Denaturation and accelerated proteolysis of sizeable heme proteins by synthetic metalloporphyrins†‡

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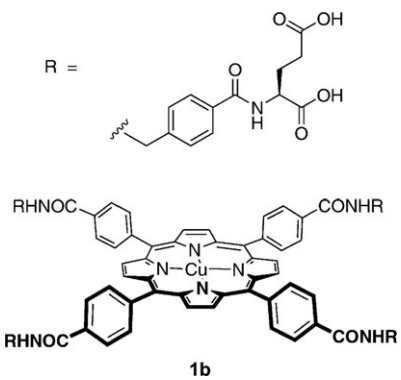
Received (in Durham, UK) 4th January 2007, Accepted 13th March 2007
First published as an Advance Article on the web 3rd April 2007
DOI: 10.1039/b700123a

A synthetic, copper porphyrin unwinds the α-helical domains of the heme proteins hemoglobin, myoglobin and cytochrome *c*, thereby catalysing trypsin-mediated proteolysis.

The design of synthetic agents that bind and change the native conformation of a protein selectively, thereby leading to a loss in protein function, is a science still in its infancy. But the possibilities that such "conformational modifiers" may offer in controlling biological function are significant. For example, these molecules may act not only by destroying the function of an active protein[1] but also by restoring the function of a misfolded (or mutant) protein by returning it to its native state, as has been demonstrated with mutated p53.[2] At physiological pH, protein denaturation normally requires high temperatures or high concentrations of chemical denaturants such as guanidinium hydrochloride or urea, conditions that are inherently non-selective. We have previously demonstrated that synthetic porphyrins containing anionic substituents on their peripheries can selectively induce the unfolding of the highly basic protein cytochrome *c* (cyt *c*: MW = 12 kDa; *p*I = 10.5) at stoichiometric quantities and at near physiological conditions (pH 7.4, 50 mM NaCl).[3–5] More recently, we have demonstrated the selective catalytic unfolding and accelerated proteolysis of cyt *c* by a class of anionic copper porphyrin dimers.[6] Rotello *et al.* have also reported the selective denaturation of cyt *c* with anionic nanoparticles, rendering the protein susceptible to proteolysis.[7]

During our studies on denaturation with synthetic agents, we observed that strong binding to cyt *c* is not a prerequisite for denaturing activity. Indeed, the stronger the binding to the native protein, the weaker any denaturing effect will be. It is likely that selective binding to the denatured state, or any of a series of non-native states, over the native state is the determining factor in the ability of a porphyrin to denature a protein. Charged peripheral groups that complement residues on the protein surface probably facilitate the denaturation process, by localizing the porphyrin scaffold close to the protein core. In this way, the charged peripheral groups may also be a source of selectivity and therefore direct denaturation. Furthermore, it is believed that charged residues at the protein surface aid in the stabilization of the tertiary structure of the protein.[8] Therefore, charge-matching of surface residues with complementary synthetic receptors may lead to a local destabilization of the protein and a concomitant reduction in the protein melting temperature.

As part of our continued interest in protein recognition, we wanted to ascertain if we could also denature the larger heme proteins (Fig. 1) myoglobin (horse skeletal: MW = 17.2 kDa; *p*I = 7.2) and hemoglobin (bovine: MW = 64.5 kDa; *p*I = 6.9). Through a structure–activity relationship study involving a series of novel, oligoanionic free base and copper porphyrins, we have identified a copper porphyrin (**1b**) that is capable of achieving this goal.



1b

The series of anionic (and one zwitterionic) porphyrins were prepared in 5 steps (Scheme 1) starting from *para*-(methylamino)benzoic acid (**2**). Cbz-protection of the amino group followed by conjugation of a range of amino acids to the benzoic acid functionality furnished amides **4–10** in yields of 47–94% for both steps. All Cbz-protected compounds were then deprotected by H_2 and 10% Pd/C, giving the free amines (**11–17**) as oils or sticky solids in nearly quantitative yields. Next, the four carboxylic acids of *meso*-tetrakis

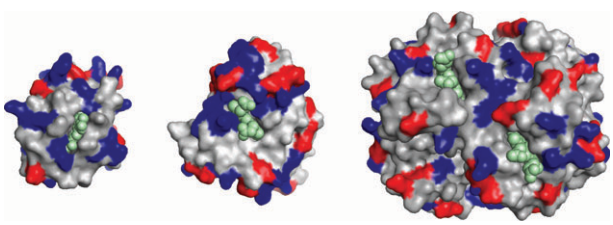


Fig. 1 The relative sizes of cyt *c* (left, MW = 12 kDa), myoglobin (middle, MW = 17.2 kDa) and hemoglobin (right, MW = 64.5 kDa); green = prosthetic heme, blue = basic residue, grey = neutral residue, red = acidic residue.

