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Kinetic and thermodynamic studies of N-acetyl-cobalt(III)-microperoxidase 8: a vitamin B12a analogue

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The Preparation of *N*-Acetyl-Co(III) Microperoxidase-8 (NAcCoMP8) and its Solution Chemistry

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Abstract

The Co(III) porphyrin octapeptide *N*-acetyl-Co(III) Microperoxidase-8 (NAcCoMP8) was prepared by the proteolytic digestion of cytochrome *c*, replacement of Fe(III) by Co(III), and protection of the N-terminal group of the peptide by acetylation. In neutral aqueous solution it is monomeric at low concentration with a Soret band at 415 nm with $\varepsilon = 1.61(3) \times 10^5$ M^{-1} cm^{-1}; higher concentrations (> 10 μM), an increase in the ionic strength of the solution, or an increase in temperature above 45 ^{o}C causes the formation of oligomeric species with a Soret band at 425 nm, usually through intermediacy of a species with a Soret band at 417 nm. Although formation of the latter can be reversed to produce the monomeric 415 nm species on dilution of the solution, or by lowering the temperature or the ionic strength, the formation of the former is irreversible and on standing eventually leads to precipitation of the metallopeptide from solution. NAcCoMP8 has five spectroscopically observable pK_as: at 1.74 ± 0.04 due to protonation and displacement of His-18 either by H_2O or a carboxylate from the peptide; at 4.08 ± 0.02 and 5.80 ± 0.05 due to ionization of the heme carboxylate groups; at 7.81 ± 0.03 is due to ionization of coordinated H_2O; and at 12.00 ± 0.03 due to ionization of His-18 to form an imidazolate. NAcCoMP8 provides a means of comparing and contrasting the chemistry of Co(III) porphyrins and corrins and so assess the influence of the macrocycle on the chemistry of the metal ion.

Keywords: Microperoxidases; cobalamins; iron porphyrins

1. Introduction

The proteolytic digestion of cytochrome *c* produces a series of water-soluble heme-containing peptides, the microperoxidases (MPs, Figure 1) [1-5] that have been used to explore aspects of the chemistry of iron porphyrins, and as biomimetics for a number of reactions catalyzed by hemoproteins such as the peroxidases and the cytochromes P450 [6, 7]. Acetylation of the N-terminus of the heme-octapeptide MP8 to form *N*-acetylmicroperoxidase-8 (NAcMP8) reduces its aggregation by preventing the α-amino group of Cys-14 from acting as an intermolecular ligand, and produces a species that is monomeric up to concentrations where it can be conveniently studied by conventional UV-vis spectroscopic methods [8, 9]. MP8 [10, 11] and NAcMP8 [9] retain the proximal His-18 ligand above pH 2; the distal coordination site is occupied by a water molecule that is readily replaced by exogenous ligands from solution (for example [12-21]).

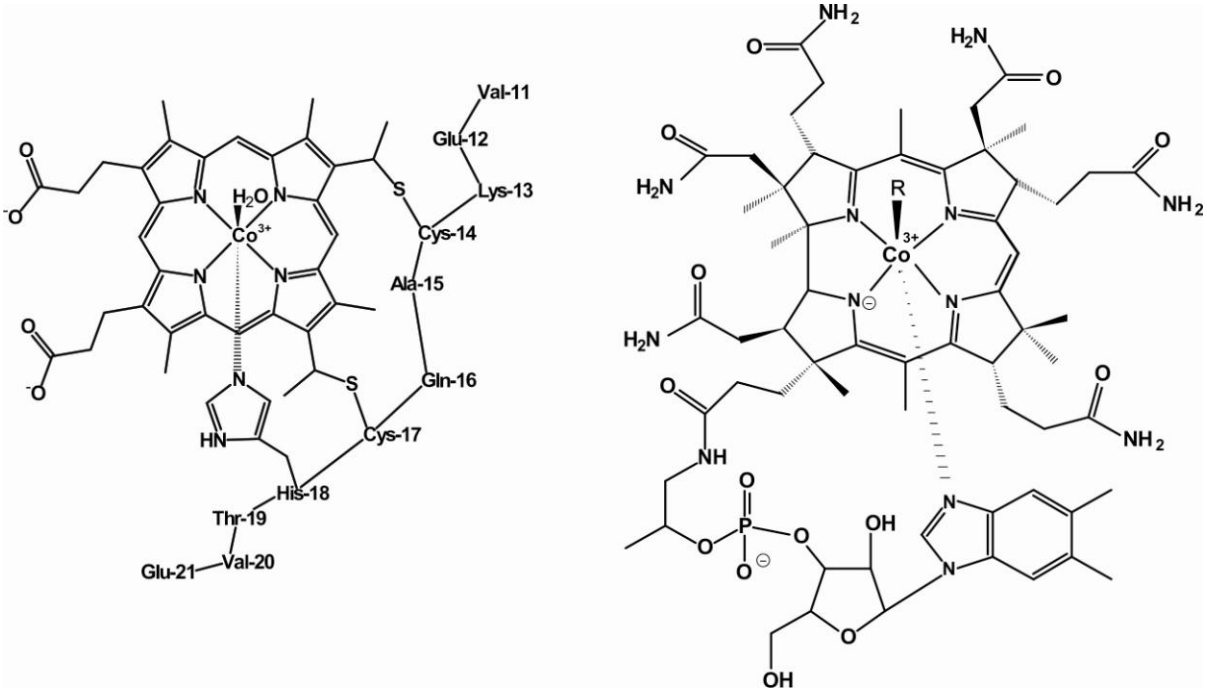


Figure 1. (A) The microperoxidases, heme-containing peptides prepared from the proteolytic digestion of the parent protein, cytochrome *c*. The amino acid residue numbering is that of the parent cytochrome *c* from horse heart. Microperoxidase-8 (MP8) retains the residues Cys-14 through Glu-21. (B) The cobalamins (Cbls, derivatives of vitamin B_{12}). In aquacobalamin (B_{12a}, H_2OCbl^+), R = H_2O. B_{12} itself has R = CN^-. The two biologically-important forms of the cobalamins have R = 5'-deoxyadenosyl (in coenzyme B_{12} or AdoCbl) and R = Me.

There are several reports concerning the removal of iron from ferric hemepeptides and the insertion of other metal ions. Demetallation of hemepeptides can be effected by

treatment with anhydrous HF at –78 ^{o}C [22] or at room temperature [23] to produce the metal-free peptido-porphyrin. Heating with manganous or zinc acetate at 40–55 ^{o}C for 2 h in aqueous solution [22, 24] or with nickel acetate at 75 ^{o}C [23] followed by purification on reverse phase chromatography [22, 24] or by dialysis [23] leads to the metallated hemepeptide.

An alternative procedure that avoids use of HF involves the reductive elimination of iron that occurs on treating a solution of the hemepeptide in deaerated cold acetic acid with ferrous chloride in concentrated HCl [25-27]. Separation of the demetallated hemepeptide from the iron salts is by size exclusion chromatography [25]. Stirring with manganous chloride at 40 ^{o}C in ammonium acetate (pH 5.5) for about 60 h leads to metallation of the porphyrin [28]. Alternatively, metals such as zinc, copper and nickel can be inserted into the metal-free peptido-porphyrin dissolved in 1:3 pyridine:acetic acid by heating with the metal sulfate or chloride at 80 ^{o}C for 10 min under N_2 followed by precipitation of the metallopeptide with acetone [26, 27].

Although there are a number of Co-containing enzymes [29], including xylose isomerase [30], methionine aminopeptidease-2 [31] and the class D β-lactamase OXA-10 [32], the biological chemistry of cobalt is dominated by the chemistry of the cobalamins, or vitamin B_{12} and its derivatives (Figure 1) [33-35]. Cobalamin is thought to have a prebiotic origin; it is made by some bacteria, is essential to humans, but seems to play no role in the metabolism of plants, fungi and most bacteria [36]. The formally Co(III) ion in the corrins is unusually labile [37]. For example, L = I^- replaces H_2O in Y—[Co(N_4)]—X , X = Y = H_2O, three orders of magnitude faster when the equatorial N_4 donor is a corrin [38] than when it is a porphyrin [39], and SCN^- nine orders of magnitude faster in corrins [38] than when the equatorial ligands are N = NH_3 (eq. 1) [40].

$$Y–[Co^{3+}(N_4)]–X + L \rightarrow Y–[Co^{3+}(N_4)]–L + X \qquad (1)$$

The electronic structure of the equatorial ligand seems to be an important factor in controlling the rate of axial ligand substitution. When L = H_2O, X = Y = Cl^-, the rate constants vary in the ratio 1:36:270 as N_4 is changed from cyclam, to *trans*[14]diene, to

cobaloxime[‡] [41] and the approximate lability ratio of the metal ion toward substitution in corrin, porphyrin, cobaloxime, and tetra-ammine systems is 10^9:10^6:10^4:1 [37, 42]. We have speculated that this kinetic *cis* effect arises from the delocalization of electron density from the equatorial ligands onto the metal, making the latter softer and imparting upon it some labile Co(II) character [37, 42, 43].

The replacement of Fe(III) in the hemepeptide NAcMP8 [9] by Co(III) would afford a Co(III) porphyrin complex in which the coordination environment of the metal is not dissimilar to that in H_2OCbl^+: the axial ligands are H_2O and an imidazole derivative (His in NAcCoMP8; 5,6-dimethylbenzimidazole in H_2OCbl^+, see Figure 1) and the equatorial ligands are porphyrin and corrin, respectively. This should therefore provide an opportunity of comparing the effect of porphyrin and corrin in two closely related systems.

We report here the synthesis and the solution behavior of NAcCoMP8. In the accompanying paper we compare and contrast the equilibrium and rate constants for the substitution of coordinated H_2O with those for H_2OCbl^+.

2. Experimental

Materials and methods. Cytochrome *c* (*ca*. 62%) was purchased from Seravac, Cape Town, South Africa, and pepsin was from Riedel-de-Häen. Reagents were of the highest purity available and used as received. Phosphoric acid and acetonitrile were from Sigma-Aldrich, and ammonium hydroxide solution (25%), ammonium sulfate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and ammonium bicarbonate were from Saarchem. Water was purified (18.1 $M\Omega.cm^{-1}$) using a Millipore Direct Q-UV3 system. Sep-Pak vac 3 cc (500 mg or 10 g) C18 cartridges were supplied by Waters. These were activated by passing slowly one cartridge-volume of 1:1 acetonitrile:water through the cartridge and then washing with five cartridge-volumes of water. The sample was loaded slowly using 1 mL aliquots to prevent leaching. The loaded sample was washed exhaustively with deionized water until no precipitation on adding $AgNO_3$ to the eluent was observed.

[‡] Abbreviations used: cobaloxime, bis(dimethylglyoximato); cyclam, 1,4,8,11-tetraazacyclotetradecane, [16-ane]N4; Cbl, cobalamin.

Sephadex G-50 (dry bead diameter 20 – 50 μm) was purchased from Sigma-Aldrich. Millex PTFE syringe filter units (0.45 μm; 13mm) were purchased from Millipore.

UV-vis spectra were recorded on a Cary Bio 300 spectrometer in 1.0 cm pathlength cells; the temperature was controlled using a Peltier device. HPLC analyses (Phenomenex C18 5 μm 4 × 3.0 mm guard column and 150 × 4.6 mm analytical column, 2.0 mL min^{-1}) were performed with a Spectra-Physics SP8800 ternary gradient pump, a Linear UV-vis 200 detector (set at 410 nm) and a Varian 4290 integrator, or on a Dionex Ultimate 3000 quaternary gradient system equipped with a diode array detector. Gradient elution was with phosphate (50 mM, pH 7, containing 0.1 mM NaCN) and acetonitrile (98:2 for 2 min then linearly to 70:30 over 10 min with a return to starting conditions after 2 additional min). MS-ESI spectra were recorded on a Thermo LXQ instrument

To determine the molar absorptivity of NAcCoMP8, the concentration of cobalt in a solution was determined on a Spectro-Ciros ICP-OES instrument using a series of calibration standards. Measurement of the UV-vis spectrum of the same solution then afforded values of the extinction coefficients.

