Caffeine Derivatives of Haematin Compounds

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1. Caffeine reacts with haematin to form a caffeine-haematin compound that has a characteristic absorption spectrum. 2. Graphical analysis of the titration of haematin with caffeine shows that 2 mol.prop. of caffeine split the dimeric haematin. 3. Thermodynamic parameters suggest that the reaction involves the making and breaking of hydrophobic bonds. 4. Graphical analysis shows dimeric haem to be split by 2 mol.prop. of caffeine to yield a compound with an unusual multibanded absorption in the Soret region. 5. It is postulated that the linkage between the haem groups of dimeric haem and the haematin groups of dimeric haematin is essentially hydrophobic in nature.

The reaction between caffeine and haem was described first by Keilin (1933) and later by Barron (1937). Both investigators believed that a caffeine haemochromogen was formed. A later study (Keilin, 1943) showed that the reaction between caffeine and certain haematin derivatives was more complex than originally supposed and it was postulated that the effects of caffeine on haem was due, not to haemochromogen formation, but to its dispersing effect on the haem. Gallagher & Elliott (1965) showed that under certain conditions pyridine haemochromogen tends to aggregate. When caffeine was added to disperse this aggregate, an absorption spectrum with a multibanded Soret region was noted. Further, the addition of caffeine to haematin solutions gave data amenable to graphical analysis.

This paper presents an analysis of the interactions between caffeine and haem and between caffeine and haematin.

MATERIALS AND METHODS

Chemicals. Baker Analyzed reagent-grade chemicals (Will-Buffalo Inc., Buffalo, U.S.A.) were used throughout.

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

Spectrophotometry. Absorption spectra and changes in extinction were recorded with the Cary model 11 recording spectrophotometer with silica cells.

Preparation of solutions. Stock solutions of haematin and caffeine were made up daily in 20 mN-NaOH and used within 6 hr. All haematin concentrations are given on the basis of dimeric haematin (i.e. 2 atoms of Fe/mol. of haematin). In a typical titration, 1 ml. of stock haematin was mixed with a given amount of stock caffeine and the mixture was diluted to 10 ml. with 20 mN-NaOH. After the mixture had stood for a few minutes the extinction was recorded to give one point on the titration curve. For the next point, a fresh 1 ml. sample of haematin was mixed with a different volume of stock caffeine and the mixture was diluted to 10 ml. as before.

To titrate haem with caffeine, haematin and caffeine were mixed, diluted to volume and reduced with the minimum

Fig. 1. Titration of 5 μ M-dimeric haematin with caffeine showing two distinct effects. The solid lines represent the splitting of dimeric haematin by 2 mol.prop. of caffeine. The dotted line represents the effect of excess of ligand.

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of solid sodium dithionite in the cuvette. Readings were taken within 3 min.

RESULTS

Titration of haematin with caffeine. This titration is represented in Fig. 1, where superimposed absorption spectra recorded at different concentrations of caffeine are shown to have an isosbestic point at $385\text{ m}\mu$. This permits analysis of the titration data on the basis of:

$$\alpha = \frac{E_x - E_0}{E_{100\%} - E_0}$$

where E_0 is the extinction of alkaline haematin,

Fig. 2. Plot of α , the fraction of haematin converted into the caffeine-haematin compound, against the caffeine concentration (on a logarithmic scale). The solid curve is the best-fitting theoretical curve for 2 mol.prop. of ligand breaking a dimer.

Fig. 3. 'Dilution test plot': plot of $M\alpha$ (on a logarithmic scale) against $M(1-\alpha)$ (also on a logarithmic scale) where M is the molar concentration of dimeric haematin at constant ligand concentration. The solid line is the best fit of a line with a slope of 0.5 through the experimental points: ●, caffeine concentration of 1 mM at 28° ; ○, caffeine concentration of 2 mM at 38° .

Fig. 4. Plot of K_D (on a logarithmic scale) of the caffeine-haematin compound against the reciprocal of the absolute temperature.

Fig. 5. Spectra of haematin (—), haematin-ethanol (-----), haematin-caffeine (----) and haematin-ethanol-caffeine (.....). All haematin concentrations were $7.65\text{ }\mu\text{M}$. The ethanol concentration was 30% (v/v) and the caffeine concentration was 60 mM.

$E_{100\%}$ is the extinction of the fully formed caffeine-haematin compound, E_x is the extinction of haematin-caffeine mixtures, and α is the fraction of haematin converted into caffeine-haematin.

The complete titration shown in Fig. 1 is presented in Fig. 2 as a plot of α versus caffeine concentration (on a logarithmic scale). The experimental points are in excellent agreement with the continuous line, which is a theoretical curve representing 2 mol.prop. of ligand splitting a dimer, as in the reaction:

At $28^\circ \text{K} = 0.16 \text{ mole l.}^{-1}$.

The best criterion for the splitting of a dimer is the 'dilution test plot' recommended by Cowgill & Clark (1952). A plot of $M\alpha$ (on a logarithmic scale) against $M(1-\alpha)$ (also on a logarithmic scale), where M is the molar concentration of dimeric haematin at constant ligand concentration, should give a straight line of a slope 0.5 if the dimer is split. Fig. 3 shows that data from two experiments, at 28° and at 38° , fit this condition. The fact that both sets of points fall along the same line is fortuitous and is due to the different caffeine concentrations used in the two experiments. Our experiments confirm the

existence of the dimeric haematin over the temperature range $8-38^\circ$. If K_D (on a logarithmic scale) is plotted against $1/T$ (Fig. 4), the slope of the resulting straight line permits calculation of ΔH of dissociation as 12.7 kcal./mole . From the relationship:

$$\Delta G = -RT \ln K_D$$

the free energy of dissociation of the complex at 28° is 1.1 kcal./mole and the entropy of dissociation is $38.7 \text{ cal./mole degree}$.

