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Tyrosine–heme ligation in heme–peptide complex: design based on conserved motif of catalase

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Abstract: On the basis of evolutionary conservation of sequence in catalases, we have designed a heme-binding peptide (Ac-RLKSYTDTQISR^{12}-(GGGG)-CRIVHC^{22}-NH_2) for the 'redox activity modulation' of heme. Heme-binding studies showed a blue-shifted Soret (369 nm) in the presence of TFE and a red-shifted Soret (418 nm) in the absence of TFE. These blue- and red-shifted Sorets suggest ligation through tyrosinate and histidine, respectively. This is the first designed peptide ligating to heme through tyrosine. NMR studies have confirmed that tyrosine ligation to heme in this heme-peptide complex occurs only in the presence of TFE. We suggest that TFE induces helicity in the peptide and brings the arginine and tyrosine in proximity, resulting in ionization of the phenolic side chain of tyrosine. In the absence of TFE, the unstructured peptide lacks the intra-molecular Arg^+Tyr^- ion pair, allowing heme binding to histidine. This peptide has significant peroxidase activity though it does not have catalase activity.

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Keywords: catalyst; conserved motif; designed peptide; peptidomimetics; Soret band

INTRODUCTION

Nature has evolved heme-binding sites within a variety of protein scaffolds to carry out such diverse tasks as electron transfer [1], substrate oxidation, metal ion storage [2], ligand sensing [3–5] and transport. The robust literature on structural and spectroscopic models of heme proteins is prepared with successful functional models [6] including the peptide-sandwiched meso-heme (heme b) [7] and functional analogue of cytochrome *c* oxidase [8]. Gibney *et al.* have designed ferredoxin–heme maquettes [9], a 16-amino acid residue peptide derived from a consensus motif of natural ferredoxins and incorporating a tetranuclear iron sulfur cluster under physiological conditions. In one larger structure, they describe a tetra-α-helix bundle that self-assembles both iron–sulfur clusters and hemes, thereby demonstrating the feasibility for the general synthesis of maquettes containing multiple, juxtaposed redox cofactors leading to a motif common to the catalytic sites of native oxidoreductases. Here we report the spectroscopic properties of heme-binding peptides designed to exhibit catalysis.

MATERIALS AND METHODS

UV-vis Spectroscopy

When a ligand binds to heme by co-ordinate bonding to iron, the Soret band of heme changes, generally by shifting the λ_{max} to higher wavelength and also increasing the molar absorbance at λ_{max}. This phenomenon has been used to determine the heme-binding constant and stoichiometry of peptide ligands [10]. All solutions are in 50 mM phosphate buffer, pH 7.4. All absorbance experiments were done in 1-cm path length quartz cuvette on a Perkin-Elmer spectrophotometer attached to a computer. The concentration of the d-cata peptide is also taken spectrometrically considering its molar absorbance $\varepsilon_{280\beta} = 1490\ M^{-1}\ cm^{-1}$. The molar absorbance for d-cata was calculated from 'expasy peptide property calculator'. The A_{214}/A_{280} absorbance ratio for d-cata is 21.23, and therefore the ε_{214} for d-cata is $\varepsilon_{214} = 31\,634\ M^{-1}\ cm^{-1}$. Since there is only one amino acid substitution in d-cata and d-pero, the d-pero concentration is measured spectrometrically considering $\varepsilon_{214} = 31\,634\ M^{-1}\ cm^{-1}$.

Synthesis of Peptides

The Rink-amide MBHA resin is used for the synthesis of peptides by solid phase Fmoc chemistry [11]. The peptides were purified by RPHPLC and their purity was checked by analytical RPHPLC (~95%). The values of mass expected for d-cata and d-pero peptides are 2449 and 2423 Da, respectively. These values are close to the observed mass values 2448.4 Da (d-cata) and 2421.4 Da (d-pero). The plan was to make intra-molecular disulphide bonds in d-cata and d-pero peptides. However, neither the air-oxidation method nor the DMSO–water method gave the desired disulphide-bonded product in good yield. All the studies presented here are that of the un-oxidized peptide. The Ellman's test for the peptide is positive (yellow), indicative of reduced cysteine present in peptide.