The acid dissociation constants of NAcCoMP8 were determined using UV-vis spectroscopy. NAcCoMP8 was dissolved in a multi-component buffer [9] (μ = 0.1 M, $NaNO_3$) in a 50 mL thermostatted cell. The pH was measured using a Metrohm 720 or 827 pH meter with a Unitrode or Biotrode electrode calibrated with standard buffers and adjusted by adding negligible volumes of either concentrated sulfuric acid or 8 M NaOH while the solution was pumped through a flow cell housed in the thermostatted cell compartment of a Cary 3E UV-vis spectrophotometer. The dependence of the absorbance A_T at an appropriate monitoring wavelength was fitted to eq. 1 using standard non-linear least-squares methods employing a Newton-Raphson procedure, where A_i is the absorbance of the i^{th} species at that wavelength, and K is an acid dissociation constant.

$$A_T = \frac{\sum_{i=0}^{n}\left[A_{i+1}[H^+]^{n-i}\prod_{j=0}^{i}K_j \right]}{\sum_{i=0}^{n}\left[[H^+]^{n-i}\prod_{j=0}^{i}K_j \right]} \qquad (1)$$

Synthesis of NAcCoMP8. It is in principle feasible to prepare NAcCoMP8 by a number of routes. Several were investigated; that which gave the highest yield involved the preparation of ferric microperoxidase-11 (FeMP11) from cytochrome *c*; the acetylation of FeMP11 to produced NAcFeMP11; its demetallation; the insertion of Co into the porphyrin; and finally the enzymatic digestion of the peptide to produce the desired compound. This procedure is described below; an account of the alternative routes explored may be found elsewhere [44].

3. Results and Discussion

3.1 The synthesis of NAcCoMP8.

Synthesis of Ferric Microperoxidase-11. FeMP11 was synthesized from horse heart cytochrome *c* as previously described [5, 9, 10, 12] with some modifications. Cytochrome *c* (300 mg) was dissolved in 0.1 M phosphate buffer (10 mL; pH 2.1) to which pepsin (15 mg; 10^4 units g^{-1}) was added. The dark brown solution was incubated at 35 ^oC for one hour after which a further 15 mg of pepsin was added. The pH of the solution (*ca.* 4) was re-adjusted to pH 2.1 by addition of phosphoric acid. Incubation was continued for a further 3 hours (a longer incubation time, up to 12 h, does not affect the yield or quality of the material). The reaction was monitored by TLC (3:5:1 MeOH:$CHCl_3$:30% NH_4OH, 0.2 mm silica gel plates). The reaction vessel was then submerged in boiling water for 5 minutes; after cooling the pH was raised to 7.0 with 30% NH_4OH to destroy peptic activity.

It is known that increasing ionic strength promotes the aggregation of porphyrins [45] and microperoxidases [9]. We therefore avoided using the salting-out step previously used in our synthesis of the microperoxidases [9], and used column chromatography instead. The bright-red solution was purified on Sephadex G50 column (3.0 cm × 40 cm) eluted with NH_4HCO_3 (0.1 M) at a rate of 10 mL h^{-1}. Two distinct bands were observed. The first was primarily FeMP11. The second (minor) band was primarily FeMP9. The samples were collected in 0.5 mL fractions with a fraction collector and those with >95 % purity (HPLC) were pooled. The fractions containing the FeMP11 band, as identified by mass spectroscopy (FeMP11 – H_2O + H^+, m/z observed 1861.93 and with Na^+, 1883.92) were loaded onto a Sep-Pak cartridge for desalting and concentrating. The concentrated FeMP11 solution was

clarified using a Millex syringe-filter and lyophilized at neutral pH to yield a bright-red spongy solid, routinely stored at –20 ^{o}C under argon. The concentration was determined spectroscopically using [12] ε_{395} = 1.76 × 10^5 M^{-1} cm^{-1}. The yield was 93%.

Acetylation of FeMP11. The acetylation procedure used was essentially as previously described for the synthesis of NAcFeMP8 [9]. The isolation steps were adapted and modified from the reports of others [46, 47]. FeMP11 (20 mg) was dissolved in 10 mL saturated sodium acetate solution in a thermostatted cell at 5 ^{o}C. A 500 molar excess of acetic anhydride was added in five aliquots at 10-minute intervals. The evolution of acetic acid *in situ* turned the solution brown-red, which is typical of microperoxidases at low pH. The reaction mixture was stirred at 5 ^{o}C overnight and then lyophilized. The lyophilized NAcFeMP11 was redissolved in sodium carbonate buffer (2 mL, 0.2 M, pH 9.5) and the scarlet compound was desalted on a Sep-Pak cartridge. Neutral NAcFeMP11 was clarified using a Millex filter before being lyophilized. The red-brown product was routinely stored at –20 ^{o}C under argon. The reaction was monitored by TLC and HPLC and the product identified by mass spectroscopy (ESI-MS positive mode, diacetylated peptide, as a monosodium adduct, *m/z* observed at 984, calculated 987, *z* = 2). The concentration was determined spectroscopically using [48] ε_{397} = 1.48 × 10^5 M^{-1} cm^{-1}. Yield 95%.

Reductive demetallation of NAcFeMP11. The demetallation procedure was adapted from Primus *et al.* [25]. Care was taken to avoid unnecessary exposure to light and air as the metal-free compound has a tendency to decompose. Oven-dried Schlenk apparatus was connected to a high-vacuum line and flooded with argon. To the reaction vessel was added NAcFeMP11 (10 mg), 10.00 mL glacial acetic acid and 166 μL concentrated HCl. The brown-red reaction mixture was frozen under argon using liquid nitrogen and deaerated by three freeze-thaw cycles, purging with argon between cycles. After the final freeze-thaw cycle, the reaction vessel was covered with aluminum foil to prevent light exposure, briefly opened under an Ar stream to add $FeCl_2$ (10 mg, 12 equiv.) and immediately sealed. The reaction was stirred for 1 hour at room temperature, resulting in a change of color from an opaque brown-red solution to a clear bright-purple solution. Purification was conducted in the dark. The reaction mixture was diluted by adding deionized water (~ 50 mL) to the Schlenk tube. The sample was immediately loaded onto a Sep-Pak cartridge for desalting and removal of acids. The neutral purple NAcMP11 was clarified using a Millex filter before

lyophilization. The light purple spongy solid was stored briefly at –20 ^{o}C under argon before metal insertion. Yield 98%.

Insertion of Co into NAcMP11. The metallation procedure was adopted from a report on the preparation of MnMP8 [25] and procedures used for insertion of Co into porphyrins [49, 50]. NAcMP11 (10 mg) was dissolved in deionized water (5 mL) in a foil-wrapped flask. The pH was adjusted to 8 using concentrated NaOH. A 100-fold excess of cobaltous acetate dissolved in a minimum volume of methanol was added to the flask and allowed to stir for two hours under reflux. After metallation was complete (UV-vis) the sample could be exposed to white light. NAcCoMP11 was purified and concentrated using a Sep-Pak cartridge. The neutral dark-pink NAcCoMP11 solution was clarified using a Millex filter and then lyophilized. The pink spongy NAcCoMP11 was routinely stored under argon at –20 ^{o}C and protected from unnecessary light. Yield 95%.

Tryptic digestion of NAcCoMP11. NAcCoMP11 (20 mg) was incubated with trypsin (30 mg; activity 12 700 units mg^{-1}) in 0.1 M phosphate buffer (20 mL) at pH 8.5 at 35 ^{o}C in a thermostatted cell for 8 hours with gentle stirring. The reaction was monitored by TLC. In this case a precipitation procedure proved adequate, obviating the need for a time-consuming chromatographic procedure. NAcCoMP8 was precipitated by lowering the pH to 3.5 with dilute H_3PO_4. The solution was placed on ice and solid $(NH_4)_2SO_4$ was added until saturation; thereafter saturated $(NH_4)_2SO_4$ was added (ca. 5 mL) and the reaction mixture was centrifuged for 45 min at 8000 rpm. If the supernatant was still colored, the salting-out step was repeated on the supernatant. The colorless supernatant was decanted and the NAcCoMP8 pellet was dissolved in 5 mL of deionized water. The pH was readjusted to 7 with NaOH and desalted using a Sep-Pak cartridge. The NAcCoMP8 solution was clarified using a Millex filter and then lyophilized. NAcCoMP8 was identified by ESI-MS in negative mode ($C_{70}H_{87}CoN_{15}O_{19}S^{3-}$, calculated 1564.51; NAcCoMP8+2H^{+} observed at 1566.56; and with loss of axial H_2O expected at 1548.52, observed at 1550.57). ICP-OES was used to determine the concentration of Co^{3+} ions in 0.210 mg NAcCoMP8, dissolved in 25 mL of 20% acetonitrile/water (v/v) and further diluted 10 times to fall within the range of the commercial ICP-OES calibration standards. The emission of Co^{3+} was detected at 237.8 nm. This standardized solution was used to determine the extinction coefficients of NAcCoMP8. $\lambda(\varepsilon/10^4\ M^{-1}\ cm^{-1})$, pH 7, 25 ^{o}C: 415 (16.1), 530 (1.67), 560 (1.51). The dark pink spongy

NAcCoMP8 was routinely stored under argon at –20 ^{o}C and protected from unnecessary light.

Figure S1 of the Supplementary Information shows the UV-vis spectrum of metal-free NAcMP8 and NAcCoMP8. Metallation causes the Soret to sharpen considerably and to shift from 394 nm to 415 nm. The four Q bands characteristic of metal-free porphyrins (502, 535, 570, 622 nm) collapse to two bands (557, 558 nm).

3.2 The aqueous solution chemistry of NAcCoMP8.

The ferric microperoxidases are notoriously prone to aggregation [12, 46, 51], and aggregates can exhibit apparent molecular masses of up to 10 kDa [4]. Aggregation arises both from π–π interactions between the porphyrins [52, 53] and through intermolecular coordination of the metal by side-chain or terminal amino groups from another molecule [54-57]. As aggregates are often ill-defined, their presence will compromise the interpretation of experimental data [7]. To circumvent this, studies are often conducted in solutions of low ionic strength [9], in the presence of detergents [58] or in mixed solvents [14, 59-61]. But by far the most effective way of minimizing (if not altogether preventing) aggregation is to block potential coordinating groups in the molecule by chemical modification. Thus, acetylation of the amino groups of the peptide to produce ferric NAcMP11 [48] and ferric NAcMP8 [9] give species that are monomeric up to at least 20 μM, which is ample for study by UV-vis spectroscopy. It is therefore not surprising that preliminary work we did on the substitution of coordinated H_2O in CoMP8 in aqueous solution gave complex and largely irreproducible data [44]. Acetylation of the N-terminal amino group significantly simplified the solution chemistry, although the species was still prone to aggregation. This necessitated a careful study of the solution behavior of NAcMP8 prior to measuring the thermodynamics and kinetics of its ligand substitution reactions.

Equilibration in solution. A preliminary investigation showed that at 25 ^{o}C samples of NAcCoMP8 in deionized water at neutral pH exhibited time-dependent and largely irreproducible changes in their UV-vis spectrum. This prompted a detailed assessment of its solution behavior. A 2 mL sample of freshly prepared NAcCoMP8 (*ca*. 1.25 μM; pH 6.89;

degassed deionized water, 25 ^{o}C) was transferred into an argon-filled 1.0 cm pathlength cuvette fitted with a septum and its UV-vis spectrum was recorded every 5 minutes for an hour. This procedure was repeated with a higher NAcCoMP8 concentration (*ca*. 10.0 μM).

At the lower concentration, there was a small blue shift in the Soret band from 417 to 415 nm with poorly defined isosbestic points at 417 and 418 nm, suggesting the presence of multiple equilibria (Figure S2 of the Supplementary Information). The 415 nm species, once formed, remained stable over several hours of observation. That there are multiple equilibria is supported by the observation that the kinetics of these spectroscopic changes showed distinct wavelength dependence. We measured apparent half-lives for the equilibration of a solution of NAcCoM8 to be either 9 min or 23 min when observed at 407 and 425, respectively. Thus, two hours at neutral pH is sufficient time to allow a dilute aqueous solution of NAcCoMP8 at 25 ^{o}C to equilibrate to the spectroscopically stable 415 nm species.

By contrast, at the higher concentration the Soret band underwent a blue shift (417 → 425 nm) with a well-defined isosbestic point at 422 nm (Figure S3). There are also distinctive shifts in the Q band region (530 → 538 nm and 560 → 568 nm, respectively) with isosbestic points observed at 533, 554, 570 nm (Figure S3, inserts). The appearance of the 425 nm species and the decay of the 417 nm species followed apparently simple first order kinetics with a half-life of between 7.2 and 8.0 min. Once formed, the 425 nm species does not revert to the 417 nm (or the 415 nm species) on dilution with water or methanol. On standing overnight, the compound precipitates from solution.

Effect of light. NAcCoMP8 is moderately light-sensitive. Exposure to ambient light over a period of three days causes a decrease in its UV-vis absorbance which appears to follow simple first order kinetics with a half-life of approximately 8 h. The final product is pale yellow with a green tinge, typical of a bilin [62]. Cobalt porphyrins are known to be light sensitive [63, 64] and are reported to undergo slow photo-oxidation [65].

Sensitivity to oxygen. Bubbling a slow stream of O_2 through a solution of NAcCoMP8 in deionized water (*ca*. 1.25 μM) causes a 10% decrease in the absorbance of the Soret band within 10 min and a 16% decrease after 1 hour. The sample has a distinct yellow-orange color that is typical of the porphyrin decomposition product, bilirubin [62].

