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Communication

NMR experiments on the transient interaction of the intrinsically disordered N-terminal peptide of cystathionine-β-synthase with heme

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ABSTRACT

The N-terminal segment of human cystathionine-β-synthase (CBS(1–40)) constitutes an intrinsically disordered protein stretch that transiently interacts with heme. We illustrate that the HCBCACON experimental protocol provides an efficient alternative approach for probing transient interactions of intrinsically disordered proteins with heme in situations where the applicability of the conventional [^{1}H, ^{15}N]-HSQC experiment may be limited. This experiment starting with the excitation of protein side chain protons delivers information about the proline residues and thereby makes it possible to use these residues in interaction mapping experiments. Employing this approach in conjunction with site-specific mutation we show that transient heme binding is mediated by the Cys^{15}-Pro^{16} motif of CBS(1–40).

1. Introduction

Intrinsically disordered proteins (IDP) and protein regions (IDPR) attached to folded protein domains have been found to be implicated in critical biological functions and in neurodegenerative and other diseases [1–5]. In contrast to covalent binding, as in many metalloproteins, IDPs have been shown to be involved in functionally important transient interactions. In many cases, heme-binding (HBM) or heme-regulatory motifs (HRM) with special amino acid combinations such as CxxHx$_{18}$H, CxxHx$_{16}$H and cysteine-proline (CP) in small sequence stretches are responsible for the heme association with moderate binding constants [6–10]. Coupled with mutagenesis, the heme-protein interaction studies are often carried out via techniques such as resonance Raman, surface plasmon resonance, NMR, circular dichroism (CD) and UV–Vis spectroscopy [11–13]. Such studies demonstrated the possibility for the IDPR to undergo conformational changes upon heme binding, e.g. due to hexa-coordination of the iron moiety in the iron-porphyrin complex with cysteine and histidine residues located at different positions in the protein sequence, leading to a reduction in the flexibility of the polypeptide chain [8–10]. A variety of techniques like chemical shift perturbation, relaxation dispersion spectroscopy and paramagnetic relaxation enhancement have been demonstrated for studies of protein-ligand interactions [14–18] and [^{1}H, ^{15}N]-HSQC experiments are often employed to map the interaction interface. However, in experimental situations leading to fast amide proton exchange with water molecules, e.g. at physiological temperature and pH, its applicability may be limited [19]. Thus, the required protocol strongly depends on the given experimental situation and the use of ^{13}C direct detection experiments [20–23] has been shown to offer an efficient alternative approach for structural investigations of uniformly enriched proteins and their complexes.

Many IDPs are involved in regulatory interactions with heme, with Cys-Pro (CP) motifs often playing an important role [8–10]. The proline residue in the CP motif assists the coordination of cysteine thiolate to the Fe(III) heme complex [12,24]. We are currently investigating the N-terminal peptide stretch (1–40) of human cystathionine-β-synthase (CBS, 551 a.a.; UniProtKB P35520) in the context of heme-IDP interaction [25–27]. In the enzyme CBS heme is bound as a cofactor via an axial coordination to cysteine-52 and histidine-65. However, NMR and UV/Vis studies

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[28] have shown that the disordered N-terminal region of CBS also contributes heme-binding capacities via a second binding site, the CP-based motif around cysteine-15 possibly extending to histidine-22. Functional assays revealed that this second heme-binding site increases the efficacy of the enzyme by ~30%.

Taking into consideration these factors, we aimed at a further robust strategy for probing the transient interaction of the N-terminal peptide stretch of human cystathionine-β-synthase (CBS (1–40)) with heme.

2. Materials and methods

CBS(1–40) (0.6 mM) was expressed via a fusion protein approach with the streptococcal protein GB1. The sample preparation followed protocols described earlier [29]. Heme was dissolved 1 mM in 20 mM NaOH and incubated for 30 min. NMR measurements on CBS(1–40) were performed in sodium phosphate buffer pH 6.9 and carried out on a Bruker 600 MHz narrow-bore Avance III NMR spectrometer equipped with pulse field gradient accessories, pulse shaping units and triple resonance cryo-probe with the sample temperature kept at 283 K and 310 K. The States procedure was used for phase-sensitive detection in the indirect dimensions [30]. DSS was used for direct ^{1}H chemical shift referencing and ^{13}C and ^{15}N chemical shifts were indirectly referenced. Details on the assignment are given in the Supplementary Material. The non-uniformly sampled multidimensional data sets were collected and processed as in our recent studies [31,32] employing the Bruker Topspin version 3.5 software.