Circular Dichroism (CD) Spectroscopy

We used a JASCO Model J-810 instrument with Peltier cooling. The scanning speed was 100 nm/min, the band width 1 nm,

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the cell length 0.2 cm and the cell volume 0.4 ml. The data is presented in units of molar residue ellipticity (MRE) in mdeg decimole^{-1} cm^2.

Peroxidase Activity Assay

The peroxidase activity of the catalyst was measured using 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) [12]. H_2O_2, ABTS and the catalyst were dissolved in 10 mM phosphate buffer, pH 7.

Nuclear Magnetic Resonance (NMR) Experiments

All NMR data were collected on a 500-MHz Brucker machine at the Indian Institute of Science, Bangalore. The sample volume was approximately 600 μl and all samples were maintained at pH 5.9 using a 50-mM phosphate buffer containing 10% D_2O and ~1 mM sodium trimethylsilylpropionate (TSP). The TOCSY spectra recorded for d-cata peptide in aqueous solution and 50% TFE had peptide concentration ~2.5 mM. Since cobalt is not paramagnetic, cobalt-containing heme derivative, cobalt (III) protoporphyrin was used for NMR studies. Iron(III) is paramagnetic. The cobalt protoporphyrin IX (cobalt heme) was procured form Frontier Scientific Company. The TOCSY spectra recorded for d-cata peptide with cobalt heme in aqueous solution had only ~0.5 mM cobalt heme (because of the limited solubility of cobalt heme in water); therefore, it was not expected to have a very good concentration of heme-bound peptide. But in presence of TFE, the solubility of cobalt heme is very good, and therefore the TOCSY spectra recorded for d-cata peptide with cobalt heme in 50% TFE had ~3 mM cobalt heme and ~1 mM peptide. In this condition, more than 50% of the peptide is supposed to be in heme-bound state. ID NMR spectrum of cobalt heme alone was taken in 50% TFE and the concentration of cobalt heme was ~2 mM.

RESULTS

Rational Design of Heme-binding Peptides

Catalase (type HPII) catalyses the disproportionation of H_2O_2 into $H_2O + O_2$. The conserved motifs of the catalase, PS00437 and PS00438 (PROSITE id), are referred to as 'heme-ligand sequence' and 'active-site sequence', respectively [13]. Both PS00437 and PS00438 are short and continuous sequence stretches. Therefore, we surmised that it may be feasible to design the heme-binding peptide for mimicking the catalase active site on the basis of these conserved signatures (Figure 1(A)). These two fragments make almost the entire local milieu of heme. So we wished to find out whether a heme in complex with such a peptide could exhibit catalase activity. The heme-ligating tyrosine comes from the helical PS00437 sequence [13] (Figure 1(A)). The catalytic residue H^{128} (in distal pocket of heme cavity) is part of PS00438. We have taken the peptide sequence corresponding to PS00437 and PS00438 from 1p7z (*E. coli.* catalase) and joined them with a tetra-glycine linker ($(R^{411}$LFSYtDTQ$^{419})^{PS00437}$???-$(GGGG)^{linker}$-$(F^{117}$dHeripERivHarGSA$^{133})^{PS00438}$). Heme is known to induce helical conformation in peptide upon binding; therefore we can expect that the designed heme–peptide complex will also assume a helical fold, similar in structure to the native enzyme. The following modifications are also made in this peptide sequence so that the assembly of peptide–heme complex can have a native-like structure, in the absence of the surrounding protein matrix: (i) Residues 117–123 FdHerip and 131–133 G-S-A have been removed from design sequence because they do not have significant stabilizing interactions with either heme or the residues included in design [14]; (ii) The fragment 420–422 I-S-R is included in the design sequence because R^{422} has extensive hydrogen bonding with heme propionate [14] though it is not part of the conserved motif; (iii) In 1p7z, F413 is involved in an inter-chain interaction responsible for tetramerization [14]. Since F413 can lead to aggregation, it has been replaced by K, which at this place is expected to salt-bridge with D417, stabilizing the designed structure; (iv) Residues 124 and 129 are replaced with C because by using the DSDBASE software [15] we found that they have distance and angle between them suitable to make a disulphide bond. It was expected that this disulphide bond would restrict the conformation of the intervening sequence to the designed loop structure (Figure 1(B)); (v) Amino and carboxyl termini have been acetylated and amidated respectively in order to eliminate charge effects. The resulting peptide sequence d-cata is Ac-RLKSYTDTQISR^{12}-(GGGG)-CRIVHC^{22}-NH_2. In the aspired structure, the tetra-glycine linker is fit to traverse the distance (of 7 Å) between the *C*-terminus of heme–ligand sequence and *N*-terminus of the active-site sequence. In another peptide d-pero (sequence: Ac-RLKSHTDTQISR^{12}-(GGGG)-CRIVHC^{22}-NH_2), we have replaced the tyrosine of the d-cata sequence with histidine for comparing the effect of tyrosine *versus* histidine on the spectroscopic properties.

