structures, primarily α-helices, preventing them from contact with the heme face. However, heme was supposed to bind in a different orientation in these CP motifs with a nearly perpendicular orientation of the α-helix to the heme plane in chloroperoxidase (PDB id 2CIW), Rev-erb (3CQV), and microsomal prostaglandin E synthase (2PBJ) and, in contrast, a parallel orientation to the heme plane in case of cytochrome P450 family members, *e.g.*, CYP154C1 (1GWI).[5]

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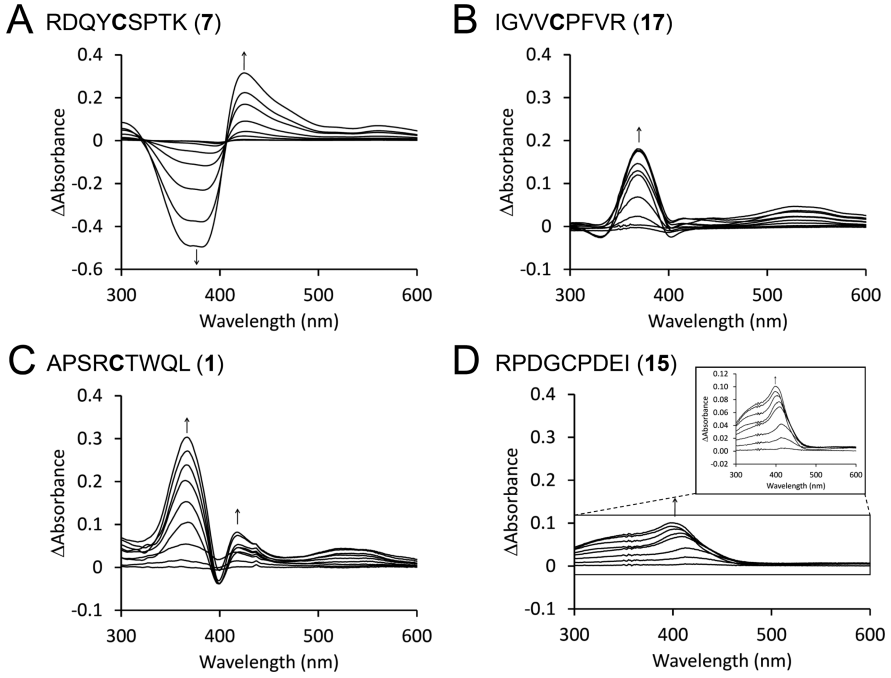

Figure 1. Difference absorption spectra of selected peptides in dependence on the Fe(III) heme:peptide ratio. Each spectrum (A–D) represents a strikingly different effect upon Fe(III) heme addition during UV–vis monitoring, which formed the basis of their classification into four groups. (A) Peptide **7**, representative of group 1, displays the well-known bathochromic Soret shift to ~420–430 nm. (B) A hypsochromic shift of the Soret band to ~370 nm was detected for peptide **17** (group 3). (C) For peptide **1** (group 2), two absorption maxima in the difference spectra at ~370 nm and ~420–430 nm were observed. (D) A class of less efficiently or differentially binding peptides (group 4) is represented by peptide **15** (inset: zoom-in).

To provide insight into the molecular mechanism of CX/CP motif binding to Fe(III) heme a set of 23 9-mer peptides was analyzed by UV–vis spectroscopy, electrophysiological competition studies, and Raman spectroscopy. Beside the characterization of the requirements for heme binding to cysteine-containing sequences, we elucidated the first structure of a CP-peptide-heme complex by homonuclear and natural abundance heteronuclear NMR. The peptide used was derived from dipeptidyl peptidase 8 (DP8: S495–G517), which we previously predicted as a typical CP-motif-containing protein.[9] Furthermore, the protein DP8 was shown to be inhibited by Fe(III) heme in a functional enzymatic assay, indicating that yet unknown heme-binding proteins can be identified taking into consideration the recognition criteria described herein.