3. Results

Initial heme-binding studies were carried out via [^{1}H, ^{15}N]-HSQC experiments. To improve the solubility of the target protein, make use of easy purification protocols, and minimize proteolytic degradations, GB1 fusion peptide samples of the N-terminal (1–40) of CBS (wild type and mutants) were employed in these investigations, and it is seen that the N-terminal peptide upon heme binding undergoes transient formation of a hexa-coordinated complex that is sparsely populated and in exchange with the free peptide [28]. Although NMR investigations could be successfully performed at 283 K, studies at the physiologically relevant temperature of 310 K could not be carried out due to considerable signal intensity losses arising from fast amide proton exchange with the solvent molecules. Another limitation in using the [^{1}H, ^{15}N]-HSQC experimental protocol is that information about the proline residues is lost. In this context, using CBS(1–40) as a model system, it is demonstrated here that the HCBCACON [21–33] experimental protocol, starting with the excitation of protein side chain protons, provides a convenient alternative approach for investigations of proline-containing sequences (e.g. CP, poly-proline or proline-rich sequence motifs).

Generation of chemical shift correlation spectra with good spectral resolution is one prerequisite for undertaking any NMR based investigation of protein-ligand interactions. Typically, in the case of IDPs, inter-residue $^{15}N_i$-$^{13}CO_{i-1}$ chemical shift correlations involving the backbone nuclei lead to spectra with relatively better spectral dispersion compared to intra-residue correlations [34,35], and this has been exploited for achieving resonance assignments. The $^{15}N_i$-$^{13}CO_{i-1}$ correlation spectrum can be generated via the HCBCACON experiment (Fig. S1) employing either the coherence transfer pathway $^{1}H^{\beta} \rightarrow {}^{13}C^{\beta} \rightarrow {}^{13}C^{\alpha} \rightarrow {}^{13}CO \rightarrow {}^{15}N$ (t1) $\rightarrow {}^{13}CO$ (t2) involving direct ^{13}CO detection or the transfer pathway $^{1}H^{\beta} \rightarrow {}^{13}C^{\beta} \rightarrow {}^{13}C^{\alpha} \rightarrow {}^{13}CO$ (t1) $\rightarrow {}^{15}N$ (t2) $\rightarrow {}^{13}CO \rightarrow {}^{13}C^{\alpha} \rightarrow {}^{1}H^{\alpha}$ (t3) involving $^{1}H^{\alpha}$ detection [36]. The advantage with the direct ^{13}CO detection approach is that the generation of $^{15}N_i$-$^{13}CO_{i-1}$ correlation spectrum involves only a simple 2D data acquisition, and the efficacy of this protocol was first tested at 283 K using only a 250 μM IDP sample.

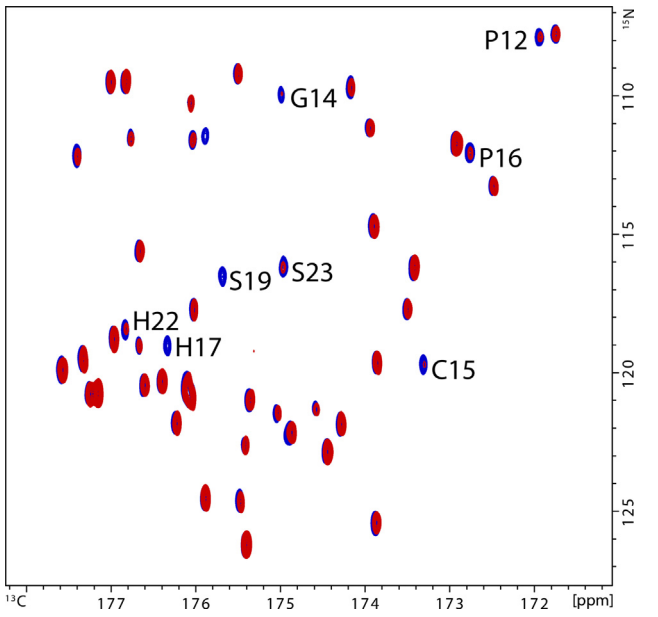

Fig. 1. Superposition of the 2D $^{15}N_i$-$^{13}CO_{i-1}$ correlation spectra of CBS(1–40) (250 μM; pH 6.9) collected via the (HCBCA)CON experiment at 283 K in D_2O without (blue) and with heme (red). Amino acids ($^{15}N_i$) involved in the interaction with heme are labeled.