Effects of Ionic Strength. An increase in ionic strength is known to promote aggregation in some metalloporphyrins [62, 66-68] and the microperoxidases [9]. The effect on solutions of deaerated (Ar) NAcCoMP8 (1.25 μM and 10.0 μM, respectively; pH 7; 25 ^{o}C) was investigated by addition of aliquots of 5 M $NaClO_4$ up to an ionic strength of 4 M and the UV-vis spectrum was monitored. The solution with the lower NAcCoMP8 concentration (equilibrated at the start to produce the 415 nm species, see above) showed a red shift of the Soret band (415 → 417 nm), which is accompanied by a very significant decrease in its intensity (Figure 2A) without any discernible isosbestic points. At the higher concentration of NAcCoMP8, there is a red shift and decrease in the intensity of the Soret band (417 → 425 nm) with a well-defined isosbestic point at 422 nm (Figure 2B). There are also distinctive shifts in the Q band region (530 → 538 nm and 560 → 568nm, respectively) with isosbestic points observed at 533, 554, 570 nm. The final solution was yellow-orange.

There are clearly different processes occurring at low and at high concentrations of NAcCoMP8, respectively. The change in intensity of the Soret in each case is distinctly sigmoidal (inserts to Figures 2A and 2B). If we assume that the 415 nm species is monomeric NAcCoMP8 (it obeys Beer's law, see below), then the simplest explanation for the observed variation in the absorbance is a dimerization process consequent on the binding of *n* cations (eqn. 1 where M is monomeric NAcCoMP8 and D is a dimer; monomeric NAcMP8 carries a net charge of –3 at neutral pH).

$$2\mathrm{M} + n\mathrm{L}^{+} \xrightarrow{K_1} \mathrm{D} \qquad (1)$$

The absorbance A_λ at a monitoring wavelength λ is given by eqn. 2 where A_m is the absorbance of the m^{th} species, and α_m is the fraction of that species in solution; α_M and α_D are given by eqns. 3 and 4, respectively.[35]

$$A_\lambda = \sum_{m=1}^{2} A_m \alpha_m \qquad (2)$$

$$\alpha_M = \frac{-1 + \sqrt{1 + 8K_1[L]^n}}{4K_1[L]^n} \qquad (3)$$

$$\alpha_D = \frac{1\text{-}[M]}{2} \qquad (4)$$

A non-linear least squares fit of the data obtained at low NAcCoMP8 concentration to eqn. 2 (Figure 2A, inset) gave a very good fit (r^2 = 0.998) with K_1 = (0.080 ± 0.008) M^{-4} and n = 3.4 ± 0.3. It is appreciated that the process in eqn. 1 is a minimal explanation for the experimental observations; the absence of well-defined isosbestic points (Figure 2A) means that in reality the process is likely to be more complex and may involve multiple intermediate species.

The ionic strength-dependent process that occurs at the higher NAcMP8 concentration is likely to be that of the aggregation of two dimers since the spectroscopic end product in the low concentration study is the same as the starting product in the high concentration study (i.e., the 417 nm species). If it is assumed that the dimeric species D forms a tetramer T on the further binding of j cations (eqn. 5), then eqn. 2 is applicable, where α_D and α_T are now given by eqns. 6 and 7, respectively. The equations give an excellent fit to the data (r^2 = 0.9998, insert, Figure 2B) with K_2 = (2.19 ± 0.09) M^{-5} and j = 3.7 ± 0.1.

$$2\mathrm{D} + j\mathrm{L}^+ \xrightarrow{K_2} \mathrm{T} \qquad (5)$$

$$\alpha_D = \frac{-1+\sqrt{1+8K_2[L]^j}}{4K_2[L]^j} \qquad (6)$$

$$\alpha_{T_L} = \frac{1\text{-}[D]}{2} \qquad (7)$$

In summary, the spectrum of NAcCoMP8 is strongly dependent on ionic strength. The simplest model consistent with the experimental data posits two equilibria. The first is the dimerization of monomeric NAcCoMP8 provided its concentration < *ca.* 1.25 μM (Soret 415 → 417 nm) which is driven by the binding of about 3 cations (n = 3.4 ± 0.3); at higher concentrations of NAcCoMP8 (*ca.* 10 μM), the equilibrium species at low ionic strength is the 417 nm dimer. This forms a tetrameric species (417 → 425 nm) on increasing the ionic strength, driven by the binding of approximately 4 cations (j = 3.7 ± 0.1).

There are clear similarities in the behavior of NAcCoMP8 when its solutions are allowed to equilibrate in pure water (see above) and when the ionic strength of the environment is increased. At high NAcCoMP8 concentrations (ca. 10 μM) there is in both cases an irreversible hypochromic red-shift in the Soret from 417 to 425 nm with a well-defined isosbestic point at 422 nm. Once formed, the 425 nm species does not revert back on dilution with methanol or other dispersing agents such as detergents; it precipitates overnight from solution. This suggests that the 425 nm species (putatively a tetramer) involves interaction between the metal of one dimeric species and the polypeptide side-chain of

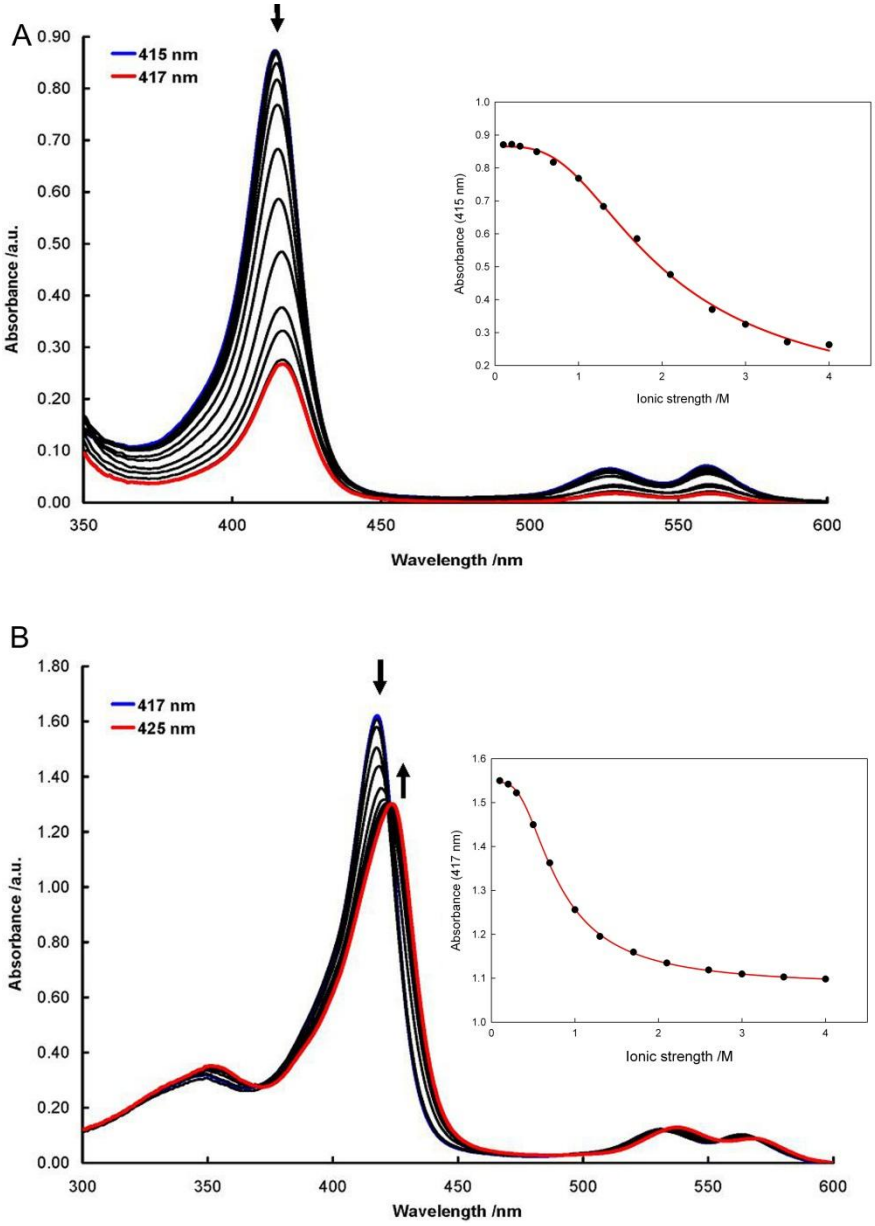


Figure 2. The variation of the UV-vis spectrum of NAcCoMP8 (neutral aqueous solution, 25 ^{o}C) with ionic strength. A: 1.25 μM NAcCoMP8. B: 10 μM NAcCoMP8. The inserts show fits to eqn. 2 of the text.

another. The diminished electrostatic repulsions due to charge neutralization on the porphyrins by the binding of cations facilitates the formation of π-π oligomers [9, 45, 69]. In a previous study on NAcFeMP8, two successive equilibria were observed as a function of ionic strength which were attributed to formation of, in the first instance, a dimer, and in the second a more tightly packed π-π dimer [9].

Our results show that to prepare a stable monomeric species of NAcCoMP8 it is not sufficient to have it at a low concentration but that low ionic strength is also required. In deionized water at 25 ^{o}C, solutions of NAcCoMP8 obey Beer's Law up to concentrations of 4.5×10^{-6} M; at higher concentrations, the Soret moves to 417 nm and deviation from Beer's Law is evident.

The Effect of Temperature. The thermal stability of NAcCoMP8 in deionized water was assessed by monitoring the UV-vis spectrum of firstly a 1.25 μM solution and secondly a 10 μM solution between 10 and 50 ^{o}C. At the lower concentration the Soret shifted from 415 to 417 nm as the temperature was increased; this was accompanied by a systematic decrease in absorbance, together with band broadening. The 417 nm species present at 50 ^{o}C reverted back to the 415 nm species when diluted with 50% methanol. At the higher NAcCoMP8 concentration the 415 nm species present at 10 ^{o}C shifted to the 417 nm species when the temperature reached 20 ^{o}C, and to the 425 nm species (with a well-defined isosbestic point at 420 nm) by 45 ^{o}C. Thus, monomeric NAcMP8 is favored at low temperature and an increase in temperature promotes its aggregation.

The Acid Dissociation Constants of NAcCoMP8. A spectrophotometric titration between pH 1 and 13 in a multi-component buffer was conducted as a function of temperature (10 – 35 ^{o}C) at constant ionic strength (0.1 M, $NaNO_3$) by titrating NAcCoMP8 (40 mL; 8 μM) with negligible volumes of either concentrated sulfuric acid or 8 M NaOH. The observed spectroscopic changes are shown in Figure S4 of the Supplementary Information.

As the solution becomes acidic, the Soret band shifts from 415 to 410 nm and broadens with a possible isosbestic point at 413 nm. The Q_0 and Q_1 bands shift from 561 and 532 nm to 559 and 526 nm, respectively, with reasonably well-defined isosbestic points at 550, 570 and 590 nm. In alkaline solution the Soret band shifts from 415 to 418 nm and the Q-bands become more intense and move to longer wavelengths (Q_0 and Q_1 shift from 559

and 526 nm at pH 7 to 567 and 535 nm, respectively, at pH 13) and gain in intensity. The variation in absorbance at 415 nm across the entire pH range is shown in Figure 3. This was fitted to eqn. 1 and required a minimum of five pK_as (solid line, Figure 3, r^2 = 0.999): 1.74 ± 0.04; 4.08 ± 0.02; 5.80 ± 0.05; 7.81 ± 0.03; and 12.00 ± 0.03. These were assigned by analogy with those of ferric microperoxidase, NAcFeMP8 [9]. The pK_a at 1.74 ± 0.04 (which may consist of more than one closely overlapping pK_as) is attributed to protonation and displacement of His-18 and its replacement in the coordination sphere of the metal either by the C-terminal carboxylate, the side-chain of Glu-21, or H_2O (in NAcFeMP8 two pK_as at 2.1 ± 0.2 and 3.12 ± 0.7 are observed). Two pK_as at 4.08 ± 0.02; 5.80 ± 0.05 are due to ionization of the heme carboxylate groups (*cf.* 4.95 ± 0.07 and 6.1 ± 0.3 in the Fe(III) system). The pK_a at 7.81 ± 0.03 is due to ionization of coordinated H_2O. In NAcFeMP8 this occurs at 9.59 ± 0.01. The greater acidity of Co(III) compared to Fe(III) is consonant with the latter's smaller ionic radius (0.545 Å for low spin Co(III) compared to 0.645 Å for high