RESULTS AND DISCUSSION

Fe(III) Heme-Peptide Association in UV–vis. The Fe(III) heme-binding characteristics of a set of 23 peptides (Table 1) containing either a CP motif or a cysteine residue as coordinating amino acid were examined (for selection criteria see Supplementary Text 1). In an earlier report[9] we demonstrated that for Fe(III) heme binding to cysteine-containing peptides (Cys is referred to position P0 in the text below) a typical shift of the Soret band to about 420 nm is observed. In Figure 1A UV–vis data for peptide 7 are shown, representative for peptides **4**, **5**, **6**, and **9**. However, because we also detected a different behavior for other peptides, we classified the corresponding members into groups. The peptides **4**–**7** and **9** thus belong to group 1. Group 2 of the peptides (Table 1, peptide **17** in Figure 1B) displayed a different shift to about 370 nm, *i.e.*, to the near-UV band;[23] such a shift was previously also observed for heme-regulated proteins, such as iron response regulator (Irr) (**11**),[24] human period circadian protein homologue 2 (hPer2) (**12**),[25] and HRI (**13**).[18] In a third group of peptides (**1**, **2**, **14**, **18**) we identified both a clear near-UV band at ~370 nm and a less intense Soret band at ~420 nm (Figure 1C). Finally, there were two peptides (**3**, **15**) without any of the characteristics found for groups 1–3 (group 4). While peptide **3** (negative control) does not bind to Fe(III) heme,[9] peptide **15** (Jak2) showed binding with a clear increase of the Soret band, but at ~400 nm (Figure 1D).

A comparison of groups 1 and 2 revealed that peptides containing a CP motif are predominant in the latter (**10**–**13**, **16**, **17**, **19**–**23**), whereas group 1 comprises cysteine-peptides only (**4**–**7**, **9**). Peptide **8** is the only one in group 2 lacking a CP motif. As already mentioned for proteins ATE1,[21] PpsR,[22] and stanniocaclin-1 (STC-1),[19] a proline in a heme-binding sequence is not necessarily required to result in the near-UV shift. However, a closer look at the corresponding sequences revealed the absence of a negative charge C-terminal to C/CP, whereas the N-terminus seems to be less important with respect to charges. Several bioinformatics-based approaches indicated the importance of hydrophobic and positively charged amino acids for heme binding to proteins with arginine, histidine, and lysine residues being responsible for anchoring of the heme propionates.[5,6,8] Indeed, 74% of our peptides contain at least one positively charged amino acid at either site of the cysteine, which predominantly occurred in the C-terminus. Our results thus represent the first experimental data to confirm the aforementioned findings on the peptide level.

Another characteristic feature is the hydrophobic C-terminus found in those peptides that completely lack a positively charged amino acid, *i.e.*, peptides **3**, **12**, **13**, **16**, **19**, and **23**. This again perfectly matches the theoretical studies mentioned above,[5,6,8] since heme-binding proteins maintain conserved interactions at the heme face involving the side chains of

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phenylalanine, tyrosine, and/or leucine, but also alanine, valine, and isoleucine. Hydrophobic aromatic interactions, in particular with phenylalanines, seem to be important with respect to van der Waals contacts with the edge of the porphyrin.[6] Beside the above-mentioned representatives, all other peptides with positively charged residue(s) also possess one or more hydrophobic amino acids: approximately 50% of the peptides contain any of the aromatic amino acids Phe, Tyr, Trp, or His at position +2 relative to Cys. This again supports the earlier theoretical studies because the heme group was found to bind in a pocket formed by numerous nonpolar side chains, in particular those of aromatic residues.[6,8] A suitable example is cytochrome P450 (PDB id 1PQ2), for which a stabilizing packing interaction with a phenylalanine was discussed.[6] There are similarities between heme-binding proteins and heme-regulated proteins concerning the coordinating cysteine and the occurrence of aromatic and/or nonpolar residues in close proximity to heme.

The importance of this finding is also reflected by the K_d values (Table 1) as the lowest values were obtained for peptides meeting the criteria described above. In particular peptides **2**, **6**, **9**, **10**, **14**, and **17** with a C-X/P-H/F motif displayed K_d values below 1 μM. The existence of nonpolar, nonaromatic amino acids (Ala, Ile, Leu, Nle, Val) at C-terminal positions in peptides **4**, **5**, **12**, **16**, **19**, **20**, and **21** led to affinities in the range of 1.37−6.43 μM. Similar binding affinities were also observed for peptides **1**, **11**, and **18**, where a tryptophane residue is present. This amino acid is not as preferred as phenylalanine and histidine.

