The Structure of Haem in Pyridine/Water Mixtures and its Implication in Studies of Haem Catabolism

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1. A study of haem spectra in pyridine/water mixtures at low pyridine concentrations revealed changes in haemochrome structure consistent with an aggregation process. No corresponding change in the structure of the haemichrome species was observed.
2. This aggregation has been correlated with a previously observed sharp decrease in the rate of coupled oxidation (degradation) of haem as pyridine concentration is decreased. The decrease appears to be due primarily to haem aggregation and not to changes in the hydrophobic nature of the solvent. The effect of ethanol and butanone addition was examined and supports this conclusion.
3. Evidence is presented that coupled oxidation occurs via the iron(II) species (haemochrome).

The formation, in pyridine/water mixtures, of complexes between pyridine and the iron(II) and iron(III) forms of protohaem is well known (Falk, 1964). These complexes, known as pyridine haemochrome and pyridine haemichrome respectively, have characteristic absorption spectra in the region 400–600 nm and have proved of considerable value in the detection and measurement of haem (Falk, 1964). Most work relating to the structure of haem in aqueous pyridine has been carried out at pyridine concentrations greater than about 20% (v/v). Under these conditions there is no doubt that both the haemochrome and haemichrome are monomeric, each haem molecule being co-ordinated to two pyridine molecules, one lying above, and the other below, the mean porphyrin plane. If the pyridine concentration is decreased to a sufficiently low value, however, aggregation of haem might be expected, since in the limit (100% water), it is well known that protohaem is strongly dimerized (Brown *et al.*, 1970). For example, a 10 μ M-ferrihaem solution in 100% water, pH 7, would be expected to be more than 95% dimerized.

The pyridine haemochrome system has been used also in attempts to establish a model system for the catabolism of haemoglobin and other haemoproteins, since haem degradation can occur very rapidly in aqueous pyridine in the presence of ascorbate and oxygen. The question as to whether haem cleavage *in vivo* is enzymic in nature (Tenhunen *et al.*, 1969) or whether it can be represented by coupled oxidation systems (O'Carra & Colleran, 1969) has been frequently discussed. Reviews by Lathe (1972) and Jackson (1974) have assembled the available evidence, but these issues remain unresolved.

One of the problems raised by studies of model systems has been the possible requirement of a hydrophobic environment for the degradation

process. Thus degradation occurs much more readily in pyridine than in water and apparently much more readily when haem is an integral part of a protein than when free in aqueous buffer (O'Carra & Colleran, 1969). Although not disputing the possible importance of the hydrophobicity of the medium, Brown *et al.* (1974) showed that haem aggregation was a major factor in model studies, monomeric haem being much more reactive than dimeric species, and that in aqueous systems haem aggregation could satisfactorily account for the low degradation rates observed.

P. O'Carra (personal communication) has suggested that although aggregation might affect degradation rates, it is unlikely to be a major factor. This conclusion is based on the observation (Colleran, 1971) that at pyridine concentrations below about 10% (v/v), there is a dramatic decrease in degradation rate, although it is stated that the haem remains in monomeric form. It was also found (O'Carra & Colleran, 1970; Colleran, 1971) that on addition of ethanol or butan-2-one to systems containing less than 10% (v/v) pyridine, high rates of degradation were re-established. The sharp fall in rate of haem cleavage was attributed therefore to changes in solvent hydrophobicity, rather than to a change in the nature of the haem.

The present work was initiated to evaluate more precisely the nature of the changes in the haem/pyridine/water system at around 10% (v/v) pyridine and to correlate them with studies on haem degradation.

Experimental

Materials

Chloroprotohaemin was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Buffer components and butan-2-one were Analytical-

Reagent grade (BDH Chemicals Ltd., Poole, Dorset, U.K.). Sodium dithionite was BDH Laboratory-Reagent grade and sodium ascorbate was obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. Pyridine was BDH Analytical-Reagent grade and was redistilled over KOH before use. Ethanol was obtained from UNALCO Ltd., Liverpool, U.K.

Preparation of solutions

To all test solutions 0.1M-phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH7.5, was added. This procedure was adopted so as to reproduce exactly the conditions used in haem-degradation studies. A series of solutions at each haem concentration was prepared as follows. An appropriate weight of protohaemin was dissolved in pure pyridine for use as a stock solution. To 1 ml of this stock solution in a 50 ml volumetric flask was added the appropriate volume of pyridine and 2 ml of buffer before making up to volume with water. For experiments at constant pyridine concentration, a similar procedure was adopted, except that various volumes of stock haem solution were used.