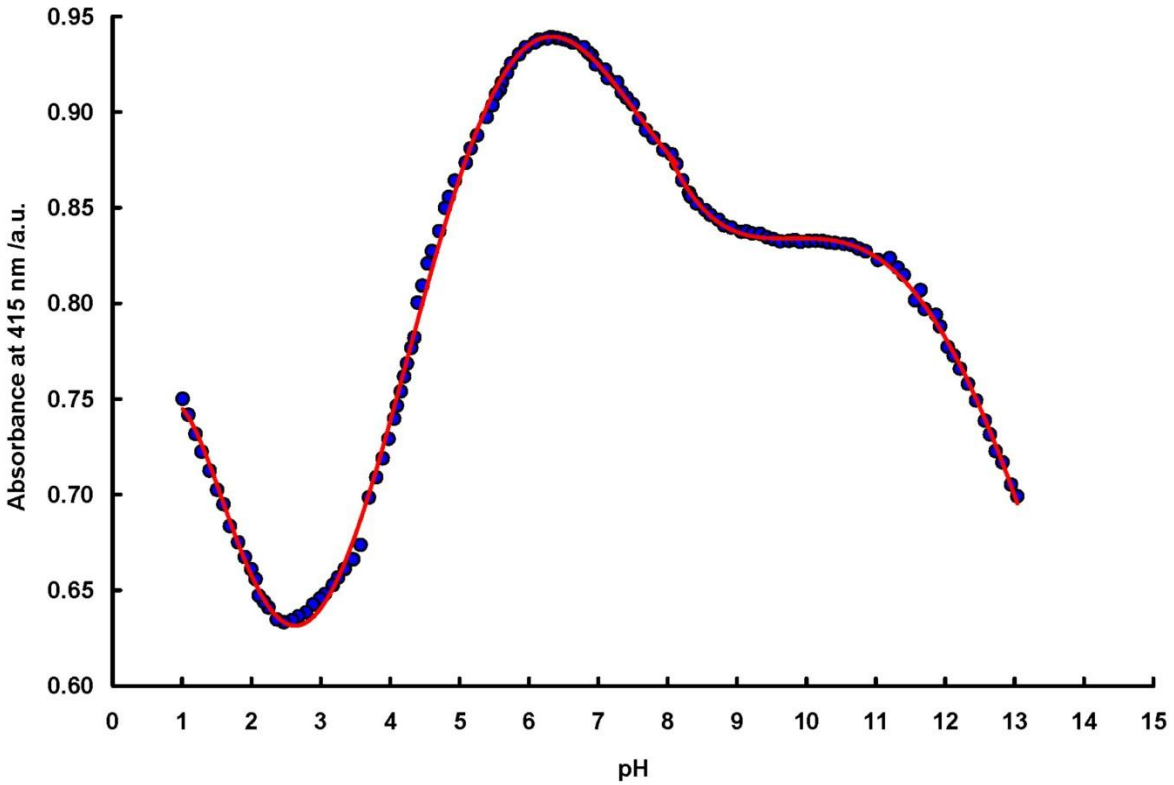


Figure 3. The change in absorbance of 1.25 μM NAcCoMP8 monitored at 415 nm as a function of pH. Fit to the data required a minimum of five acid dissociation constants (see text).

spin Fe(III) [70]). A pK_a of 7.81 at μ = 0.1 M is not dissimilar to that observed in H_2OCbl^+, viz., 8.1 in 1.0 M KCl [71, 72] and 7.462 ± 0.007 at μ = 1.5 mM [73]. The pK_a at 12.00 ± 0.03 is due to ionization of His-18 to form an imidazolate (12.71 ± 0.05 in NAcFeMP8 [9] and 12.40 ± 0.03 in NAcFeMP11 [48]). Figure 4 gives a visual summary of these assignments.

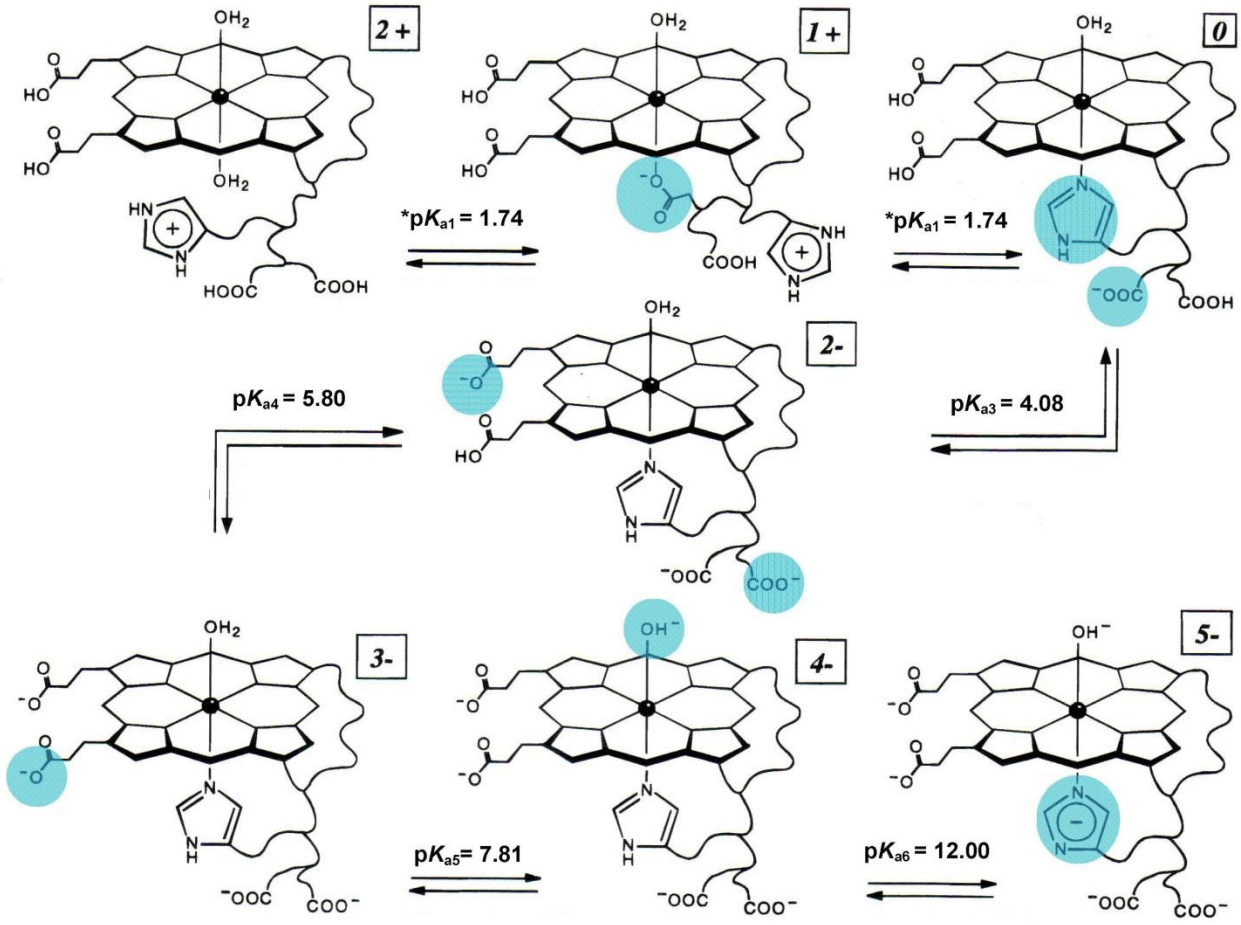


Figure 4. The acid dissociation constants of NAcCoMP8 from a spectrophotometric titration monitored at 415 nm. *A single pK_a is discernible below pH 2. There may be more than one overlapping pK_as. It is uncertain whether this corresponds to displacement of His-18 by the side-chain of Glu-21 (as in NAcFeMP8), or by water

The Temperature Dependence of the Ionization of Coordinated H_2O in NAcCoMP8. Knowledge of the temperature dependence of the pK_a of coordinated H_2O is important in order to correct equilibrium and rate constants for the reaction of NAcCoMP8 with

exogenous ligands for the effect of pH; without this a meaningful comparison cannot be made with analogous data for H_2OCbl^+.

Two factors compromised the results we obtained. Firstly, to ensure that we were dealing with the monomeric, 415 nm species, low concentrations of NAcCoMP8 had to be used; this inevitably meant that the spectroscopic data were noisier than might have been hoped for. Secondly, there is a relatively small change in the spectrum on ionization of the axially coordinated H_2O (Figure S5 of the Supplementary Information); this is in contrast to B_{12a} itself, where the spectroscopic changes are quite marked, or ferric NAcFeMP8 where ionization is accompanied by a spin change of Fe(III). In NAcCoMP8 the ionization occurs with a shift of the Soret band from 415 nm to 418 nm with an increase in its intensity. Above pH 9.1, the intensity of the Soret decreases and it undergoes a red shift as His-18 ionizes to form an imidazolate.

In order to average out the noise, the spectral changes at each 1 nm interval about 10 nm on either side of the isosbestic point near 415 nm were fitted between pH 6 and 9 to eqn. 1 with a single pK_a. The values obtained from the typically 15 to 20 such fits (Figure S5 shows an example) at each temperature were then averaged, with each value weighted as the reciprocal of the relative percentage of the standard error of the estimate. The results are listed in Table 1.

Table 1. The pK_a of coordinated H_2O in NAcCoMP8 as a function of temperature.

Temp /^{o}C	**pK_a**	**Temp /^{o}C**	**pK_a**
10.0	7.51(5)	30.1	7.81(3)
15.0	7.60(6)	34.9	7.27(3)
20.0	7.66(9)	39.5	7.41(3)
25.0	7.51(2)		

There is no clear dependence of pK_a on temperature; this suggests that the temperature dependence is small compared to the uncertainty in its measurement. We therefore used an average value, *viz.*, 7.5, for the acid dissociation constant of coordinated H_2O in NAcCoMP8.

4. Conclusions

The peptic digestion of cytochrome *c* yields the hemeundecapeptide FeMP11; treatment with acetic anhydride produces the acetylated hemepeptide NAcFeMP11 which was reductively demetallated and Co inserted into the porphyrin. Tryptic digestion of this gives the cobalt porphyrin octapeptide NAcCoMP8. NAcCoMP8 is moderately sensitive to light and oxygen and undue exposure to either causes bleaching of the spectrum. In neutral aqueous solution NAcCoMP8 exists as a monomeric species at low concentration with a Soret band at 415 nm. Under these conditions Beer's Law is obeyed up to at least concentrations of 4.5 μM. At higher concentrations it forms a transient dimeric species with a Soret 417 nm which can lead to further aggregation and formation of a species with a Soret at 425 nm. Once formed, that species does not revert back to the 417 or 415 nm species on dilution with water or methanol and, on standing, may precipitate from solution. Increasing the ionic strength of a 1.25 μM solution of NAcCoMP8 causes the transformation of the 415 nm species into the 417 nm species; the spectroscopic data for this process is consistent with the formation of a dimer on binding 3.4 ± 0.3 cations from solution. A solution of 10 μM NAcCoMP8 is largely present at the 417 nm species; increasing its ionic strength causes its dimerization to the 425 nm species with binding of 3.7 ± 0.1 cations. The monomeric 415 nm species is favored at low temperature (10 ^{o}C) and increasing the temperature above 45 ^{o}C produces the 425 nm species. NAcCoMP8 has five spectroscopically observable pK_as at 1.74 ± 0.04 (which may consist of more than one closely overlapping pK_a) is attributed to protonation and displacement of His-18 either by H_2O or a carboxylate from the peptide; at 4.08 ± 0.02 and 5.80 ± 0.05 due to ionization of the heme carboxylate groups; at 7.81 ± 0.03 is due to ionization of coordinated H_2O and at 12.00 ± 0.03 due to ionization of His-18 to form an imidazolate. The pK_a for the ionization of coordinated H_2O does not change significantly with temperature between 10 and 40 ^{o}C, and has an average value of 7.5 ± 0.2 in this temperature range.