However, a few peptides (**1**, **7**, **8**, **11**, **13**, **15**, **18**, **22**, **23**, and control peptide **3**) did not fit into this scheme and need to be discussed in another context. The most striking impact on binding affinity was detected for peptide **22** (48.40 ± 2.73 μM). Although **22** possesses exactly the same amino acids (CPIKE) C-terminal to cysteine as peptide **20**, the latter showed a higher Fe(III) heme-binding affinity (1.42 ± 0.24 μM). Concerning the N-terminal amino acids in both sequences, the occurrence of a large number of negatively charged residues is found for **22** (EDKD) compared to **20** (SDFK). The influence of negative charges on binding affinity is compensated if the C-terminal arrangement meets the criteria for heme association as observed for peptides **13** (DESACPYVM, 3.27 ± 1.44 μM) and **23** (DESACPVYM, 13.26 ± 1.44 μM). While both share the same N-terminal sequence, obviously "YVM" in **13** is preferred over "VYM" in **23**. This again confirms the importance of an aromatic residue in P+2 relative to cysteine.

Peptide **15** (derived from Jak2) with a K_d value of 0.50 ± 0.23 μM showed a different behavior compared to all other peptides because a shift to ∼400 nm was observed (Figure 1D). The sequence is rather atypical with respect to its amino acid content, since it contains aspartic acid at two positions, P−2 and P+2. However, it possesses the requirements of a positively charged residue (Arg) and a C-terminal nonpolar amino acid (Ile) for a good binding affinity. The different behavior regarding the Soret band and/or the near-UV shift may be due to the existence of two proline residues that are not present in this arrangement in other peptides of the series with the exception of **14**. However, in peptide **15** the second proline residue is close to Arg that is important for the recognition process.

Raman Spectroscopy for Cys-Peptides. In contrast to proteins belonging to group 1, structural information for proteins belonging to either group 2 or 3 is missing in the literature. A selection of peptides (**2**, **7**, **8**, **13**, **14**, **20**, **21**) was thus subjected to Raman spectroscopy studies with the aim to clarify the discrepancies in their UV−vis spectra. Raman spectra of samples containing the Fe(III) heme-peptide complex or Fe(III) heme alone showed four prominent bands in the spectral region between 1300 and 1700 cm^{-1} (Figure 2, Supplementary Figure 1). These bands were assigned to vibrations within the porphyrin ring (Figure 2) and shown to vary with porphyrin core size.[26,27] Excitation at 413 nm was chosen to make use of the resonance Raman effect in close vicinity of the Soret band and thus selectively enhance vibrational bands of the Fe(III) heme moiety. The Raman signal derived from the sample containing Fe(III) heme and peptide **2** was more intense than the signals from samples containing either pure Fe(III) heme or Fe(III) heme and peptide **20**. In the presence of **2**, the absorption of the Fe(III) heme-peptide complex was shifted toward the excitation wavelength (see UV−vis data), causing an increased resonance Raman effect. This supports the findings from UV−vis studies. Iron coordination in heme complexes is known to be reflected by the ν_3-vibrational band caused by $C^{\alpha}-C^{\beta}$ stretching[28] due to its core size sensitivity.[27] For peptide **20**, this band occurs at 1490 cm^{-1} similar to pure Fe(III) heme (Figure 2). This clearly indicates that the iron ion rests in the pentacoordinated state in the presence of peptide **20**. Ishikawa *et al.* attributed the ν_3-vibrational band occurring at 1492 cm^{-1} to pentacoordinated Cys-ligated Fe(III) heme in a bacterial iron-regulated protein.[24] In samples possessing hexacoordinated iron ions, this band shifts considerably to higher wavenumbers as reported earlier.[24,27] We found an upshift for peptide **2** (Figure 2) with the ν_3-vibrational band appearing at 1499 cm^{-1} (for further examples see Supplementary Figure 1). The position of the band reflecting ν_2-vibration further supports the conclusions drawn for the coordination states concerning peptides **20** and **2**. The ν_2-vibration is also known to be sensitive for coordination of the central iron ion, however, to a lesser extent.[27,29] It occurred at the same position (1569 cm^{-1}) for samples containing either pure Fe(III) heme or Fe(III) heme and peptide **20**, whereas it was shifted to 1585 cm^{-1} for the Fe(III) heme-peptide **2** complex (Figure 2). A proof for the interaction between Fe(III) heme and peptide **20** can be given by the form of the ν_2-derived band, which changed depending on whether **20** was present. A shoulder assigned to ν_{11} and