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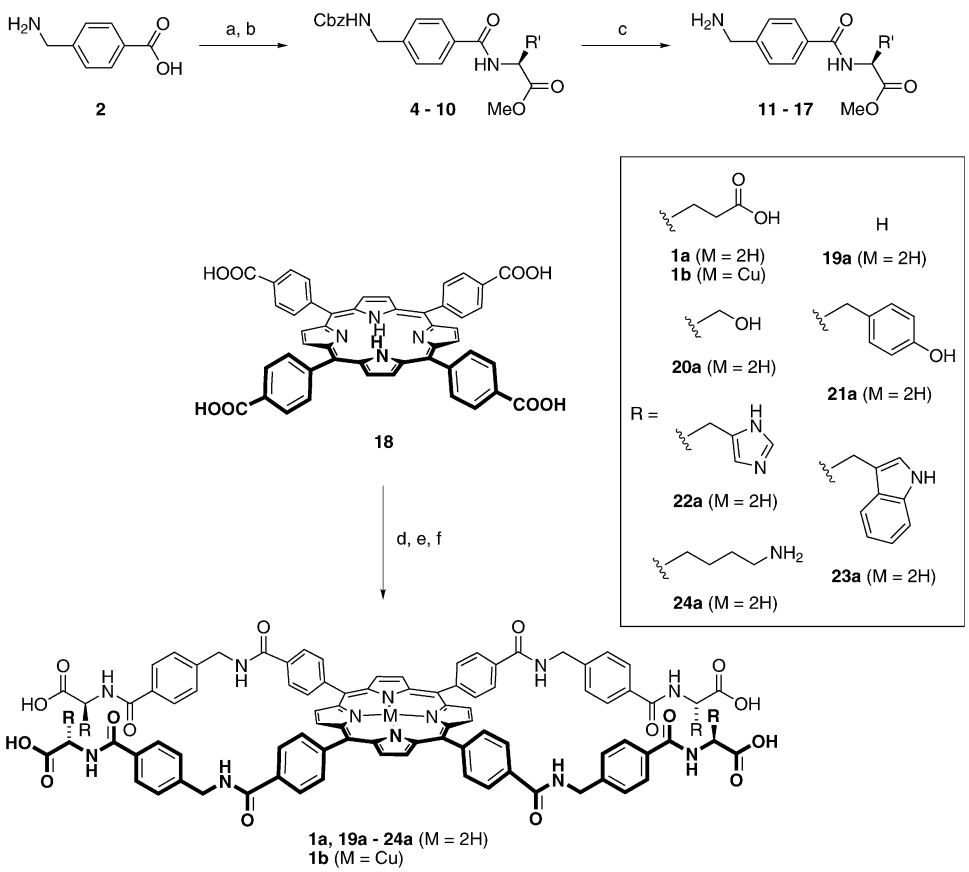
† Dedicated to Professor George Gokel on the occasion of his 60th birthday.
‡ Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b700123a



Scheme 1 (a) CbzCl, NaOH, dioxane–H_2O, 0 °C → RT, 16 h, 97%; (b) (i) *i*BuOCOCl, *N*-methylmorpholine, THF, 0 °C, 2 min; (b) (ii) $HCl \cdot H_2NCH(R')CO_2Me$, *N*-methylmorpholine, 0 °C → RT, 16 h, 48–97%; (c) H_2, 10% Pd/C, MeOH, RT, 1 h, 90–98%; (d) (i) $(COCl)_2$, cat. DMF, CH_2Cl_2, 3 h; (d) (ii) **11–17**, DIPEA, CH_2Cl_2–THF, 0 °C → RT, 16 h, 45–75%; (e) either (i) TFA–CH_2Cl_2, 1 : 1, RT, 4 h, 85–99%, or (ii) $LiOH \cdot H_2O$, MeOH–THF–H_2O, 3 : 1 : 1, RT, 4 h, then 1 N HCl, 95–99%; (f) $CuCl_2$, Δ, MeOH, 3 h, 55%.