Acknowledgements

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References

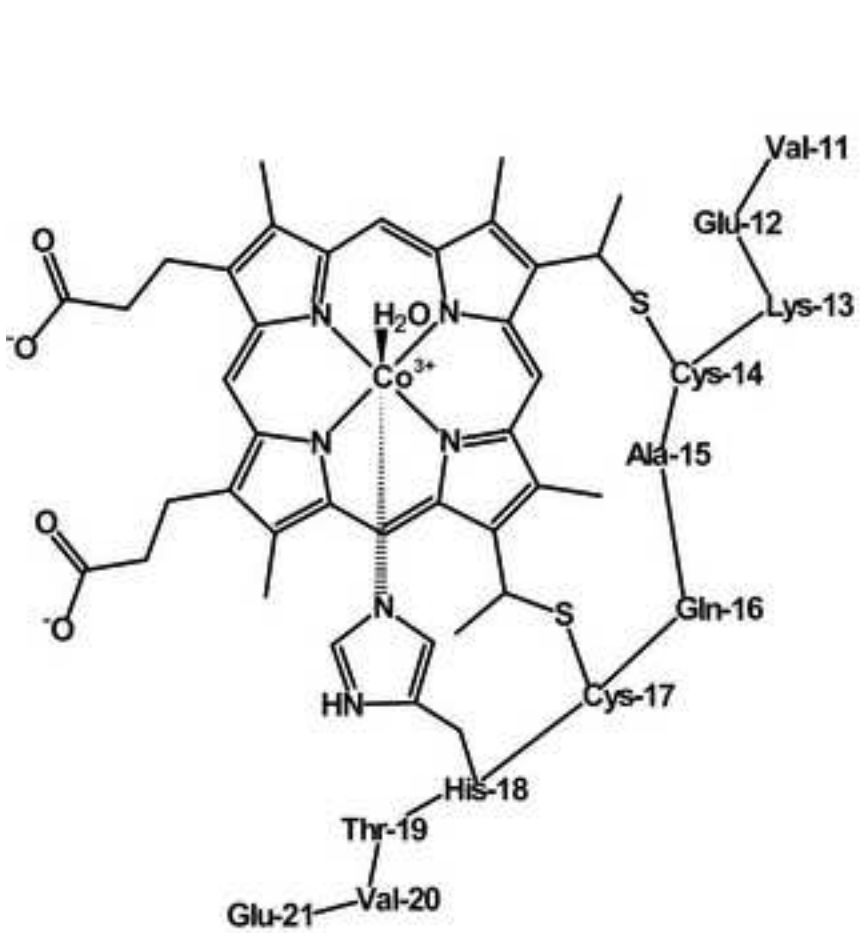
[1] C.L. Tsou, Nature, vol. 164, 1949, pp. 1134.
[2] C.L. Tsou, Biochem. J., vol. 49, 1951, pp. 362.
[3] H. Tuppy, S. Paleus, Acta Chem. Scand., vol. 9, 1955, pp. 353.
[4] A. Ehrenberg, H. Theorell, Acta Chem. Scand., vol. 9, 1955, pp. 1193.
[5] H.A. Harbury, P.A. Loach, J. Biol. Chem., vol. 12, 1960, pp. 3640-3645.
[6] P.A. Adams, D.A. Baldwin, H.M. Marques, in: R.A. Scott, A.G. Mauk (Eds.), Cytochrome c: A Multidisciplinary Approach, University Science, Mill Valley, CA, 1996, pp. 635-692.
[7] H.M. Marques, Dalton Trans., 2007, pp. 4371-4385.
[8] E.K. Yang, K. Sauer, in: C. Ho (Ed.), Electron Transport and Oxygen Utilization, Elsevier, North Holland, 1982, pp. 89-94.
[9] O.Q. Munro, H.M. Marques, Inorg. Chem., vol. 35, 1996, pp. 3752-3767.
[10] J. Aron, D.A. Baldwin, H.M. Marques, J.M. Pratt, P.A. Adams, J. Inorg. Biochem., vol. 27, 1986, pp. 227-243.
[11] D.A. Baldwin, H.M. Marques, J.M. Pratt, J. Inorg. Biochem., vol. 27, 1986, pp. 245-254.
[12] M.T. Wilson, R.J. Ranson, P. Masiakowski, E. Czarnecka, M. Brunori, Eur. J. Biochem., vol. 77, 1977, pp. 193-199.
[13] T. Shimizu, T. Nozawa, M. Hatano, J. Biochem. (Tokyo), vol. 91, 1982, pp. 1951-1958.
[14] H.M. Marques, D.A. Baldwin, J.M. Pratt, J. Inorg. Biochem., vol. 29, 1987, pp. 77-91.
[15] A.B.M. Sadeque, T. Shimizu, M. Hatano, Inorg. Chim. Acta, vol. 135, 1987, pp. 109-113.
[16] J.W. Owens, C.J. O'Connor, R.J. Kassner, Inorg. Chim. Acta, vol. 151, 1988, pp. 107-116.
[17] M.M.M. Saleem, M.T. Wilson, Inorg. Chim. Acta, vol. 153, 1988, pp. 99-104.
[18] M. Byfield, J. Pratt, J. Chem. Soc., Chem. Commun., 1992, pp. 214-215.
[19] H.M. Marques, M.P. Byfield, J.M. Pratt, J. Chem. Soc., Dalton Trans., 1993, pp. 1633-1639.
[20] C.B. Perry, T. Chick, A. Ntlokwana, G. Davies, H.M. Marques, J. Chem. Soc., Dalton Trans., 2002, pp. 449-457.
[21] P.R. Vashi, H.M. Marques, J. Inorg. Biochem., vol. 98, 2004, pp. 1471-1482.
[22] J.E. Kim, M.A. Pribisko, H.B. Gray, J.R. Winkler, Inorg. Chem., vol. 43, 2004, pp. 7953-7960.
[23] J.-G. Ma, J.M. Vanderkooi, J. Zhang, S.-L. Jia, J.A. Shelnutt, Biochemistry, vol. 38, 1999, pp. 2787-2795.
[24] D.W. Low, S. Abedin, G. Yang, J.R. Winkler, H.B. Gray, Inorg. Chem., vol. 37, 1998, pp. 1841-1843.
[25] J.-L. Primus, M.G. Boersma, D. Mandon, S. Boeren, C. Veeger, R. Weiss, I.M.C.M. Rietjens, J. Biol. Inorg. Chem., vol. 4, 1999, pp. 274-283.
[26] T.P. Henning, T.G. Spring, in: WIPO (Ed.), USA, 1996.
[27] T.P. Henning, T.G. Spring, Proc. Proceedings - Electrochemical Society: 99-5 (New Directions in Electroanalytical Chemistry II)1999144-151.
[28] J.L. Primus, S. Grunenwald, P.-L. Hagedoorn, A.-M. Albrecht-Gary, D. Mandon, C. Veeger, J. Am. Chem. Soc., vol. 124, 2002, pp. 1214-1221.

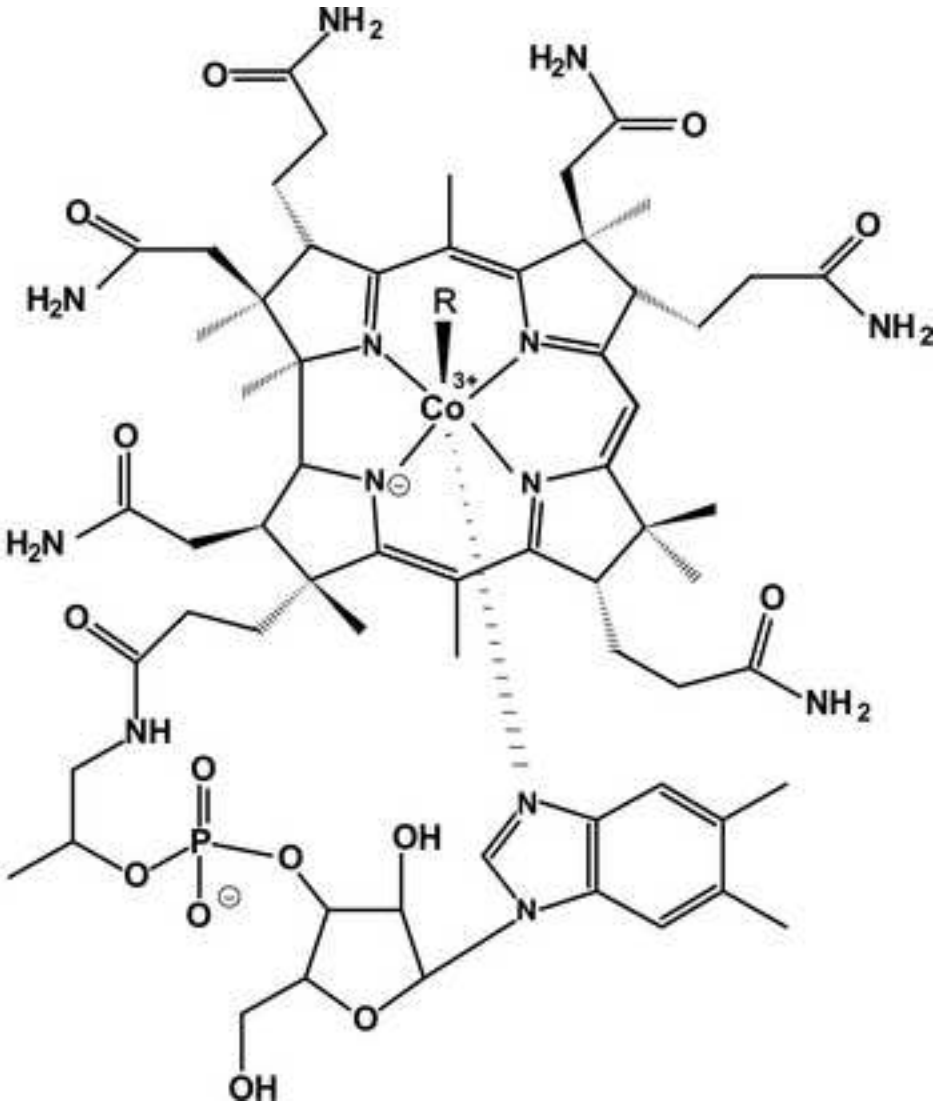
[29] M. Kobayashi, S. Shimizu, Eur. J. Biochem., vol. 261, 1999, pp. 1-9.
[30] S.M. Gaikwad, M.B. Rao, V.V. Deshpande, Enzyme Microb. Tech., vol. 14, 1992, pp. 317-320.
[31] S. Liu, J. Widom, C.W. Kemp, C.M. Crews, J. Clardy, Science vol. 282 1998, pp. 1324-1327.
[32] M. Paetzel, F. Danel, L. de Castro, S.C. Mosimann, M.G.P. MPage, N.C.J. Strynadka, Nature Struct. Biol., vol. 7, 2000, pp. 918-925.
[33] R. Banerjee (Ed.), Chemistry and Biochemistry of B_{12}, Wiley, New York, 1999.
[34] K.L. Brown, Chem. Rev., vol. 105, 2005, pp. 2075-2149.
[35] B. Kräutler, Biochem. Soc. Trans., vol. 33, 2005, pp. 806-810.
[36] J.R. Roth, J.G. Lawrence, T.A. Bobik, Annu. Rev. Microbiol., vol. 50, 1996, pp. 137-181.
[37] L. Knapton, H.M. Marques, Dalton Trans., 2005, pp. 889-895.
[38] E.A. Betterton, University of the Witwatersrand, Johannesburg, 1982.
[39] K.R. Ashley, M. Berggren, M. Cheng, J. Am. Chem. Soc., vol. 97, 1975, pp. 1422-1426.
[40] W.G. Jackson, S.S. Jurisson, B.C. McGregor, Inorg. Chem., vol. 24, 1985, pp. 1788-1790.
[41] C.K. Poon, Coord. Chem. Rev., vol. 10, 1973, pp. 1-35.
[42] K.L. Brown, S. Cheng, X. Zou, J.D. Zubkowski, E.J. Valente, L. Knapton, H.M. Marques, Inorg. Chem., vol. 36, 1997, pp. 3666-3675.
[43] C.B. Perry, M.A. Fernandes, K.L. Brown, X. Zou, E.J. Valente, H.M. Marques, Eur. J. Inorg. Chem., 2003, pp. 2095-2107.
[44] S. Mathura, vol. PhD, University of the Witwatersrand, Johannesburg, 2012.
[45] J.A. Shelnutt, M.M. Dobry, J.D. Satterlee, J. Phys. Chem., vol. 88, 1984, pp. 4980-4987.
[46] A.D. Carraway, M.G. McCollum, J. Peterson, Inorg. Chem., vol. 35, 1996, pp. 6885-6891.
[47] E.N. Kadnikova, N.M. Kostić, J. Org. Chem., vol. 68, 2003, pp. 2600-2608.
[48] H.M. Marques, C.B. Perry, J. Inorg. Biochem., vol. 75, 1999, pp. 281-291.
[49] K.R. Ashley, S. Au-Young, Inorg. Chem., vol. 13, 1976, pp. 1937-1939.
[50] M. Lin, L.G. Marzilli, Inorg. Chem., vol. 33, 1994, pp. 5309-5315.
[51] S. Othman, A. Le Lirzin, A. Desbois, Biochemistry, vol. 32, 1993, pp. 9781-9791.
[52] D.W. Urry, J.W. Pettegrew, J. Am. Chem. Soc., vol. 89, 1967, pp. 5276-5283.
[53] D.W. Urry, J. Am. Chem. Soc, vol. 89, 1967, pp. 4190-4196.
[54] A.D. Carraway, R.S. Burkhalter, R. Timkovich, J. Peterson, J. Inorg. Biochem., vol. 52, 1993, pp. 201-207.
[55] A. Carraway, S. Povlock, M. Houston, D. Johnston, J. Peterson, J. Inorg. Biochem., vol. 60, 1995, pp. 267-276.
[56] J.S. Wang, A.L. Tsai, J. Heldt, G. Palmer, H.E. Van Wart, J. Biol. Chem., vol. 267, 1992, pp. 15310-15318.
[57] J.S. Wang, H.E. Van Wart, J. Phys Chem., vol. 93, 1989, pp. 7925-7931.
[58] S.A. Kazmi, M.A. Mills, Z.W. Pitluk, R.A. Scott, J. Inorg. Biochem., vol. 24, 1985, pp. 9-12.
[59] D.C. Blumenthal, R.J. Kassner, J. Biol. Chem., vol. 255, 1980, pp. 5859-5863.
[60] R. Santucci, H. Reinhard, M. Brunori, J. Am. Chem. Soc., vol. 110, 1988, pp. 8536.

