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REDOX POTENTIALS OF THE PYRIDINE-HAEM *c* SYSTEM

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SUMMARY

1. Haem *c* was synthesized and purified. It was shown unequivocally that the method gives a product with the cysteine residues on the α -carbon atoms at the 2 and 4 positions of the haem.

2. Redox potentials of haem *c* in the presence of 2.5 M pyridine were determined in the pH range 1.5–13; it was found necessary to add cetyl trimethyl ammonium bromide (CTAB) to prevent precipitation in the acid range below about pH 4. The E_m vs pH curve shows three slopes ($-dE/dpH$) of value, 0.18, 0.01 and 0.06 with points of inflexion at pH 3.8 and 10.6. The potentials are intermediate between those of protohaem and mesohaem obtained under similar conditions.

3. With constant haem *c* concentration (a) 10^{-4} M and (b) 10^{-5} M and varying pyridine concentration (0.12–5 M) it was found at pH 9.0 that E_m values increased as the pyridine concentration was increased and there was a tendency to reach a plateau value. The explanation appears to be that pyridine binds more firmly to ferroporphyrin *c* than to ferriporphyrin *c*.

4. When the pyridine concentration was kept constant (2.5 M) and the haem *c* concentration was varied in the range $7 \cdot 10^{-4}$ – $7 \cdot 10^{-6}$ M, it was found that a decrease in haem *c* concentration brought about an increase in redox potential. The results are explained as being due to dimerization of the oxidized form.

5. The results are discussed in comparison with a number of related haem systems.

INTRODUCTION

One of the distinguishing features of the *c*-type cytochromes is that the haem is bound in thioether linkage to the protein moiety; a binding which is in effect the addition of cysteine residues to the vinyl groups of protohaem. Due to the stability of this binding it is possible to bring about extensive changes in the protein moiety without loss of the haem and in this way to obtain important information with regard to the influence of the environment on the redox potential of the haem. Thus Harbury and Loach^{1,2} observed, with the undeca- and octapeptide haem *c* containing fragments, considerably lower potentials than those of native cytochrome *c*. They also observed

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Abbreviation: CTAB, cetyl trimethyl ammonium bromide.

under certain conditions, potential *vs* pH relationships which were different from those of cytochrome *c* and of protohaem when liganded to simple nitrogenous bases. There still remained, however, the question as to what effect, if any, the sulphur atoms themselves were having on the redox potential of the haem and this could not be answered by use of the relatively complex haem-peptide systems. Accordingly, haem *c*, the iron complex of porphyrin *c* (2,4-dicysteinyl-ethyl porphyrin), was synthesized and its redox potential when liganded to pyridine examined under conditions of varying concentration and pH. This paper presents the results of this study and their interpretation in comparison with the redox potentials of a number of related haem systems.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade unless otherwise mentioned.

Elementary analysis

Oxygen and sulphur contents were determined by the Australian Microanalytical Service, C.S.I.R.O. and University of Melbourne. Iron was determined by the *o*-phenanthroline method described by Henderson and Rawlinson³.

Spectrophotometry

A Cary Model 14 recording spectrophotometer was used.

Preparation and purification of porphyrin c

Porphyrin *c* was prepared by the method of Neilands and Tuppy⁴. The crude product was purified by the following procedure: 100 mg of crude porphyrin *c* was dissolved in 16 ml of 70 % (v/v) acetic acid; this solution was then diluted to 700 ml with water and applied to a column (3 cm × 15 cm) prepared from Amberlite CG-50 (acid form) suspended in water. The porphyrin formed a concise band at the top of the column. After washing the column with 600 ml of distilled water, elution of the porphyrin was commenced using 1 M ammonium acetate solution (pH 8.5). Porphyrin *c* separated from a smaller band of unidentified slower moving brown material. The pH of the eluted porphyrin solution was adjusted to pH 4.7–4.8 with glacial acetic acid and was then applied to a fresh column (3 cm × 15 cm) of Amberlite CG-50 prepared in the same way as above. The porphyrin *c* again became bound to the resin. The column was washed with 5 % (v/v) acetic acid until the eluate was free from NH_4^+ (by test with Nessler's reagent) following which the porphyrin *c* was eluted with 70 % (v/v) acetic acid. This solution was evaporated to a small volume in a rotary evaporator at 40 °C and was finally taken to dryness over NaOH, under vacuum in a desiccator. (Found: O, 20.8 %; S, 7.20 %; porphyrin *c* diacetate ($\text{C}_{44}\text{H}_{56}\text{N}_6\text{O}_{12}\text{S}_2$) calcd: O, 20.8 %; S, 6.94 %). Absorption maxima, absorbance ratios and extinction coefficients were close to those already reported^{4,5}.

