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Initial characterization of the ferric H175G cytochrome *c* peroxidase cavity mutant using magnetic circular dichroism spectroscopy: phosphate from the buffer as an axial ligand

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Abstract

Electronic absorption and magnetic circular dichroism (MCD) data are reported for the H175G cavity mutant of yeast cytochrome *c* peroxidase (H175G CCP) in its ligand-free ferric state at 4 °C under slightly acidic conditions (pH 5.9). Initial analysis of the MCD spectrum of this state suggested a five-coordinate structure with ligation by an aspartate or glutamate. However, examination of the crystal structure of wild-type CCP revealed *no* plausible carboxylate-containing amino-acid residues close enough to the heme iron to serve as the ligand. An alternative interpretation of the MCD data is that the phosphate ion from the buffer is bound to the ferric heme iron. A phosphate group is similar in electronic character to a carboxylate and could give an MCD signal similar to that of a carboxylate-bound heme. Phosphate coordination to the ferric heme iron, albeit trans to imidazole, has recently been seen in a 2.0-Å-resolution crystal structure of imidazole-bound ferric H175G CCP in phosphate buffer [Biochemistry 40 (5) (2001) 1265]. To investigate the validity of phosphate ligation, the pH 5.9 species in the 100 mM phosphate buffer was exchanged into the pH 5.9, 100 mM MES (2-[*N*-morpholino]ethanesulfonic acid) buffer. The MCD spectrum of the species in the MES buffer was spectrally distinct from that in the phosphate buffer, indicating that the original spectrum depends on the presence of a phosphate ion. We conclude that in the phosphate

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buffer, the exogenous ligand-free ferric state of the H175G CCP cavity mutant is actually coordinated by a phosphate ion from the buffer.
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1. Introduction

The proximal ligand is believed to play an important role in controlling the chemistry and, therefore, the function of heme proteins. Globins, heme oxygenases, and the majority of peroxidases utilize histidine as their proximal ligand [1,2]. Catalase has a tyrosinate axial ligand, while a cysteinate ligand is found in cytochrome P450 (P450) [3], *C. fumago* chloroperoxidase [4], nitric oxide synthase [5], and the CO-sensing CooA protein [6]. As the function of each protein is thought to be partially attributable to the proximal ligand, the differences in the ligands themselves are of interest. Numerous studies have shown that the degree of electron donation from the proximal ligand to the iron regulates its reduction potential [7], promotes reactions, such as dioxygen bond cleavage at the sixth position [8,9], and stabilizes high-valent iron intermediates such as compound I and compound II [10,11].

Recent attention has focused on replacing the proximal ligand with a smaller, non-coordinating residue, such as glycine or alanine, thus forming an artificial cavity on the proximal side of the heme. Termed cavity mutants, these proteins have the unique ability to bind exogenous ligands in the cavity in order to reconstitute their wild-type activity. This approach was pioneered by Barrick for sperm whale myoglobin [12] and has since been accomplished for cytochrome *c* peroxidase (CCP) [13], heme oxygenase [1], and horseradish peroxidase (HRP) [14]. Studies of cavity mutants to date have focused on the dynamics of binding unnatural ligands [15–18] and the resulting effects on catalytic activity [15]. Before these systems can be evaluated in the presence of exogenous ligands, it is vital to discern the coordination structure of the exogenous ligand-free complex. Understanding the nature of these derivatives is an essential part of the complete characterization of the cavity mutant systems and is expected to be the subject of future studies as more cavity mutant proteins are engineered.

