# STUDY OF HEMATIN-GLOBIN LINKAGE. DETERMINATION OF EQUILIBRIUM CONSTANTS

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The linkage between hematin and globin in hemoglobin molecules has always been considered to be extremely strong. A reversible equilibrium has sometimes been imagined, but no attempts have till now been made to determine the magnitude of the equilibrium constant.

We intend, in this report, to outline the principles of a method which we believe to have succeeded in this direction. It has been possible (1) to demonstrate that methemoglobin and metmyoglobin dissociate reversibly in solution at neutral pH, (2) to determine the magnitude of the equilibrium constant in the case of metmyoglobin, and (3) in the case of methemoglobin, to evaluate approximately the degree and nature of interaction between the hematin-linking sites.

The ability of certain imidazole-containing bases to form characteristic coordination complexes with hematin is well known. Clark and collaborators (1940, 1952) report their high affinity for the coordination centres around the iron atom in the metalloporphyrin. The method used here is based on the observation that some of these bases, used in sufficiently high concentrations, can strip off metalloporphyrin prosthetic groups from the hemoprotein molecules, to give rise to a mixture of apoprotein and hematin-base complex. The concentration of the complex thus formed can be measured by spectrophotometry. Consideration of quantitative data justifies the hypothesis of the existence of a reversible dissociation equilibrium. The equilibrium constant can be calculated by estimating the fraction of metalloporphyrin transferred from apoprotein to base under given conditions of hemoprotein and base concentrations, provided the association constant of the hematin-base complex be known from independent studies.

For the hematin-base reaction, the equilibrium is known to correspond to the equation

$$(M_{OH})_2 + 4L + 2\,H^+ \rightleftharpoons 2\,ML_2 + 2\,H_2O \qquad (1)$$

$$K_1 = [ML_2]^2 / [(M_{OH})_2]\,[L]^4\,(H^+)^2$$

$(M_{OH})_2$ representing a dimer of hematin, L being the base in its dissociated form. The value of $K_1$ was determined for two bases found suitable for this study, namely histidylhistidine and pilocarpate, at 2°C and at 25°C.

For the hematin-apoprotein reaction, we admit the stœchiometric relations to be, for methemoglobin : $G + 2(M)_2 \rightleftharpoons GM_4$ (2) ; for metmyoglobin : $2\,g + (M)_2 \rightleftharpoons 2\,gM$ ; $K = [gM]^2/[g]^2[(M)_2]$ (3), where G and g represent the corresponding apoprotein moieties, $(M)_2$ , $GM_4$ and gM represent respectively a dimer of metalloporphyrin and the molecules of methemoglobin and metmyoglobin without reference to their ionisation states.

For metmyoglobin, it can be shown that the association constant K is related to the association constant $K_1$ (hematin-base), total base concentration $S_L$ and the fraction ($\beta$) of metalloporphyrin transferred to base under given conditions of pH and temperature by the relation :

$$\log K = \log K_1 + 4 \log S_L - 4 \log (1 + (H^+)/K_{ab}) - 2 \log S_M \beta$$
$$- 2 \log \beta/(1-\beta) - 2 \log (1 + (H^+)/K_i) - 2\,pH \qquad (4),$$

where $S_M$ = total concentration of hematin, $K_i$ = ionisation constant of hematin, $K_{ab}$ = ionisation constant of the base.

The values of apoprotein-hematin association constants as calculated from measurements of $\beta$ at varying concentrations of base and at different values of pH are summarized in fig. 1.

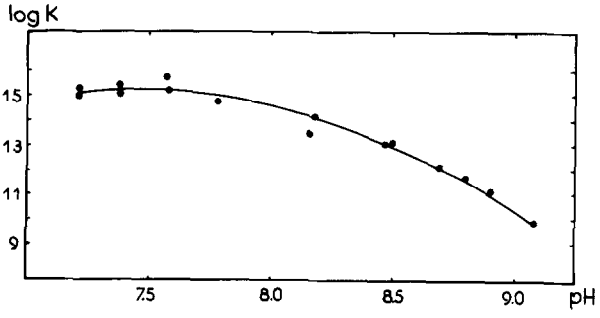


Fig. 1 - Hematin-globin association constant for metmyoglobin (as defined by equation (3)) at various pH, at 25°C.

It should be pointed out that the constants are those defined by equation (3), two molecules of apoprotein reacting with a dimer of hematin. It is necessary to represent hematin as a dimer since it appears to react as such even in very dilute solutions. Attempts to determine an equilibrium constant for a supposed dimer $\rightleftharpoons$ monomer equilibrium were without effect.

The decrease in association constant with increasing pH may be shown to be related to the ionisation of metmyoglobin ($pK \sim 9.0$), caused by the departure of a proton from the molecule of water coordinated to the iron. Two association constants of different magnitude can thus be assigned and calculated, one for the conjugated acid form and the other for the ionised form. The hematin-globin linkage is found to be less strong in the ionised form of the molecule.

Methemoglobin. Methemoglobin shows analogous behaviour in the presence of hematin-binding bases. It is not possible, however, to calculate an overall association constant from data corresponding to a partial and relatively small transfer of hematin from apoprotein to base ; an eventual interaction between the hematin-binding sites is to be considered. It has been found convenient to represent the data in the manner envisaged by Edsall et.al.(1954) for the combination of several molecules of a ligand to the same site or to sites that might eventually show mutual interaction.