Spectrophotometric measurements

All measurements were made at 25°C and were obtained within 4 h of preparation of solutions, although there was no evidence of instability. Spectra between 370 and 570 nm were recorded by using a Unicam SP.1800 double-beam spectrophotometer. Stoppered cuvettes with path lengths of 1, 5 or 10 mm were used according to the different haem concentrations. Spectra of reduced haem were obtained by adding a small quantity of solid dithionite, shaking vigorously in the cuvette, and then allowing the excess of dithionite to settle. The spectrum was then obtained, more dithionite added, and the spectrum re-run. This technique was essential to obtain complete reduction and effectively prevented re-oxidation of the haemochrome. In a few solutions similar measurements were made with sodium ascorbate as reductant. These measurements were complicated, however, by the sensitivity of haem to coupled oxidation by ascorbate and molecular oxygen. All measurements using ascorbate were therefore made on solutions purged with O_2 -free N_2 . Under these conditions, results obtained by using ascorbate were almost identical with those obtained by using dithionite, and because of its experimental advantage the latter reagent was used to obtain all the data quoted below.

Results and Discussion

Spectra obtained by varying the pyridine concentration at constant haem concentration revealed significant changes as shown in Fig. 1. At pyridine

concentrations above about 9–10% (v/v), e.g. Fig. 1(a), the well-known haemichrome and haemochrome spectra were obtained with Soret-band maxima at 408 and 419 nm respectively. However, Fig. 1(b)

Fig. 1. Spectra of reduced and oxidized haem in pyridine/water mixtures

All measurements refer to 5 mm cuvettes at 25°C. The haem concentration was $18.7 \mu\text{M}$. The pyridine concentrations were (a) 9.0% (v/v), (b) 6.5% (v/v).

Fig. 2. Dependence of the Soret-peak wavelength ($\lambda_{\max}$) on pyridine concentration for reduced and oxidized haem

All measurements refer to 25°C. The haem concentrations were: $\blacktriangle$, 93.8 μM ; $\bullet$, 18.7 μM ; $\circ$, 3.75 μM .

shows that when the pyridine concentration is decreased, although the spectrum of the oxidized haem remains unchanged, that of the reduced form changes significantly, with a shift in maximum from 419 to 436 nm. Although these changes must reflect a change in haem structure, these results alone do not distinguish between, on the one hand, a change characteristic of the solvent system and consequent change in haem solvation and, on the other hand, the occurrence of haem aggregation. The above procedure was therefore repeated at different fixed haem concentrations. These results are shown in Fig. 2, in which the variation in the wavelength of the Soret-band maximum is plotted against pyridine concentration for each of three fixed haem concentrations. These plots show that the spectral changes are extremely sharp, occurring over a very narrow range of pyridine concentration. The sharp nature of the changes and their associated spectra are very similar to changes observed in aqueous porphyrin systems and attributed to micellization (Brown *et al.*, 1976). Further, the critical pyridine concentration was found to vary significantly with the haem concentration, i.e. the higher the haem concentration, the higher the critical pyridine concentration (Fig. 2). This variation is clearly not consistent with a change characteristic only of the solvent system itself, since in this event a haem dependence would not be expected, but it is compatible with a mechanism involving haem aggregation. Further, the sharpness of the change suggests aggregation more than simply dimerization and it is possible that the process might be better

Fig. 3. Dependence of the Soret-peak wavelength ($\lambda_{\max}$) on haem concentration for reduced ($\bullet$) and oxidized ($\circ$) haem

All measurements refer to 25°C. The pyridine concentration was 8% (v/v).

classified as micellization (cf. Brown *et al.*, 1976). In this event, the critical pyridine concentration would be expected to decrease as the haem concentration decreases, as observed.

More detailed evidence in support of these ideas was obtained by observing spectral changes obtained by varying haem concentration at constant pyridine concentration (Fig. 3). Again the sharp changes in spectrum were observed. This could not be due to any change in the solvent, whether from considerations of hydrophobicity or otherwise, since the solvent composition remained constant throughout and the haem concentration, although varying, was so small that it could not possibly perturb the solvent structure.

