chromatography was described by Wilchek (1972). A single stage peptic digestion at pH 1.5 was conducted at room temperature for 14 hours. A one step isolation of the neutralised product on bovine or human serum albumin coupled to Sepharose 4B gave 90 per cent recovery. Both MP-11 and MP-8 apparently bind selectively to the albumin, while cytochrome c and other digest products do not. The method was extended to the purification of MP-8 (Gerber et al., 1975) prepared by a tryptic digestion of MP-11 in the same reaction mixture in which the peptic digestion took place (without prior separation). Purity of the MP-8 product, assessed by paper chromatography and gel isoelectric focusing, appeared high.

A further step, gel chromatography, was added to the Harbury method to improve the purity of the final product (Tu et al., 1968). But Kraehenbuhl et al. (1974) replaced Harbury's partition chromatographic method (used twice in the preparation of MP-8) by gel chromatography on Biogel P-6 (200-400 mesh). The same Biogel column was used to separate MP-11 from undigested cytochrome c and haem-free peptides after a one stage peptic digestion at pH 1, and to remove undigested MP-11 and smaller haem-free peptides from MP-8. Final purification of MP-8 was by countercurrent distribution using an automatic apparatus (1000 tubes) and a two-phase butanol/pyridine/0.1% acetic acid [5:3:11] system. Gel chromatography was also employed by Hallenbeck (1978) in a four-stage purification procedure for the peptic hydrolysis product of cytochrome c_1.

It has been suggested that ambiguity in results of studies on MP-11 using ESR, NMR, Mössbauer and electronic spectroscopy, could be due to variations in the purity of samples used (Peterson et al., 1980). The Peterson method for the preparation of MP-11 differs from earlier methods [illegible] the peptic digestion is conducted at pH 2.6, which a[illegible]duction of two species of undecapeptide in solution (du[illegible] hydrolysis of glutamine 12 and glutamine 16 (Jehanli et al., [illegible]6). A two-stage purification scheme was used: precipitation of the peptide by ammonium sulphate was followed by chromatography on Sephadex G-15, using water as the eluant.

In the method devised for the preparation of MP-8 in this study, the best features of the various methods reported in the literature were combined to give greater efficiency in the digestion step and a product of higher purity. These modifications are discussed below and the advantages of the procedure in its entirety in the discussion [Section 6.6.].

6.2 Experimental procedure

The reagents and enzymes used, methods of chromatography, buffer preparation, TLC, visible spectroscopy, pH measurement and lyophilisation, are described in Chapter 2.

6.3 The preparation of MP-8

A procedure based largely on the method of Kraehenbuhl et al. (1974) was used.

6.3.1 Modifications to the Kraehenbuhl method

The modifications made to the method of Kraehenbuhl et al. (1974) are as follows:

1. the peptic digestion was carried out at pH 2.6 in preference to the lower pH conventionally used (pH 1.5) (Harbury and Loach, 1960a). It was hoped to avoid the production of two undecapeptide species, one with one glutamine and two glutamate residues (pI 4.75), the other with three glutamate residues (pI 4.6), due to the conversion of a glutamine residue to a glutamate in acid conditions (Jehanli et al., 1976);
2. a double digestion of cytochrome c with pepsin was employed to ensure complete digestion (Tuppy and Paleus, 1955; Harbury and Loach, 1960a);
3. a substantially larger quantity of pepsin was utilised to avoid the production of two undecapeptide species (Peterson et al., 1980) and to facilitate complete digestion;
4. the duration of the peptic digestion exceeded the Kraehenbuhl digestion time by ca. 10 hours;
5. tryptic activity was destroyed by heating the solution to 96°C for three minutes at the completion of the tryptic digestion (Harbury and Loach, 1960a);
6. purification of the haem-octapeptide by countercurrent distribution, was replaced by chromatography on Sephadex G-50 Superfine, a simpler and more rapid method.

6.3.2 Preparation of Microperoxidase-8

1 g of horse-heart cytochrome c and 25 mg of twice recrystallised pepsin were dissolved in 50 mℓ of deionized water. The pH was adjusted to 2.6 with 1 M hydrochloric acid and the stoppered solution incubated at 25°C for 16 hours. A further 25 mg pepsin was then added, the pH readjusted to 2.6 (if necessary) and incubation continued for 16 hours. Peptic activity was destroyed by raising the pH to 8.5 with concentrated ammonia solution. The product was recovered as the solid by lyophilisation.

The solid product was divided into five equal portions. Each was dissolved in a minimum amount of water and introduced separately onto a 4 x 100 cm Biogel P6 column. The column was equilibrated and eluted with 0.1 M NH_4HCO_3 and 20 mℓ$cm^{-2}hr^{-1}$ by means of a Pharmacia Model P-1 peristaltic pump. An ISCO Model 1850 fraction collector was used to collect 2.0 mℓ fractions. A sample from each fraction was diluted with 0.1 M NH_4HCO_3 and the absorbance measured in a Cary 2300 spectrophotometer at 423 nm (the wavelength employed by Krauhenbuhl (1974)). A typical elution profile is shown in Figure 6.2.

The major product, haem-undecapeptide (MP-11: $M_r \sim 1879$) was separated from undigested cytochrome c (eluted in the void volume), smaller haem-peptides and haem-free peptides. The method of testing for haem-free peptides eluted after MP-11, that of monitoring the absorbance of the eluant at 280 nm (Kraehenbuhl et al., 1974), is unreliable since a relatively large absorption of the haem also occurs in this region (Margoliash and Frohwirt, 1959). The main band in this initial separation step was somewhat diffuse. Fractions falling within a 90 per cent confidence interval of a Gaussian band superimposed on the actual peak were pooled. The other fractions were discarded. The yield of MP-11 in terms of haem units was about 80 per cent.

The pooled fractions of 5 runs were lyophilised and the mass of the product recorded. The product was dissolved in 50 mℓ of 0.1 M NH_4HCO_3 and an amount of trypsin equivalent to ca. 10 per cent of the mass of MP-11 added. The solution was incubated for a total of 36 hours at 36°C: at 12 hour intervals an additional amount of trypsin equivalent to half the initial amount was added, giving a total of three trypsin additions. Finally tryptic activity was destroyed by heating the solution to 96°C for three minutes. The solution was lyophilised and the resulting solid was divided into four equal portions; each was dissolved in a minimum amount of water and chromatographed on the same column of Biogel P6. The conditions used were identical to the previous separation, except that fractions of 2.75 mℓ were collected. Figure 6.2 shows the elution profile, monitored at 423 nm.

The haem-octapeptide (MP-8: $M_r \sim 1502$) was separated from MP-11 (eluted first) and a smaller haem containing peptide. The yield was about 80 per cent. The MP-8 fractions from all four runs were pooled, as described previously for MP-11, and lyophilised. In a final purification step, the solid was dissolved in a minimum of concentrated ammonia solution, and chromatographed on a 30 x 1.5 cm Sephadex G-50 Superfine column, equilibrated and eluted with deionized water, at 4 $m\ell cm^{-2}hr^{-1}$. Fractions of 2.0 mℓ were collected. The method takes advantage of the polymerisation of haem-peptides in non-liganding alkaline solutions, and the accentuated adsorption of aromatic substances to dextran gels in alkaline solution (Hallenbeck, 1978).

The elution profile is shown in Figure 6.2. A small peak distinct from the MP-8 peak and running ahead of it probably represents MP-11, inadequately separated in the Biogel chromatography. The yield was 80-90 per cent in this step, giving an overall yield of ca. 50 per cent. The MP-8 fractions were pooled and lyophilised to yield a red, fibrous solid. The use of volatile buffers throughout the preparation precluded the desalting step usually required before running TLC plates. The solid was stored at -20 °C. The solid has independently been shown to be stable at room temperature for three weeks (Marques, 1986).

6.4 The TLC system and R_{B12} values

In previous studies the integrity of the peptic digest products were determined by amino acid analysis (e.g. Harbury and Loach, 1960a). The purity and structural reproducibility of the haem-peptides have been assessed by a variety of techniques:

1. Paper electrophoresis: Kraehenbuhl et al. (1974) assumed that the appearance of a single band at pH 2.0 in 20% acetic acid (2 hours at 100 Vcm^{-1}) was evidence of a pure MP-8 product.