The exogenous ligand-free ferric cavity mutants investigated thus far have been reported to be ligated by oxygen-donor ligand(s) with the exception of H170A HRP at pH 6.0 [14]. Recent magnetic circular dichroism (MCD) and resonance Raman analysis of H93G Mb determined that the cavity mutant has spectrally distinct species at pH 5.0, 7.0, and 10.0. All three are ligated by an oxygen-donor ligand(s) with the acidic form being five-coordinate water-bound, the alkaline form being five-coordinate hydroxide-bound and the neutral form being a mixture of five and six coordination [19,20]. Similarly, exogenous ligand-free ferric H25A heme oxygenase appears to be five-coordinate high spin with an oxygen-donor ligand as determined by resonance Raman and MCD analysis [1,19]. However, comparison of the MCD spectrum of H25A heme oxygenase with the spectra of H93G Mb indicated that the former is not ligated by just water or hydroxide, as reported for the latter [19]. Instead, spectral comparison with carboxylate-ligated models indicated that ferric H25A heme oxygenase is likely ligated by glutamate or aspartate [19]. Finally, the exogenous ligand-free ferric H175G CCP has been crystallized from unbuffered water and structurally characterized at 2.3-Å resolution [13,21]. Resonance Raman studies at pH 5.9 and 7.2 led to the conclusion that the protein is predominately five-coordinate high spin and ligated by water or hydroxide, respectively [21]. However, the electronic absorption spectra at pH 5.9 and 7.2 do not agree well with those of the acidic or alkaline ferric H93G Mb (ligation by water and hydroxide, respectively [19]), indicating a potential difference in axial ligation.

As the MCD spectra of iron porphyrins are very sensitive to the nature of the heme iron axial ligands, the technique is especially well suited for investigations of axial ligation in structurally undefined heme iron proteins [22]. Ligation assignments are made through comparisons of the spectra of the structurally uncharacterized heme protein with those of structurally defined heme iron centers. In this manner, MCD spectroscopy has been used to characterize the coordination structure of numerous heme proteins including P450 [23], *C. fumago* chloroperoxidase [23], *Amphitrite ornata* dehaloperoxidase [24], *Notomastus lobatus* chloroperoxidase [24], and heme oxygenase [25]. We present herein spectroscopic investigations of exogenous ligand-free ferric H175G CCP in the context of known ferric five-coordinate oxygen-donor complexes in order to establish its coordination structure and, in so doing, to refine the application of MCD spectroscopy to distinguish various types of oxygen-donor ligation encountered in natural and engineered proteins and synthetic model systems.

2. Materials and methods

2.1. Materials

H175G CCP was expressed and purified in the presence of 10 mM imidazole as previously described [13]. Samples were received as crystal suspensions, which were washed with water three times discarding the supernatant following each wash thereby removing the exogenous imidazole. The crystals were then dissolved in 500 mM potassium-phosphate buffer at the appropriate pH forming the stock protein solutions. The samples were handled at 4 °C in the 100 mM potassium-phosphate buffer unless otherwise stated. When necessary, samples were exchanged into 100 mM MES (pH 6.0 or 7.0) on a Biorad P6DG gel filtration column. Heme protein concentrations were determined by the pyridine hemochromogen method [26].

2.2. Spectroscopic techniques

Electronic absorption spectra were recorded with a Cary 210 spectrophotometer interfaced to an IBM PC. The MCD spectra were measured at 4 °C using a JASCO J500-A spectropolarimeter equipped with a JASCO MCD-1B electromagnet (1.41 T) and interfaced to a Gateway 2000 4DX2-66V PC through a JASCO IF-500-2 interface unit as previously described [25]. All spectral measurements were performed at 4 °C. Data

acquisition and manipulation has been described elsewhere [25]. Electronic absorption spectra were recorded before and after the MCD measurements to verify the sample integrity.