Consistent with the conclusions reached via heme titration studies employing [^{1}H, ^{15}N]-HSQC spectra, the ^{15}N-^{13}CO correlation spectrum (Fig. 1) recorded with the addition of ~100 μM heme (Fe^{3+}) shows significant reduction in the intensity of peaks involving the amino acid residues P12, G14, C15, P16, H17, S19, H22, and S23, reflecting the spatial proximity of these residues to the paramagnetic center of the heme. The fact that the cross-peaks involving P16 and P12 are affected significantly provides

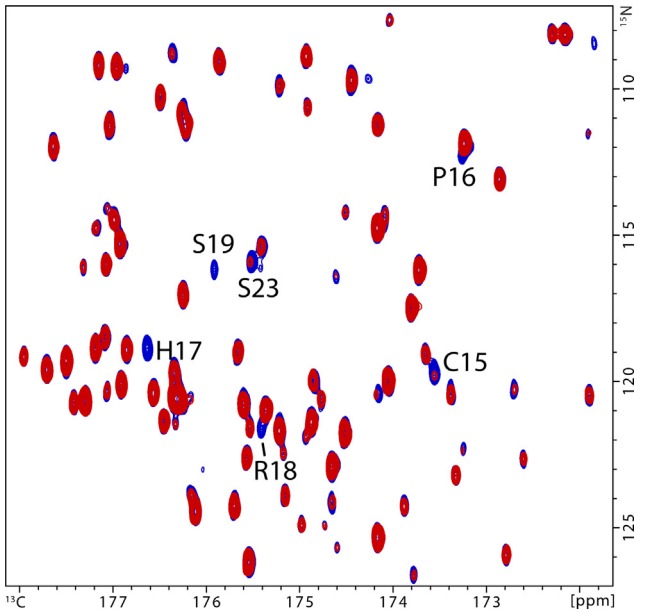

Fig. 2. Superposition of the 2D $^{15}N_i$-$^{13}CO_{i-1}$ correlation spectra of CBS(1–40) (250 μM) collected via the (HCBCA)CON experiment at 310 K in D_2O without (blue) and with heme (red). Relevant residues ($^{15}N_i$) involved in interaction with heme are labeled.