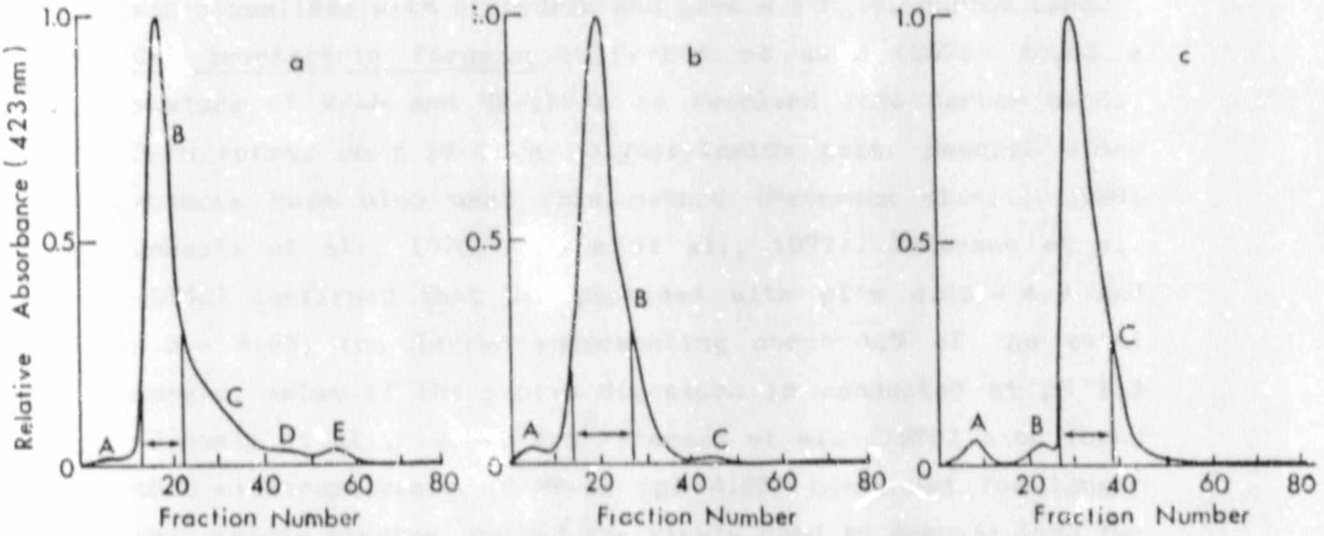


Fig. 6.2 Preparation of MP-8: column chromatography elution profiles. Solvent: 0.1 M NH_4HCO_3, room temperature. Pooled fractions are indicated by arrows. (i) Peptic digestion product of cytochrome c on 4 x 100 cm Biogel P6 at 20 mℓcm^{-2}hr^{-1}, 2.00 mℓ fractions: A = undigested cytochrome c, B = MP-11, C-E = smaller unidentified haem-peptides. (ii) Tryptic digestion product of MP-11, as in (i) but with 2.75 mℓ fractions: A = undigested MP-11, B = MP-8, C = unidentified haem-peptide. (iii) Purification of MP-8 on 1.5 x 30 cm Sephadex G-50 Superfine, 4 mℓcm^{-2}hr^{-1}, 2.00 mℓ fractions: A,B = MP-11 and an unidentified impurity; C = MP-8.

2. Thin layer chromatography: Hallenbeck (1978) quoted an R_f value of 0.56 for the cytochrome c_1 peptic digest product, in butanol/acetic acid/water [4:1:5], the TLC plate used was not specified. Gerber et al. (1975) reported R_f values of 0.34 and 0.56 for MP-8 and MP-11, respectively, in the same solvent. Smith and McLendon (1980) claim to have verified the purity of their MP-8 using silica gel TLC: no R_f values were quoted and the solvent system was not specified.
3. Paper chromatography: Gerber et al. (1975) used a butanol/pyridine/0.1% acetic acid [5:3:11] solvent system. MP-8 was visualised with ninhydrin and gave a single, narrow band.
4. Gel isoelectric focusing: Gerber et al., (1975) found a mixture of MP-8 and MP-11 to be resolved into narrow bands, 3 mm apart, on 6 cm long polyacrylamide gels. Several other authors have also used this method (Peterson et.al., 1980; Jehanli et al., 1976; Wilson et al., 1977). Peterson et al. (1980) confirmed that two peptides with pI's 4.85 - 4.9 and 4.6 - 4.65, the latter representing about 10% of the total sample, arise if the peptic digestion is conducted at pH 1.5 (Jehanli et al., 1976). But Peterson et al. (1980) also found that electrophoresis of MP-11 (pI 4.85) continued for longer than thirty minutes, caused the single band to degrade into two other species.
5. The absence of lysine in MP-8 preparations is a useful indicator to exclude an MP-11 impurity (Gerber et al., 1975); this will not, however, exclude the presence of other impurities.

The deficiencies of these methods were noted during this study. Isoelectric focusing is a fairly complicated technique and has been shown to cause degradation of the sample (Peterson et al., 1980). TLC in the butanol/acetic acid/water [4:1:5] solvent on silica gel gives a very diffuse spot with heavy streaking and fails to separate commercial MP-8 (Sigma) into components, in contrast to the TLC solvent system developed in this study. Paper chromatography, while useful for qualitative analysis, is a time-consuming technique; TLC is more rapid and gives more reproducible R_f values (Skoog and West, 1976).

A rapid and simple process is clearly required to assess the purity of MP-8 from its precursors and further degradative products formed in the digestion. To this end, a TLC solvent system was developed which gave good separation of MP-8 from cytochrome c and MP-11, and from a smaller haem-peptide (eluted after MP-8, see Figure 6.2c). The most suitable solvent system was found to be water/sec-butanol/0.88 M ammonia/solid KCN [88 mℓ/88 mℓ/0.2 mℓ/0.114g].

Silica gel TLC plates were equilibrated for an hour in the tank head space and developed for 1.5 - 2 hours. As the R_f value is sensitive to temperature, solvent mixture and time of development, R_f values were standardised against cyanocobalamin (Vit. B_{12}): $R_{B12} = R_f$ (sample)/R_f (cyanocobalamin).

The species of MP-8 in this solvent is probably the mono-cyanide. Cytochrome c is not displaced from the baseline, and

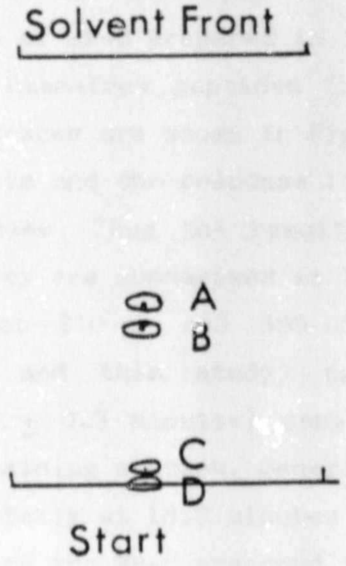


Fig. 6.3 The separation of a mixture of haem-peptides on silica gel TLC in the solvent system 88 mℓ water/88 mℓ sec-butanol/0.2 m 0.88 M NH_3/0.114 g KCN; A = unidentified haem-peptide impurity found in commercial MP-8; B = MP-8; C = MP-11; D = cytochrome c.

MP-11 remains close to the baseline. MP-8 (R_{B12} = 2.3) moves at least 30 per cent of the solvent front distance. The commercial product (Sigma) always shows an additional faint spot (R_{B12} = 3.1 - 3.3); this spot is effectively removed by the Biogel column used in this study. Figure 6.3 shows the separation of a mixture of haem-peptides.

The optimal concentration of potassium cyanide and percentage of water for the TLC solvent were determined by varying their proportions. A cyanide concentration of 1×10^{-2} M and a volume of water, 50 - 60 per cent of total volume, were found to be the most satisfactory. Extending the development time beyond 2 hours gave rise to streaking and decreased resolution of spots. Other solvent systems gave streaking and failed to separate the Sigma MP-8 into components viz. *sec*-butanol/acetic acid/water [4:1:5] and butanol/pyridine/0.1 per cent acetic acid [5:3:11].

6.5 **HPLC results for microperoxidase-8**

An HPLC (high pressure liquid chromatography) method,* using a reversed phase column, was developed in order to obtain a more quantitative analysis of MP-8 prepared in this study, and to assess its separation from haem-free peptides (not apparent in the TLC analysis). The HPLC traces are shown in Figure 6.4. The results are based on area counts and the response factors of all species are unlikely to be the same. Thus the results should be regarded as semi-quantitative (they are summarised in Table 6.1).