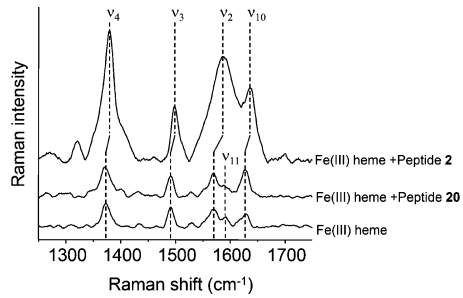

Figure 2. Resonance Raman spectra of the high frequency fingerprint region of pure Fe(III) heme and Fe(III) heme complexed with peptides. For the complex of Fe(III) heme-peptide **20** (SGGLPA-PSDFKCPIKEEIAITSG), prominent bands were assigned to normal modes: 1627 cm^{-1}, ν_{10}; 1591 cm^{-1}, ν_{11}; 1569 cm^{-1}, ν_2; 1490 cm^{-1}, ν_3; 1373 cm^{-1}, ν_4. For Fe(III) heme-peptide **2** (SSIPCLHYK) the following normal modes were assigned: 1635 cm^{-1}, ν_{10}; 1585 cm^{-1}, ν_2; 1499 cm^{-1}, ν_3; 1378 cm^{-1}, ν_4.

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visible in the spectrum of the pure Fe(III) heme almost disappeared upon addition of peptide **20** (Figure 2). Since both ν_2 and ν_{11} were shown to represent $C^{\beta}-C^{\beta}$ stretching vibrations,[28] a change in their vicinity is likely. Furthermore, there is a broadening of the band assigned to the ν_4-vibration at 1373 cm^{-1} in the peptide **20** sample compared to the respective band in the spectrum of pure Fe(III) heme (Figure 2). The ν_4-vibrational band was described to reflect the occupancy of HOMOs and LUMOs of the porphyrin skeleton.[27,28] The broadening was thus considered to be caused by peptide **20**-induced fluctuations in orbital occupancy. Peak position (between 1373 and 1378 cm^{-1} for the three samples) confirms the presence of ferric iron, since this band is sensitive for iron oxidation state.[29] A change in the coordination state is excluded for peptide **20** by the ν_3-vibrational band position; interaction with Fe(III) heme might thus be procured at the porphyrin ring site. The ν_{10}-vibrational band is representative for $C^{\alpha}-C^{\mu}$ vibrations.[28] It is not that instructive for studies of Fe(III) heme-peptide interaction and was thus not considered further.

Competition with Slo1 BK Channels. In order to validate the binding potency of the peptides to Fe(III) heme in a physiological setting, we performed a functional competition assay based on the Fe(III) heme dependence of human calcium-activated potassium channel subunit α-1 (Slowpoke homologue, hSlo1) BK channels as described earlier.[9] Application of Fe(III) heme to the intracellular face of membrane patches containing Slo1 BK channels results in a reduction of current because Fe(III) heme binds to a CxxCH motif in the channel protein and thereby closes the ion permeation pathway (Figure 3A, top).[30] We hypothesized that Fe(III) heme would leave the channels largely unaffected if co-applied with a peptide with strong Fe(III) heme-binding properties. As shown for two examples in Figure 3A (bottom), currents recorded at 250 mV were subjected to an equimolar mixture of Fe(III) heme and peptide (100 nM) for about 250 s. Upon a period of wash with control saline, 100 nM Fe(III) heme was applied without peptide to ensure Fe(III) heme sensitivity of that particular membrane patch. As indicated in Figure 3B, the block efficiency of Fe(III) heme was clearly diminished by peptides **2** and **17**−**19**, suggesting high affinity Fe(III) heme-peptide interaction. Peptides **7**, **12**−**14**, and **23** competed with the channel less well.

NMR Structure of a Heme-Peptide Complex. The resonance assignment of the 23-mer peptide **20** (sequence S495-G517 of DP8) in free and Co(III) protoporphyrin IX and Ga(III) protoporphyrin IX bound form is given in the Supplementary Table 3. On the basis of natural abundance [^{1}H,^{13}C]-HSQC spectra, also the carbon atoms in the three different samples were almost completely assigned.

