A functional mimic of natural peroxidases: synthesis and catalytic activity of a non-heme iron/peptide hydroperoxide complex

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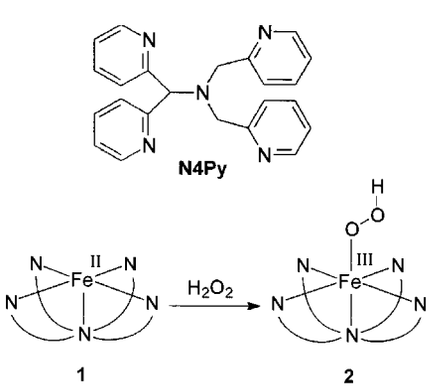
Site-selective attachment of unprotected peptides to a non-heme iron complex is achieved by displacing two halides on the catalyst by peptide caesium thiolates. This coupling approach should be compatible with any peptide sequence provided there is only a single reduced cysteine. The oxidation activity with hydrogen peroxide of the dipeptide–catalyst complex in water is retained, and shows similarities with oxidation mechanisms observed for natural oxidizing enzymes. The results pave the way for the future design of peroxidase mimics where the activity of the catalyst will be modulated by a designed protein matrix.

The field of *de novo* protein design, *i.e.* the design from first principles of protein sequences which fold into predicted structures, has recently matured sufficiently so that not only structure, but also the means for rudimentary function, can now be incorporated into designed proteins.[1] Function can arise solely from sequence, as in the case of synthetic ion channel proteins,[2] or from the incorporation of prosthetic groups such as hemes.[3] We intend to use the latter approach to mimic natural water-soluble peroxidases by designing and synthesizing functionalized proteins in which a protein matrix will eventually enclose, and modulate the activity of, a small molecule catalyst. The catalyst is a non-heme iron complex of a tetrapyridyl ligand ('N4Py') with Fe^{II} ('N4PyFe'; **1**)[4] which reacts with H_2O_2 to generate a purple-coloured species proposed to be an Fe^{III} hydroperoxide complex.

The catalytic oxidation of several organic compounds by this Fe^{III} hydroperoxide complex in acetone or methanol has been demonstrated.[4,5] Recent qualitative evidence indicated that the N4PyFe **1** complex retains at least some oxidative capacity when in aqueous media. To data, the *de novo* designed proteins which have been most successful (*i.e.* which have folded into predicted, uniquely packed structures) have been water-soluble, antiparallel four-helix bundles.[6] Superimposing the crystal structure of N4PyFe onto the backbone of α_2,[7] a *de novo* designed four-helix bundle, suggests (Fig. 1) that the size and shape of the catalyst is conducive to incorporation into a designed four-helix bundle matrix. Prior to embarking on cycles



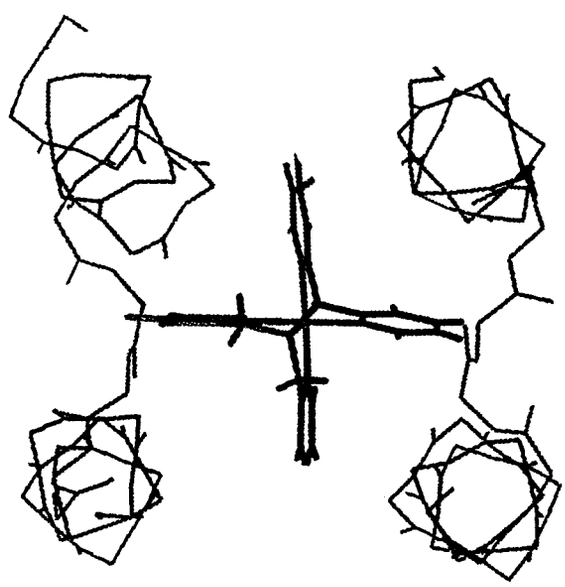


Fig. 1 A top view of the crystal structure of N4PyFe[4] centred on the backbone of the α_2[7] homodimer

of protein design and synthesis, it was necessary to ascertain whether (a) unprotected peptides can be controllably linked to specific sites on the catalyst and (b) if the N4PyFe^{III}OOH complex with covalently attached peptides retains activity.