Peaks observed at 210 nm and 395 nm are comparable. Both preparations (Sigma and this study) contain a haem impurity (retention time 13.8 ± 0.3 minutes) constituting 0.7 to 2.9 per cent of all haem-containing species. Generally, impurities found in the Sigma product (notably at 18.8 minutes retention time) are much diminished or absent in the MP-8 prepared in this study.

---

* HPLC was conducted by Dr. Paul Adams, University of Cape Town, and results are reported here with his kind permission (Aron et al., 1986).

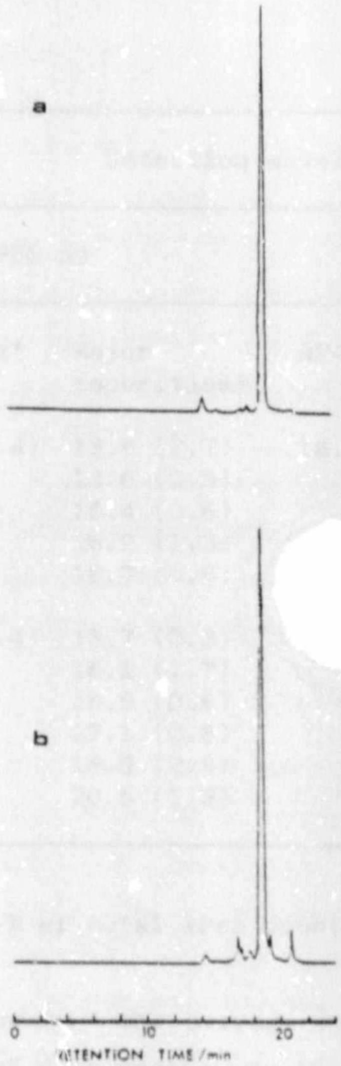


Fig. 6.4 HPLC analysis of MP-8 on Vydac C-18 at room temperature. 25 - 50 µℓ injection of 1 mg/mℓ sample gradient eluted; A = TEAP buffer, pH 2.25, B = 60/40 acetonitrile in A: 10% B increased linearly to 60% B over 20 min and then to 95% B over 0.5 min. Detection at 395 nm: a) This work, final purification on Sephadex G-50; b) Commercial MP-8 (Sigma).

TABLE 6.1: HPLC analysis of commercial MP-8 samples and those prepared as described in the text.

Source of MP-8	Detection wavelength			
	395 nm		210 nm	
	MP-8 Peak*	Major impurities*	MP-8 Peak*	Major impurities*
MP-8 prepared in this work	18.1 (94.4)	13.6 (2.7) 14.6 (0.5) 16.4 (0.6) 16.9 (1.3) 18.7 (0.6)	18.1 (96.1)	13.6 (2.8) 14.6 (0.4) 16.5 (0.5) 18.7 (0.3)
Commercial MP-8	18.1 (92.4)	13.7 (0.3) 16.1 (1.7) 16.5 (0.6) 17.1 (0.8) 18.8 (2.3) 20.5 (1.8)	18.2 (88.4)	13.7 (0.7) 16.2 (2.3) 16.5 (1.1) 17.2 (1.6) 18.9 (3.0) 20.4 (2.9)

* Retention time in min (% of total area count).

Retention times varied daily; the average retention time for the MP-8 peak over a period of some months was 18.3 ± 0.3 min.

6.6 Discussion

6.6.1 Preparation

The preparative method for MP-8 described in this chapter, incorporates time saving procedures, and measures to effect an efficient digestion of cytochrome c. It has several advantages over previously reported methods.

(a) The methods of Harbury and Loach (1960a)

The time consuming dialysis procedures employed by Harbury and

Loach (1960a), and the product loss they necessarily entail, are avoided. Peterson et al. (1980) claim to have doubled the MP-11 yield on replacing the partition chromatographic method of purification (with its dialysis procedures) by gel chromatography. The purification procedures in the method used in this work in fact comprised three separate gel chromatographic separations. Recovery from these columns was over 80 per cent in each case, and resulted in a fairly pure MP-8 product (HPLC analysis). Harbury and Loach did not produce a pure product according to Kraehenbuhl et al. (1974); and it was not subjected to as stringent a test of purity as HPLC. Elution of the product prepared in this work was usually carried out overnight using an automatic eluting and collecting apparatus: this in itself gives a considerable time advantage over the partition chromatographic method.

(b) <u>The method of Gerber et al. (1975)</u>

The method of purification by affinity chromatography, developed by Wilchek (1972) for MP-11, and extended to MP-8 by Gerber et al. (1975), is novel and possesses several advantages. It is a rapid, single-step purification procedure, and selective binding of MP-11 or MP-8 facilitate concentration of the product from large volumes. However, the purity of the MP-8 product is in doubt. Gel-isoelectric focusing, used to assess purity, is an unreliable technique [see Section 6.4]. The reported absence of precursor haem-peptides (cytochrome c and MP-11), and further degraded peptides, is astonishing, given that both tryptic and peptic digestions were carried out in the same reaction mixture. Even with the benefit of two chromotographic separations <u>before</u> the final purification, longer digestion times, and a greater proportion of pepsin enzyme used in a double addition, compared to the Gerber preparation, the MP-8 produced in this work showed traces of MP-11 on the Sephadex G-50 Superfine column (Figure 6.2). The Gerber preparation was also conducted in more acidic conditions, which would be expected to give two haem-undecapeptides (Jehanli et al., 1976), and hence two haem-octapeptide species. Finally, that both MP-11 and MP-8 (and presumably other similar haem-peptides) selectively bind to bovine or human serum albumin, does not auger well for their complete separation. This could perhaps be a very useful technique if a further gel chromatographic step was included.

(c) The method of Kraehenbuhl et al. (1974)

The preparative method of Kraehenbuhl et al. (1974) was extended and improved in several ways [Section 6.3]. A simpler final purification step than countercurrent distribution was ultimately used, for this procedure is time consuming. Some of the improvements made to the Kraehenbuhl method i.e. the longer digestion times, conducting the peptic digestion at a higher pH than 1.5 and a three stage purification by gel chromatography, represent improvements to most of the reported methods.

6.6.2 Purification

A rapid test of purity which succeeds in separating the commercial product into a number of components, was developed. After the final chromatographic purification on a Sephadex G-50 Superfine column, TLC on silica gel in the solvent system: 88 mℓ water/88 mℓ sec-butanol/0.2 mℓ 0.88M NH_3/0.114 g KCN, gave rise to one spot (with no sign at all of impurities behind or ahead of the spot), indicating a product of reasonable purity. The HPLC results confirmed the purity of the MP-8 product [Section 6.5]. This is the first instance where HPLC has been employed to test the purity of MP-8 (or MP-11).

The MP-8 prepared in this study appeared to be free of MP-11, which under the HPLC conditions used, gave a retention time of 15.5 minutes. The MP-8 also seemed to be of consistent purity. An impurity which is eluted after MP-8 on reversed phase HPLC with a retention time of 18.7 minutes, is present in both preparations, though considerably reduced in concentration in the MP-8 prepared in this study, compared to the Sigma product. The impurity could correspond to the faint spot which travels ahead of MP-8 on silica-gel TLC plates. The impurity is more hydrophobic than MP-8, has a lower molecular mass and is a haem-peptide. It displays peroxidatic activity. Its identity has not yet been established. A combination of the second Biogel P6 separation, and the Sephadex

G-50 separation, virtually eliminates this impurity.

The nature of the other impurities less hydrophobic than MP-8, eluted before MP-8 on the reversed-phase HPLC column (see Table 6.1), is difficult to assess. If they are smaller in molecular mass than MP-8, a further purification step on Sephadex G-25 Superfine (fractionating in the range $M_r \sim 1\,000 - 5\,000$) could be advocated. It is unlikely that any of these impurities is due to MP-8 with a hydrolysed Glu-16 residue: Peterson et al. (1980) report only one undecapeptide species after digestion at pH 2.6.