Heme Binding of d-pero and d-cata

Our peptides have histidine and tyrosine as potential heme-ligating residues; therefore we have studied the heme-binding properties of free amino acid tyrosine and histidine also. We have found the heme-binding constant of histidine, $k = 0.00025$ mM^{-2} (Supplementary information) and stoichiometry of the binding, heme: histidine = 1 : 2 ($\lambda_{max} = 409$ nm). The tyrosine axially ligated to the heme in catalases is negatively charged [16]. The pK of the tyrosine side chain is 10.5; therefore we have studied the heme binding of tyrosine at pH 11. The λ_{max} of the Soret band in heme optimally ligated to tyrosine is blue-shifted to 369 nm. The detailed heme-binding study is done with phenol instead of tyrosine (Supplementary information) because the solubility of tyrosine in phosphate buffer at pH 11 is approximately 5 mM only. The Soret is blue-shifted to $\lambda_{max} = 369$ nm upon the phenolate ion binding to heme (Figure 2(A)).

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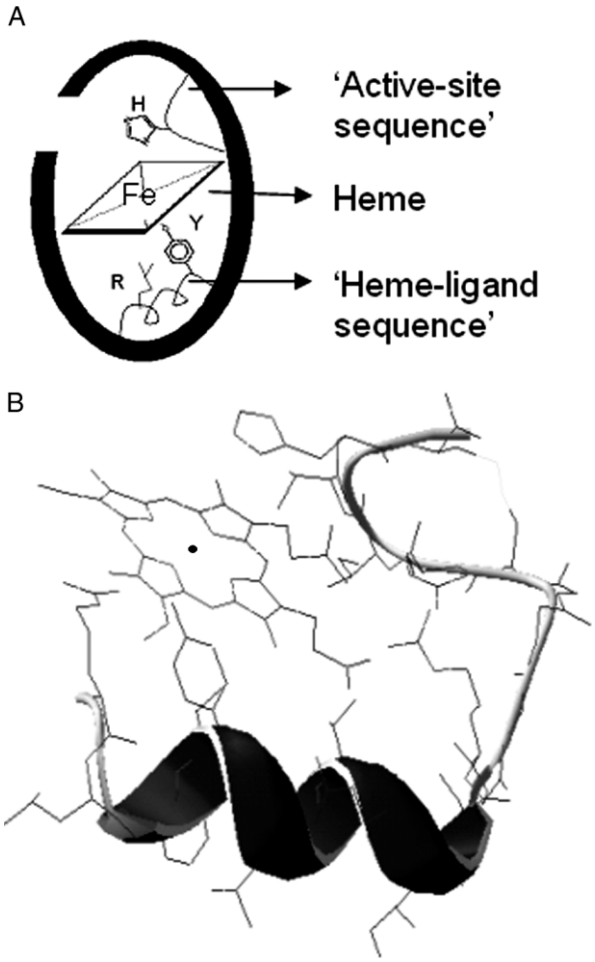