We have previously[8] coupled unprotected peptides containing a single reduced cysteine to a dibromoacetamide-porphyrin *via* thiolate displacement of the bromides. Specific thioether linkage of the peptide to the porphyrin through the cysteine sulfur was generally attained in high yield; however, peptides containing histidine in addition to the unique cysteine were found to give a complex mixture of reaction products,[8] probably because histidine also acts as a nucleophile. We present here a simple and general strategy for the site-specific attachment of single cysteine unprotected peptides to a derivative of N4Py, and show that the catalyst–dipeptide complex retains full activity in water.

Results and discussion

The bis(chloromethyl)-substituted N4Py ligand **3** containing

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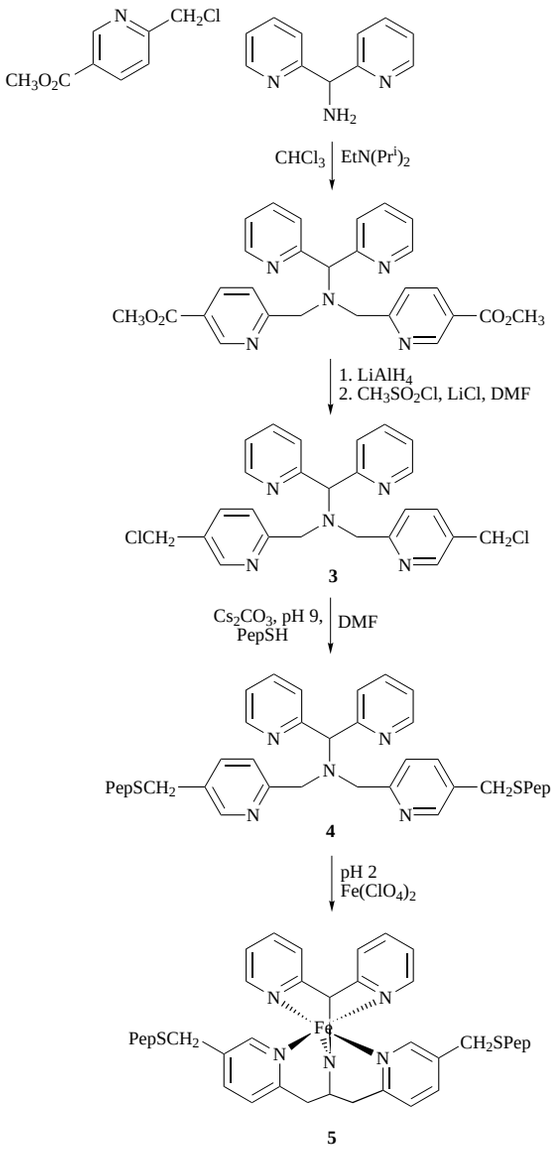
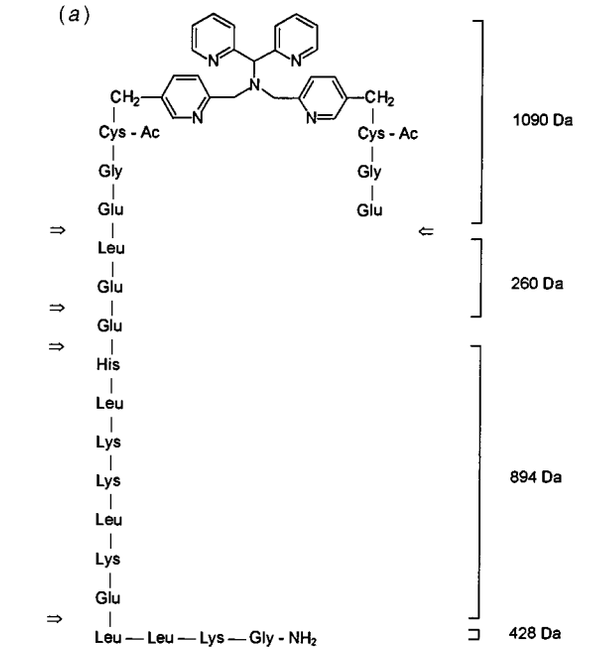
two peptide attachment sites, was synthesized in a three step sequence from bis(2-pyridyl)methylamine.[9] Two-fold alkylation with methyl 6-chloromethylnicotinate was followed by reduction of both ester groups and conversion of the resulting alcohol moieties into dichloro derivative **3** (Scheme 1).



