Alkaline Haematin and Nitrogenous Ligands

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1. Pyridine reacts with alkaline haematin to form a dipyridine dihydroxy dimeric haematin in which there is no competition between pyridine and OH^- for co-ordination positions on the iron of haematin. 2. Imidazole competes directly with OH^- to form the monomeric imidazole parahaematin in alkaline media. 3. The monomeric imidazole parahaematin aggregates readily to form a species that is precipitated on standing. 4. A structure is proposed for the haematin-pyridine compound in alkaline media.

The classical view of the reaction between haematin and a nitrogenous ligand such as imidazole, near neutral pH, is that a monomeric di-imidazole-haematin complex is formed in which the two imidazole molecules are bound by nitrogen-iron bonds at the fifth and sixth co-ordination positions of the haematin iron atom.

Keilin (1926) proposed the name parahaematin for this class of compound; thus the imidazole-haematin compound would be called imidazole parahaematin. He reported that on dilution of parahaematin with alkali the compound dissociated to give free ligand and alkaline haematin.

Barron (1937) presented evidence for an interaction between pyridine and haematin in alkaline media and this was confirmed by Lemberg (1943) and Shack & Clark (1947).

The present paper is concerned with the reactions of nitrogenous ligands with haematin in alkaline media and is the third in a series concerned with the relationship between the structures of haematin compounds and their absorption spectra (Gallagher & Elliott, 1965, 1967).

MATERIALS AND METHODS

Chemicals. Spectrophotometric grade pyridine was purchased from Matheson, Coleman and Bell, East Rutherford, N.J., U.S.A. All other chemicals were Baker Analyzed reagent grade (Will-Buffalo Inc., Buffalo, N.Y., U.S.A.).

Haemin. Two commercial samples of recrystallized haemin, one (lot 1043) from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and the other (lot 52352) from General Biochemical Corp., Chagrin Falls, Ohio, U.S.A., were used throughout without detectable difference in behaviour.

Preparation of solutions. Fresh haematin solutions were

made up daily and used within 6 hr. All haematin concentrations are given on the basis of a dimeric haematin (i.e. 2 atoms of iron/mol. of haematin). Crystalline haemin was dissolved in 1N-NaOH and diluted to a volume that would yield the desired NaOH concentration.

Spectrophotometry. Absorption spectra and changes in extinction were recorded with a Cary model 14 recording spectrophotometer with silica cells.

Spectrophotometric measurements. Each point on the titration curve (Fig. 1) was determined by mixing a given amount of stock haematin solution with the desired amount of ligand and then diluting to the desired volume. Readings were made at $400\text{ m}\mu$ after the solutions had stood for at least $\frac{1}{2}$ hr. The same procedure was used in Figs. 2 and 3 except that the whole absorption spectrum was recorded for each ligand concentration. In Fig. 2 the pyridine concentration ranged from 0 to 1M and in Fig. 3 the imidazole concentration ranged from 0 to 0.2M.

Stopped-flow apparatus. The Durrum stopped-flow

Fig. 1. Spectrophotometric titration of $28.35\text{ }\mu\text{M}$ -dimeric haematin in 2mN -NaOH with pyridine. Analysis of the lower part of the curve shows that two molecules of pyridine combine with the dimeric haematin. A second pyridine effect is noted above 2M -pyridine: this appears to be a solvent effect. The light-path was 1 cm.

Fig. 2. Superimposed absorption spectra obtained during titration of alkaline haematin with pyridine, showing several isosbestic points. The dotted line, which is the absorption spectrum in 30% (v/v) pyridine, illustrates the solvent effect of the pyridine. Absorption spectra in the visible region were taken with a 10 mm. cell and those in the Soret region were taken with a 2 mm. cell.

Fig. 3. Superimposed absorption spectra of the titration with imidazole of $31 \mu\text{M}$ -dimeric haematin in 20 mM-NaOH . Absorption spectra in the visible region were taken with a 10 mm. cell and those in the Soret region were taken with a 2 mm. cell.

apparatus (Durrum-Gibson Co., Palo Alto, Calif., U.S.A.) was used to follow the initial rapid changes occurring on the mixing of imidazole and alkaline haematin solutions.