Figure 1 (A) Model of the catalase active site. The structure of the conserved motif surrounded by protein matrix is shown here. (B) Model of the designed heme–peptide complex. Sequence of the peptide is: Ac-RLKSYTDTQISR12-(GGGG)-CRIVHC22-NH2. Y5 is ligated to the heme. Residues 1–12 are shown in helical conformation (heme–ligand sequence). Residues 17–22 are shown as loop (active-site sequence).

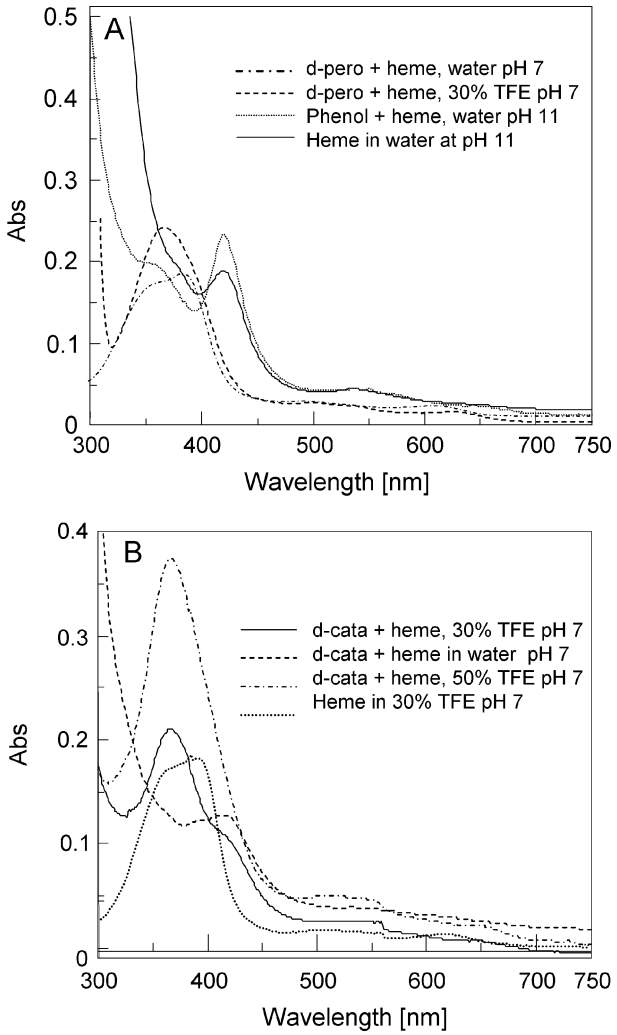

Figure 2 (A) Heme bound to phenolate (pH 11) with a blue-shifted Soret peak at 369 nm but heme alone at pH 11 with no peak at 369 nm. This peak at 369 nm is not present in the d-pero–heme complex in 30% TFE or in aqueous solution. (B) The d-cata–heme complex has no peak at 369 nm in an aqueous medium, but in 30% TFE there is a peak at 369 nm which becomes more prominent in 50% TFE. This peak at 369 nm is not present in heme alone in 30% TFE.

The stoichiometry of binding is 1 : 1 and the binding constant is $k = 0.0337\ \text{mM}^{-1}$. The d-pero–heme complex shows (Figure 2(A)) merged α, β peaks at 550 nm, which is the indicative of a bis-ligated heme, and therefore it has a bis-His ligated heme–peptide complex [17]. It can also be a mixed population of mono- and bis-His ligated heme–peptide complexes. The heme-binding constant of d-pero is $k = 4.4\ \text{mM}^{-1}$ (data not shown) and λ_{max} is shifted to 422 nm at pH 7 (Figure 2(A)). Heme bound to d-cata at pH 7 has a Soret λ_{max} at 418 nm. The stoichiometry is 1 : 1 and the binding constant k is $104\ \text{mM}^{-1}$ (Supplementary information). The poorly resolved α and β peaks may indicate a mixed population of mono-ligated and bis-ligated heme [17]. When heme binds to a peptide, it induces helical conformation in the peptide [6]. We found by CD spectroscopy that both d-pero and d-cata peptides show a trend of increasing helical content with increasing concentration of the added heme (Supplementary information).