Cleavage of the thioether bonds of porphyrin c and identification of the products

There are two possibilities for the addition of the sulphhydryl group at each vinyl group of protoporphyrin. Whereas Sano *et al.*⁵ using a different method of

preparation had shown that addition of the sulphydryl groups was to the α -carbon atoms by their procedure, Neilands and Tuppy⁴ had not similarly examined their product. The material prepared for the present study, essentially by the latter procedure, was therefore examined in the following way: 30 mg of porphyrin *c* was dissolved in 35 ml of 30 % (v/v) acetic acid and was then stirred with 25 ml of 0.32 % (w/v) Ag_2SO_4 at 80 °C for 25 min. The buff coloured precipitate which formed was separated and then suspended in 0.3 M HCl after which H_2S gas was passed through the suspension for 10 min. The silver sulphide which formed was separated by centrifugation. The supernatant liquid was concentrated to a small volume and shown by paper chromatographic procedures to contain cysteine as its single constituent. The porphyrin left in the supernatant liquid following the Ag_2SO_4 treatment was extracted with ether, esterified with 20 % (w/v) HCl in methanol and purified by chromatography on an alumina column. The porphyrin was shown to be haematoporphyrin dimethyl ester by comparison of its chromatographic properties and its dehydration rate on heating with values found for authentic haematoporphyrin dimethyl ester. Thus the addition of the cysteine residues was at the α -carbon atoms in the 2 and 4 positions of the haem.

Preparation of haem c

Iron was incorporated into porphyrin *c* free acid by the method of Warburg and Negelein⁶. The haem was purified by the resin-column procedure described for porphyrin *c* except that the haem was eluted from the second column using 60 % (v/v) acetic acid. The alkaline elution from the first column ensured the removal of all excess iron. (Found: Fe, 5.57 %; acetatoferriporphyrin *c* diacetate ($\text{C}_{46}\text{H}_{57}\text{N}_6\text{O}_{14}\text{S}_2\text{Fe}$) calcd: Fe, 5.40 %.) Absorption maxima, absorbance ratios and extinction coefficients of the haem in buffer solutions and when liganded with pyridine were in close agreement with published values⁷.

Oxidation-reduction potentials

Redox potentials were determined by potentiometric titration with the reduced sodium salt of anthraquinone-2-sulphonate in an atmosphere of purified N_2 . The apparatus and titration technique used were essentially that described by Henderson and Rawlinson³. The above titrant was chosen because of its low redox potential ($E_m = -0.23$)⁹ relative to the system being investigated and its ease of preparation and storage in the reduced state. E_m values were assessed by graphical analysis and in some cases by the rectification method of Reed and Berkson⁸. In all cases the E_m values reported have been referred to the standard hydrogen electrode.

RESULTS

Redox potentials of the pyridine-haem c system

Dependence on pH

The ferri- and ferroporphyrin *c* system (haem concentration 10^{-4} M) in the presence of 2.5 M pyridine was found to be electromotively active without the addition of a mediator. Stable potentials were quickly obtained (1–2 min) after each addition of titrant. In the pH range 5–12.5 symmetrical titration curves were obtained with n values close to 1. A typical titration curve is shown in Fig. 1.