[61] V.J. Razumas, A.V. Gundavičius, J.D. Kazlauskaite, J.J. Kulys, J. Electroanal. Chem, vol. 271, 1989, pp. 155.
[62] L.R. Milgrom Journal (1997) Pages.
[63] R.F. Pasternack, M.A. Cobb, Biochem. Biophys. Res. Commun., vol. 51, 1973, pp. 507-511.
[64] S. Mosseri, P. Neta, A. Harriman, P. Hambright, J. Inorg. Biochem., vol. 39, 1990, pp. 93-100.
[65] K. Hatano, K. Usui, Y. Ishida, Bull. Chem. Soc. Japan, vol. 54, 1981, pp. 413-419.
[66] C. Endisch, J.H. Fuhrhop, J. Buschmann, P. Luger, U. Siggel, J. Am. Chem. Soc., vol. 118, 1996, pp. 6671-6680.
[67] L. Ruhlmann, A. Nakamura, J.G. Vos, J.H. Fuhrhop, Inorg. Chem., vol. 37, 1998, pp. 6052-6059.
[68] T. Sawaguchi, T. Matsue, K. Itaya, I. Uchida, Electrochim. Acta, vol. 36, 1991, pp. 703-708.
[69] W.I. White, R.A. Plane, Bioinorg. Chem., vol. 4, 1974, pp. 21-35.
[70] R.D. Shannon, Acta Cryst., vol. A32, 1976, pp. 751-767.
[71] W.W. Reenstra, W.P. Jencks, J. Am. Chem. Soc., vol. 101, 1979, pp. 5780-5791.
[72] H.M. Marques, K.L. Brown, D.W. Jacobsen, J. Biol. Chem., vol. 263, 1988, pp. 12378-12383.
[73] C.F. Zipp, S. Mathura, J.P. Michael, M.A. Fernandes, H.M. Marques, unpublished results.

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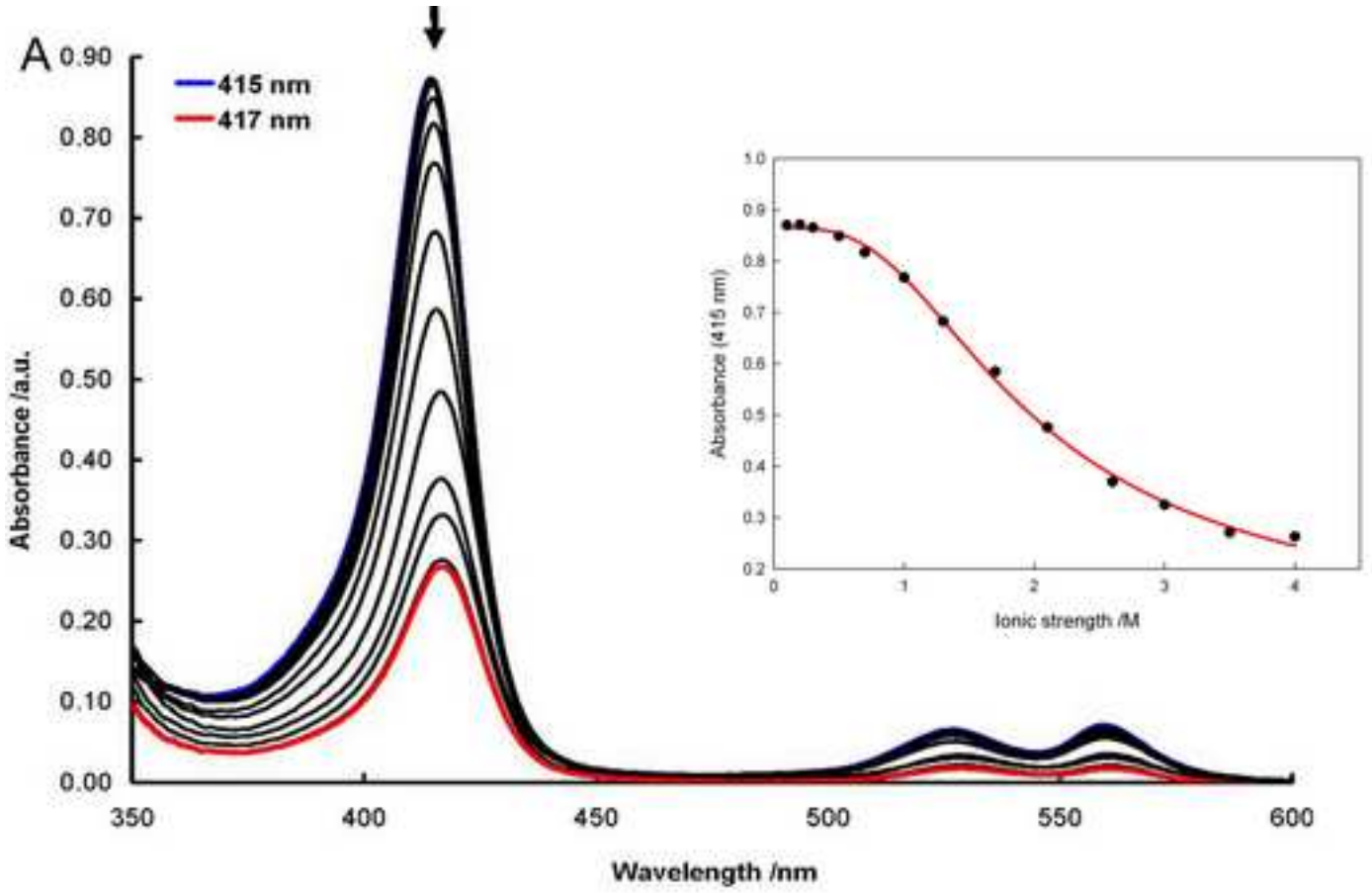
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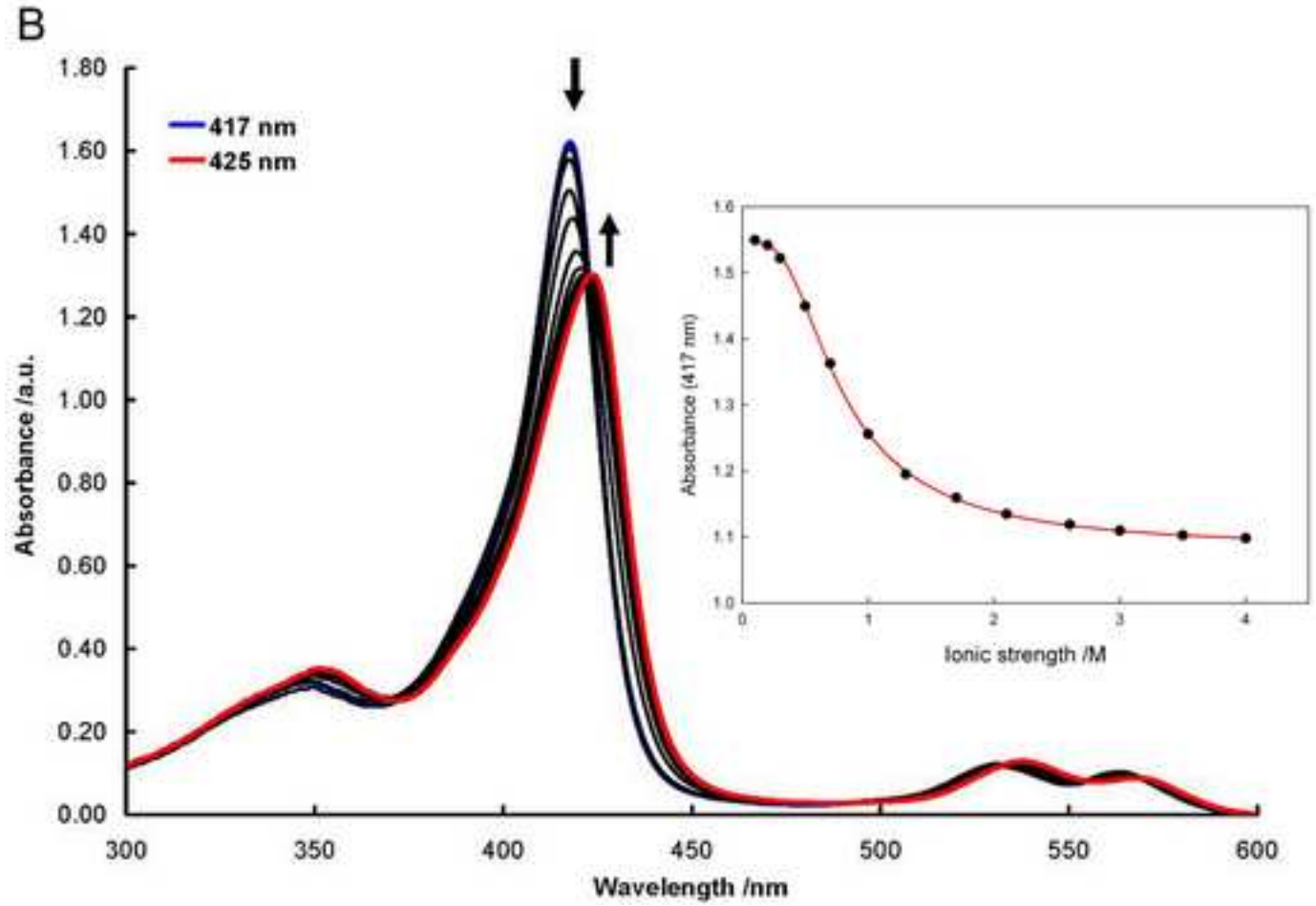