When the haematin-caffeine mixture in 20 mM-sodium hydroxide is diluted to contain 30% (v/v) ethanol, the titration kinetics show that the haematin is in a monomeric form. Fig. 5 shows the spectra of haematin, haematin-ethanol, haematin-caffeine and haematin-caffeine-ethanol mixtures all at the same haematin concentration. The spectra of the caffeine-haematin compound and the alkaline pyridine-haematin compound are compared in Fig. 6, and the spectra of the reduced forms of these compounds, the caffeine-haem compound and the pyridine haemochromogen, are given in Fig. 7.

Titration of haem with caffeine. Dimeric haem reacts with 2 mol.prop. of caffeine in the same manner as the dimeric haematin (Fig. 8). The 'dilution test

Fig. 6. (a) Spectra of haematin (-----), haematin-pyridine (.....) and haematin-caffeine (—). All haematin concentrations are $5 \mu\text{M}$, and the pyridine and caffeine concentrations are both 90 mM . The ligand concentrations were chosen to be just below saturation level for caffeine (about 0.1 M). (b) Spectra as in (a) except that all haematin concentrations are $38 \mu\text{M}$ -dimeric haematin. The caffeine concentration is 0.1 M (saturated). The pyridine concentration is 20% (v/v) to ensure full formation of the pyridine-haematin compound.

Fig. 7. Spectra of reduced haem compounds: haem (-----), caffeine-haem compound (.....), and pyridine haemochromogen (—). The concentrations of caffeine, pyridine and haem were respectively 90 mM, 90 mM and 5 μ M.

plot' gives the expected slope of 0.5 at low concentrations of haem, but at higher concentrations the slope decreases and becomes zero.

DISCUSSION

The work of Hogness, Zscheile, Sidwell & Barron (1937), confirmed and extended by Shack & Clark (1947), has given clear evidence for dimeric haematin in alkaline media. Our present data on the caffeine-haematin reaction in 20mM-sodium hydroxide give further support for the dimeric haematin and also clarify the stoichiometry of the reaction between caffeine and haematin (O'Hagan & Barnett, 1958).

Comparison of the absorption spectra of the caffeine-haematin compound and the alkaline pyridine-haematin compound shows a marked similarity in the Soret region (Fig. 6a). In both compounds the main haematin band at 385m μ has been strengthened and shifted to 400m μ , and the 360m μ band of haematin has been very much weakened. Excess of ligand in both cases gives a general increase in the absorption spectra, as

Fig. 8. Plot of α , the fraction of haem converted into caffeine-haem compound, against concentration of caffeine (on a logarithmic scale). ●, With 5 μ M-haem; ○, with 8.4 μ M-haem. The solid lines represent the theoretical reaction of 2 mol. prop. of caffeine splitting a dimeric haem.

shown by the dotted line for the caffeine-haematin compound in Fig. 1.

The absorption spectra in the visible region for these two compounds are superimposed in Fig. 6(b). Both spectra are clearly distinct from that of haematin and differ from each other in that the relative intensities of their α - and β -bands are reversed. The nature of the alkaline pyridine-haematin reaction has never been satisfactorily demonstrated, but the existence of a pyridine-haematin interaction in alkaline media has been claimed (Lemberg & Legge, 1949; Drabkin, 1961). Our work on this reaction gives clear evidence for such a compound, but the high concentrations of pyridine required to complete the titration change the medium so that the precise stoichiometry of the reaction is not clear. At pyridine concentrations as high as 90% (v/v) in alkaline media the classical pyridine parahaematin, normally formed near neutral pH, is not formed.

Aside from the possibility of a pyridine interaction with the porphyrin ring of the haematin, the alkaline pyridine-haematin compound might be pictured as involving a direct interaction of the pyridine nitrogen with the iron of the haematin while the remaining co-ordination position is occupied by an OH⁻ ion (Lemberg & Legge, 1949).

The presence of a free nitrogen in the caffeine molecule along with a certain similarity in the absorption spectra of the two compounds might encourage proposal of a similar structure for both compounds. The failure of the reduced caffeine-haematin compound to give the classical haemochromogen spectrum argues against this interpretation, as was noted by Keilin (1943). Further, the titration of haem with caffeine shows only 1 molecule of caffeine reacting/monomeric haem group (Fig. 8).

The thermodynamic parameters of the caffeine-haematin reaction, the relatively low ΔG value and

the high entropy of dissociation are compatible with hydrophobic bonding (Scheraga, 1963), and the overall evidence is consistent with the caffeine-haematin bond's having a large hydrophobic component owing to an overlap in the two ring systems.

It is probable that the binding between the haematin of dimeric haematin and the haem groups of dimeric haem is also hydrophobic in nature. Both dimers titrate as monomers in solvents such as ethanol and ethylene glycol (Gallagher & Elliott, 1965), which reduce the dielectric constant of the medium, and both dimers are split by caffeine at rather low concentrations of the ligand. The haematin dimer is split more easily because of the strong electrostatic forces involved in the haematin nuclei, whereas higher concentrations of caffeine are required to produce the same degree of compound formation when titrating haem. Apparently, an excess of an alternative hydrophobic surface is being offered, and one hydrophobic bond is being substituted for another. This hypothesis is attractive in that hydrophobic bonding probably occurs between the dimers of haem, resulting in its rather low solubility and failure to agree with the 'dilution test plot' except at rather low concentrations of haem (about 1–3 μM -dimeric haem).

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