Scheme 1 Summary of the synthesis of the dichloride-substituted tetrapyridyl ligand **3**, the iron-free ligand-dipeptide **4** and N4PyFe-dipeptide **5**

The catalyst–dipeptide complex was prepared by employing caesium thiolates ‡,[10] of single cysteine peptides to displace chlorides at positions C13 and C22[4] of an N4Py derivative (Scheme 1). These sites were chosen for the CH_2Cl 'handles' as this would position the peptides away from the N–Fe ligation sites and prevent interference with $Fe^{III}OOH$ formation. The sequence of the test peptide, Ac-C-G-E-L-E-E-H-L-K-K-L-K-E-L-L-K-G-NH_2, was chosen for its representative content of nucleophilic amino acid side chains (histidine, lysine, glutamic acid) and was not designed to interact with the catalyst in any way. In the presence of Cs_2CO_3, two equivalents of the unprotected test peptide were coupled *via* the *N*-terminal cysteine to the dichloride-substituted ligand **3** to afford the iron-free



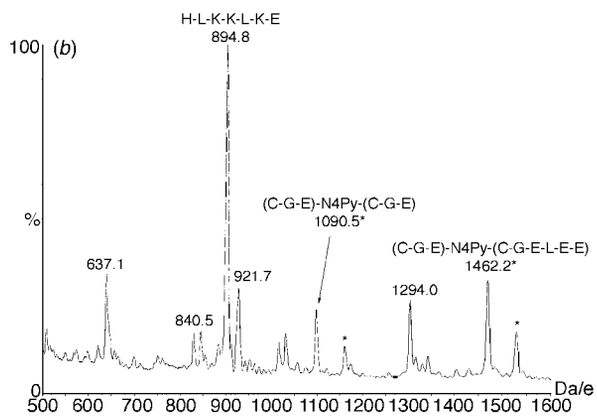


Fig. 2 (*a*) Schematic representation of **4**, showing the expected Protease V8 digestion sites and corresponding masses of the generated fragments; (*b*) MALDI–TOF spectrum of the **4** protease digest

ligand–dipeptide **4**. The reaction mixture was acidified and a slight molar excess of $Fe(ClO_4)_2 \cdot 6H_2O$ was added. A single pass over a reversed-phase HPLC column afforded N4PyFe-dipeptide **5** in approximately 95% purity as judged by reinjection onto the HPLC column and electrospray mass spectrometry.

That the peptides are attached to **3** solely through the cysteine residues was established unequivocally by digesting the test peptide and **4** with *Staphylococcus aureus* Protease V8, which cleaves peptide bonds specifically after glutamic acid residues. Incubation of **4** with the protease would generate specific fragments [Fig. 2(*a*)] identifiable by Edman degradation and mass spectrometry. Peptide fragments corresponding to the 894, 428 and 260 Da fragments were sequenced from the two digests and found to be present in similar relative amounts. Since the cysteine is acetylated, the C-G-E fragment from the test peptide digest was not detected by Edman degradation. Laser desorption mass spectrometry of the **4** digest [Fig. 2(*b*)] confirmed that peptide attachment in **4** occurs uniquely through the cysteine, since the only masses observed in the **4** digest, but not observed in the test peptide control digest, corresponded to cysteine-ligated fragments of N4Py-dipeptide.

The comparative ability of **1** and **5** to oxidize substrates in aqueous media was first determined using the chromogen [2,2′-

‡ Caesium forms weakly-associated salts with certain organic groups which exhibit unusual reactivity; for example, in the case of caesium thiolates the sulfur is converted into an excellent nucleophile well suited for S_N2 displacement of primary and secondary halides.[10]