RESULTS

Pyridine-haematin. A typical spectrophotometric titration of alkaline haematin with pyridine is shown in Fig. 1. Graphical analysis of the first part of the curve showed it to fit the ideal curve for two molecules of ligand reacting with a dimeric haematin without splitting the dimer as in eqn. (1):

In Fig. 2 the superimposed absorption spectra of an alkaline haematin-pyridine titration are shown. The isosbestic points are evidence for only two chromophores in the titration. At concentrations of pyridine above 1M the superimposed spectra no longer passed through these isosbestic points, indicating a second effect of pyridine.

Application of the 'dilution test' recommended by Cowgill & Clark (1952) indicated no splitting of the dimeric alkaline haematin by pyridine at concentrations as high as 5M-pyridine.

When pyridine (0.5M) and haematin concentrations (29 μM -dimeric haematin) were held constant and the sodium hydroxide concentration varied from 2mN to 200mN there was no change in the absorption spectrum of the pyridine-haematin compound in the visible region.

Dilution of alkaline haematin in 20mN-sodium hydroxide with ethylene glycol (50%, v/v) yielded a typical alcohol-alkaline haematin absorption spectrum (Maehly & Akeson, 1958; Gallagher & Elliott, 1967) that was enhanced by the addition of pyridine. Pyridine did not react with alkaline haematin in this solvent.

Imidazole-haematin. The superimposed absorption spectra from the imidazole-haematin titration in alkaline medium are given in Fig. 3. These spectra were unexpected for a para-haematin, in that

Fig. 4. (a) Superimposed absorption spectra showing the partial conversion on standing of 22 μM -monomeric imidazole para-haematin (highest Soret band) into the aggregated compound. The light-path was 1 cm. (b) Visible spectra of 39 μM -dimeric haematin with an excess of imidazole. The lower curve was measured in the solvent system 20mN-NaOH-ethylene glycol (2:3, v/v). The upper curve shows the enhanced absorption accompanying the shift to longer wavelengths of the aggregated para-haematin in 20mN-NaOH. The light-path was 1 cm.

all the absorption bands were shifted to longer wavelengths, and the ratio of the absorption of the Soret band to that of β -band was about 4 : 1 instead

Fig. 5. Stopped-flow analysis of the imidazole-haematin reaction. The upper curve shows an oscilloscope tracing showing the rapid formation of the classical imidazole parahaematin over an elapsed time of 0.5 sec. The decreasing voltage reading represents a decrease in the percentage of light transmitted. The lower curve (total elapsed time of 5 sec.) shows the first reaction on a tenfold-contracted time-base and illustrates the second phase representing the aggregation step. Both curves were followed at 430 m μ and the final concentrations at pH 11 were 7.5 μ M-dimeric haematin and 116 mM-imidazole. The zero time-point of the lower curve was raised 2 v above that of the upper curve to insure that the tracing would remain on the oscilloscope face.

of the expected 10 : 1 (see Fig. 4 for a typical parahaematin absorption spectrum). Graphical analysis of the data gave a steep titration curve that approximated to the ideal curve for a reaction in which four molecules of ligand react with the dimeric haematin without splitting the dimer.

At lower concentrations of haematin, the reaction with imidazole in alkaline media yielded a classical parahaematin absorption spectrum (Fig. 4a) that was slowly converted into the second form on standing. The kinetics of this conversion were followed by stopped-flow measurements and are summarized in Fig. 5.

When haematin and imidazole concentrations

Fig. 7. Proposed model for dihydroxy dipyridine dimeric haematin, allowing free access of ligands to all co-ordination positions of iron. The relative positions of pyridine and OH⁻ ion in this model might well be reversed.

Fig. 6. Superimposed spectra showing the conversion of imidazole-haematin aggregate into free ligand and dimeric haematin on dilution with alkali. The imidazole concentration was 20 mM, the dimeric haematin concentration was 28 μ M and the light-path was 2 mm. Concentrations of NaOH were: curve 1, 2 mN; 2, 7 mN; 3, 10 mN; 4, 100 mN; 5, 300 mN; 6, 600 mN.

were held constant and the sodium hydroxide concentration was increased, the imidazole-haematin absorption spectrum was replaced by the absorption spectrum of alkaline haematin. This transition (Fig. 6) indicated the release of the ligand, not the formation of a di-imidazole dimeric haematin compound analogous to the dipyridine dimeric haematin compound. The effect of increasing the concentration of alkali was very marked in the Soret region.