The NMR data for free peptide **20** showed a low dispersion of amide resonances, indicating the absence of a fully defined structure. With exception of the region Asp9−Cys12, all amides were within a margin 0.25 ppm around the random coil chemical shift.[31] Extending the chemical shift analysis to the (later assigned) carbon atoms using the random coil index[32] also supported the lack of a defined secondary structure. The structure calculations with CYANA using 118 distance constraints from the NOESY spectra resulted in a backbone rmsd of only 5.38 Å. However, segmental superposition revealed that the segment Gly3−Ser8 is conformationally defined (backbone rmsd 0.99 Å), most probably due to the presence of a structurally restrictive element of two prolines at positions 5 and 7 separated only by residue Ala6 (Supplementary Figure 2C). In addition, at the C-terminal end, residues Glu1−Thr21 form a turn-like element, although less well-defined (backbone rmsd 1.37 Å; Supplementary Figure 2D). For complete structure statistics of free peptide **20** and its bound form see Supplementary Table 4.

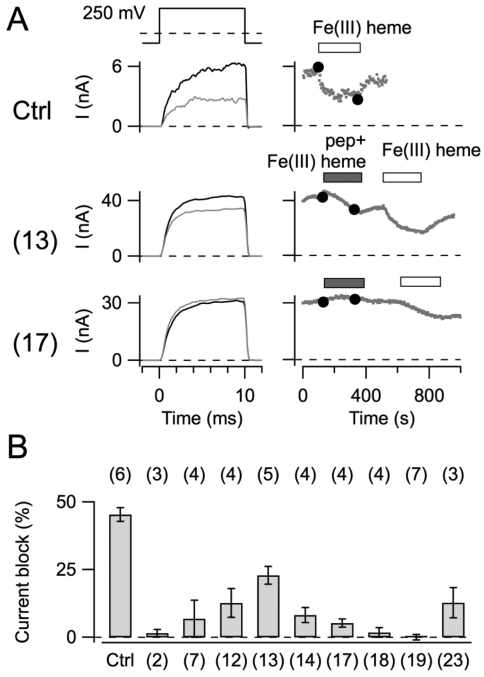

Figure 3. CP motif peptides compete with hSlo1 BK channels for Fe(III) heme. (A, top) Currents mediated by hSlo1 BK channels were measured during depolarizations to 250 mV before (black) and after (gray) application of Fe(III) heme (100 nM) (left); time course of the maximum currents at 250 mV (right). The white bar indicates application of Fe(III) heme. (A, bottom) Similar experiments as in the top panel but with the first application (gray bar) being an equimolar mixture of Fe(III) heme plus the indicated peptide (100 nM each); upon wash with control saline Fe(III) heme alone was applied (100 nM, open bar). The black symbols indicate data points from the current traces shown on the left. (B) Mean block of hSlo1 BK currents upon 250 s application of Fe(III) heme and the indicated peptide (100 nM each). "Ctrl" refers to application of Fe(III) heme (100 nM) only. Data are mean ± SEM with (n) indicated in parentheses at the top. In the presence of peptide the blocking potency was smaller than under control conditions in all cases ($P < 0.01$). Control application of peptides (100 nM) alone did not inhibit BK channels; instead, in some cases an immediate current increase of about 10−15% was observed. By means of the kinetics this effect could be clearly discriminated from the slow current block induced by hemin.

To reveal whether heme binding leads to structural changes of peptide **20**, we performed NMR measurements on samples of the peptide incubated with Co(III) protoporphyrin IX and Ga(III) protoporphyrin IX. Whereas in the spectra recorded with Co(III) protoporphyrin IX a signal broadening for residues Cys12 and Ile14, which did not allow to assign the resonances, was observed, in the Ga(III) protoporphyrin IX complex the corresponding resonances could be partially assigned. Interestingly, comparison to the random coil chemical shift amide values showed the highest differences (~0.5 ppm) for the residues Phe10, Lys11, Cys12, and Ile14. Superimposition of the [^{1}H,^{13}C]-HSQC spectra for the three samples (Supplementary Figure 2A) revealed that the C^{α}/H^{α} as well as the C^{β}/H^{β} resonances of Phe10, Lys11, and Cys12 shift strongly or vanish. In addition, the C^{δ}/H^{δ} resonances of