Emergence of the Tyrosine Binding Peak in Heme Binding of d-cata in a TFE–Water Solution

We studied the heme binding of d-cata in 30% TFE at pH 7, expecting that TFE will induce helicity in d-cata facilitating strong binding of the peptide. There is a slight increase in the heme affinity of d-cata from $0.6\ \text{mM}^{-1}$ in 0% TFE to $7.6\ \text{mM}^{-1}$ in 50% TFE (Supplementary information). Surprisingly, we found some new spectral features in the heme binding of d-cata in 30% TFE. There is a shifted Soret peak at 422 nm as observed earlier in the heme binding of d-cata in aqueous solution, but there is an additional shifted Soret peak at 369 nm also. This 369 nm peak is likely due to tyrosine binding to heme in a sub-population of the heme–peptide

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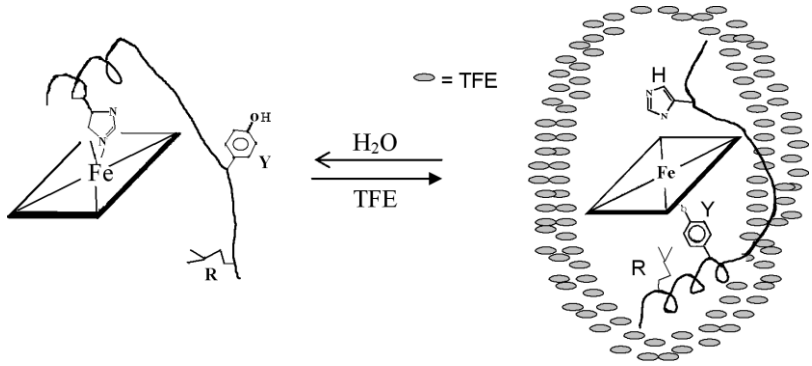

Figure 3 Left: The protonated side chain of the tyrosine in water showing that it is incapable of co-ordinate bonding with the heme iron. Addition of TFE (right) induces a helical fold in d-cata, facilitating ionic interaction between the side chains of tyrosine and arginine. Consequently, the ionized tyrosine can ligate to heme.

complex. Upon increasing the TFE concentration to 50% in d-cata–heme binding at pH 7, the peak at 422 disappears and the tyrosine-binding peak becomes more prominent (Figure 2(B)). Probably the peak at 422 is due to heme binding to histidine alone, and in 30% TFE some molecules are binding through histidine and the rest of them are binding through tyrosine, and that is why we can observe both the peaks in 30% TFE. TFE is favouring the binding through tyrosine instead of histidine. Without TFE, histidine is binding and tyrosine is not binding, or not binding effectively. The heme binding of d-pero in 30% TFE does not give this tyrosine-binding peak (Figure 2(A)) because in place of tyrosine in d-cata there is histidine residue in d-pero. Instead, the histidine-binding peak at 422 becomes more prominent in 30% TFE, compared to the case without TFE. The protein matrix in catalase forces the arginine and tyrosine to come close, leading to the formation of a phenolate–guanidinium ion pair complex. The anionic side chain of tyrosine makes a co-ordinate bond to the Fe(III) of heme (Figure 1(A)). But in the absence of this protein matrix, in our designed heme peptide complex, the tyrosine is not tightly coupled to arginine. Thus it is not ionized and cannot co-ordinate-bond to Fe(III) effectively. In this situation, the histidine ligates to heme axially (Figure 3 (left)). TFE can induce helicity in the peptide d-cata. Helix induction by TFE brings Y and R together and TFE also provides hydrophobic confinement for the interaction between Y and R such that the resulting negatively charged Y makes a co-ordinate bond with Fe(III) of heme (Figure 3 (right)). When tyrosine is optimally ligated to Fe(III), the histidine cannot ligate on the sixth valence of Fe(III) because the size of the tetra-glycine linker will be insufficient for that. For the same reason, when histidine is ligated to heme, it makes difficult for tyrosine to ligate. We cannot perfectly mimic the restraint of a large protein matrix in this small peptide, but the motive was to hold the histidine back from co-ordinate-bonding to heme. A small percentage of molecules (heme–d-cata complexes) may have bis-ligated heme. In presence of TFE, tyrosine–heme ligation is preferred over histidine–heme ligation because affinity of heme for ionized tyrosine is more than that for histidine. The heme-binding constant of phenolate ion is $k = 0.0337\ \text{mM}^{-1}$, which is more than the heme–histidine binding constant ($k = 0.00025\ \text{mM}^{-2}$) (Supplementary information). Though the units of two constants given are different, still they can be compared on the basis of the concentration required for the half saturation of 1 μM heme. The tyrosine required for half saturation of the 1 μM heme is 30 mM, whereas that required for histidine is 63.25 m M.