(4-carboxyphenyl)porphine (**18**) were activated as their acid chlorides and coupled to one of the amines **11–17**. After purification by silica gel chromatography, the resulting symmetrical porphyrins were deprotected either by treatment with TFA, or by treatment with LiOH and then precipitated as their free acids with 0.1 M HCl, or by treatment with TFA. Lyophilisation then gave compounds **1a** and **19a–24a** as green powders. All yields were close to quantitative. Copper incorporation was achieved by refluxing a dilute solution (80 μM) of porphyrin **1a** in MeOH with a 10-fold weight excess of copper(II) chloride. After 3 h, when UV spectroscopy indicated the reaction was complete, the red solution was reduced under vacuum to a minimal volume in MeOH, then the brick red copper porphyrin **1b** was precipitated with 0.01 M HCl and collected by centrifugation.

Initial thermal denaturation experiments (see ESI‡) on the heme proteins in this study were conducted by monitoring the α-helical CD signal at 222 nm in the presence of the synthetic porphyrins. It is important to note here that the chirality of the porphyrins had negligible effects on the protein CD signals at 222 nm. These preliminary experiments suggested that **1a** was the most potent denaturant across the heme proteins in this study, and that copper derivative **1b** was more potent still, consistent with previous reports.[6] Porphyrin **1b** was therefore chosen for further study.

Copper(II) porphyrins are known to dimerise in water[9] through enhanced π–π stacking interactions,[10] and similar observations have been reported by us previously.[4,6] Various equivalents of porphyrin **1b** were incubated with a 20 μM solution of cyt *c* in 5 mM NaH_2PO_4 buffer, pH 7.4. As shown in Fig. 2, just 2 equiv. of **1b** reduced the melting temperature of cyt *c* (T_m = 85 °C) by around 50 °C, consistent with previous findings on related porphyrins.[6]

Next, we investigated myoglobin, and observed that 2 equiv. of **1b** reduced the melting temperature of the protein (T_m = 83 °C) by approximately 20 °C, and 4 equiv. reduced the melting temperature further still to around 47 °C (Fig. 3). With this result in hand, we next investigated the effect of **1b** on hemoglobin, a protein that exhibits quaternary structure, and whose α and β subunits share substantial tertiary structure similarity both with each other and with myoglobin.[11]

CD thermal denaturation experiments of **1b** with hemoglobin did not lead to any significant change in the melting temperature of the protein (T_m = 64 °C). However, the α-helical content at 25 °C was reduced by around 20 mdeg for every 2 equiv. of **1b**, as monitored by the CD signal at 222 nm (Fig. 4), suggesting a marked unravelling of the protein. We conducted similar denaturation experiments with other proteins, including azurin (MW = 13.9 kDa; *p*I = 5.6) and α-lactalbumin (MW = 14.2 kDa; *p*I = 4.5); the thermal

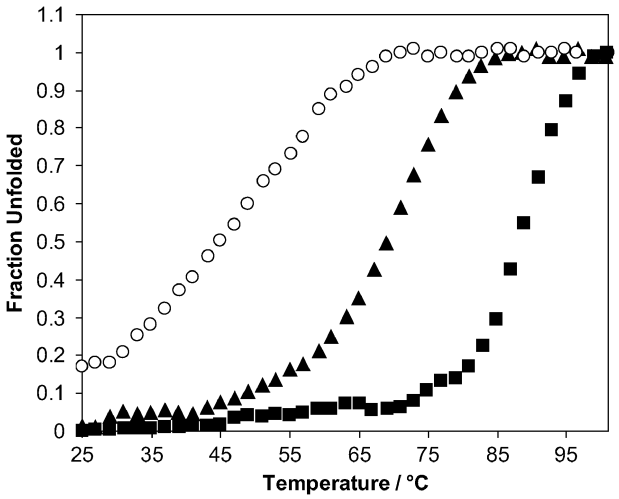


Fig. 2 CD-monitored thermal denaturation profile (λ = 222 nm) of cyt *c* in the presence and absence of **1b**, in 5 mM NaH_2PO_4 buffer, pH 7.4. ■: cyt *c* (20 μM); ▲: cyt *c* + **1b** (20 + 20 μM); ○: cyt *c* + **1b** (20 + 40 μM).

stabilities of these proteins were unaffected by **1b** (see ESI‡). These results encouraged us to further investigate the effects of **1b** on the heme proteins in this study.