These authors have defined a function Q by the relation $Q = \bar{\nu}/(N-\bar{\nu})[M]$, where [M] denotes the concentration of free ligand molecules (in this case hematin), N the total number of sites and $\bar{\nu}$ the mean number of sites occupied. In the case of methemoglobin N = 4. Different values of Q represented as a function of $\bar{\nu}$ results in a curve which shows whether the bound ligand molecules interact or not. The intrinsic association constants $\chi_1$ and $\chi_4$, corresponding to the first and fourth molecule of hematin bound may be calculated, since

$$\lim_{\bar{\nu} \to 0} Q = \chi_1 = K_1/4 \quad ; \quad \lim_{\bar{\nu} \to 4} Q = \chi_4 = 4\,K_4$$

where $K_1$ and $K_4$ are respectively the corresponding association constants.

We have adopted this representation, with the reserve that [M], the concentration of free hematin is deduced from data on the proportion of hematin transferred from apoprotein to another hematin-binding body of known hema-

tin affinity. The imidazole bases used in the case of metmyoglobin were not adequate since, even in near-saturation concentration, they can strip off only about 40 p.100 of metalloporphyrin from methemoglobin ($5.10^{-5}$ M). This corresponds to less than two units of hematin out of four. We have, instead, used a solution of apomyoglobin, prepared as usual by splitting off hematin from metmyoglobin. Considered as a hematin-binding agent, the apomyoglobin is a thousand times more efficient than the imidazole bases as shown from the association constant determined earlier in this report ; it can split off all the four hematin groups from methemoglobin at reasonable concentrations. The percentage transformation can be measured by spectrophotometry, though the absorption spectra of methemoglobin and metmyoglobin are very close ; one takes advantage of the fact that the ionisation constant of metmyoglobin (pK$\sim$9.0) is different from that of methemoglobin (pK = 8.3), the spectra are quite separated at pH 8.75.

Experiments performed with varying concentrations of apomyoglobin furnish directly the proportion of hematin transferred from apohemoglobin to apomyoglobin under given conditions. Since the association constant for the hematin-apomyoglobin reaction is known, the concentration of free hematin at equilibrium in its dimeric form is deduced. Free hematin concentration in its monomeric form is linked to it through a postulated equilibrium constant ($K_d$) for the monomer $\rightleftharpoons$ dimer reaction. The results may be expressed in terms of a function $Q/\sqrt{K_d}$ against $\bar{\nu}$ instead of Q against $\bar{\nu}$ as in Edsall representation, but since $Q/\sqrt{K_d}$ is proportional to Q , the curve obtained is parallel to the one that would have resulted by plotting Q against $\bar{\nu}$ .

From the results obtained at pH 8.75 and at 2°C (fig.2), the existence of interactions is obvious. In the absence of interactions, the curve $\log (Q/\sqrt{K_d})$ against $\bar{\nu}$ would have been a straight line parallel to the $\bar{\nu}$ axis. The interactions are not simple either. Fixation of the first molecule of hematin enhances the affinity for the second ; there seems to exist a negative interaction for the fixation of the third molecule. The fourth and the last is captured far more easily than the others.

It is interesting to note that these interactions run almost parallel to those observed in the case of oxygenation of hemoglobin. According to Roughton et al. (1955), the ratio of the successive association constants for the fixa-

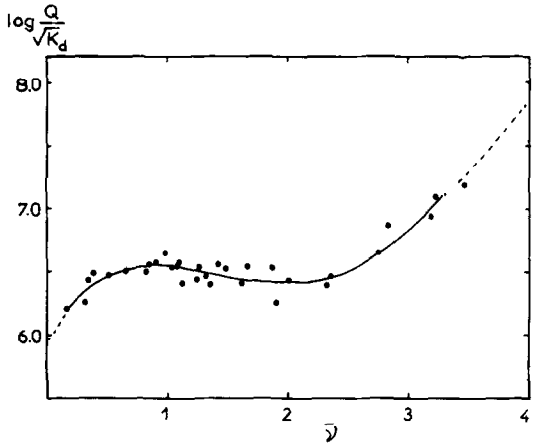


Fig. 2 - showing interaction of hematin-binding sites in methemoglobin.

tion of oxygen to sheep hemoglobin (19°C, pH 9.1) $k_1 : k_2 : k_3 : k_4$ = 1 : 1.76 : 1.31 : 17.8. This corresponds to the ratio of intrinsic constants $\chi_1 : \chi_2 : \chi_3 : \chi_4$ = 1 : 4.68 : 7.85 : 285. Having regard to the scatter of experimental points, we have judged prudent not to calculate all the intrinsic or association constants. Only $K_1$ and $K_4$ have been calculated, in order to have an order of magnitude.

$$\log K'_1 = 12.46 \quad : \qquad \log K'_4 = 15.17$$
$$\log \chi'_1 = 11.86 \quad ; \qquad \log \chi'_4 = 15.78.$$

These constants correspond, as in the case of metmyoglobin, to the reaction of a dimer of hematin with two identical groups from two molecules of apoprotein.

The interactions are in the same sense as in the case of oxygenation, except in the middle range : they are negative for the fixation of the third molecule of hematin ; for oxygenation they are always positive. It is known, however, that apohemoglobin is split into two sub-units with M. W. around 34,000. The molecule of reconstituted methemoglobin regains the original dimensions, junction of the two halves having occurred at some step during the successive fixation of hematins. It is not unlikely that this step be related in some manner with the observed negative interaction in the middle of the saturation curve.

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