The effect of ethanol and butan-2-one on haem spectra in pyridine was also investigated. For pyridine concentrations above the critical point, addition of small amounts of ethanol had no effect on the spectrum. However, for pyridine concentrations just below the critical point, replacement of 50% (v/v) of the water content by ethanol was sufficient to restore the pyridine haemochrome peak, i.e. a shift in $\lambda_{\max}$ from 419 to 408 nm was observed. Similar effects were observed by replacement of 15% (v/v) water by butan-2-one. These findings clearly correlate with the observations of O'Carra & Colleran (1970) and Colleran (1971) that addition of ethanol or butan-2-one at low pyridine concentrations can re-establish high rates of haem degradation.

Fig. 4 shows the dependence of the critical point on both pyridine and haem concentrations, for the reduced species. A zone in which haem is monomeric is separated from a zone in which haem is aggregated by this curve. By reference to this graph, it is therefore possible to predict immediately the state of the haem

Fig. 4. Aggregation of reduced haem in pyridine/water mixtures

All measurements refer to 25°C. The graph is a profile which permits the division of the pyridine/water/haem system into two distinct zones corresponding to monomeric haem and aggregated haem. The state of the haem in any solution is thereby readily predicted.

for any solution of haem in pyridine/water. Fig. 4 further reveals that above about 10% (v/v) pyridine, an increase in haem concentration no longer produces aggregation. This explains why much previous work at pyridine concentrations above 10% (v/v) has been unaffected by haem aggregation.

As a final check on the above findings, several haem solutions in pyridine, above and below the critical concentration, were tested for haem-degradation reactivity with ascorbate/oxygen. In all cases, haem solutions below the critical concentration were rapidly degraded, whereas those above were unreactive.

General discussion

The present work has demonstrated the occurrence of aggregation in solutions of ferrohaem in pyridine/water mixtures. The extent of aggregation is unknown, but because of the sharpness of the changes at the critical concentration, it may represent several haem molecules condensing into the aggregate. It is therefore likely that haem aggregation accounts for the sharp decrease in rates of haem degradation in such solutions. This conclusion is in agreement with the results from previous studies (Brown *et al.*, 1974) demonstrating the lack of reactivity of dimeric species towards coupled oxidation. It is possible therefore that the chief role of pyridine in haem degradation is to ensure the monomerization of the otherwise largely dimeric haem. Supporting this

possibility is the observation (Brown *et al.*, 1974) that coupled oxidation also proceeds rapidly in dimethyl sulphoxide, a solvent which, though quite unlike pyridine in structure, binds strongly to haem and also acts as a 'dimer breaker'.

In spite of the above evidence, it would be surprising if the rate of degradation was completely independent of solvent. It is quite possible that haem in water reacts more slowly than haem in pyridine or hydrophobic solvents such as benzene (O'Carra & Colleran, 1970), even after allowances are made for differences in aggregation. However, such comparisons must be made with care, since other factors may also change with the solvent. For example, oxygen solubility is much greater in benzene than in water and this could lead to a higher rate of degradation, even though the rate constant may not increase proportionately.

A major conclusion of this and other work (Brown *et al.*, 1974) is that haem aggregation must be taken into account in any consideration of model systems for haem catabolism. In the haemoproteins themselves, however, direct haem-haem aggregation is not possible and degradation occurs readily under coupled oxidation conditions. In systems designed to assay the ability of animal tissues to cleave haem (Tenhunen *et al.*, 1969), haem alone is a poor substrate, but on addition of albumin, significant rates of cleavage are obtained. It seems likely that the chief effect of albumin is to bind monomeric haem (Beaven *et al.*, 1974), thus displacing the monomer-dimer equilibrium towards the readily degradable monomeric species. A further corollary of these ideas is that if a free haem pool exists *in vivo*, the haem may be protected against the effects of haem-cleaving systems by virtue of its aggregation.

The present results also make a contribution to the issue of whether coupled oxidation of haem proceeds via the iron(II) or iron(III) species. Although the iron(II) form has been favoured, direct proof has not until now been obtained. However, since the spectrum of the pyridine haemichrome remains virtually unchanged on variation of the pyridine concentration in the region where degradation rates decrease, whereas the spectrum of the haemochromes changes dramatically, there now seems little doubt that degradation proceeds via the iron(II) form.

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