NMR Evidence of Tyrosine Binding to Heme in Presence of TFE

We have only two aromatic residues in the d-cata peptide sequence: tyrosine and histidine. So it is very easy to identify the aromatic CH peaks of tyrosine in the aromatic CH region (6.5–7.7 ppm) of spectrum. The cobalt heme also does not have any peaks in this region. The chemical shift values for 2H and 4H of histidine in a random coil structure is 8.12 and 7.14 ppm, respectively. Generally the 4H peak of histidine is not observed; therefore, the tyrosine and histidine peaks are well resolved [18]. The 3H and 5H aromatic ring protons of tyrosine are equivalent and have a chemical shift value 6.83 ppm (for random coil structure). Likewise, 2H and 6H are equivalent and have a chemical shift value 7.12 ppm. In the TOCSY (Figure 4(A)) of d-cata in water, there is a tyrosine CH cross peak (at 6.83, 7.15 ppm). The same cross peak is observed for the samples d-cata in 50% TFE (Figure 4(B)) and d-cata with cobalt heme in aqueous solution (Figure 4(C)). The co-ordinate bonding of tyrosine to heme causes a shift in chemical shift of the aromatic CH of tyrosine. In the TOCSY of d-cata with cobalt heme in presence of 50% TFE (Figure 4(D)), we observed an additional cross peak (at 6.9, 7.25 ppm) of bound tyrosine besides the unbound tyrosine cross peak. Here, we have observed