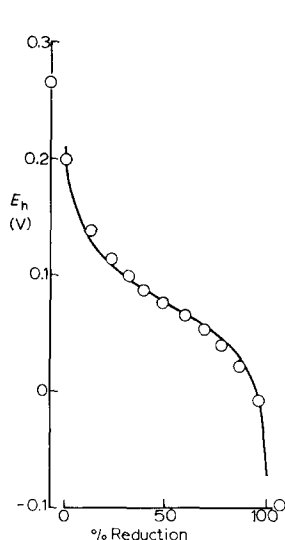


Fig. 1. E_h vs per cent reduction curve for pyridine-haem *c* system. \circ , experimental points obtained from reductive titration of 10^{-4} M ferriporphyrin *c* in the presence of 2.5 M pyridine at pH 8.21. Solid line is for theoretical curve where: $E_m = 0.076$ V and $n = 1$.

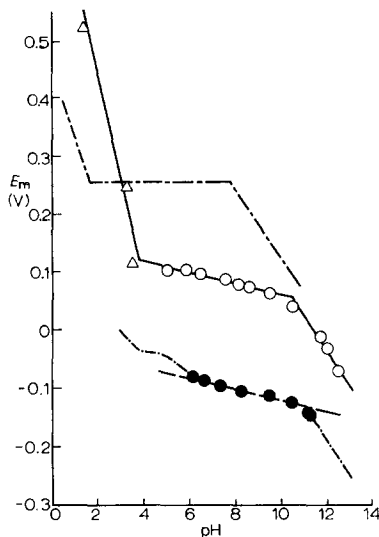


Fig. 2. Comparison of redox potential data obtained for the pyridine-haem *c* system with data from other systems. \circ , experimental points obtained for haem *c* in the presence of 2.5 M pyridine at various pH values; Δ , experimental points obtained for haem *c* in the presence of 2.5 M pyridine with CTAB added; ---, E_m vs pH curve for haem undecapeptide from cytochrome *c* in the presence of 0.12 M pyridine, from the data of Harbury and Loach²; \bullet , some of the experimental points obtained by Harbury and Loach²; -.-, dashed line drawn through data of Harbury and Loach²; ---, E_m vs pH curve for cytochrome *c* from data of Rodkey and Ball¹⁴.

Attempts were made to carry out titrations under these conditions at pH values below 5 but precipitation of pyridine ferroporphyrin *c* commenced at about pH 4.5. It was found, however, that addition of cetyl trimethyl ammonium bromide (CTAB) (1 mg/ml of haem solution) prevented this precipitation. Check titrations in the presence and absence of CTAB at pH values at which precipitation did not occur, showed that there was no effect on the potentials due to the presence of CTAB and titrations were therefore carried out in its presence in the pH range 3.6 to 11.5. The potentials so obtained were much less stable than those obtained in the higher pH range; it was possible, however, to assess points of inflexion and hence to obtain E_m values from the resultant curves but reliable assessment of n values was not possible. The E_m vs pH relationship for the pyridine-haem *c* system in the pH range 1.5–12.5 is included in Fig. 2. The curve shows three clearly defined slopes ($-dE/dpH$) of 0.18, 0.01 and 0.06 with points of inflexion at pH 3.8 and 10.6.

Dependence on pyridine concentration

This investigation was carried out at pH 9.0 as this value is sufficiently removed from the pH value of the point of inflexion in the E_m vs pH curve to ensure that only one molecular species was present. The redox potentials of pyridine-haem *c* determined at pyridine concentrations in the range 0.12–5 M and with haem concentrations maintained at (a) 10^{-4} M and (b) 10^{-5} M are graphed in Fig. 3. Unstable poten-

tials were obtained when the pyridine concentration was lowered below 0.12 M. It can be seen from Fig. 3 that there is in general an increase in E_m with increase in pyridine concentration and a tendency to form a plateau potential at high pyridine concentration.