Protein secondary structures, such as the α-helix, confer a degree of resistance to proteolysis by restricting access of the proteolytic enzymes to the backbone amide bonds.[12] If **1b** is denaturing cyt *c*, myoglobin and hemoglobin as Fig. 2, Fig. 3 and Fig. 4 suggest, then proteolysis of these proteins may be expected to be facilitated in the presence of a proteolytic enzyme. Trypsin cleaves at the C-terminus side of the basic residues lysine and arginine, hence has no effect on the amide bonds in porphyrin **1b**. After 5 h in the presence of trypsin (1 mg ml^{-1}) at 37 °C and pH 7.4, minimal digestion of cyt *c* was detected by SDS-PAGE analysis (Fig. 5a, lane C). However, in the presence of 4 equiv. of **1b** under the same

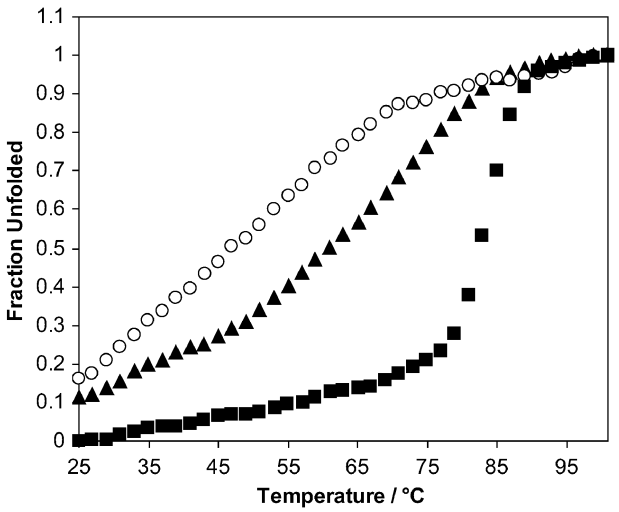


Fig. 3 CD-monitored thermal denaturation profile (λ = 222 nm) of myoglobin in the presence and absence of **1b**, in 5 mM NaH_2PO_4 buffer, pH 7.4. ■: myoglobin (20 μM); ▲: myoglobin + **1b** (20 + 40 μM); ○: myoglobin + **1b** (20 + 80 μM).

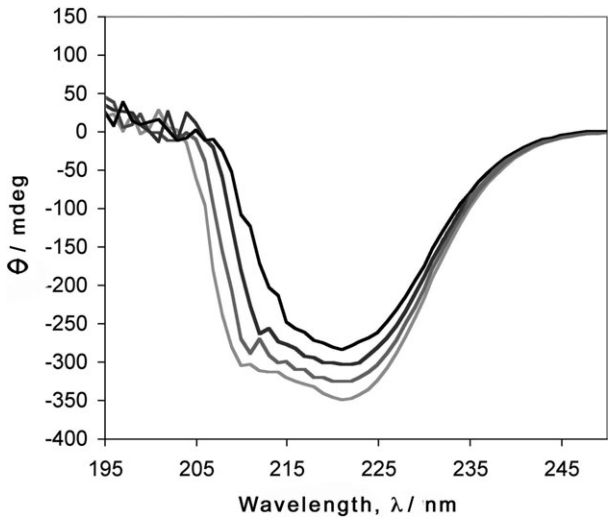


Fig. 4 Wavelength scan of hemoglobin in the presence of increasing equivalents of **1b**, in 5 mM NaH_2PO_4 buffer, pH 7.4, 25 °C. Light grey: hemoglobin (20 μM); mid-grey: hemoglobin + **1b** (20 μM + 40 μM); dark grey: hemoglobin + **1b** (20 μM + 80 μM); black: hemoglobin + **1b** (20 μM + 120 μM).

conditions, cyt *c* was digested within 15 min (Fig. 5a). Similarly, myoglobin proved resistant to tryptic proteolysis after 20 h alone (Fig. 5b, lane C), whereas 4 equiv. of **1b** led to digestion of the bulk of the protein within 2 h (Fig. 5b). Hemoglobin is also resistant to proteolysis, as shown by SDS-PAGE analysis at 20 h (Fig. 5c, lane C).[13] In contrast, in the presence of 4 equiv. of **1b**, the bulk of the protein was digested within 4 h (Fig. 5c).