6.6.3 Possible improvements to the method

A great deal of time is consumed in the lyophilisation procedures. It has been shown that the digestion proceeds without alteration if a ten-fold smaller volume of solution is used in the initial peptic digestion; this volume could then be applied directly to the Biogel column prior to lyophilisation (Marques, 1986). Similarly, the volume reduction to yield solid MP-11 prior to the tryptic digestion is unnecessary, provided that the correct mass of trypsin to be added is known. Ultrafiltration using a Diaflo UM2 membrane for volume reduction (Marques, 1986), has been used as an alternative to lyophilisation. Lyophilisation in this work was frequently carried out overnight [Section 2.2.4] and proved useful for large batch preparations.

6.7 Summary

An improved method was developed for the preparation of microperoxidase-8 (MP-8) in 50 per cent yield by peptic and tryptic hydrolysis of cytochrome c. Purification by three gel permeation chromatographic separations replaced the time consuming dialysis and affinity chromatographic procedures conventionally used. A TLC system was developed as a rapid test of purity and gave good separation of MP-8 ($R_{B12} = 2.3$), MP-11 (close to baseline) and a smaller haem-peptide impurity ($R_{B12} = 3.1 - 3.3$). HPLC analysis of the product showed it to be of superior purity to the commercial product (Sigma).

CHAPTER 7: EQUILIBRIUM STUDIES WITH MICROPEROXIDASE-8 IN AQUEOUS METHANOL SOLUTION

7.1 Introduction

In Chapter 1 it was suggested that the potential usefulness of models for the haemoproteins includes a) elucidation of the controlling effect of the protein on the activity of the metal site, b) modelling the in vivo function of the active site, and c) clarification of the structure of the haemoprotein. The isolation of various haem-peptides from cytochrome c with the peptide chain length varying from 6 to 80 amino acids, and their potential use for clarifying the rôle of the protein, was discussed in Section 6.1.1. The haem-peptides have been used as models for the anion binding of haemoproteins (Blumenthal and Kassner, 1979; 1980; 1981), the haem-haem interaction between cytochrome c and its physiological partner, cytochrome reductase, and the cooperative effects in haemoglobin (Urry, 1967; Urry and Pettigrew, 1967), the peroxidatic function of the peroxidases (e.g. Baldwin et al., 1985b) and for cytochrome c function (Wilgus et al., 1978; Yang and Sauer, 1982). Knowledge of the structure of the cytochrome c has been enhanced by model studies with haem-peptides [Section 1.3.2.]. The preparation of MP-8 (Figure 7.1) from cytochrome c was described in the previous chapter.

In this chapter, the potential of MP-8/sulphur adducts to act as models for the iron-methionine (Fe-S) bond interaction in cytochrome c is examined in binding studies with N-acetyl-D,L-methionine and D,L-methionine. Donor-acceptor interactions between MP-8 and the drugs caffeine, tryptophol, barbital and chlorpromazine are examined for comparison with the binding of these drugs to cytochrome c (Chapter 5), and for the parallels they may show to the binding of drugs and other molecules as substrates for cytochrome P-450, in its detoxifying rôle in vivo.

7.1.1. Haem-peptide/thioether complexes as models for cytochrome c

The importance of the Fe-S bond in cytochrome c was discussed in

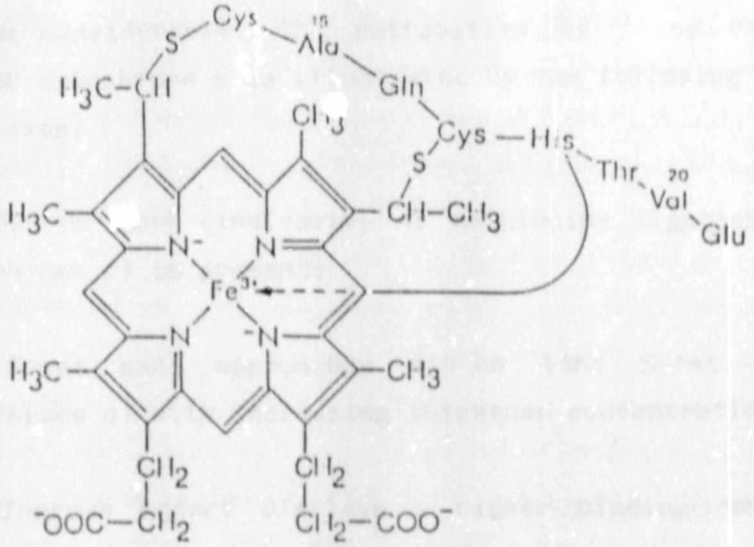


Fig. 7.1 The structure of microperoxidase-8.

Chapter 5. The presence of the bond is required by physiologically active cytochrome c; although it is weak, when the bond is intact, the haem crevice maintains its Type III closed conformation. The first sulphur compounds of haems were observed by Falk (1961) with mercapto ethanol. Harbury et al. (1965) showed that N-acetyl-D,L-methionine, its methyl ester, 2,2'-thiodiglycol, ethyl sulphide and β-methylmercaptopropionic acid formed haemochromes with haem and MP-8. The spectra obtained on complexation with either the ferrous or ferric MP-8 were characteristic of low-spin species; but in contrast to imidazole binding, the thioethers bound less well to the oxidised species. The oxidation-reduction potential in ca. 2 M N-acetyl-methionine was about 150 mV more positive than that for MP-8 (-207 mV). The complex formation was maintained in acid solution. On the basis of these results, Harbury et al. (1965) suggested that the sixth ligand to the haem was the sulphur of methionine 80. The MP-11 adduct with N-acetyl-methionine was later shown by Schechter and Saludjian (1967) to exhibit the 695 nm band characteristic of cytochrome c methionine ligation. X-ray studies (Dickerson et al., 1971) confirmed that methionine was the sixth ligand in cytochrome c. Interest in haem-peptide/thioether complexes as models for cytochrome c has thus been considerable. The suitability of these complexes as models for cytochrome c is illustrated by the following features of the complexes:

(1) the 695 nm band (indicative of methionine ligation to ferric cytochrome c) is present;

(2) the Soret band approaches 410 nm (the Soret maximum of cytochrome c) with increasing thioether concentration;

(3) the ferrous adduct displays a higher binding constant with methionine than the ferric adduct (as in the case with cytochrome c);

(4) the oxidation-reduction potential is raised from ca. -207 mV to about -50 mV on thioether binding (the value for cytochrome c is +260 mV).

These features of similarity have been refined and extended in later studies.

A comparison of the properties of the model complexes with cytochrome c has enhanced the understanding of the effect of the protein on the metal site. Yang and Sauer (1982) in optical and magnetic susceptibility studies, demonstrated the existence of a thermal spin-state equilibrium for the MP-8/methionine complex. The value of μ_{eff} was 4.0 μ_B, larger than the expected low-spin value for native cytochrome c (ca. 2.2-2.3 μ_B) and corresponded to a 63 per cent low-spin character. This was confirmed by an estimated 35 per cent high-spin character for the intensity of the 695 nm band. The ESR spectrum of the model complex gave a low-spin ground state with g- values distinctly different from cytochrome c. This was interpreted as reflecting a longer Fe-S bond distance in the model complex than in cytochrome c. The protein was seen as altering the geometry of the bound axial ligand to achieve a ligand field environment giving rise to the desired redox properties. An independent CD, NMR and ESR study (Smith and McLendon, 1981) found that the molar ellipticity of the methionine complex of MP-8 does not resemble cytochrome c and that a 29 per cent increase in overall spin density is present for the complex. It appeared that the rigid orientation of the methionine in the native protein exerted the main influence on electron spin distribution: the orientation of methionine in the model complex was suggested to be random. Lemberg and Barrett (1973) have also suggested that the positioning of methionine 80 in the protein probably results in a closer contact of its sulphur with the iron than in model complexes, and that this feature is responsible for the high oxidation-reduction potential in eukaryotic cytochromes c, and for the low-spin iron. The slight deviation of the α-band of the haem c- methionine haemochrome at 552.5 nm instead of 550 nm(cytochrome c) (Nanzyo and Sanyo, 1968) has also been explained in this manner. A further example of the control exerted by the protein was demonstrated by McLendon and Smith (1981): the electron transfer reactivity of an MP-8/N-acetyl-methionine complex was found to exceed that of cytochrome c (self-exchange rates were K_{11} (model complex) $\sim 10^{6} M^{-1} S^{-1}$ and K_{11} (cytochrome c) $\sim 10^{3}-10^{4} M^{-1} S^{-1}$). The protein structure appears to decrease the reactivity by maintaining the haem in a hydrophobic crevice, and control the reactivity by providing a more polar environment for the ferric species. The results of X-ray studies confirm this suggestion: in the ferric species, the haem iron is displaced out of the hydrophobic crevice by 0.16 Å and certain buried water molecules lie close to the iron atom (Takano and Dickerson, 1981a; 1981b).