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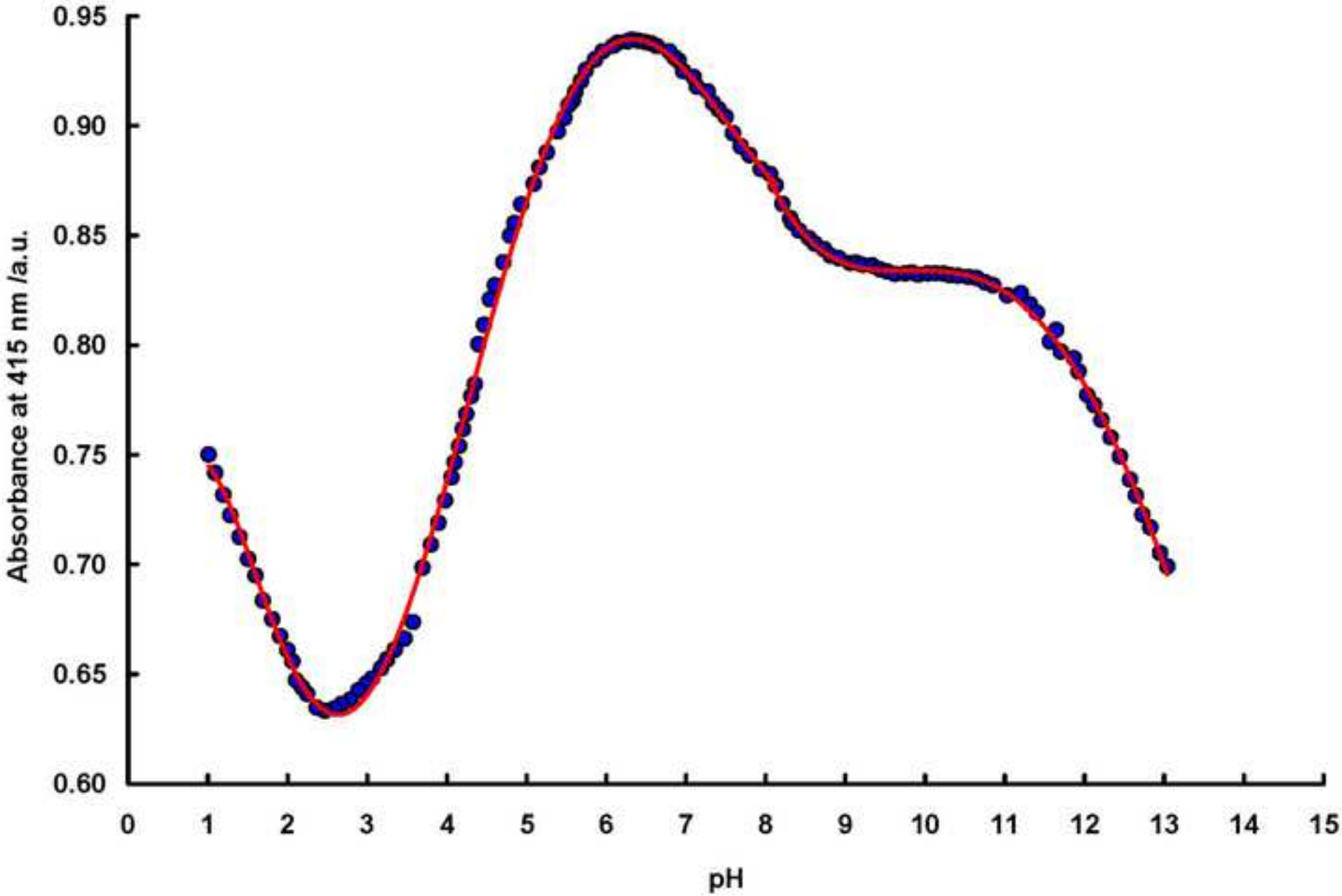
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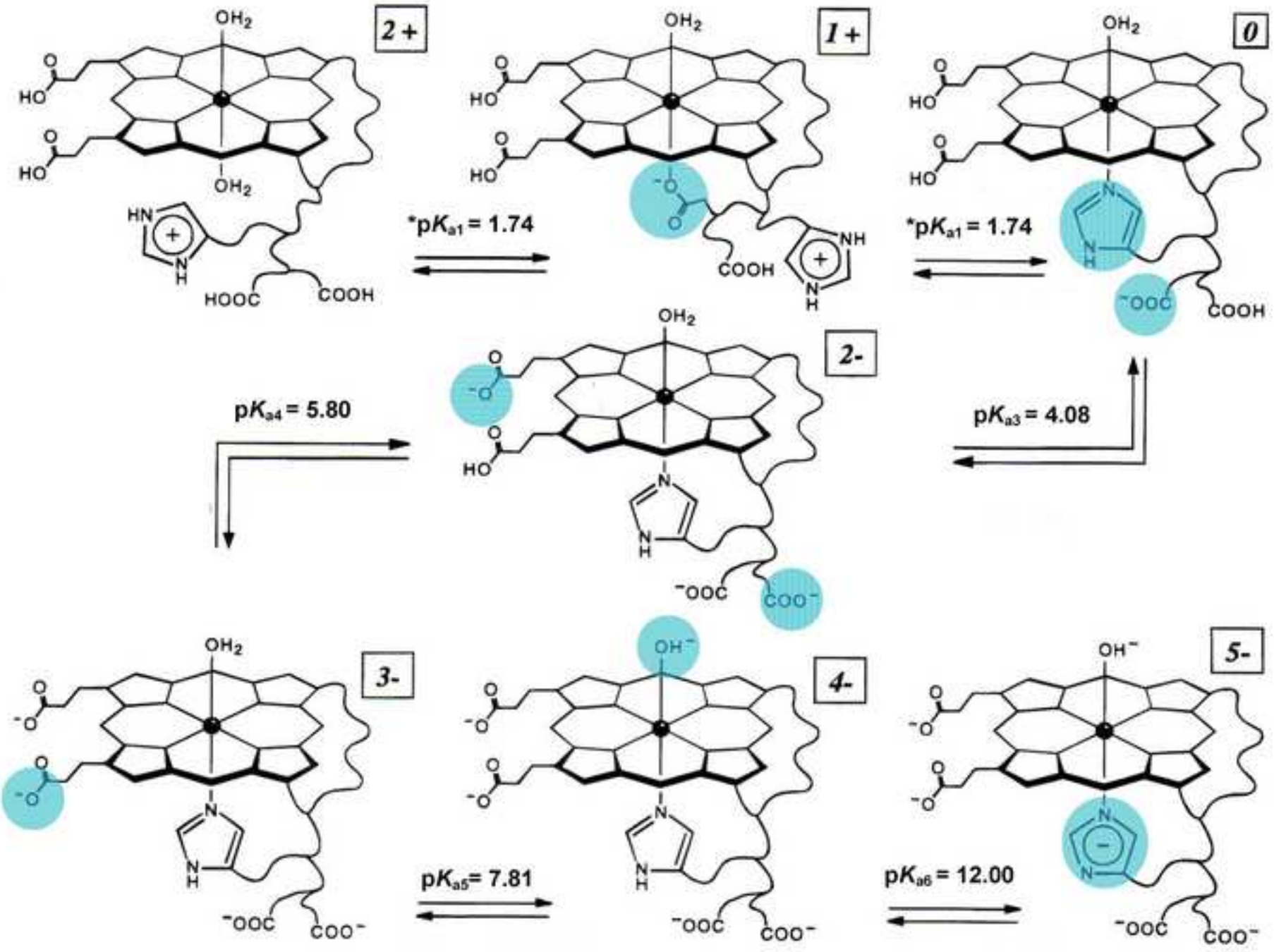
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The Preparation of N-Acetyl-Co(III) Microperoxidase-8 (NAcCoMP8) and its Solution Chemistry

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Supplementary Information

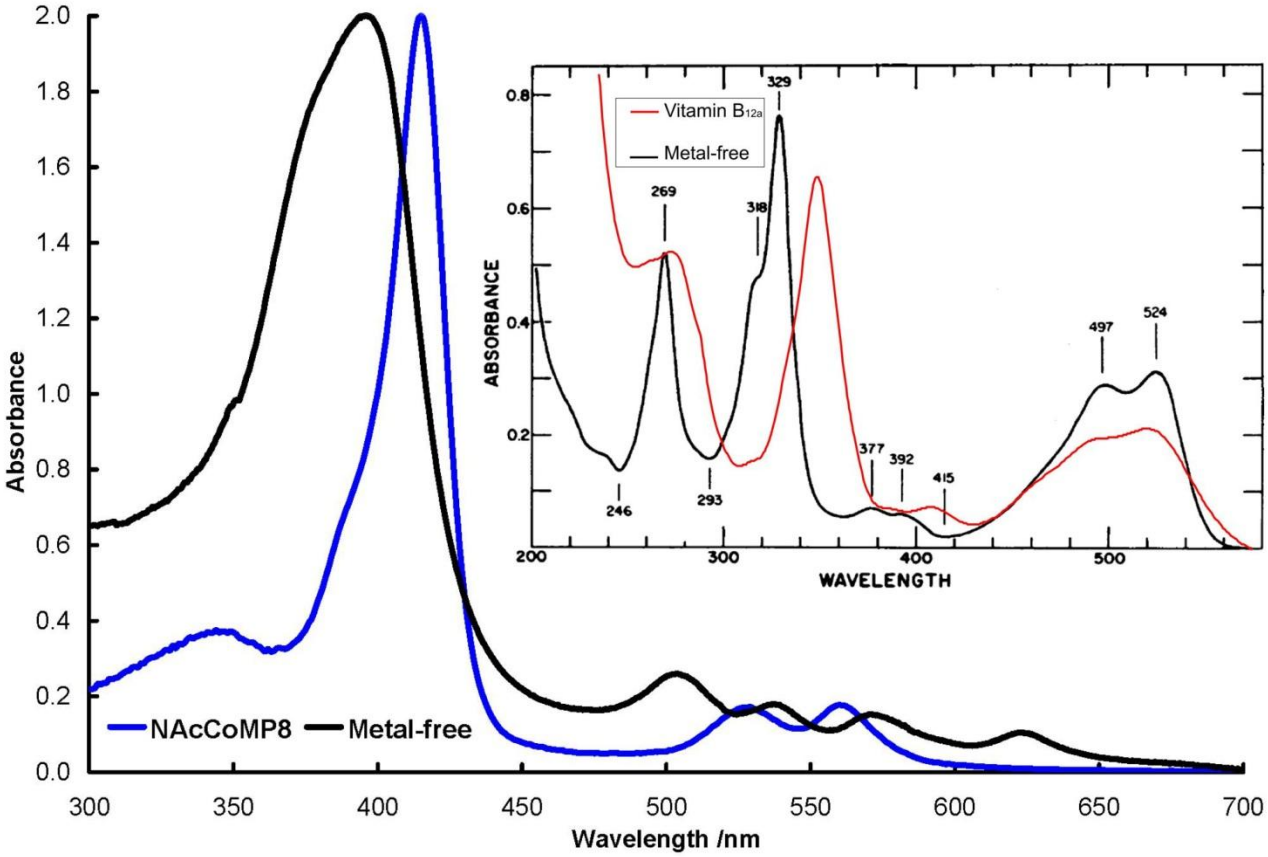


Figure S1. A comparison of absorbance spectra of metal-free NAcMP8 (black) and NAcCoMP8 (blue) in neutral aqueous solution. The insert shows (black) the spectrum of the metal-free corrinoid from *Chromatium*, adapted from Toohey [1] overlaid with the spectrum of H_2OCbl^+ (red). The concentration of the two species in each case is probably different.

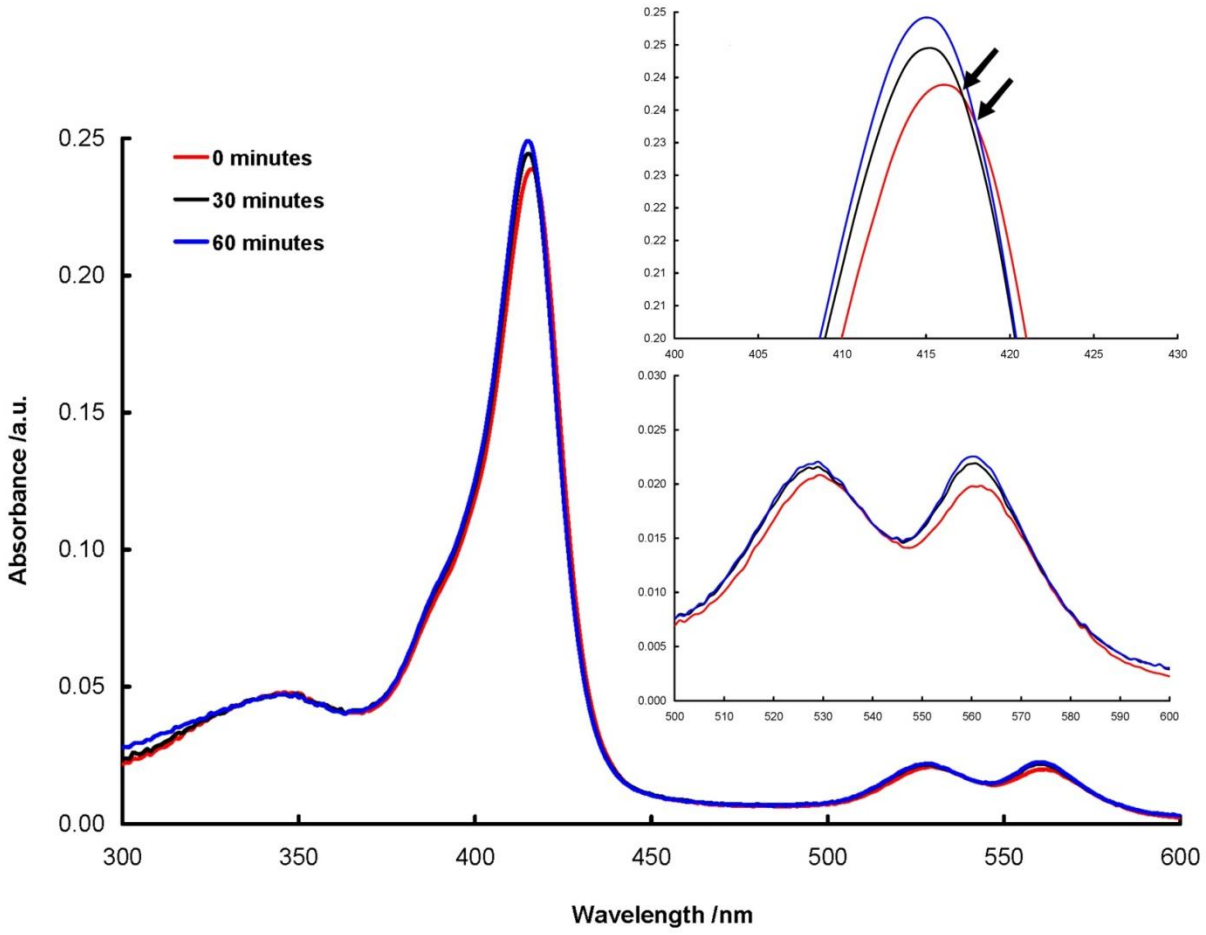


Figure S2. The effect of equilibration time on NAcCoMP8 at low (*ca*. 1.25 µM) concentration in deionised water pH 6.89.