Kinetic measurements of the relative digestion rates were determined by monitoring the α-helical CD signal at 222 nm.

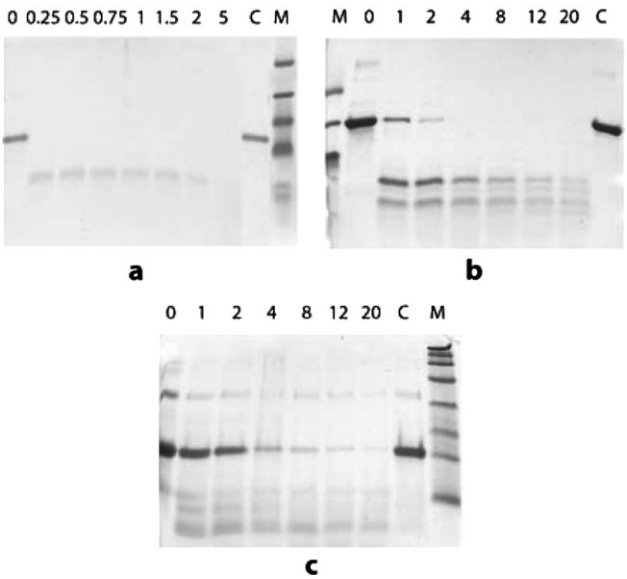


Fig. 5 SDS-PAGE analysis of the tryptic digestions of (a) cyt *c*, (b) myoglobin and (c) hemoglobin, in the presence of 4 equiv. of **1b**. Digestion times shown in hours. C = digestion in the absence of **1b** after (a) 5 h, (b) 20 h and (c) 20 h. M = molecular weight marker (ESI‡).

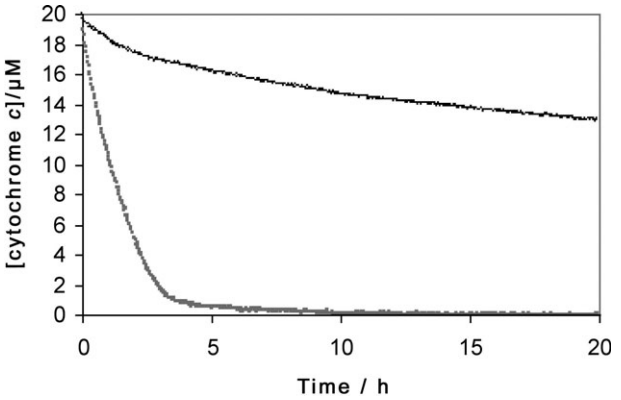


Fig. 6 Kinetics of the tryptic proteolysis of cyt *c* (20 μM), as measured by CD at 222 nm, 37 °C, 5 mM NaH_2PO_4, 50 mM NaCl, trypsin (1 mg ml^{-1}) with 0 equiv. (black) and 0.25 equiv. (grey) of **1b**.

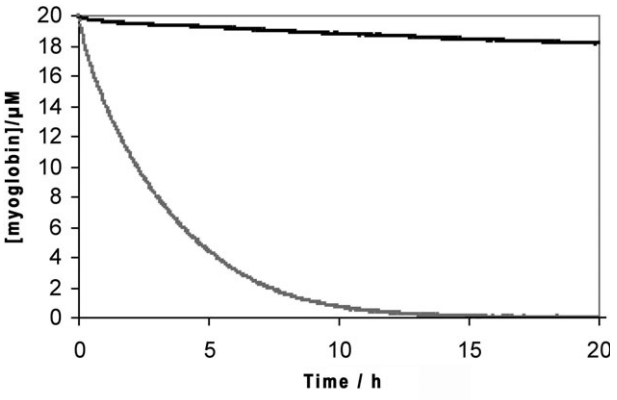


Fig. 7 Kinetics of the tryptic proteolysis of myoglobin (20 μM), as measured by CD at 222 nm, 37 °C, 5 mM NaH_2PO_4, 50 mM NaCl, trypsin (1 mg ml^{-1}) with 0 equiv. (black) and 1.5 equiv. (grey) of **1b**.