An explanation of the shape of the curves obtained can be made in terms of an equation derived by Clark⁹ which links E_m with the dissociation constants K_o and K_r of the ferri- and ferroporphyrin ligand complexes and the ligand concentration:

$$E_m = E_1 + \frac{RT}{nF} \ln \frac{K_o}{K_r} + \frac{RT}{nF} \ln \frac{K_r + [L]^q}{K_o + [L]^p}$$

p and q represent the number of ligand molecules (L) bound to the oxidant (O) and reductant (R), respectively. $[L]$ is the concentration of free ligand and is approximately equal to the total concentration $[S_L]$ when $[\text{haem}] = 10^{-4}$ – 10^{-5} M, and $S_L = 0.12$ – 5 M. The increase in E_m with pyridine concentration seen in Fig. 3 is predicted from this equation if $K_o > K_r$ and S_L is significant compared to K_r . From the equation it can be seen that when $S_L \gg K_o$ and K_r , and $q = p$ the third term is removed and the E_m would reach a final plateau potential defined by

$$E_m = E_1 + \frac{RT}{nF} \ln \frac{K_o}{K_r}$$

However, it was impracticable to perform titrations with pyridine concentrations above 5 M so it was not determined whether a true plateau could be reached. Thus an adequate explanation for dependence of the redox potential of this system on pyridine concentration is that the more stable complex is formed between ferroporphyrin *c* and pyridine. This is predicted in complexes of this type, as ferrous iron complexes are stabilized by a greater degree of back donation with π bonding ligands such as pyridine, than are the ferric complexes^{10, 11}.

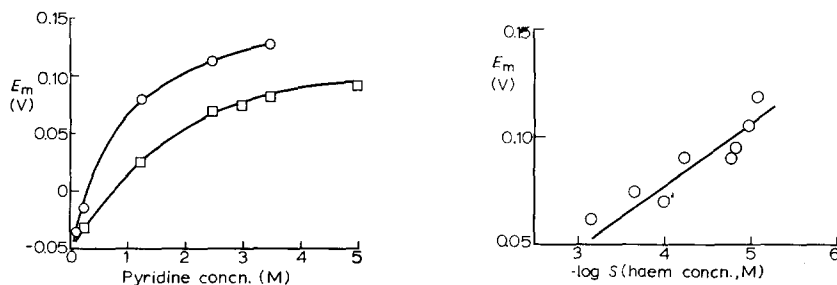


Fig. 3. Plots of E_m vs pyridine concentration. \circ , E_m values obtained when haem *c* (10^{-5} M), pH 9.0; \square , E_m values obtained when haem *c* (10^{-4} M), pH 9.2.

Fig. 4. Oxidation-reduction potentials of pyridine haem *c* plotted against $-\log$ haem concentration ($S = \text{haem concentration (M)}$). E_m values obtained from titration curves when haem *c* concentration was varied between $7 \cdot 10^{-4}$ – $7 \cdot 10^{-6}$ M and with a constant pyridine concentration of 2.5 M. Smooth curve, line of best fit to the data, slope 0.03.

Dependence on haem c concentration

Redox potentials were determined as the haem *c* concentration was varied in the range $7 \cdot 10^{-4}$ – $7 \cdot 10^{-6}$ M while the pyridine concentration was kept constant at 2.5 M. Fig. 4 shows a plot of the E_m values so obtained against values of $-\log S$, where S is the haem concentration. Titrations were attempted at lower haem concentrations but the potentials were unstable and reliable E_m values could not be obtained. At haem concentrations of 10^{-4} M and higher, values of n were found to be close to 1; as the haem concentrations were decreased below 10^{-4} M, however, n values of < 1 were obtained until at the lowest concentration tested ($7 \cdot 10^{-6}$ M) a value of 0.6 was obtained.

As can be seen from Fig. 4, a decrease in haem *c* concentration brings about an increase in redox potential. Such a relationship in haem systems is frequently due to a change in the state of aggregation of the oxidant. An equation was derived by Clark⁹ which relates E_m to the concentration of the haem for the case in which there is dimer formation in the oxidant. Where one electron is involved in the conversion of oxidant to reductant and when K_{do} (the formation constant for a dimer of the oxidant) is $> 10^6$ and S (the haem concentration) is in the range 10^{-3} – 10^{-5} M, this equation can be simplified to:

$$E_m = E' - 2.303 \frac{RT}{2F} \log K_{do} - 2.303 \frac{RT}{2F} \log S$$

In Fig. 4 E_m was plotted *vs* $-\log S$. A line of slope $2.303RT/2F$ or 0.03 at 25 °C has been drawn as a line of best fit through the points. Although there is some scatter in the points the trend in the data clearly follows this line. Thus the simplest explanation of the concentration dependence of the redox potential is that the pyridine ferriporphyrin *c* forms a dimer. Shack and Clark¹² found a concentration dependence for the pyridine protohaem system at pH 11 which along with other data showed that pyridine ferriprotoporphyrin was dimeric at pH values above the inflexion in the E_m *vs* pH curve at pH 9 but monomeric at lower pH values. The pyridine ferriporphyrin *c* system is different in this respect as it appears to be dimeric at pH 9 which is well below the inflexion at pH 10.6 in the E_m *vs* pH curve.