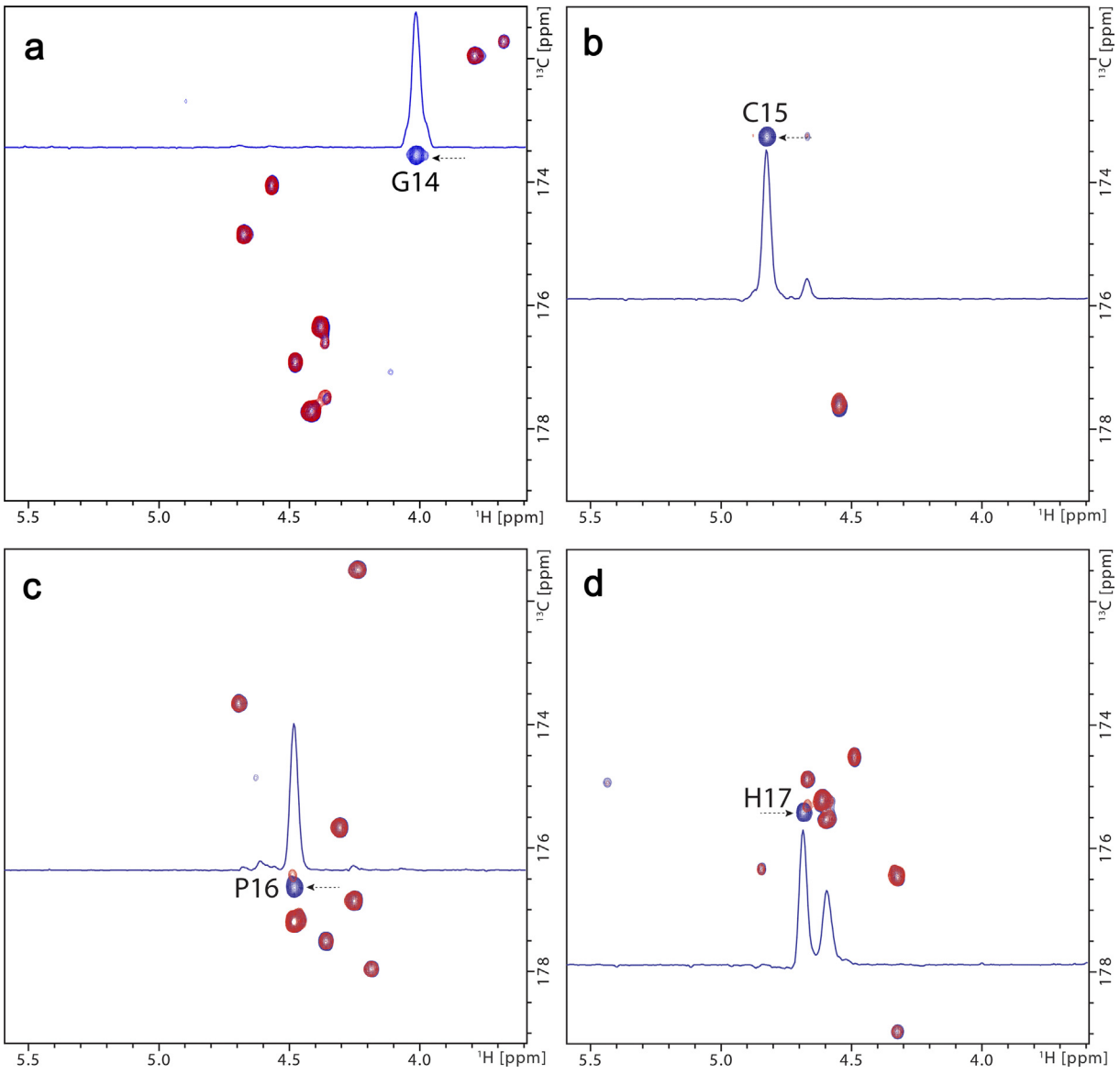

Fig. 3. Superposition of the 2D $^{1}H^{\alpha}_{i-1}$-$^{13}CO_{i-1}$ cross-sections from 3D HCBCACON spectra of CBS(1–40) (250 μM) at 310 K in D_2O generated without using linear prediction in the indirect dimensions and collected without (blue) and with heme (red, 100 μM). Other experimental details are as given in Figure caption S4. The cross-sections were taken at the $^{15}N_i$ positions corresponding to the residues of C15 (a), P16 (b), H17 (c) and R18 (d). 1D cross-sections taken from the spectrum collected without heme and at the positions indicated by the arrow are also given to indicate spectral quality.

additional confirmation for the involvement of C15 in heme binding. In spite of the low protein concentration, it is seen that a triple resonance cryo-probe tailored for ^{1}H detection allows to collect ^{15}N-^{13}CO correlation spectra of good quality within a reasonable amount of time. An additional advantage in carrying out IDP-heme interaction studies via ^{15}N-^{13}CO correlations is that in cases where the IDP system is investigated as a fusion peptide, as in our case a GB1 fusion, the HCBCACON protocol at 283 K leads to spectra in which signals from the GB1 domain are largely eliminated, in contrast to the situation with the conventional [^{1}H, ^{15}N]-HSQC experiment (Fig. S2). This leads to spectra with less overlap and the convenient mapping of the protein-heme interaction interface. Although these studies can also be carried out via other experimental schemes such as HCBCACO [37], Fig. S3, good spectral resolution observed in the ^{15}N-^{13}CO correlation makes the ^{13}CO-detected HCBCACON experiment an attractive approach for the study of heme-protein interactions.