Various equivalents of **1b** were pre-incubated with 20 μM solutions of each of cyt *c*, myoglobin and hemoglobin in 5 mM NaH_2PO_4, 50 mM NaCl, pH 7.4 at 37 °C for 30 min (total volume = 400 μl). Next, 8 μl of a 1 mg ml^{-1} solution of trypsin in 0.1 N HCl were added to each sample, immediately followed by brief mixing and transfer to cuvettes pre-warmed to 37 °C.

In the SDS-PAGE analyses of the proteolysis experiments, there was no accumulation of large fragments, suggesting that the initial fragments were further digested quickly. This allows for a continuous, quantitative measurement of the concentration of the undigested proteins during the kinetic proteolysis reactions. Hence, we observed that just 0.25 equiv. of **1b** effected total digestion of cyt *c*, in the presence of trypsin, in less than 4 h, at 37 °C and pH 7.4. However, in the absence of the porphyrin, less than 20% of cyt *c* was digested by trypsin in the same time (Fig. 6). With 1 equiv. of **1b**, tryptic digestion was complete within 30 min. A plot of initial rates of proteolysis of cyt *c* *versus* equivalents of **1b**, then extrapolation to 2 equiv. gives a rate enhancement of approximately 76-fold over trypsin digestion in the absence of **1b**. In accordance with Wang and Kallenbach,[14] this represents a stabilisation of the conformation(s) required for initial proteolytic attack of approximately 11.2 kJ mol^{-1} at 37 °C and pH 7.4.

Porphyrin **1b** (1.5 equiv.) catalyzed the complete digestion of myoglobin, in the presence of trypsin, in approximately 10 h, at 37 °C and pH 7.4, whereas in the absence of the porphyrin, less than 7% of myoglobin was digested by trypsin in the same time (Fig. 7). Further CD kinetic studies of the proteolysis of myoglobin revealed that 2 equiv. of **1b** led to an 11-fold rate enhancement, relative to trypsin digestion in the absence of the metalloporphyrin. This represents a stabilisation of the conformation(s) required for initial proteolytic attack of around 6.18 kJ mol^{-1} at 37 °C and pH 7.4.

Similarly, porphyrin **1b** (1.5 equiv.) effected total digestion of hemoglobin, in the presence of trypsin, in around 15 h, at 37 °C and pH 7.4. Under the same conditions but in the absence of **1b**, less than 20% of hemoglobin was digested by trypsin (Fig. 8). Proteolysis of hemoglobin with 2 equiv. of **1b** furnished an 8-fold acceleration in the digestion of hemoglobin. This rate enhancement corresponds to a reduction in the ΔG value for the initial unfolding required for proteolytic attack of 5.36 kJ mol^{-1} at 37 °C and pH 7.4. Importantly, as observed in the thermal denaturation studies, neither azurin nor α-lactalbumin were rendered more susceptible to proteolytic attack by trypsin in the presence of **1b** (see ESI‡).

Although the molecular basis for the denaturation of the heme proteins with copper porphyrin **1b** is yet to be determined, CD studies indicate that **1b** leads to an unravelling of the α-helical regions of these proteins, facilitating proteolytic attack through the stabilisation of certain non-native states. The observation that **1b** has little effect on the thermal stability of hemoglobin yet accelerates its trypsin-mediated digestion is presumably a reflection of the size differences between hemoglobin and the smaller proteins myoglobin and cyt *c*. The hemoglobin melting temperature is influenced by hydrophobic forces within and among the four monomers, such that reductions in α-helical content may have a lesser impact on the thermal stability of the protein. It is likely that an initial step of **1b**-mediated denaturation involves interaction with positively-charged domains on the proteins' surfaces. There are a number of sites on the heme proteins studied,