Fractional n values are not unusual in haem systems and can sometimes be explained in terms of aggregation effects⁹. In the present case, however, it is difficult to explain the apparent increase of the effect, *i.e.* a reduction of n values as the dilution is increased.

DISCUSSION

The potentials of the pyridine–haem *c* system can be seen (Fig. 2) to be intermediate between those of the pyridine–protohaem¹² and pyridine–mesohaem systems¹³. This can be most simply explained as being due to an inductive effect of the sulphur atoms on the π electron system of the haem which results in an electron withdrawal from the iron atom; the effect being less than that brought about by the vinyl groups of protohaem.

The pyridine–haem system has, in common with all of the systems shown in

Fig. 2, two well defined sections to the E_m vs pH curve in the pH range 7 (or less in some cases) to about 13. There is considerable variation in the positions of the inflexions but after these, as the pH value increases, there is in each case a 0.06 slope. Such a slope can be due to the addition of a hydroxyl or the removal of a proton from the oxidized form so that there is a direct effect on the iron atom. As the pK value for the dissociation of the pyridinium ion is 5.2, there is no question of the involvement of proton in this pH range with the simple systems pyridine-protohaem and pyridine-mesohaem. This means that interaction with hydroxyl ion must be responsible for the 0.06 slope in these cases. We obtained no spectral evidence for the liganding of the primary amino groups of the cysteine residues of haem *c* with the haem iron (either Fe^{2+} or Fe^{3+}) at either pH 7 or 11.5. This in conjunction with the demonstrated dependence of the potential of this system on the pyridine concentration and the finding of Nanzyo and Sano⁷ that more of the ferri form but not of the ferro form of the pyridine-haem *c* system exists in the highspin state as the pH increases after about pH 8 leads us to the conclusion that the 0.06 slope with this system is also due to the replacement of pyridine by hydroxyl.

It should be pointed out that this simple explanation cannot be extrapolated to the more complex pyridine-haem *c* undecapeptide² and cytochrome *c*^{14,15} systems where the spectral data at high pH are at variance with replacement of an iron ligand directly by hydroxyl.

The mid-part of the pyridine-haem *c* curve (Fig. 2) has the unusual slope of 0.01. It is interesting that a line of best fit drawn through the data of Harbury and Loach² also shows a 0.01 slope which, as in the above case, inflects as the pH is increased and becomes a 0.06 slope. In the equation derived by these workers to describe the curve for the pyridine-haem *c* undecapeptide system it was found necessary to include for this part of the curve, two dissociation constants in the oxidized form and one in the reduced form. The latter and one of the former were postulated as being ϵ -amino groups of lysine. We derived a similar equation for the pyridine-haem *c* system. The fit was not as good as desired, however, and there was also the difficulty of giving a physical meaning to the dissociation constants in view of the absence of amino acid residues other than the cysteines which were found not to ligand to haem *c* either in the ferro or ferri forms. Furthermore Nanzyo and Sano⁷ showed that pyridine was the most firmly bound to both ferro- and ferri-haem *c* of all the nitrogenous bases examined. An explanation for the 0.01 slope of the pyridine-haem *c* system is therefore probably best sought in the demonstrated aggregation of the oxidized form (Fig. 4) which it might be expected would vary with pH.

The increase in slope of the pyridine-haem *c* curve (Fig. 2) at pH values below 3.8 is indicative of a protonation of the oxidized component of the system⁹ and almost certainly involves the pyridine molecules liganded to each haem *c* molecule. Addition of more than one proton is indicated by a slope value (0.18) which is in excess of that required for a one proton addition (0.06)⁹.

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