While sensitivity considerations limited the applicability of [^{1}H, ^{15}N]-HSQCs at 310 K, 2D ^{15}N-^{13}CO correlation data could be conveniently collected, without and with the addition of heme. Significant variations (Fig. 2) in the intensity of many of the peaks corresponding to the amino acid residues that were found to be involved in heme interaction at 283 K clearly reveal the presence of transient heme binding even at the elevated and physiologically relevant temperature. However, unlike the situation at 283 K, cross-peaks originating from the folded GB1 domain are also seen with considerable signal intensities (Fig. 2) leading to spectral crowding in the 2D ^{15}N-^{13}CO correlation spectrum. Spectral overlaps can also be expected when one deals with IDP systems of much larger size. It is also important to mention that these studies typically employ low protein concentrations (~250 μM) so as to minimize the concentration of the heme required and thereby heme aggregation and high solvent PREs. Hence, it will be difficult to carry out the ^{13}CO detection-based measurements using con-

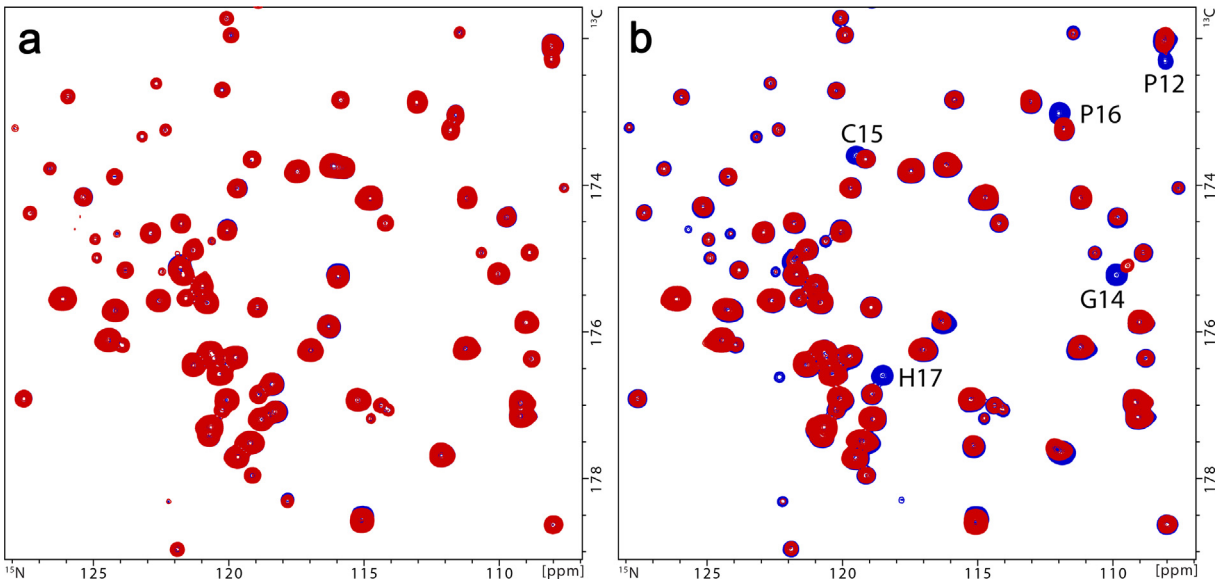

Fig. 4. Superposition of the 2D $^{15}N_i$-$^{13}CO_{i-1}$ projections from 3D HCBCACON spectra of CBS(1–40) (250 μM) mutants C15S (a) and H22L (b) generated without using linear prediction in the indirect dimensions at 310 K in D_2O and collected without (blue) and with heme (red, 100 μM) employing 16 transients per t1 increment. Other experimental details are as given in figure caption S7. Relevant amino acid residues ($^{15}N_i$) involved in interaction with heme are indicated.

ventional room-temperature NMR probes optimized for ^{1}H detection.

It is conceivable that heme-protein interaction studies would require experimental protocols with good sensitivity facilitating the collection of higher dimensional data sets within a reasonable amount of time. Thanks to the introduction of powerful non-uniform data sampling and processing procedures and to better sensitivity provided by direct proton detection, this can be realized via the HCBCACON protocol using $^1H^\alpha$ detection (see above). Fig. S4 shows ω_{12} spectral projections from a 3D HCBCACON experiment carried out in absence and in presence of heme at 310 K (see Fig. S5 for other spectral projections). Improved spectral resolution achieved via 3D data acquisition is demonstrated by a few representative ω_{13} spectral cross-sections given in Fig. 3.