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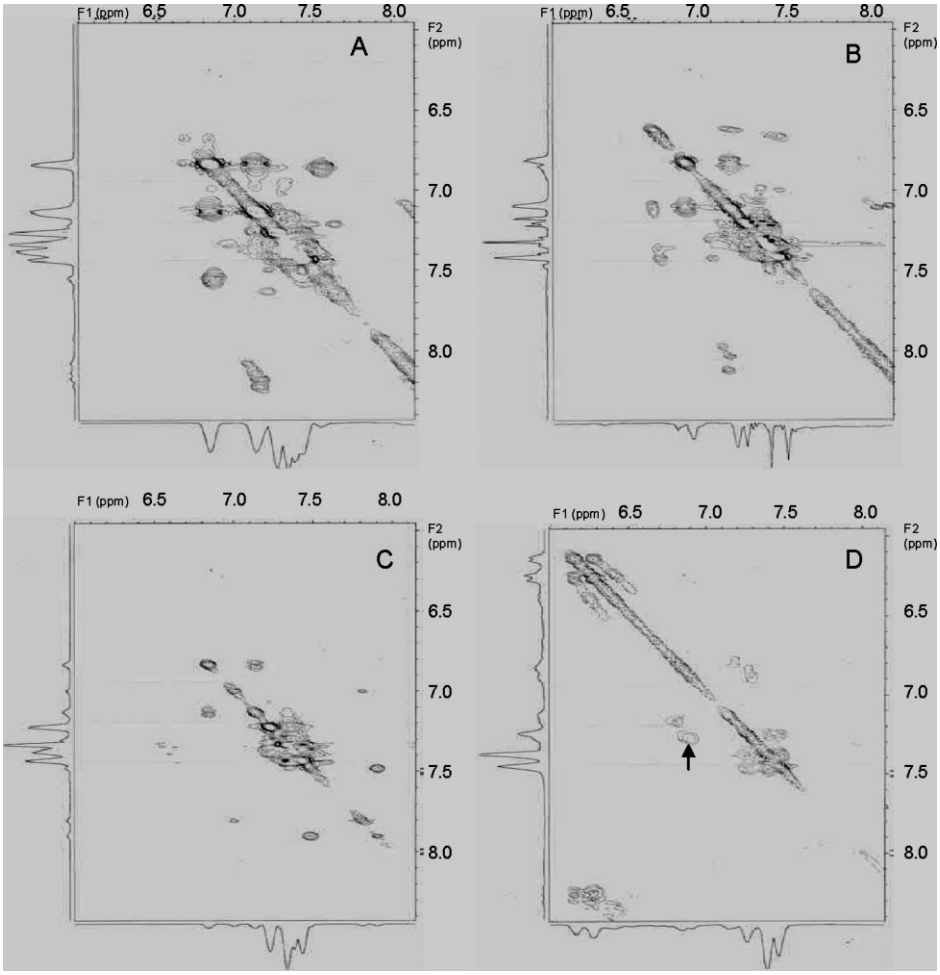

Figure 4 Aromatic CH region of the TOSCY spectra of (A) d-cata in aqueous, (B) d-cata in 50% TFE, (C) d-cata with cobalt heme in aqueous solution, (D) d-cata with cobalt heme in 50% TFE. (A), (B) and (C) have only one cross peak at (6.83, 7.15 ppm) corresponding to unbound tyrosine, whereas (D) has an additional cross peak (at 6.9, 7.25 ppm, see arrow) of bound tyrosine besides the unbound tyrosine cross peak.

the unbound tyrosine cross peak also because some population of the peptide is still not bound to cobalt heme. So the co-ordinate bonding to cobalt heme has downfield-shifted the 5H and 6H of tyrosine by 0.07 and 0.1 ppm, respectively. In all other conditions, except d-cata with cobalt heme in 50% TFE, we have not observed this additional cross peak of bound tyrosine. This confirms our earlier observation that TFE causes the co-ordinate bonding of tyrosine to heme.

Redox Activity of Heme–Peptide Complexes

The designed peptides are expected to alter the redox activity of heme upon binding to it. The modulation of heme redox activity by peptide binding has been assayed by catalase activity assay and peroxidase activity assay. Catalase activity was assayed by monitoring decomposition of H_2O_2 spectrophotometrically at 240 nm [19]. Neither d-pero nor d-cata has shown any catalase activity in presence of heme, but there is considerable peroxidase activity in both. The increasing peroxidase activity in the heme–peptide complex with increasing TFE concentration also confirms the role of TFE in inducing the native-like contacts in the designed catalase active site. The reduction potential of the peroxidase enzyme is −306 mV, which is not very different from that of bis-imidazole-ligated heme (−235 mV) [6]. This is why it should be easier to achieve peroxidase activity compared to catalase activity where the requirement of the reduction potential (∼ − 500 mV) is significantly higher [6]. There is some peroxidase activity in heme also, and peptide binding increases this peroxidase activity. The peroxidase activities of heme–peptide complexes, horse radish peroxidase (HRP) and heme are roughly compared using their initial rate of reaction, keeping the same concentrations of substrates (ABTS and H_2O_2) in all experiments. Here, the peptide concentration used is more than that of heme to ensure binding of most of the heme. The heme–d-cata complex (3 μM) gives an initial reaction rate of 12 μM/min (Table 1). HRP (14 nM)