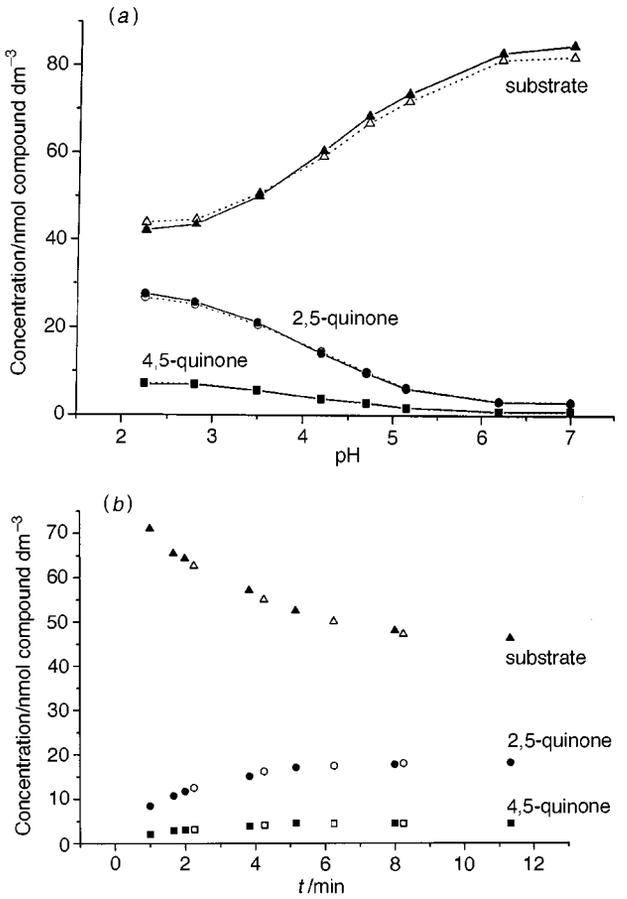


Fig. 3 Oxidation of 1,2,4,5-tetramethoxybenzene by **1** (solid symbols) and **5** (hollow symbols) determined by measuring the substrate and product concentrations (*a*) at various pHs after 4 min incubation at room temperature; (*b*) at pH 4.0 after various incubation times at room temperature. For both data sets, substrate : catalyst : H_2O_2 ratio was 14 : 1 : 28.

azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS).[11] Oxidation of this substrate by **1** in aqueous buffer was found to occur most rapidly at pH values of 4 or less, similar to the oxidation of ABTS by natural peroxidases.[12] There was no difference in the oxidation of ABTS by **1** or **5**, either in initial rate of ABTS cation formation or in final number of ABTS molecules (turnover ~40) oxidized by the catalyst. The results indicate that the covalent attachment of two 17-residue peptides to **1** has no detrimental effect on the efficiency of the tetrapyridyl–Fe complex to catalyze the single electron oxidation[11] of ABTS.

A number of organic compounds were screened for their suitability as substrates for **1** and **5**, and 1,2,4-trimethoxybenzene and 1,2,4,5-tetramethoxybenzene[13] were chosen for further studies. Methoxybenzenes have been used as substrates for some natural ligninases,[14] laccases[15] and peroxidases[16] and, for some substrates and enzymes, the reaction products have been elucidated and reaction mechanisms postulated.[15–17] We observed that both 1,2,4-trimethoxybenzene and 1,2,4,5-tetramethoxybenzene were oxidized by **1** and **5** to the same two products (4,5-dimethoxy-*o*-benzoquinone and 2,5-dimethoxy-*p*-benzoquinone) in the same relative quantities (approximately 1 : 4, respectively). The products were easily separated from the methoxybenzene by reversed-phase HPLC and identified; their identity was confirmed by independent synthesis.

We observed that 1,2,4,5-tetramethoxybenzene was oxidized marginally faster by **1** and **5** than was 1,2,4-trimethoxybenzene, and thus it was the substrate of choice for further oxidative studies. The oxidation of 1,2,4,5-tetramethoxybenzene by **1** and **5** at various pHs [Fig. 3(*a*)] and at pH 4.0 for various incubation times [Fig. 3(*b*)] showed that (i) acidic conditions (pH 4 or less) are required by **1** and **5**; (ii) the attachment of two peptides to the catalyst has no effect on its pH preference and (iii) five minutes after initiation of the reaction with H_2O_2, catalysis by both **1** and **5** at pH 4 has essentially terminated.