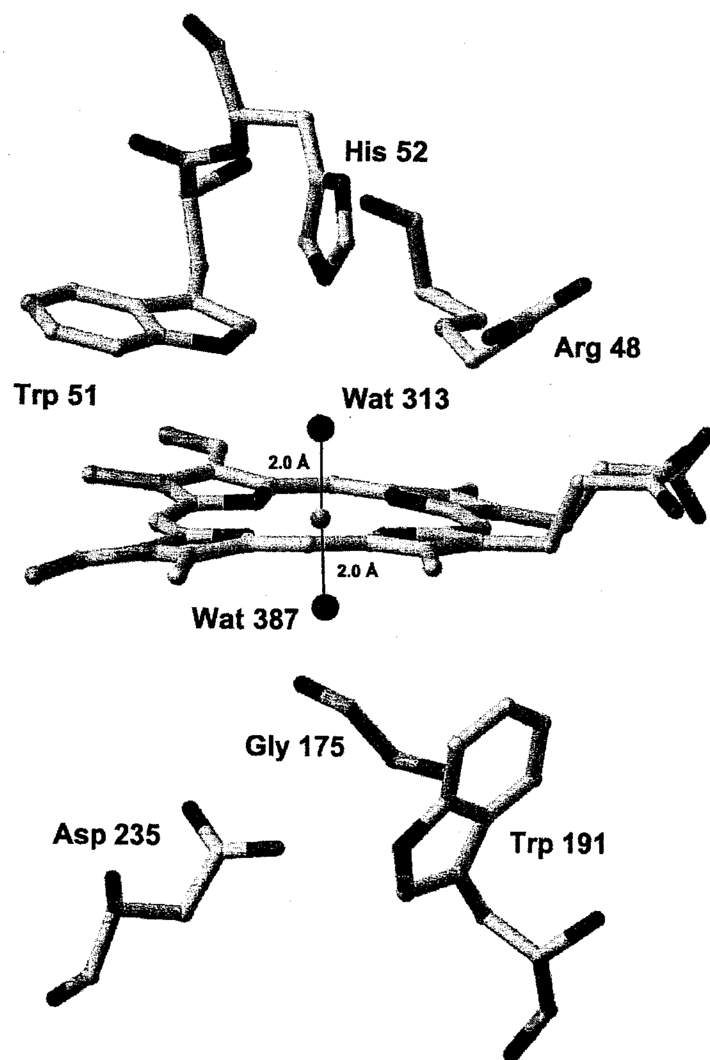

Fig. 1. Active site of exogenous ligand-free ferric H175G CCP (taken from Protein Data Bank, PDB Code 1CCE [13]).

3. Results and discussion

Fig. 1 illustrates the heme active site of H175G CCP as initially determined by x-ray crystallography to a 2.3-Å resolution [13]. Refinement of the H175G CCP structure resulted in the placement of the iron in the heme plane with two solvent molecules at equal distances of 2.0 Å from the iron [13]. However, due to the low resolution of the structure, it was uncertain whether one or both of these waters was strongly coordinated, their respective protonation states, and whether coordination was maintained in solution. Later resonance Raman studies indicated that only one of the waters actually serves as a ligand and that it is deprotonated as the pH is increased from pH 5.9 to 7.2 [21]. This behavior would suggest that the complex at pH 5.9 is predominantly five-coordinate water ligated, and at pH 7.2 is predominantly five-coordinate hydroxide ligated. As these two coordination complexes have been previously observed and characterized by MCD for acidic (pH 5.0) and alkaline (pH 10.0) ligand-free ferric H93G Mb [19,20], respectively, an MCD study of ferric H175G CCP at pH 5.9 was initiated with the expectation that the data obtained would be similar to that already reported for acidic H93G Mb [19,20].

The electronic absorption and MCD data for ferric H175G CCP at pH 5.9 are listed in Table 1 together with the data for acidic (pH 5.0) and alkaline (pH 10.0) exogenous ligand-free ferric H93G Mb. The spectral data for H175G CCP at pH 5.9 are quite different from those of either H93G Mb species. This implies that the electronic and, therefore, the

Table 1
Magnetic circular dichroism and Electronic absorption data for ferric exogenous ligand-free heme-containing proximal cavity mutants

Protein	pH	Absorbance		MCD		Refs.
		λ_{max} (nm)	ε (mM cm)$^{-1}$	λ_{max} (nm)	$\Delta\varepsilon/H$ (mM cm T)$^{-1}$	
H175G CCP	5.9	406.5	84.2	401	4.6	this work
100 mM Phos		500	8.3	410	0	
		524	7.7	422	− 6.9	
		623	4.5	550	− 3.2	
				644	− 3.2	
H175G CCP	5.9	409.5	80.9	404	9.2	this work
100 mM MES		530	8.5	417	0	
		559	7.2	430	− 9.3	
		620	3.1	550	3.7	
				568	− 5.0	
				639	− 1.1	
H93G Mb	5	368	82.1	358	− 3.1	[19]
		506	10.4	427	− 1.8	
		641	4.2	561	− 4.1	
				662	− 1.9	
H93G Mb	10	403	104	397	13.9	[19]
		482	10.9	534	− 1.9	
		600	8.05	621	− 5.2	
H25A HO	6–10	400	96.1	392	5.98	[19]
		492	10.0	549	− 1.55	
		620	5.16	641	− 1.93	

structural characteristics of the complexes are different, ruling out five-coordinate water or hydroxide ligation, respectively, for exogenous ligand-free ferric H175G CCP at pH 5.9. This interpretation suggests that a different oxygen-donor ligand is bound to the heme iron and is responsible for the observed spectral properties.