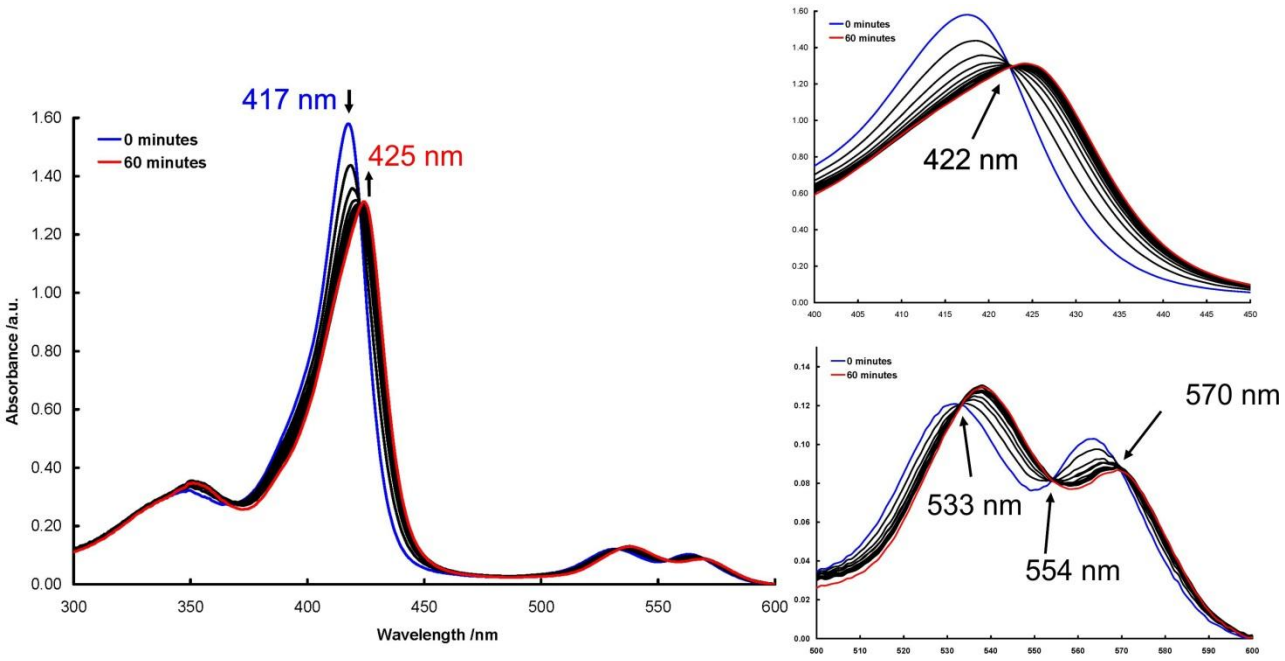


Figure S3. The effect of equilibration time on NAcCoMP8 at high (*ca*. 10 µM) concentration in deionised water pH 6.89.

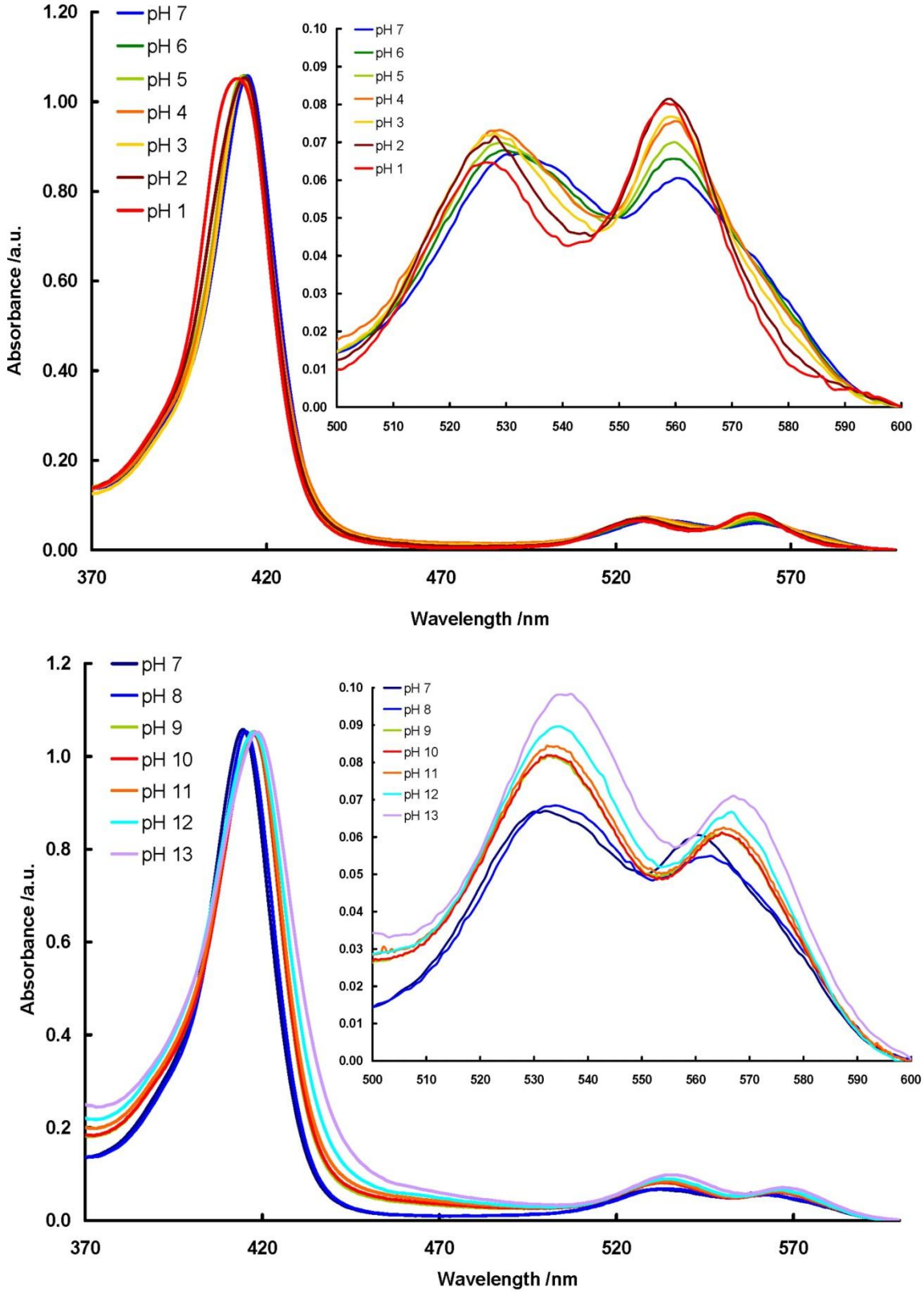


Figure S4. Spectroscopic changes observed in a 8 μM solution of NAcMP8 as a function of pH at 25 ^{o}C, μ = 0.1 M

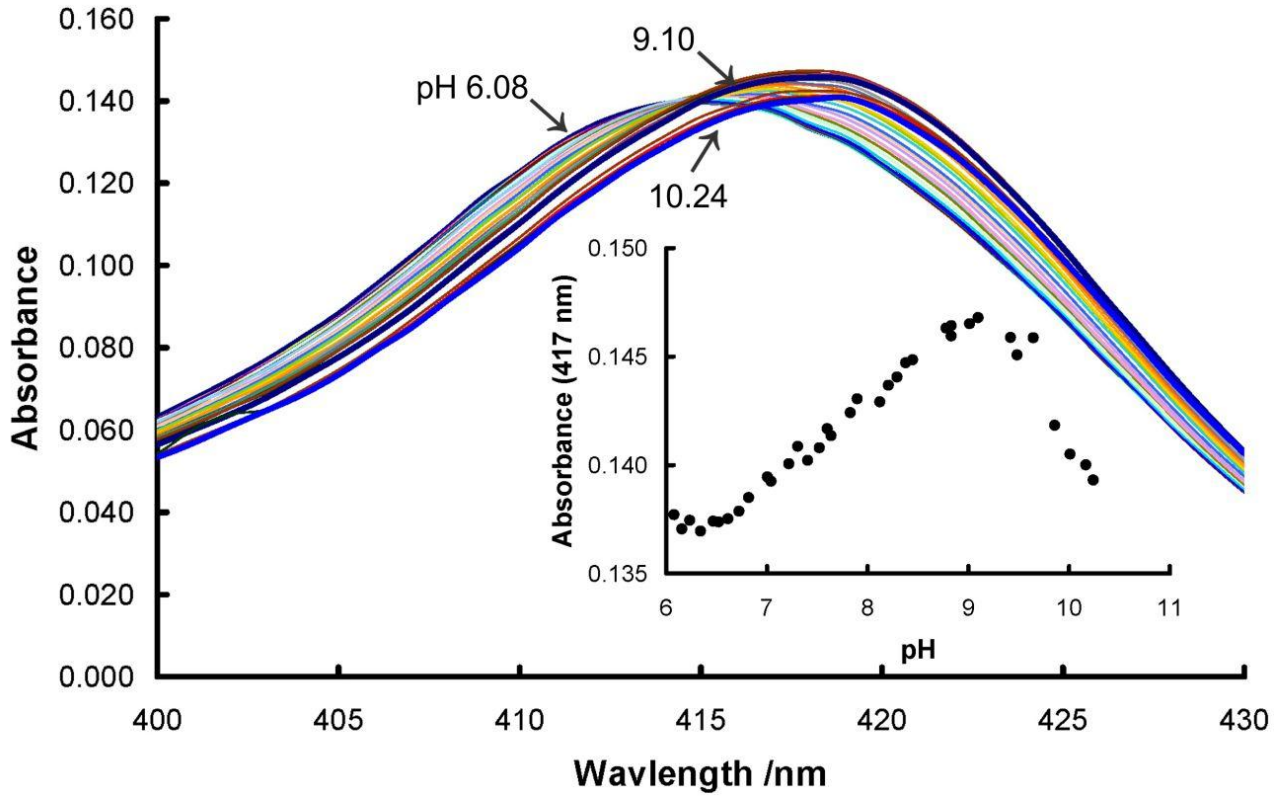


Figure S5. Example of the dependence of the Soret region of NAcMP8 on pH. The data were obtained at 30 ^{o}C. The insert shows the absorbance at 417 as function of pH.

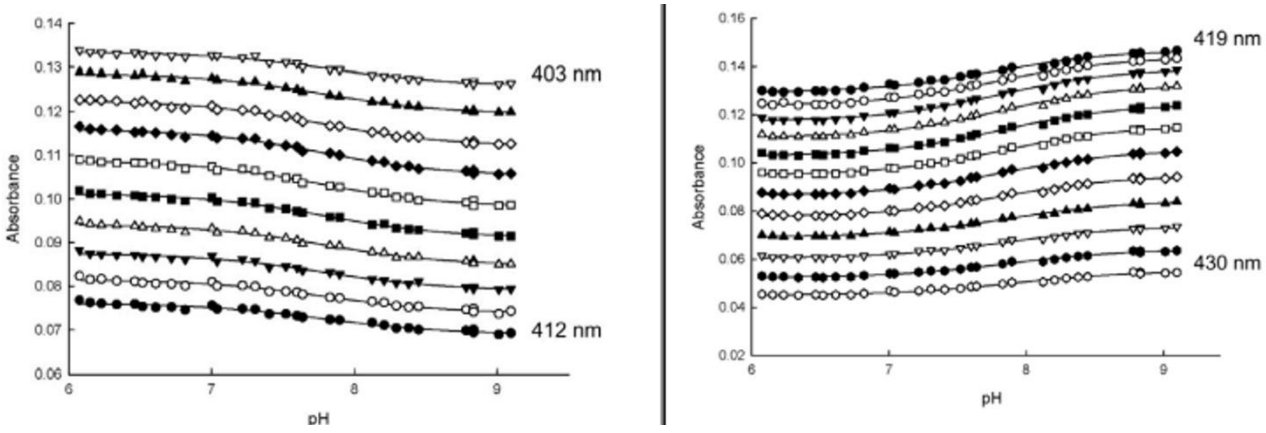


Figure S6. Fits of the change in absorbance between 403 and 412 nm, and between 419 and 430 nm, for NAcMP8 at 30 ^{o}C to an equation involving a single pK_a

Reference

[1] J. I. Toohey, Proc. Natl. Acad. Sci. U.S.A. , 54 (1965) 934.