The dependence of activity of natural peroxidases, ligninases and laccases on acidic (<pH 4) environments has been attributed to the need to stabilize intermediates unstable at higher pH.[15,18] In particular, it has been reported that horseradish peroxidase oxidizes 1,2,4,5-tetramethoxybenzene to 4,5-dimethoxy-*o*-benzoquinone and 2,5-dimethoxy-*p*-benzoquinone (the same products formed by **1** and **5**) and that this reaction proceeds optimally at pH 3.[16] The postulated reaction mechanism for oxidation of this substrate by horseradish peroxidase involves the formation of a 1,2,4,5-tetramethoxybenzene radical cation observable by EPR spectroscopy.[16] Under the same reaction conditions, we found that the identical radical cation formed by horseradish peroxidase is formed by the oxidation of 1,2,4,5-tetramethoxybenzene by **1**, suggesting that **1** employs the same reaction mechanism as horseradish peroxidase for the oxidation of this substrate. Interestingly, this appears not to be the case for the oxidation of 1,2,4-trimethoxybenzene; whereas oxidation by **1** results in the selective production of 4,5-dimethoxy-*o*-benzoquinone and 2,5-dimethoxy-*p*-benzoquinone, oxidation by horseradish peroxidase results in the formation of four major products observable by HPLC.

The observation that catalytic activity in water is retained after the attachment of two linear 17-residue peptides onto the tetrapyridyl-Fe catalyst is consistent with the catalyst being relatively insensitive to electronic effects experienced on its periphery. With a catalytically active N4PyFe-dipeptide complex in hand, we are in a position to undertake the challenge of designing a stable helical bundle protein around the tetrapyridyl catalytic core. In later design stages we foresee altering the reactivity of the catalyst by controlling the pH environment in the vicinity of the catalytic Fe *via* the peptide sequence, and eventually using the structure of the protein to regulate access of the substrate to the catalytic site.

Experimental

Synthesis of the diester

The diester was prepared from 1.125 g (6.08 mmol) bis-(2-pyridyl)methylamine,[9] 2.88 g (15.5 mmol) methyl 6-chloromethylnicotinate[19] and 6.0 g (46.5 mmol) ethyldiisopropylamine in 3.0 ml chloroform under reflux in a nitrogen atmosphere for 2 days. The reaction was followed by ^{1}H NMR spectroscopy (δ 5.33 disubstituted, 5.13 monosubstituted). The reaction mixture was concentrated under vacuum and the residue was purified by flash chromatography on Al_2O_3 act. III (Merck) to yield the diester as a viscous liquid (2.02 g, 69%); δ_H(200 MHz, $CDCl_3$) 9.05 (d, *J* 1.7, 2H), 8.54 (dd, *J* 4.3, *J* 1.3, 2H), 8.7 (dd, *J* 8.1, *J* 2.1, 2H), 7.67 (d, *J* 8.5, 2H), 7.6 (m, 4H), 7.12 (m, 2H); δ_C(50 MHz, $CDCl_3$) 52.18 (CH_3), 57.44 (CH_2), 72.42 (CH), 122.19 (CH), 122.45 (CH), 123.88 (CH), 124.14 (C_q), 136.25 (CH), 137.23 (CH), 149.23 (CH), 150.13 (CH), 159.56 (C_q), 164.41 (C_q), 165.65 (C_q); *m/z* (electrospray) 484 (M + 1).

Synthesis of the diol

The diester (0.73 g, 1.5 mmol) was dissolved in 10.0 ml of THF and stirred while 2.26 ml of a 1 M $LiAlH_4$ solution in THF (2.26 mmol) was added dropwise at 0 °C. After 18 h at room temperature, 10.0 ml of THF and 4.0 ml of 1 M NaOH were added. The clear brown supernatant solution was decanted, and 10.0 ml of THF was added to the viscous precipitate and boiled for several minutes. After decanting, the procedure was repeated, the combined fractions were concentrated under vacuum, and the residue was stripped with toluene under vacuum giving a brown viscous oil. Purification by flash chromatography on polyamide (Woelm) yielded 0.53 g (82%) of the diol as a viscous

liquid; δ_H(200 MHz,$CDCl_3$) 8.53 (d, *J* 4.3, 2H), 8.23 (s, 2H), 7.72 (d, *J* 7.7, 2H), 6.65 (m, 2H), 7.46 (dd, *J* 7.9, 2.0, 2H), 7.37 (d, *J* 7.7, 2H), 7.15 (m, 2H), 5.33 (s, 1H), 4.60 (s, 4H), 3.89 (s, 4H); δ_C(50 MHz, $CDCl_3$), 57.97 (CH_2), 61.84 (CH_2), 73.91 (CH), 122.37 (CH_2), 123.29 (CH_2), 123.97 (CH_2), 134.87 (C_q), 135.47 (CH_2), 136.65 (CH_2), 147.34 (CH_2), 149.18 (CH), 158.22 (C_q), 159.91 (C_q); *m*/*z* (electrospray) 428 (M + 1).