Previous resonance Raman characterization of the heme oxygenase cavity mutant (H25A heme oxygenase) indicated that the ligand-free complex was five-coordinate with either a solvent molecule or a carboxylate group from a nearby glutamate served as the fifth ligand [1]. Further characterization of this mutant by MCD ruled out water ligation and was consistent with carboxylate ligation [1]. This assignment was based on a positive comparison of the MCD spectra of H25A heme oxygenase [1] and that of the *p*-nitrophenolate and acetate adducts of Fe(III)PPIXDME [27]. To investigate potential carboxylate ligation in the H175G CCP system, the MCD spectrum of H25A heme oxygenase is compared to that of H175G CCP at pH 5.9 in Fig. 2. The similarity of the MCD spectra of these two complexes argues that they have closely related coordination structures.

To further explore the possibility of carboxylate ligation in ferric CCP, the crystal structure of H175G CCP (see Fig. 1) was examined for potential glutamate or aspartate residues within bonding distance of the heme. An aspartate (Asp235) is present in the proximal pocket, but is ~ 6.6 Å away [13] from the heme iron, too far to bind to the heme iron. Additionally, comparison of the electronic absorption data for the pH 5.9 H175G

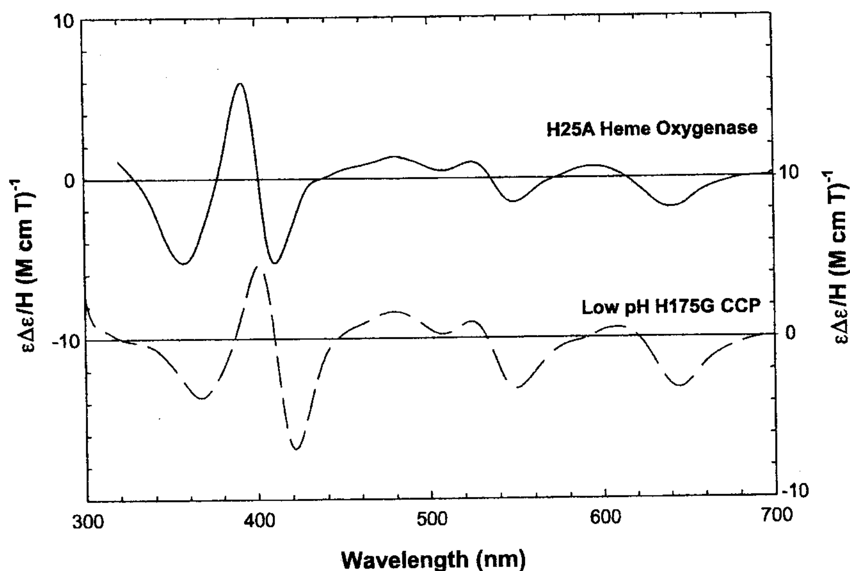

Fig. 2. The MCD spectra for the exogenous ligand-free ferric state of H25A heme oxygenase (pH 7.0) (top) and of H175G CCP (pH 5.9) (bottom). The H25A heme oxygenase spectrum is replotted from data in Ref. [19]. The spectra were measured in the 100 mM potassium phosphate buffer at 4 °C.

CCP species with that of a glutamate-ligated CCP mutant (H175E) [28,29] showed significant differences in the absorption band positions especially in the location of the charge transfer transition in the 600–650 nm region (~ 623 nm for H175G CCP and 637 nm for H175E CCP). Together, these observations make carboxylate ligation in the H175G CCP system unlikely. A different ligand with electronic characteristics similar to a carboxylate group must give rise to MCD spectra of the H175G CCP species.