An impressive assessment of the effect of the protein was carried out by Wilgus et al. (1978) by comparing the characteristics of various haem-peptide complexes with cytochrome c. Haem-peptide fragments 14-21 (MP-8), 11-21 (MP-11), 11-26 (hexadecapeptide), 1-38, 1-65 and 1-80 [see Section 6.1.1] were found to form non-covalent complexes with the haem-free peptide 1-104 (i.e. cytochrome c apoprotein). The following conclusions were reached:

(1) Uncomplexed haem-peptides 1-65 and 1-80 (residues 65 and 80 are methionine) did not display sulphur coordination.

(2) The adducts (haem-peptide: apoprotein) 1-38:1-104, 1-65:1-104, 1-80:1-104 and 1-65:66-104 (66-104 a haem-free peptide fragment) displayed the 695 nm absorption band, and Soret values approached 410 nm. The MP-8 and MP-11 complexes with peptide 1-104 did not display this band although Wilgus et al. (1978) postulated sulphur ligation to methionine 80 in these complexes. It is more likely that high aggregation impeded sulphur ligation and that the binding of a histidine residue gave rise to the reported spectra (a Soret maximum shift to 406 nm (see Baldwin et al. (1986)).

(3) The formation constant for the reduced 1-38: 1-104 methionine 80 complex at pH 7 ($7.36 \pm 2.54 \times 10^4$ M^{-1}), exceeded the value for the ligation to reduced peptide 1-38 of N-acetyl-methionine methylamide, by about 4 orders of magnitude (7.0 ± 1.4 M^{-1}).

(4) The formation constant for the methionine 80 complexation (using 1-104) in the reduced haem-peptides exceeded that of oxidised haem-peptides by 1 to 2 orders of magnitude.

(5) The oxidation-reduction potentials of the adducts in (1) which displayed the 695 nm band approached the value for cytochrome c (e.g. E°(1-65) is -108 mV, E°(1-65: 1-104) is 196 mV).

(6) Electron transfer abilities of the non-covalent complexes displaying the 695 nm band also approached that of cytochrome c (1-65: 1-104 has 91 per cent electron acceptance ability from the reductase and 89 per cent electron donation ability to the oxidase, relative to cytochrome c).

The conclusion of these studies was that the burial of haem in a hydrophobic crevice in these models serves to approximate the structure and function of cytochrome c. The important rôle of covalent interpeptide interactions in increasing the local concentration of methionine about the haem and orienting it for ligation was emphasised.

Two schools of thought concerning the raised oxidation-reduction potential of cytochrome c relative to protein-free models, are apparent. First, a decrease in the Fe-S bond length in cytochrome c is implicated (e.g. Yang and Sauer, 1982), and secondly, the hydrophobic haem environment provided by the protein in cytochrome c (Wilgus et al., 1978). The first assertion is supported by the independent observation that a decrease of 0.1 Å in Fe-S bond length could raise the oxidation-reduction potential by ca. 400 mV (Moore and Williams, 1977). The second is supported by the theoretical and experimental studies of Kassner (1972; 1973) which show that the oxidation-reduction potential of haem iron is affected by the dielectric constant of the surrounding medium.

7.1.2 Donor-acceptor interactions in model compounds

Hydrophobic interactions and charge-transfer or donor-acceptor interactions have been little studied compared to other modes of bonding. They are of importance in (1) the binding of the haem moeity to the protein in haemoproteins (Argos and Matthews, 1975), (2) the dimerisation of porphyrins in solution (Brown and Hatzikonstantinou, 1979; Viscio and La Mar, 1978)), (3) the binding of drugs, such as the aromatic compound, chlorpromazine (Cann, 1965; 1967a; 1967b; 1969) and of anaesthetics, for example xenon (Karreman et al., 1959), to the iron porphyrin in haemoproteins, and (4) the binding of substrates including aniline, anaesthetics, insecticides and carcinogens to cytochrome P-450 as a necessary first step in the hydroxylation reaction in detoxification (Coon and White, 1979; Adams et al., 1984). The π-electron cloud from conjugated rings in aromatic substances probably interacts with one or more pyrrole rings of the haem porphyrin.

There appears to be no reported example of donor-acceptor interactions with MP-8, save in its dimerisation (Urry and Pettigrew, 1967). The binding of chlorpromazine to haemin in aqueous solution, pH 6, produced spectral effects which were attributed to the formation of a donor-acceptor complex (Cann, 1965; 1967a; 1967b; 1969). A qualitative study with haemin at pH 8 established that a μ-oxo spectrum is produced in the α-β spectral region on addition of chlorpromazine (Aron, 1982). Caffeine has been shown to bind to haemin, splitting the dimer to form a monomeric haemin/caffeine adduct (Campbell, 1980). The interaction between caffeine and haemin was attributed to π-π (donor-acceptor) bonding. The addition of caffeine to bis-cyanohaemin showed that spectral changes on the binding of caffeine arise largely from splitting the dimer; similarly the titration of monomeric haemin with caffeine in the solvents ethanol, DMSO and formamide gave rise to changes in the intensities, but not positions, of the UV-visible bands. The probable donor-acceptor interactions in the binding of caffeine, tryptophol, barbital and chlorpromazine to cytochrome c were discussed in Chapter 5.

7.2 Experimental procedure

MP-8 was prepared from horse-heart cytochrome c (Sigma Type VII) by peptic and tryptic hydrolysis, according to the procedure laid out in Chapter 6. Phosphate buffers in the pH range 6.0 - 7.4 were prepared according to Long (1971). The stock solutions of MP-8 dissolved in deionized water were stored at -20°C. The concentration of the stock solutions was determined by the pyridine-haemochrome method [Section 2.2.8]. Other reagents used in this chapter are listed in Section 2.1.3.

Titrations of MP-8 were conducted in 20% MeOH:H_2O (v/v) solutions to ensure the presence of monomeric MP-8 [see Section 7.3]. Solutions were prepared by diluting the MP-8 stock solution to ca. 2 x 10^{-6} MP-8 ($\geqslant$97% monomeric (Aron et al., 1986)) in 20% methanol/phosphate buffer solution, and phosphoric acid was used to adjust the pH. The pH electrode was immersed in deionized water between measurements.

Titrations were conducted with freshly made up solutions. The Soret absorbance band of MP-8 at 397.2 nm was monitored by titration of 2.0 mℓ ca. 2 x 10^{-6} M MP-8 (397.2 nm) in 20% MeOH:phosphate buffer (v/v) solution. Aliquots of 1.64 M N-acetyl-methionine solution (pH 7.0), 0.22 M methionine solution and 1 x 10^{-4} M caffeine solution were added to the MP-8 solution using 10 uℓ and 100 uℓ Hamilton syringes, then mixing thoroughly. Titrations with sparingly soluble ligands (methionine, caffeine) were completed by adding weighed portions of the solid ligand directly to the 1 cm cuvette; the entire titration with tryptophol employed the solid ligand. Absorbance values at 397.2 nm, and ligand concentrations, were corrected for dilution, where appropriate. Visible spectra, 650 - 300 nm, in the 0 - 0.5 absorbance range, were recorded on a Cary 2300 UV-Vis-NIR spectrophotometer. The cell compartment was thermostatted at 25.0 ± 0.2°C. The reference cell contained phosphate buffer for all titrations. Since the addition of solid ligand to the cuvette sometimes produced turbidity, the absorbance was adjusted to zero at 750 nm (where no absorbance due to MP-8 is expected) before recording spectra.

A Beer's Law plot of $A_{397.2}$ against MP-8 concentration at 25°C, pH 7.4 (phosphate, μ = 0.1) was obtained by titrating 2.0 mℓ and 20.0 mℓ volumes of 20% MeOH:H_2O solution in 1 cm and 10 cm cells, respectively, with aliquots (10 - 100 uℓ) of MP-8 stock solution (ca. 2×10^{-4} M). Absorbance values were corrected for dilution and were normalised where 10 cm cells were employed. A limiting gradient at low MP-8 concentration was obtained below 1×10^{-7} M in 10 cm cells.