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Table 1 Peroxidase activity of heme–d-cata complex in 30% and ~100% TFE. Sample composition and the initial rate of reaction (μM/min) are given. All samples have the same concentration of substrate (10 mM H_2O_2, and 0.5 mM ABTS)

Sample	Reaction rate
60 nM heme, (100% TFE)	6.3
14 nM HRP	28.3
102.6 μM d-cata, 750 nM heme, (30% TFE)	82.6
750 nM heme, (30% TFE)	5.2
102.6 μM d-cata, 60 nM heme, (100% TFE)	38.0
102.6 μM d-cata, (100% TFE)	0

gives a reaction rate 28.3 μM/min, so for the reaction rate to be nearly equal to that of HRP, approximately 500 times d-cata–heme complex will be required. The peptide and heme are not strongly bound, so both heme and peptide are susceptible to H_2O_2 oxidative damage. Probably this is the reason why we do not observe peroxidase activity after 5–6 min. The peroxidase activity of heme itself is increased by TFE, still the increase in peroxidase activity of the heme–peptide complex in 30% and ~100% TFE is very significant. The initial rate of the reaction catalysed by heme in 30% TFE is ~3 times that in aqueous solution. Heme (0.75 μM) in 30% TFE or heme (60 nM) in ~100% TFE gives a reaction rate equal to 3 μM heme in aqueous solution (Supplementary information). The initial rate of reaction of heme–d-cata in 30% TFE is ~15 times more compared to the same amount of heme in 30% TFE (Table 1). For the reaction rate to be equal to that of HRP, approximately 50 times d-cata–heme complex in 30% TFE (or 4 times in 100% TFE) is required. Similar results are found for the d-pero–heme complex also (Supplementary information).

DISCUSSION

We have explored a strategy of using an evolutionarily conserved sequence in designing a heme–peptide complex to mimic the active site of the catalase. Using this approach, we have been able to make native-like contacts in the designed catalase active site along with a promising redox activity of heme for catalyzing the peroxidase reaction. We have used the conserved motif of catalases for mimicking some structural features of active site and redox activity only, but the parts of sequence not included in our design but present in the parent protein must be important for other functions such as substrate specificity, efficient folding of the protein, funneling of the substrate to the active site, safety from intermediates of reaction, cellular localization of the catalase, etc. By a coarse estimate, we can say that in order to have activity comparable to that of HRP, 4 times more of peptide–heme complexes with d-cata or d-pero are required (initial rate of reaction of 14 nM HRP is approximately equal to 60 nM heme–peptide complex (60 nM heme with excess of d-cata peptide in TFE)). Here we have assigned the 369 nm Soret band to the binding of heme to the phenolic side chain of tyrosine. Even as the catalase has the heme axially ligated to tyrosine [20,21], it shows the λ_{max} of the Soret band at 405 nm. This is probably because the constraints of protein matrix distort the tyrosine–heme co-ordinate bonding and histidine in distal pocket also may be partially ligated [22] to heme. Our study is the first reported designed peptide that shows ligation of heme through tyrosine. The requirement of TFE for inducing helicity in d-cata and then co-ordinate bonding of tyrosine to heme gives a glimpse of the folding process of the catalase and reaffirms that the tyrosine (i^{th} residue) has to be charged by arginine (i – 4th residue) for its ligation to heme. Insertion of dehydrophenyl-alanine (ΔF) or aminoisobutyric acid (Aib) between R^1 and Y^5 in the d-cata sequence may allow tyrosine's axial ligation to heme in the absence of TFE because ΔF or Aib will induce helicity in the peptide and that will bring R and Y in proximity. Further improvements on this design hold the promise of giving industrially and medically [23] useful, small, cheap and stable redox-active catalysts.

Supplementary Material

Supplementary electronic material for this paper is available in Wiley InterScience at: http://www.interscience.wiley.com/jpages/1075-2617/suppmat/

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