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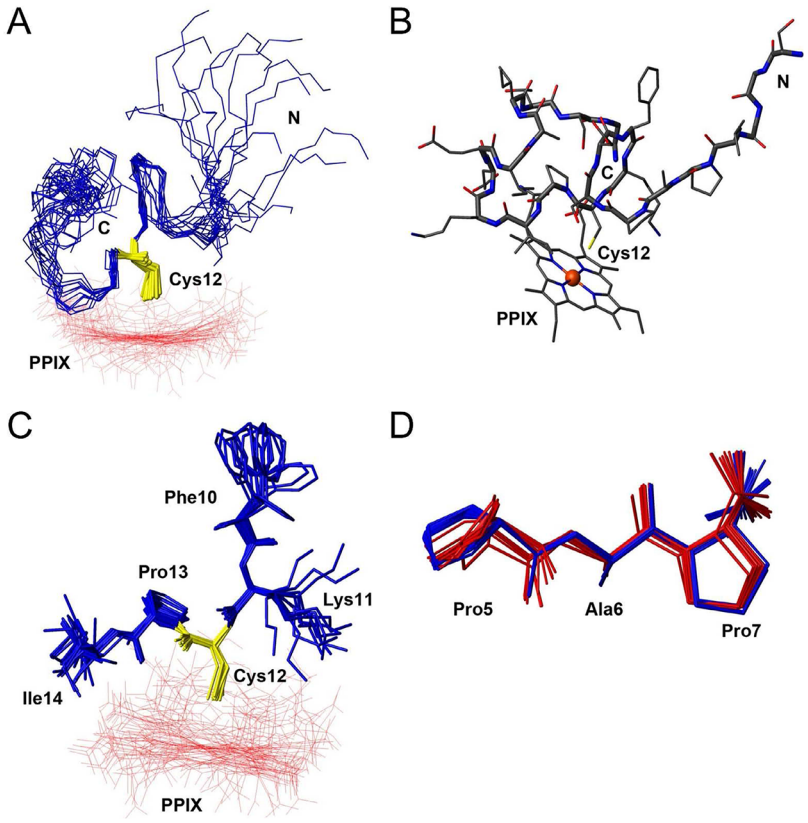

Figure 4. NMR structural analysis was performed for peptide **20** in the free and the complexed state. (A) Backbone view of the 15 structures with lowest target function of the complex of DP8(S495-G517) (blue) and Ga(III) protoporphyrin IX (red). The coordinating cysteine is indicated in yellow. (B) Heavy atom view of the structure closest-to-mean, colored by atom type. (C) Superimposition of residues Phe10−Ile14 (blue) and the bound PPIX moiety (red), heavy atom view. (D) Detail view of the superimposition of the Pro5−Pro7 stretch for the free DP8(S495-G517) (blue) and the Ga(III) protoporphyrin IX-bound form (red). The 15 structures with lowest target function are shown in heavy atom view.

proline-13 were affected. These findings indicate that the binding of the protoporphyrin IX (PPIX) unit directly involves the CP motif and also influences the neighboring residues from Phe10 to Ile14.

Figure 4A−C shows the structural details of peptide **20** in the protoporphyrin-bound form, which was calculated using 182 NOE derived distance constraints (see Supplementary Table 4). The overall picture of the peptide still resembles the properties of a flexible peptide (Figure 4A). However, upon complex formation, structural definition is introduced in the central portion of peptide **20** (residues 10−14; Figure 4C) as indicated by the backbone rmsd of 0.29 Å. Upon binding to protoporphyrin, the central part of peptide **20** forms a looplike structure between residues Ser8 and Lys11, which positions the backbone almost in line with the $C^{\beta}-S^{\gamma}$ bond of the cysteine. The conformation of proline-13 with its ring oriented in parallel to the protoporphyrin plane ensures a sufficient interspace of the further backbone to the PPIX ring system (Figure 4C). Due to this placeholder, only at Ile14 the backbone regains the flexibility normally observed in shorter peptides. At the same time, a loss in structural definition can be observed for the N-terminus and C-terminus. This can be explained by the fact that the protoporphyrin moiety upon binding to the cysteine side chain would cause steric contacts to the termini when backfolded as in the free form (Supplementary Figure 2C and D). Consistently, NOE cross signals between residues 1−3 are missing in the NMR spectra of the bound form. The motif Pro5-Ala6-Pro7 with its gross structural feature is also retained in the peptide **20** complex (Figure 4D).