The equilibrium constant, K_{app}, for the binding of n ligands, L, to MP-8:

$$\text{MP-8} + n\text{L} \rightleftharpoons \text{MP-8.L}_n \quad ,$$

$$\text{where } K_{app} = \frac{[\text{MP-8.L}_n]}{[\text{MP-8}][\text{L}]^n} \quad ,$$

was derived from a plot of $\ell og|A_o - A|/|A - A_\infty|$ against $\ell og[L]$ [see Appendix (b)], where A is the absorbance at 397.2 nm. The value of A_∞ was obtained from a plot of $1/|A_o - A|$ against $1/[L]$ [see Appendix (b)]. The induction of dimerisation by added ligands was evaluated by plotting $\ell og(|A_o - A|/2)/|A - A_\infty|[M]$ (where [M] is the concentration of the monomeric species) against $\ell og[L]$ [see Appendix (b)]. Least squares analysis was conducted using the SAS procedure on an IBM 3083 computer.

7.3 A monomeric system

To exploit the haem peptides as models for haemoproteins, it is necessary to develop a system whereby they can be studied as the monomeric species. For even where a particular haemoprotein may contain more than one iron porphyrin (e.g. four in haemoglobin or catalase) there is no evidence that they are ever present except as discrete monomeric complexes.

It is known that protein-free iron porphyrins are subject to aggregation in aqueous solution (White, 1978). Haemin dimerises in aqueous alkaline solution by interactions between the rings to give ring-ring dimers (O'Keefe et al., 1975) which may be hydrophobically bonded (Fuhrhop, 1976) and may form $\pi-\pi$ donor-acceptor complexes (Foster, 1969; Slifkin, 1971). The aggregation of haem-peptides derived from cytochrome c has been reported (Smith and McLendon, 1981; Urry 1967) and characterised for MP-11 (Urry, 1967) and MP-8 (Urry and Pettigrew, 1967). The MP-8 aggregation produced stacked or oblique conformation rather than a head to tail alignment.

Low porphyrin concentrations and the addition of ligands disperse the MP-8 aggregates (Harbury and Loach 1960; 1966; Urry and Pettigrew, 1967). Alcohol:water mixtures are known to disperse porphyrin oligomers (Wagner and Kassner, 1975) probably because of the decrease in polarity of the solvent (White, 1978). In some binding studies with MP-8, 50% ethylene glycol:water (v/v) mixtures have been used to minimise haem-haem interactions (Blumenthal and Kassner, 1979; 1980; 1981).

Working at very low MP-8 concentrations in pure aqueous solution to ensure the presence of the monomeric species ($<2 \times 10^{-7}$ M (Aron et al., 1986)) is complicated by a low Soret absorbance (below 0.015): 10 cm cells for measurements in the absorbance range 0 - 0.1 are required. Ligand absorbance in the Soret region and turbidity effects are magnified under these conditions. Yet it is important to study non-aggregated MP-8: the results of various studies of MP-8 and MP-11 are subject to doubt as cognizance was not taken of the aggregative properties of porphyrins (e.g. Yang and Sauer, 1982; Smith and McLendon, 1980). In this study 20% MeOH:H_2O (v/v) mixtures were employed (the coordination of methanol to porphyrins is probably minimal (White, 1978)), where below ca. 2×10^{-6} M MP-8 the haem-peptide exists predominantly ($\geqslant 97\%$) as a monomer (Aron et al., 1986). Three independent pK's for MP-8 have been determined and 4.43, 8.90 and 10.48 at 25°C, reported (Baldwin et al., 1986). Thus between pH 5 and 8.0, MP-8 can be studied as a monomeric species in aqueous methanol solution, using 1 cm cells in the 0 - 0.5 absorbance range. A further advantage of using alcohol/water mixtures is that the solubility of caffeine, tryptophol and methionine is enhanced.

In the Beer-Lambert law: $I = I_o \exp(-\alpha c \ell)$, the intensity of radiation, I, falls off exponentially with the thickness of the sample, ℓ, and depends on both the concentration of the absorbing species and its ability to absorb light at the frequency employed (α is the absorption coefficient). The conventional form of the Beer-Lambert law uses logarithims to the base 10 and $\epsilon = \alpha/2.303$ is the extinction coefficient: $\ell og(I/I_o) = A = -\epsilon c \ell$. Thus a plot of A against c should be linear for an absorbing species. Deviation from 'Beer's Law' for porphyrins indicates that aggregation of the absorbing species is occurring (White, 1978). At low concentrations of MP-8 ($<8 \times 10^{-6}$ M), a simple monomer-dimer equilibrium has been demonstrated (Baldwin et al., 1986). A Beer's Law plot at pH 7 in 20% MeOH:H_2O (v/v) solution showed adherence to the theoretical curve expressing the equilibrium. In this work, the Beer's Law plot was confirmed at pH 7.4 (the pH predominantly employed for titrations) in 20% MeOH:H_2O (v/v) solution in duplicate experiments (see Figure 7.2). Deviation from the limiting line occurred at ca. 2×10^{-6} M MP-8. The MP-8 solutions used for titrations with thioethers and drugs were $< 2 \times 10^{-6}$ M ($\geqslant$97% monomeric).

7.4 **MP-8/thioether complexes as models for cytochrome c**

A number of studies have examined the formation of thioether complexes with haem-peptides derived from cytochrome c:-

(1) (i) complexes of ferric and ferrous MP-8 (ca. 1×10^{-5} M) in aqueous solution with various thioethers detected by optical spectroscopy (Harbury et al., 1965),

(ii) an MP-8/N-acetyl-methionine complex (ca. 6×10^{-6} M MP-8) in aqueous solution (a formation constant of ca. 0.5 M^{-1} is quoted) detected by ESR, NMR and optical spectroscopy (Smith and McLendon, 1981),

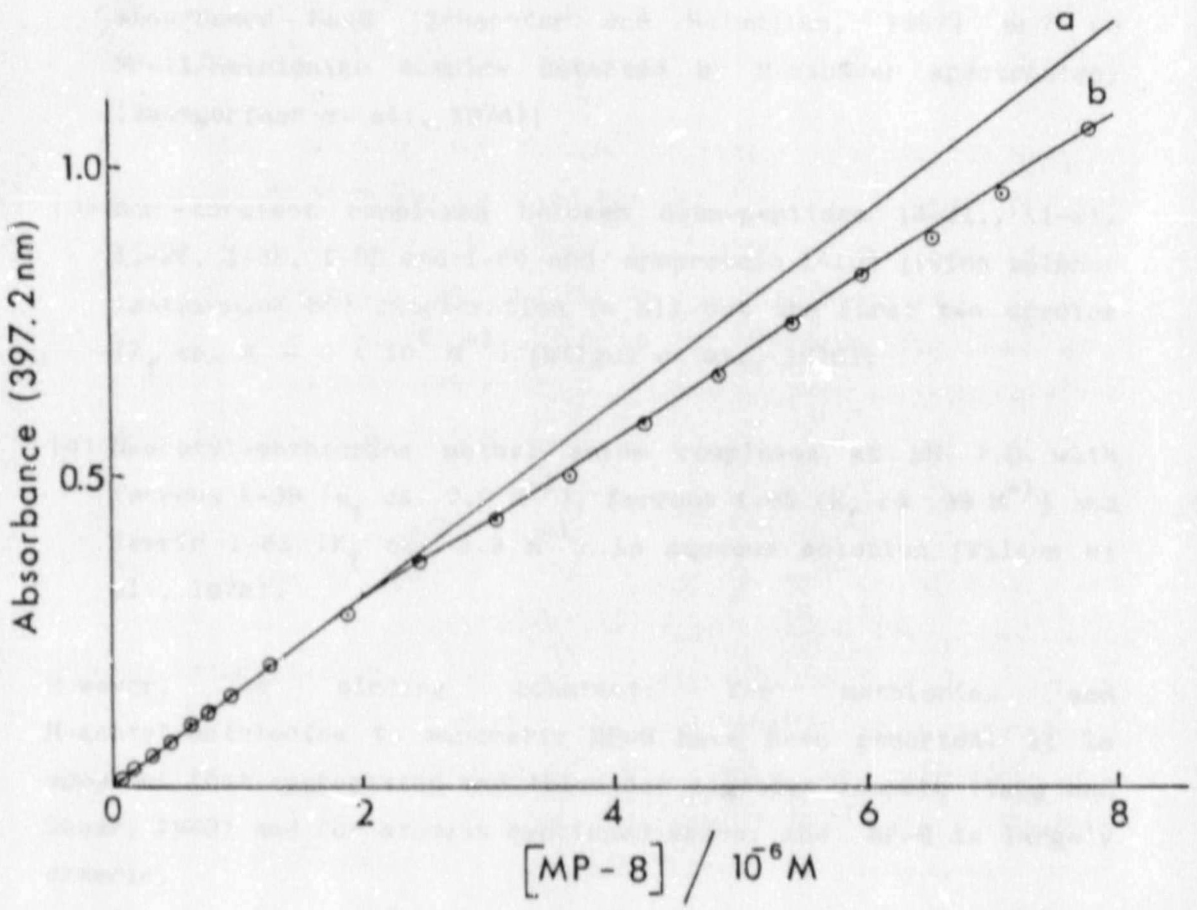


Fig. 7.2 Beer's Law plot for MP-8 at 25°C, $\mu = 0.1$, pH 7.4 (phosphate) in 1 cm and 10 cm cells in 20% MeOH: H_2O (v/v). The ordinate values are normalised for 1 cm cells.