Synthesis of 3

To a mixture of 0.333 g (0.78 mmol) of the diol, 72.0 mg (1.56 mmol) of LiCl (dried at 120 °C) and 0.23 ml (1.72 mmol) of 2,4,6-collidine, dissolved in 3.0 ml dry DMF and cooled in an ice–salt bath, 0.14 ml (1.72 mmol) methanesulfonyl chloride was added dropwise and the mixture was stirred for 2 h. Work-up with 10% aqueous Na_2CO_3 and chromatography over Al_2O_3 90 act. II-III (Merck) gave the pure dichloride **3** (28.9 mg, 8% yield). Note that the low yield was caused by decomposition during chromatography; δ_H(200 MHz, $CDCl_3$) 8.55 (d, *J* 4.8, 2 H), 8.45 (s, 2 H), 7.62 (m, 8 H), 7.13 (dd, *J* 4.9, 8.6, 2H), 5.33 (s, 1 H), 4.52 (s, 4 H), 3.97 (s, 4H); δ_C(50 MHz, APT, $CDCl_3$) 159.96 (C_q), 159.71 (C_q), 149.11 (CH), 148.55 (CH), 136.39 (CH), 136.14 (CH), 131.03 (C_q), 123.71 (CH), 122.75 (CH), 122.00 (CH), 72.30 (CH), 57.05 (CH_2), 42.87 (CH_2); *m*/*z* (electrospray) 464 (M + 1) (the isotope ratio is in agreement with the calculated isotope ratio).

Peptide synthesis

The peptide was synthesized by standard automated methods on an Applied Biosystems 433A peptide synthesizer using Fmoc-protected amino acids[3] and was purified using a Hewlett Packard 1050 HPLC instrument equipped with a Vydac C_{18} reversed-phase column. The reduced peptide was determined to be homogeneous by analytical HPLC and electrospray ionization mass spectrometry. To prevent reoxidation of the cysteine, the purified peptide was stored under vacuum until use.

Synthesis of dipeptide-N4Py 4 and dipeptide-N4PyFe 5

In a 1 ml screw-cap conical vial fitted with a triangular stirrer bar, the test peptide (6.86 mg, 3.4×10^{-6} mol) in 280 μl Ar-purged DMF was titrated to approximately pH 9 with Cs_2CO_3 (stock solution approx. 10 mg 100 μl^{-1} water). The dichloride ligand **3** in 22 μl DMF (0.32 mg, 6.9×10^{-7} mol; peptide concentration 2.5 times in excess over **3** coupling site concentration) was added, the vial was flushed with Ar, and the contents stirred at room temperature for 3 h. The reaction mixture was diluted twofold with water +0.1% TFA, and $Fe(ClO_4)_2$ (0.28 mg, 7.6×10^{-7} mol) was added. After 5 min stirring, the reaction mixture was chromatographed on a Hewlett Packard 1050 HPLC instrument using a Vydac C_{18} reversed-phase column and employing a gradient of 28–38% solvent B (0.25% per min; solvent A = water + 0.1% TFA, solvent B = 90% acetonitrile, 10% water, 0.1% TFA). The effluent was monitored at 220 and 450 nm; unreacted peptide would absorb only at 220 nm, whereas N4PyFe compounds would absorb at both wavelengths. No unreacted **3** was observed, but two major new peaks were collected and lyophilized: one eluting at 20 min and absorbing at both monitoring wavelengths, and one eluting at 25 min and absorbing only at 220 nm. Electrospray mass spectrometry confirmed the identity of the yellow product (eluted at 20 min) as **5** (*M* + H requires 4463.0. Found MH^+, 4462.5 ± 1) and the white product to be oxidized test peptide. From the reaction mixture, 1.08 mg (35% recovery) of **5** was obtained. The purity of **5** was checked by reinjection onto the C_{18} column; a symmetric peak eluting at 20 min with <5% visible contaminants at 220 nm was observed.