The titration of alkaline haematin with imidazole was also performed in 20mM-sodium hydroxide diluted with ethylene glycol (50%, v/v) and gave a smooth titration in close agreement with that expected for four molecules of imidazole splitting a dimeric haematin. The absorption spectrum in ethylene glycol was that of a typical parahaematin, with slight displacement of the absorption bands towards the red due to the different solvent (Fig. 4b).

DISCUSSION

Our results confirm the earlier observations of Barron (1937) and Lemberg (1943) that a stable pyridine-haematin compound exists in alkaline media. Our stoichiometry is in excellent agreement with that of Shack & Clark (1947) and indicates that the compound is a dipyridine dimeric haematin. Further, Shack & Clark (1947) and Drabkin (1961) have shown that this compound contains two hydroxyl groups that must be titrated with acid before the dimer is split, giving pyridine parahaematin.

We consider these data are evidence for a structure of alkaline pyridine-haematin compound as pictured in Fig. 7. The dimer is held together by hydrophobic bonding in the overlap of the porphyrin rings, but the overlap is not sufficient to block the fifth and sixth co-ordination positions of the haematin iron. The spectral changes due to ethylene glycol and other alcohols (Maehley & Akeson, 1958) interacting with haematin in alkaline media probably result from a similar binding of the oxygen of the alcohol opposite the OH^- of haematin iron without splitting the dimer. The failure of pyridine to react with alkaline haematin in 50% (v/v) ethylene glycol suggests competition between the two ligands.

The second effect of the pyridine, noted in Fig. 1, is not due to splitting of the dimer, but more likely represents a solvent effect that makes it difficult to determine the stoichiometry of weakly reacting ligands, such as alcohols, with haematin.

In alkaline solution two spectrophotometrically distinct imidazole-haematin compounds are formed neither of which is analogous to the dipyridine dihydroxy dimeric haematin. The imidazole-

haematin compound formed at lower concentrations of haematin appears to be a classical parahaematin. This compound is unstable and aggregates to a second compound that is much less soluble. This interpretation of the two spectra is based on several observations. First, the clear haematin-imidazole mixtures become opalescent on standing, and eventually yield fine precipitates. Secondly, ethylene glycol, which is known to act as a dispersing agent (Gallagher & Elliott, 1965), prevents aggregation. Thirdly, the time-course of the reaction, as noted with the stopped-flow apparatus, is biphasic. Fourthly, a red shift of the absorption bands accompanies the change from one compound to the other (Keilin, 1926).

The nature of the aggregate is unclear. The formation of the aggregate involves splitting of the dimeric haematin to form the monomeric imidazole parahaematin followed by a very rapid dimerization to yield the chromophore responsible for the absorption spectra shown in Fig. 3. Further aggregation does not significantly affect the absorption spectrum until the precipitation stage is reached; this causes a marked red shift.

Similar phenomena were observed in studies on the formation of pyridine haemochromogen (Gallagher & Elliott, 1965). Thus the monomeric pyridine haemochromogen that appeared at low concentrations of reagents was unstable and tended to aggregate with consequent shift of absorption bands to longer wavelengths. Further, there was a decrease in the intensity of the Soret band and an increase in the intensity of the bands in the visible region in a manner perfectly analogous to the imidazole parahaematin. In the present study, however, it is the parahaematin rather than the haemochromogen of imidazole that shows the strongest tendency to aggregate, though the imidazole haemochromogen will aggregate under some conditions.

The failure of the imidazole to form a di-imidazole dihydroxy dimeric haematin confirms the earlier observation of Keilin (1926) that dilution with alkali causes dissociation of the parahaematin into free ligand and alkaline haematin. This suggests the incompatibility of certain mixed pairs of ligands on the iron of haematin, in this case the OH^- ion and the imidazole molecule, which would explain the previously noted conflicting observations on the reactions of haematin with nitrogenous ligands in alkaline media.

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