(iii) an MP-8/N-acetyl-methionine complex (MP-8 ca. 2.11×10^{-3} M) in 2.5 M N-acetyl-methionine, by ESR spectroscopy (Yang and Sauer, 1982);

(2) an MP-11/N-acetyl-methionine complex yielding the 695 nm absorbance band (Schechter and Saludjian, 1967) and an MP-11/methionine complex detected by Mössbauer spectroscopy (Baumgartner et al., 1974);

(3) non-covalent complexes between haem-peptides 14-21, 11-21, 11-26, 1-38, 1-65 and 1-80 and apoprotein 1-104 giving sulphur (methionine 80) complexation in all but the first two species (K_f ca. $4 - 8 \times 10^{5}$ M^{-1}) (Wilgus et al., 1978);

(4) N-acetyl-methionine methyl amide complexes at pH 7.0 with ferrous 1-38 (K_f ca. 7.0 M^{-1}), ferrous 1-65 (K_f ca. 99 M^{-1}) and ferric 1-65 (K_f ca. 3.9 M^{-1}) in aqueous solution (Wilgus et al., 1978).

However, no binding constants for methionine and N-acetyl-methionine to monomeric MP-8 have been reported. It is apparent that aggregation and thioether ligation compete (Yang and Sauer, 1982) and for studies mentioned above, the MP-8 is largely dimeric.

The binding of methionine and N-acetyl-methionine in 20% MeOH:H_2O (v/v) solution at pH values 7.4 and 6.7 is examined below.

(a) N-acetyl-D,L-methionine

The binding of N-acetyl-methionine to ca. 2×10^{-6} M MP-8 was monitored at 397.2 nm (Soret region) in 20% MeOH:H_2O (v/v) solution in the absorbance range 0 - 0.5, at pH 7.4 and 6.7, at 25°C. The method of titration is outlined in Section 7.2. Equilibrium was rapidly achieved and no pH changes occurred during the titration. The Soret maximum decreased in intensity and displayed a red shift (characteristic of the formation of a low-spin species) during the course of the titration (isosbestic point at 403 nm). The α-β region showed a shift of the peak from ca. 495 nm to ca. 525 nm, a characteristic of sulphur-haem ligation forming a low-spin species. The plots yielding K_{app} are shown in Figure 7.3 [see Section 7.2]. Three titrations were carried out at each pH. K_{app} is pH-dependent (see Table 7.1) and the slopes (n = 1) indicate a 1:1 adduct. Wilgus et al. (1978) noted a pH-dependence for the binding of N-acetyl-methionine methyl amide with the haem-peptide 1-65: binding constants increased as the pH decreased, as in this work.

The presence of the 695 nm absorbance band due to sulphur ligation of MP-8 was verified for ca. 2×10^{-5} M MP-8 in 1.6 M N-acetyl-methionine, pH 7.0 (0 - 0.1 absorbance range). Figure 7.4 illustrates the suitability of the MP-8/N-acetyl-methionine adduct as a model for cytochrome c: the complex mimics the spectral characteristics of the native protein in the Soret and near infra-red spectral regions.

(b) Methionine

The binding of methionine to ca. 2×10^{-6} M MP-8 in 20% MeOH:H_2O (v/v) solutions at pH 7.4 was monitored spectroscopically at 397.2 nm, at 25°C. The method of titration is presented in Section 7.2. Equilibrium was established rapidly and no pH changes occurred during the titration. The spectral changes characteristic of the formation of a low-spin sulphur/MP-8 adduct are shown in Figure 7.5 (isosbestic point at 403 nm). The α - β region gave similar changes to (a) above. Due to the limited solubility of methionine, it was not possible to demonstrate the presence of the 695 nm band, nor to reach completion in the titrations.

Titrations at pH 6.0 and 6.7 failed to show binding of methionine; dimerisation of MP-8 appeared to be induced with isobestic points of 335 and 408 nm and a decreased Soret intensity, but no shift in maximum. The plot yielding K_{app} at pH 7.4 (for five separate titrations) is shown in Figure 7.6; the slope (n = 1) shows that one ligand is bound per molecule of MP-8 (Table 7.1).

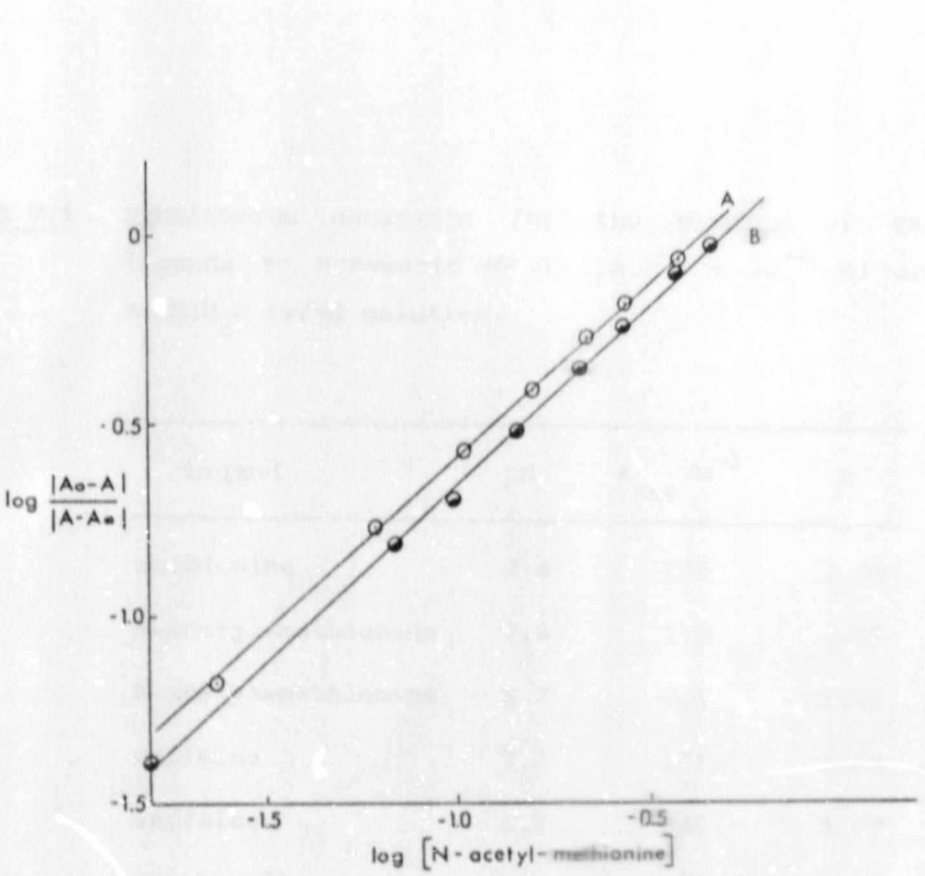
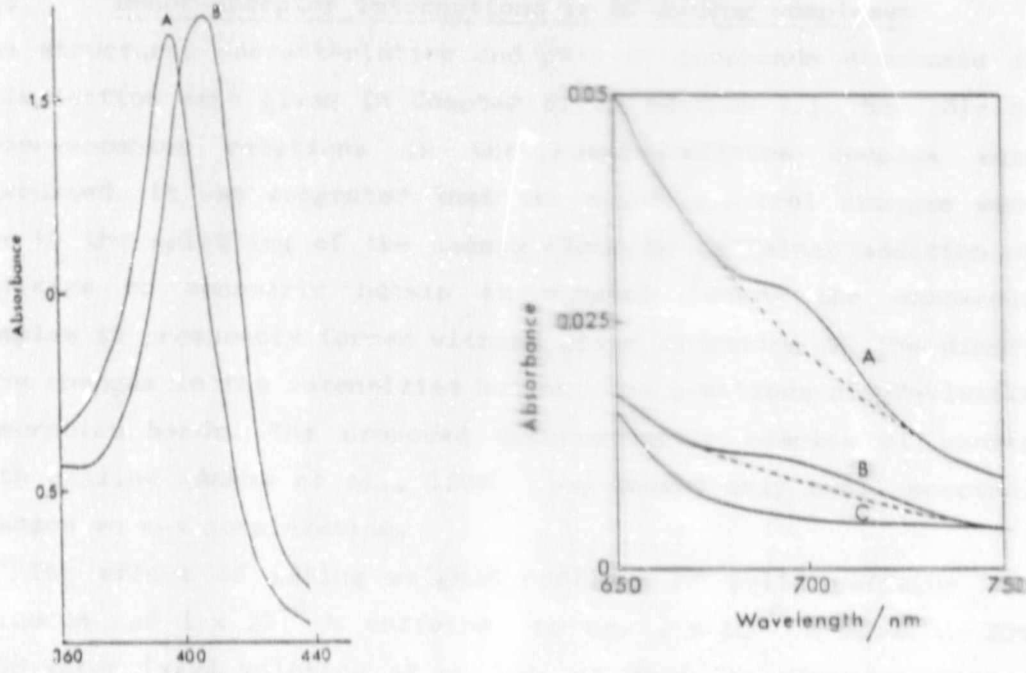