Verification of cysteine attachment in 4

Identification of side chains attached covalently to the tetrapyridyl ligand was established using **4**, since metals can interfere with the activity of Protease V8. Protease V8 (Endoproteinase Glu-C, sequencing grade) was purchased from Boehringer Mannheim Biochemica and enzymatic digestions were performed on equal concentrations of test peptide and **4** according to established methods.[20] Amino acid sequences were determined by Edman degradation using an Applied Biosystems pulse-liquid protein sequencer model 477A, on-line connected with a model 120A PTH-analyzer. Sequence determination of the peptide fragments in the crude digest mixture was possible because of the cleavage specificity of the protease employed, and because the sequence of the intact peptide was known. All the phenylthiohydantoin (PTH) amino acid derivatives found in each cycle of Edman degradation were in agreement with the expected sequences of the peptide fragments and the specificity of the protease. These analyses were performed by Eurosequence B.V. (Groningen, The Netherlands). Mass determinations of the digest fragments were performed with a Micromass VG TofSpec E MALDI–TOF mass spectrometer using angiotension-I, rennin and insulin B as calibration standards and 2-cyano-3-(4-hydroxyphenyl)propenoic acid as matrix.

Reaction conditions for oxidation of ABTS by 1 and 5

ABTS has an absorption maximum at 340 nm and is oxidized by peroxide to a radical cation which has an absorption maximum at 412 nm (pH 4.4, molar absorption coefficient ε 32 400 M^{-1} cm^{-1}). The reaction has a stoichiometry of one mole of H_2O_2 to two moles of ABTS. ABTS (Sigma) was dissolved in water (77.05 mg in 9.3 ml, 1.5×10^{-2} M solution). Buffer solutions of 0.1 M sodium acetate–acetic acid were prepared at pH values of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 6.0; buffers at pH 7.0 and 8.0 were prepared using 0.1 M 2-[*N*,*N*-bis(2-hydroxyethyl)-amino]ethanesulfonic acid (BES).[21] Solutions of ABTS in each of these buffers were prepared by diluting 1.0 ml of ABTS stock solution with 30.3 ml of buffer (5×10^{-4} M buffered ABTS solutions). N4PyFe **1** stock solution (16.20 mg in 70.2 ml water, 3.3×10^{-4} M) was prepared by sonication for 5 min. N4PyFe-dipeptide **5** has a true molecular mass of 4463 Da, but a 'practical' or 'weighing' mass of 5033 Da due to the association of a TFA molecule (from the HPLC elution buffer) with each histidine and lysine in the peptide. A stock solution of **5** (3.60 mg in 0.24 ml water, 3.3×10^{-4} M) was prepared. Oxidation of ABTS by non-N4Py iron (background reaction) was checked by using a solution of $Fe(ClO_4)_2$ (1.197 mg in 10.0 ml water, 3.3×10^{-4} M). A commercial stock solution of 10 M H_2O_2 was diluted to 0.1 M in water.

Reactions at room temperature were conducted in a 1 × 1 cm quartz cuvette fitted with a micro stirrer bar. Generation of the ABTS radical cation was monitored at 5 s intervals on a Hewlett Packard 8452 UV-visible diode array spectrophotometer at 412 nm. Each reaction solution consisted of 1.5 ml (7.5×10^{-7} mol) of ABTS at the appropriate pH, 4.55 μl of a 3.3×10^{-4} M solution of the iron compound [either **1**, **5** or $Fe(ClO_4)_2$; 1.5×10^{-9} mol] and the reaction was initiated by the addition of 15 μl 0.1 M H_2O_2 (1.5×10^{-6} mol). Thus, the ratio of the reactants ABTS : iron compound : peroxide was 500 : 1 : 1000. The formation of the radical cation was monitored for 15 min, and then the number of moles of ABTS oxidized was calculated. At each pH, blank reactions consisting of ABTS oxidized solely by H_2O_2 (no iron compound present) were subtracted from the experimental data sets. Note that inorganic Fe oxidized the substrate at approximately 10% the rate of **1**.

Identification of methoxybenzene oxidation products

To 5 ml of 10 mM 1,2,4-trimethoxybenzene (56.3 μl, 5×10^{-5} mol) in 0.1 M acetate buffer, pH 4.0, 212 μl of a solution of **1** (4.05 mg in 1.75 ml water, 3.3×10^{-3} M, 7×10^{-7} mol) and 10 μl 10 M H_2O_2 (1×10^{-4} mol) was added. After stirring at room temperature for approximately 2 h, the products were purified on a C_{18} reversed-phase HPLC column; the eluting buffers were

as above, and the gradient was 1% B per min. The minor product eluted at 17% B, and the major at 18% B; unreacted 1,2,4-trimethoxybenzene eluted at 40% B.