This data also confirms transient heme interactions at the physiological temperature and clearly illustrate the potential of 3D HCBCACON experiments for protein-ligand interaction studies. It is worth mentioning that: 1. While the [^{1}H, ^{15}N]-HSQC protocol may become difficult at 310 K due to fast amide proton exchange with water, elevated temperatures are ideally suited for carrying out the HCBCACON experiment as faster molecular tumbling leads to reduction in relaxation losses and thereby to improved signal intensities. 2. In situations where the H^α_{i-1}-$^{15}N_i$ and $^{13}CO_{i-1}$-$^1H^\alpha_{i-1}$ correlation data have sufficient spectral resolution it is not necessary to carry out the HCBCACON experiment in the 3D mode and simple 2D data collections realizable in a few hours could be good enough to map the protein-ligand interface (Fig. S6).

Our initial heme interaction studies via the [^{1}H, ^{15}N]-HSQC protocol were carried out using both the wild-type and CBS(1–40) mutants. NMR data were consistent with UV/Vis that indicated heme binding to wild-type CBS(1–40), possibly forming a hexa-coordinated heme-protein complex as indicated by a shift of the Soret band to 420 nm, and no heme binding to the C15S mutant.

Results from the 3D HCBCACON experiment at 310 K with the C15S mutant are in agreement with these data. Unlike the case with the wild-type, the ^{15}N-^{13}CO spectral projections obtained without and with the addition of ~100 μM heme (Fe^{3+}) show no significant variations in the intensity of the correlation peaks (Fig. 4a). Thus, heme binding is abolished in the C15S mutant.

UV/Vis data of the heme-incubated H22L mutant indicated the presence of a penta-coordinated heme-IDP complex as suggested by a maximum at 370 nm, as earlier shown for heme-coordinated CP motifs [8,12,24,28]. The [^{1}H, ^{15}N]-HSQC experiments carried out at 283 K on the H22L mutant of CBS(1–40) showed no effect on the relative intensities of the cross-peaks upon heme addition, possibly the binding was too weak to be detected by the experimental protocol or the exchange rate between the heme bound and free IDP was not in the favorable range on the PRE timescale. However, the ^{15}N-^{13}CO spectral projections of the HCBCACON with H^α detection (as well as the data employing ^{13}CO-direct detection, Fig. S7) obtained without and with the addition of ~100 μM heme (Fe^{3+}) showed significant intensity variations of the correlation peaks, e.g. for residues P12, G14, C15, P16, H17 (Fig. 4b) consistent with the heme-IDP interaction detected by UV/Vis.

4. Discussion and conclusions

The objective of this study was to evaluate the heme-binding of the non-crystallizable N-terminal peptide stretch (1–40) of human cystathionine-β-synthase, an intrinsically disordered protein region. As the conventional approach via [^{1}H, ^{15}N]-HSQC interaction mapping experiments was hampered by fast HN exchange, the HCBCACON protocol was adapted as an alternative strategy. This experiment has the combined advantages of being HN exchange-independent and of adding proline correlations as additional probe for the interaction mapping. Such an approach was of particular interest as CP motifs were described as preferential heme interaction sites [8–10] and in the case of CBS(1–40) the supposed heme binding could involve the $C^{15}P^{16}$ motif of the intrinsically disordered N-terminal peptide stretch. The data using site-specific mutants unambiguously showed that H22L still binds heme while heme binding is abolished in the C15S mutant. Thus, in CBS(1–40) the heme interacts with the $C^{15}P^{16}$ motif resulting in a penta-coordinated heme-IDP complex as the contribution of H17 in formation of a hexa-coordinated complex can be excluded by steric considerations. The data presented might contribute to antagonize pathological CBS conditions via therapeutic intervention at this less-well studied molecular region.

Considering that interactions leading to hexa- or penta-coordinated heme-protein complexes frequently involve the side-chains of His and Cys residues, this study demonstrated that the HCBCACON protocol starting with the excitation of side-chain atoms and involving either ^{13}CO detection [38] or H^{α} detection can be effectively used in heme-IDP interaction studies as a powerful complementary procedure to the conventional [^{1}H, ^{15}N]-HSQC experiment, especially at elevated temperatures with limited applicability of the canonical mapping approach. Thus, the presented approach can be used for protein-ligand or drug-screening studies and allows also for heme-IDP investigations under cell-like crowded environment. Initial results (data not shown) indicate that CBS(1–40) is still disordered and its transient interactions with heme at 310 K stay intact under molecular crowding effected by 200 g/l of PEG-3500 and 150 g/l of Ficoll-70.

Compliance with ethical standards

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Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmr.2019.07.048.

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