Taken together, we observed a change in the conformational features of peptide **20** upon protoporphyrin binding including (a) a rearrangement of the N-terminus, (b) an increase in structural definition in the binding region around the CP motif, and (c) loss of structural definition in the C-terminus. The conformation of the CP motif is in line with the observation of Li *et al.* from a survey of CP dipeptides found in heme-binding proteins.[5] Here, it was described that the proline residues introduce a bend steering the following part of the peptide chain away from the heme face.

In contrast to heme proteins binding the heme moiety by burying it into clefts and thereby reducing the solvent accessibility,[8] the short version of peptide **20** does not allow for the formation of such an extended tertiary structure binding motif. The "surface binding" of peptide **20** DP8(S495−G517) lacks a complete set of nonbonded contacts normally present in binding clefts, which leads to a reduced binding constant and a transient binding in pentacoordinated state as observed in the Raman spectroscopy.

Fe(III) Heme Binding Regulates DP8 Activity. Dipeptidyl peptidase 8 (DP8) was previously identified in a library screen as a potential heme-binding protein.[9] It is a member of the serine proteases and catalyzes the hydrolysis of dipeptides from the N-terminus of substrates with specificity for H-X-A/P-$(X)_n$-COOH.[33,34] As mentioned before, the heme-binding capability of the peptide sequence S495−G517 (**20**) was unequivocally confirmed by UV−vis, Raman, and NMR spectroscopy. To investigate the effect of Fe(III) heme on protein function, an enzymatic activity assay was performed

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employing H-Ala-Pro-pNA as the substrate and Fe(III) heme as potential effector (Figure 5B).[33,34] As demonstrated in

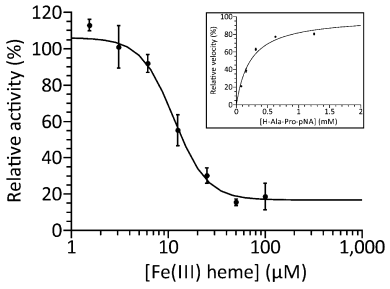

Figure 5. Fe(III) heme-dependent inhibition of dipeptidyl peptidase 8 yielded an IC_{50} value of 11.5 ± 1.2 μM (mean ± SEM). The enzyme was treated with 1.6−100 μM of Fe(III) heme, and the remaining activity (change of absorbance per second) was determined and normalized to the basal activity of the noninhibited control after subtracting autocatalytic degradation effects of the substrate at 405 nm. Inset: K_m value determination for the substrate H-Ala-Pro-pNA incubated with dipeptidyl peptidase 8 resulted in 226.02 ± 47.2 μM (mean ± SEM).

Figure 5B (inset), the substrate cleavage by DP8 follows Michaelis−Menten kinetics with a K_m value of 226.0 ± 47.2 μM, which is in good agreement with literature data.[33,34] This setup was then used to examine the impact of varying Fe(III) heme concentrations (~1−100 μM) on substrate conversion (Figure 5B). Indeed, the enzyme's activity was inhibited by Fe(III) heme with an IC_{50} value of 11.5 ± 1.2 μM. Thus, it is very likely that the catalytic activity of DP8 is affected through association to Fe(III) heme, probably *via* binding to the proposed stretch represented by peptide **20**. Since it was required to use Ga(III) protoporphyrin IX in the NMR experiments, the inhibition assay with DP8 was additionally performed with this effector. A similar result as for Fe(III) heme was obtained with a slightly smaller IC_{50} value (8.7 ± 1.8 μM) (Supplementary Figure 3).

Conclusion. The present report contributes essentially to the understanding of the function of Fe(II)/Fe(III) heme in protein regulation through an in-depth analysis of the binding of Fe(III) heme to cysteine-containing peptides. This approach represents an extension to earlier strategies for the identification of heme-regulated proteins, which initially were based on findings for individual proteins and confirmed by studies on the protein and peptide level, respectively.[19,30] A step toward the opposite direction, namely, starting from potential heme-binding peptides and subsequent transfer of knowledge to confirmed as well as predicted proteins, is the central concern of this study.