The products were characterized by UV–VIS[22] NMR spectroscopy; δ_H(major compound) 5.85 (s, 1H), 3.85 (s, 3H); δ_H-(minor compound) 5.77 (s, 1H), 3.90 (s, 3H); δ_C(major compound) 181.51 (C=O), 159.41 (C_q), 105.34 (CH), 56.51 (CH_3); δ_C(minor compound) 178.89 (C=O), 163.59 (C_q), 102.84 (CH), 56.88 (CH_3) and mass spectroscopy [both compounds gave *m*/*z* (electrospray) 169 (M + 1)] [HRMS (major compound): Found M^+, 168.042. Calc. for $C_6H_8O_4$, 168.042] [HRMS (minor compound): Found M^+, 170.058. Calc. for $C_8H_{10}O_4$, 170.058]. The major compound was identified as 2,5-dimethoxy-*p*-benzoquinone and the minor as 4,5-dimethoxy-*o*-benzoquinone. Both compounds were synthesized independently.[23] The spectroscopic data of the isolated and synthesized quinones proved to be identical and a co-injection of isolated and synthesized material gave a single peak on HPLC.

Reaction conditions for the oxidation of 1,2,4,5-tetramethoxybenzene by 1 and 5

The synthesis of 1,2,4,5-tetramethoxybenzene from 2,5-dimethoxy-*p*-benzoquinone was achieved by reduction to the hydroquinone followed by methylation. Due to the broad UV–VIS spectrum of the purple intermediate (N4PyFe^{III}OOH),[5] it was not possible to follow the oxidation of methoxybenzenes spectroscopically. The response of the HPLC diode array detector at 285 nm to absorption by 1,2,4,5-tetramethoxybenzene, 2,5-dimethoxy-*p*-benzoquinone and 4,5-dimethoxy-*o*-benzoquinone was calibrated by preparing standard solutions of the authentic compounds and injecting triplicate samples onto a Vydac C_{18} reversed-phase column using the HPLC autosampler. The chromatograms were developed as above. An internal standard was not used due to its possible oxidation by H_2O_2. The amount of remaining substrate at each data point was measured, since the quinone products were observed to be unstable under the reaction conditions.

The effect of pH on the rate of 1,2,4,5-tetramethoxybenzene oxidation was determined by preparing 5 mmol solutions of the substrate in 0.5 M sodium acetate–acetic acid buffers (due to the relatively high catalyst concentrations used, a high buffering capacity was required in order to neutralize the TFA adhering to **5**). Stock solutions of **1** and **5** (3.3×10^{-3} M) and H_2O_2 (1 M) were used for both the pH and time course studies. To 100 μl (5×10^{-7} mol) stirring substrate solution, 10.8 μl (3.6×10^{-8} mol) catalyst was added and the reaction was initiated by the addition of 1 μl (1×10^{-6} mol) peroxide. For the pH studies, the reaction mixture was stirred for 3 min at room temperature and was then transferred to the HPLC autosampler and an aliquot was injected; total reaction time until the sample was injected onto the column was 4 min. For the time course study, it is assumed that the reaction continued until the moment of injection onto the HPLC column.

EPR Measurements

Measurements were obtained using a Bruker ECS 106 EPR spectrometer operating at a frequency of 9.76 GHz, employing 50 kHz field modulation and a sweep of 3350–3550 G. The sample [1 ml (5 mM) 1,2,4,5-tetramethoxybenzene in 0.1 M acetate buffer pH 2.5, 100 μl (3.3×10^{-3} M) **1** and 10 μl (0.1 M) H_2O_2] was mixed, immediately transferred to an EPR sample tube and the spectrum was scanned at room temperature. The experiment was repeated using horseradish peroxidase in place of **1**.

Acknowledgements

Partial financial support to B. L. F. was provided by Unilever Research (Vlaardingen, The Netherlands). We thank Dr R. Hage and Mr R. Naasz of Unilever Research for assistance with the EPR measurements, and helpful discussion with Dr Hage and Dr L. Que (Dept. of Chemistry, University of Minnesota) are gratefully acknowledged.

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Paper 7/06416K
Received 2*nd September* 1997
Accepted 28*th November* 1997