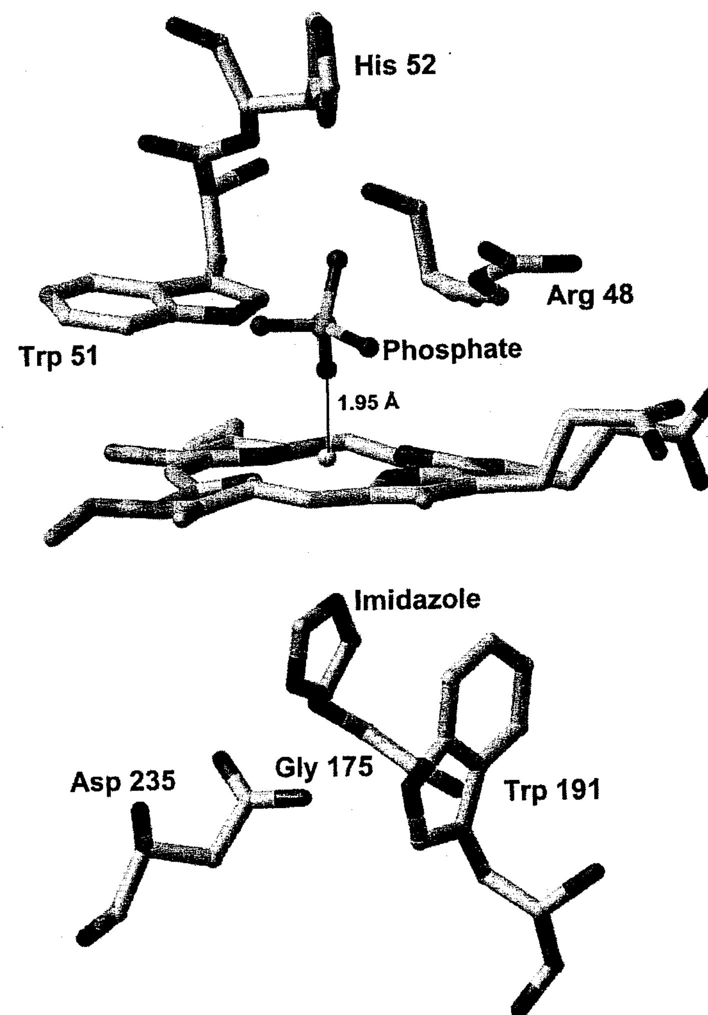

Fig. 3. Active site of imidazole-bound ferric H175G CCP in the presence of potassium phosphate (taken from Protein Data Bank, PDB Code 1DSE [30]).

The ligand assignment for H25A heme oxygenase was based on the MCD spectral similarity to the *p*-nitrophenolate and acetate adducts of Fe(III)PPIXDME examined by Nozawa et al. [27]. This group determined that ligands with nonoccupied π^* orbitals have the effect of lowering the energy of the porphyrin to iron charge transfer transition as a result of interaction of the iron $d\pi$ orbital with the fifth ligand nonoccupied π^* orbital.