The 9-mer peptides described herein represent suitable tools to elucidate heme-binding events that have been earlier reported for distinct proteins, *e.g.*, Irr,[24,35] HRI,[12,18] Hap1,[10] and iron regulatory protein 2 (IRP2).[36] This can be demonstrated by the fact that highly similar amino acid sequences display different effects in the UV−vis experiment. Beside the known shift of the Soret band (to ~420−430 nm) upon binding of Fe(III) heme to a suitable peptide ligand, some representatives revealed a shift to 370 nm, while others showed bands at both wavelengths. Up to now, the typical shift of the Soret band was primarily associated with a coordination to tyrosine and histidine, respectively,[23,30,37] and in case of a cysteine coordination it was significantly more often described for sequences lacking the proline C-terminal of the cysteine residue.[38,39] The results reported herein confirm the latter finding. In contrast, the shift to 370 nm is currently much less understood and less structurally investigated, though few examples point to a pentacoordination of the central iron ion in the complex.[12,24] Concerning this near-UV band, the specificity for CP-containing peptides that are predominant in this group is obvious from our results. Pentacoordination seems to be preferred for these examples. For the third group of peptides displaying a shift to both wavelengths the cause of these two maxima in the UV−vis spectra still needs to be clarified. Raman spectroscopy revealed hexacoordination for representatives of this group (SSIPCLHYK and TPILCPFHL). In our opinion, for these particular cases at least two binding modes are possible: (1) both coordination sites are within one peptide sequence and (2) coordination sites (*e.g.*, two cysteine residues) from two peptide molecules associate on either side of the protoporphyrin ring system. The maximum at 370 nm was generally more intense than the band at 420−430 nm; whether this is an effect of concentration or a different binding mode right from the start of the titrations needs to be clarified with structural studies. Nevertheless, these peptides definitively bind to Fe(III) heme in a different way than those displaying a single band at either 370 or 420−430 nm. Our results suggest distinct Fe(III) heme-binding modes for cysteine-containing sequences with CP-peptides predominantly found in a pentacoordination and a typical shift to the near UV band. In contrast, motifs containing cysteine without any other proline, histidine, and/or tyrosine residue currently remain to a large extent unexplored. The features regarding amino acid composition of C/CP-containing sequences are suggested to be representative for temporary Fe(II)/Fe(III) heme binding as known for heme-regulatory motifs (HRMs). However, also heme-binding proteins may reflect these sequence requirements, since large heme-binding cavities allow for the involvement of residues, which are far away from the coordination site but upon folding are close enough to stabilize the complex. This approach is therefore applicable to numerous cysteine-containing proteins whose sequences meet the criteria for heme association described in this report.

A further proof of this statement was obtained from the detailed structural and functional investigation performed with the 23-mer CP stretch and/or the full-length protein of dipeptidyl peptidase 8. Beside information from UV−vis and Raman spectroscopy, methods that were also applied earlier by other groups,[24,37,39−41] the NMR solution structure in complex with Ga(III) heme was analyzed. This is the first structural investigation of a CP motif containing sequence bound to a heme ligand. Heme-regulatory events are generally discussed to occur preferentially at the protein surface, such as for mouse period circadian protein homologue 2 (mPer2).[12] We demonstrated a clear effect of Fe(III) heme on the activity of enzyme dipeptidyl peptidase 8, confirming our earlier suggestion that potential heme-regulated proteins can be predicted provided that the requirements of recognition and fast association/dissociation are met. This may stimulate further studies on earlier predicted proteins,[9] which emerged from a combinatorial peptide library screen and subsequent database search using the identified sequence criteria. Structural aspects such as three-dimensional arrangements and, in turn, resulting accessibilities of potential heme interaction sites might however lead to ambiguities between results on the peptide and the protein level, respectively. Nevertheless, one may speculate

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from the results reported that, apart from transcription regulation, heme metabolism, and ion channel regulation, also other biochemical processes such as protein degradation or immune response may be affected by heme. In this way, the role of heme in physiological and pathophysiological processes is to be expanded to a wider range of activities.

METHODS

A detailed description of the materials and methods (peptide synthesis and purification, UV−vis, Raman and NMR spectroscopy, biochemical and electrophysiological studies) is provided as Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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DEDICATION

This article is dedicated to the memory of Professor Ivano Bertini.

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