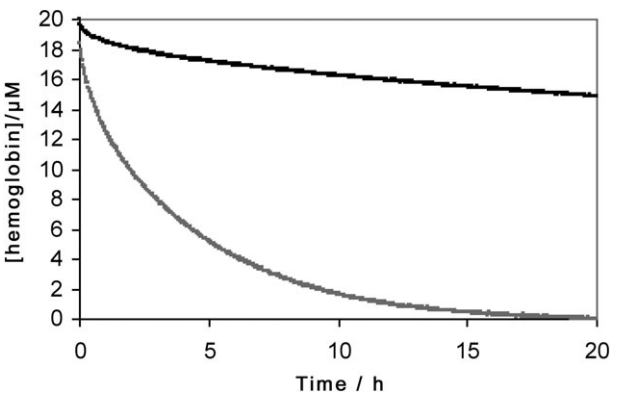


Fig. 8 Kinetics of the tryptic proteolysis of hemoglobin (20 μM), as measured by CD at 222 nm, 37 °C, 5 mM NaH_2PO_4, 50 mM NaCl, trypsin (1 mg ml^{-1}) with 0 equiv. (black) and 1.5 equiv. (grey) of **1b**.

particularly those close to the heme crevices, where there are hydrophobic patches surrounded by positively-charged residues that well complement the structural features of **1b**.[3]

We have demonstrated that a catalytic quantity of **1b** can not only accelerate the trypsin-mediated digestion of cyt *c* but also catalyse the digestion of the larger protein myoglobin and the much larger quaternary protein hemoglobin. To the best of our knowledge, the denaturation and accelerated proteolysis of myoglobin and hemoglobin by synthetic agents was hitherto unknown. In addition, porphyrin **1b** exhibited no effect on the thermal stabilities of azurin and α-lactalbumin, nor did **1b** enhance the susceptibility of these proteins to proteolytic attack by trypsin, demonstrating selectivity for the heme proteins in this study. To better understand the denaturing properties of synthetic porphyrins, further studies with our series of anionic metalloporphyrins, including an analogous series of cationic porphyrins, with a larger range of heme proteins are currently under way in our laboratories.

Acknowledgements

The authors wish to thank the National Institutes of Health (GM35208) for financial support of this research. Dr Steven Fletcher would like to thank Debarati M. Tagore for assistance with SDS-PAGE, Dr Patrick Gunning for assistance with the figures and William Katt for the abstract artwork.

References

1 J. R. Peterson, R. S. Lokey, T. J. Mitchison and M. W. Kirschner, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 10624–10629.
2 B. A. Foster, H. A. Coffey, M. J. Morin and F. Rastinejad, *Science*, 1999, **286**, 2507–2510.
3 R. K. Jain and A. D. Hamilton, *Angew. Chem., Int. Ed.*, 2002, **41**, 641–643.
4 A. J. Wilson, K. Groves, R. K. Jain, H. S. Park and A. D. Hamilton, *J. Am. Chem. Soc.*, 2003, **125**, 4420–4421.
5 T. Aya and A. D. Hamilton, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 2651–2654.
6 K. Groves, A. J. Wilson and A. D. Hamilton, *J. Am. Chem. Soc.*, 2004, **126**, 12833–12842.
7 J. W. E. Worrall, A. Verma, H. Yan and V. M. Rotello, *Chem. Commun.*, 2006, 2338–2340.
8 A. Wada and H. Nakamura, *Nature*, 1981, **293**, 757–758.
9 R. F. Pasternack, L. Francesconi, D. Raff and E. Spiro, *Inorg. Chem.*, 1973, **12**, 2606–2611.
10 C. A. Hunter and J. K. M. Sanders, *J. Am. Chem. Soc.*, 1990, **112**, 5525–5534, and the references therein.
11 D. L. Nelson and M. M. Cox, *Lehninger Principles of Biochemistry*, 3rd edn, pp. . 210–212.
12 M. Ehrmann and T. Clausen, *Annu. Rev. Genet.*, 2004, **38**, 709–724.
13 In Fig. 5c, lane 0, the highest band corresponds to the hemoglobin tetramer, but the most intense band corresponds to the hemoglobin monomer unit. In order to quench the tryptic digestion experiments, we heated the samples to 95 °C, which led to hemoglobin denaturation. In addition, the SDS-PAGE gel is inherently denaturing, separating hemoglobin into its four subunits. Regardless, the band corresponding to the monomer unit is lost during the tryptic digestions, confirming the proteolysis of hemoglobin.
14 L. Wang and N. R. Kallenbach, *Protein Sci.*, 1998, **7**, 2460–2464.