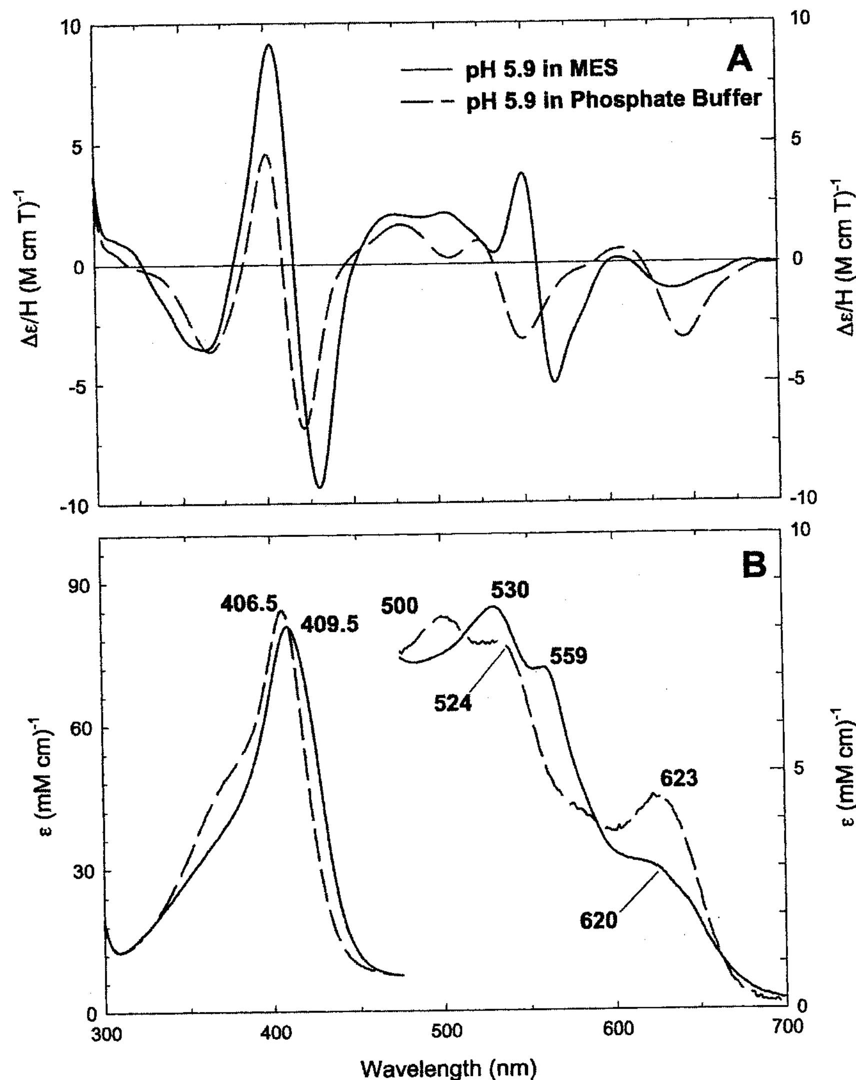

Fig. 4. The MCD (A) and electronic absorption (B) spectra of ferric H175G CCP in pH 5.9, 100 mM potassium phosphate buffer (dashed) and in 100 mM MES buffer (dashed).

Furthermore, Nozawa et al. [27] suggested that this lowered energy is, in turn, translated to a lower energy requirement for the $p\pi$ to $d\pi$ charge transfer transition and a red shift of the absorption and MCD features resulting from this transition. It also appears that this interaction results in an MCD spectrum with less A-term character (i.e., less derivative-shaped character) than the spectra of those complexes with ligand lacking vacant π^* orbitals such as water and hydroxide. In natural protein systems, the only available amino acids with nonoccupied π^* orbitals are the carboxylates of aspartate and glutamate. However, phosphate from the potassium-phosphate buffer represents a potential ligand that has these nonoccupied π^* orbitals.

The consideration of phosphate ligation for the pH 5.9 species derives from the recent 2.0-Å crystallographic data of the imidazole-bound complex of H175G CCP [30]. Previous crystallographic characterization of exogenous ligand-free H175G CCP at a 2.3-Å resolution was based on crystals grown in *unbuffered water*; therefore, no phosphate was present [13]. For the imidazole bound complex of H175G CCP, two sets of low temperature crystallographic data were collected for crystals grown in the presence of either potassium phosphate or the MES buffer. Comparison of the electron density within the active site for the two crystal preparations showed a clear difference on the distal side of the heme, indicating the presence of a heme ligand in the presence of the potassium-phosphate buffer, but not the MES buffer [30]. This electron density feature is well modeled by a heme-coordinated phosphate ion with an iron to oxygen distance of 1.95 Å (Fig. 3). The ability of phosphate to bind to this complex suggested that ferric H175G CCP at pH 5.9 in the phosphate buffer might be a five-coordinate phosphate-ligated complex.

If phosphate in the buffer is the ligand in ferric H175G CCP, the absorption and MCD spectra of the protein should change in the absence of phosphate. The protein was exchanged into 100 mM, pH 5.9 MES buffer and its electronic absorption and MCD spectrum compared to those in the phosphate buffer (Fig. 4). Removal of phosphate leads to a slight red shift of the Soret absorption peak to 409.5 nm, a red shift of the visible features to 530 and 559 nm and a significant decrease in the intensity of the charge transfer transition at 620 nm. In the MCD spectrum, the derivative-shaped feature in the Soret region has increased in intensity and red-shifted to 417 nm. Additionally, the two troughs in the visible region have been replaced with a second-derivative shaped band at 558 nm. The significant difference in the MCD spectrum upon removal of phosphate from the H175G CCP sample is consistent with phosphate coordination when phosphate is present. Further investigations will focus on the coordination structure of H175G CCP in the absence to phosphate and as a function of pH.

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