Fig. 7.3 The binding of N-acetyl-D,L-methionine to ca. 2 x 10^{-6} M MP-8 in 20% MeOH:H_2O (v/v) solution at 25°C (μ = 0.1 (phosphate)): A = pH 6.7; B = pH 7.4.



(i) (ii)

Fig. 7.4 Absorption spectra of ca. 2 x 10^{-5} M MP-8 in 1.6 M N-acetyl- D,L-methionine, pH 7.0: (i) Soret region A: MP-8; B: MP-8/N-acetyl-methionine adduct; (ii) near infra-red region A: cytochrome c; B: MP-8/N-acetyl-methionine adduct; C: MP-8.

TABLE 7.1 Equilibrium constants for the binding of various ligands to monomeric MP-8 (ca. 2 x 10^{-6} M) in 20% MeOH:H_2O (v/v) solution.

Ligand	pH	K_{app}/M^{-1}	n
methionine	7.4	155	1.09
N-acetyl-methionine	7.4	1.9	0.95
N-acetyl-methionine	6.7	2.1	0.91
caffeine	7.4	151	1.03
caffeine	6.7	240	1.07
tryptophol	7.4	60	1.01

7.5 Donor-acceptor interactions in MP-8/drug complexes

The structural characteristics and pK's of compounds discussed in this section were given in Chapter 5. In Section 7.1, the rôle of donor-acceptor reactions in the haemin-caffeine complex were discussed. It was suggested that the major spectral changes were due to the splitting of the haemin dimer by caffeine: addition of caffeine to monomeric haemin in ethanol (where the monomeric complex is presumably formed without prior splitting of the dimer) gave changes in the intensities but not the positions of UV-visible absorption bands. The proposed donor-acceptor complex of haemin with aniline (Adams et al., 1984) also showed only small spectral changes on $\pi-\pi$ complexation.

The effect of adding weighed portions of solid caffeine (or aliquots of 1 x 10^{-4} M caffeine) to ca. 2 x 10^{-6} M MP-8 in 20% MeOH:water (v/v) solution at pH 7.4, at 25°C, is shown in Figure 7.7. The Soret maximum is decreased (without being shifted) with an isosbestic point at 418 nm. In the α-β region, the α-band increased and β-band decreased in intensity, without being shifted. Equilibrium was achieved rapidly after mixing and no pH change during the titration occurred.

Initially it was thought that this effect might be due to

Fig. 7.5 Spectral changes for a titration of ca. 2×10^{-6} M MP-8 in 20% MeOH:H_2O (v/v) solution, pH 7.4 at 25°C, with D,L-methionine.

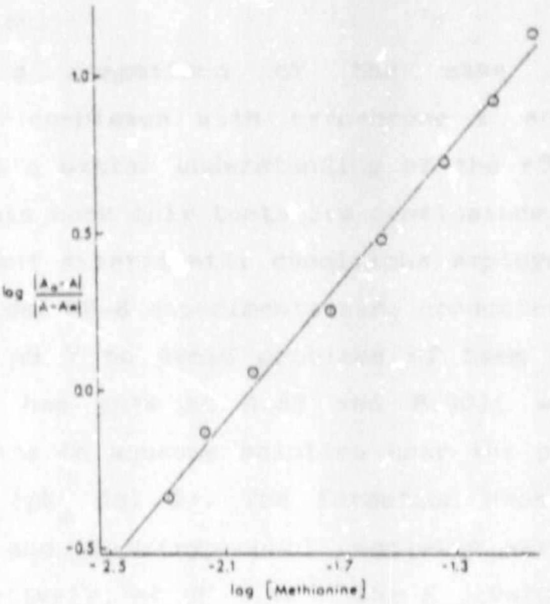


Fig. 7.6 The binding of D,L-methionine to ca. 2×10^{-6} M MP-8 in 20% MeOH:H_2O (v/v) solution at 25°C (μ = 0.1 (phosphate)) at pH 7.4.

dimerisation, although this seemed unlikely given the monomerising effect of caffeine on haemin. Indeed the data did not fit an analysis of dimerisation [Section 7.2].

Accordingly, the data were analysed for binding by caffeine: the plot of ℓog K_{app} against ℓog[caffeine] is given in Figure 7.8 for the titration at pH 7.4 (four titrations were conducted). The slope suggests that one caffeine molecule is bound per MP-8 molecule at each pH and the value of K_{app} appears to be pH-dependent (Table 7.1). By analogy with haemin, it is likely that donor-acceptor interactions govern MP-8/caffeine complexation.

Two determinations of the binding of tryptophol to ca. 2×10^{-6} M MP-8 in aqueous-methanol solution at pH 7.4 gave a 1:1 adduct with $K_{app} = 60\ M^{-1}$ (Table 7.1) (see Figure 7.9 for a plot of ℓog K_{app} against ℓog[tryptophol]).

Chlorpromazine, known to give a donor-acceptor complex with haemin at pH 6.0 and 8.0 [Section 7.1], gave qualitatively similar changes to caffeine and tryptophol with MP-8 in the Soret region. A quantitative study was precluded by experimental difficulties, outlined in Chapter 5.

Finally, titrating MP-8 with barbital at pH 6.0 gave rise to the same spectral changes seen on formation of the MP-8/caffeine donor-acceptor complex, with isosbestic points at 421 and 341 nm in the Soret region.

Ideally, a comparison of the ease of formation of donor-acceptor complexes with cytochrome c and the model MP-8 should lead to a better understanding of the rôle of the protein. However, in this work only tentative conclusions may be reached due to the different experimental conditions employed in cytochrome c and MP-8 studies. MP-8 experiments were conducted in 20% MeOH:water solution near pH 7 to avoid problems of haem aggregation and pH effects (MP-8 has pK's at 4.43 and 8.90); while cytochrome c experiments were in aqueous solution near the pK_a of the alkaline isomerisation (pK_a ca. 9). The formation constants for the 1:1 MP-8/caffeine and MP-8/tryptophol complexes were ca. 150 M^{-1} and 60 M^{-1}, respectively, at pH 7.4. The K_{eq} values for the binding of caffeine (1:1) and tryptophol (1:2) to cytochrome c were $6 \times 10^{-4}\ M^{-1}$ and $9 \times 10^{-2}\ M^{-1}$, respectively. Despite the pH-dependence of MP-8/caffeine complex formation (which probably

Author Aron Janine
Name of thesis The Coordination Chemistry Of Some Cobalt Corrinoid And Iron Porphyrin Complexes. 1986

PUBLISHER:

University of the Witwatersrand, Johannesburg