Biochem. J. (1977) **163**, 153–158
Printed in Great Britain

The Kinetics of the Recombination Reaction between Apomyoglobin and Alkaline Haematin

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(*Received* 29 *September* 1976)

Apomyoglobin was prepared by an extremely mild modification of the acid/butanone technique, and the kinetics of the recombination reaction between this preparation and alkaline haematin were studied. The recombination has been shown to be precisely second-order and mono-phasic. Rate constants obtained from the study are in good agreement with values obtained previously by an indirect technique not involving separation of haem and apoprotein.

Interest in the recombination reaction between apohaemoproteins and haemin (and derivatives) has centred on the question of the number of kinetic stages which occur in the formation of the native haemoprotein. Adams (1976) has put forward evidence which suggests that the recombination reaction between apomyoglobin and haemin can, under certain circumstances, take place as a single-stage second-order reaction between folded apoprotein and monomeric haemin. The technique used to study the recombination kinetics involved splitting the myoglobin with dilute HCl, followed by immediate recombination initiated by addition of pH7.4 buffer; no physical separation of apoprotein and haem was involved.

In the present paper the recombination kinetics of apomyoglobin, prepared by an extremely mild modification of the acid/butanone technique (Teale, 1959), and alkaline haematin (maintained at pH13 until the instant of recombination) have been studied at pH7.2. A critical kinetic analysis of the results has been carried out with haem and apoprotein concentrations varying between 0.5 and 3 μM. The conditions were selected so that the molecular state of the haematin was a mixture of monomer and dimer, the latter being in a readily dissociable state. The rate of dissociation of the dimers is more rapid than the recombination between the monomeric haematin and the apomyoglobin.

The results obtained are compared with previous studies, and the relevance to the mechanism of the recombination process *in vivo* is discussed.

Experimental

Ferrimyoglobin

Ferrimyoglobin was purified from commercial sperm-whale myoglobin (Miles Laboratories, Cape Town, S. Africa, and Sigma Chemical Co., St. Louis, MO, U.S.A.) by a slight modification of the method of Yonetani & Schleyer (1967).

Commercial myoglobin was dissolved in water (approx. 5mg/ml) and oxidized with a fivefold molar excess of $K_3Fe(CN)_6$ to ensure complete conversion of all iron into the ferric form. The ferrimyoglobin solution was then treated with solid $(NH_4)_2SO_4$, and the protein which precipitated between 0.71 and 0.85 saturation collected. At least two recrystallizations were carried out on all samples of myoglobin used, the purity index (A_{409}/A_{280}) of the recrystallized protein varying between 5.3 and 5.77, whereas that of the commercial product (oxidized, dialysed, but not recrystallized) varied between 4.5 and 4.9. All myoglobin solutions were dialysed against 5 litres of distilled deionized water before use.

Apomyoglobin

Apomyoglobin was prepared from recrystallized ferrimyoglobin by a modification of the acid/butanone method (Teale, 1959; Yonetani, 1967). In my modification, extraction of the haem from weakly acidic ferrimyoglobin solution is effected by using diethyl ketone in place of ethyl methyl ketone. The main advantage of this procedure is that the solubility of diethyl ketone in water at 0°C is approx. 5% (v/v), whereas that of ethyl methyl ketone under the same conditions is approx. 20% (v/v). Use of diethyl ketone results therefore in lower contamination of apoprotein solution with organic solvent, and a more efficient extraction of haem from the aqueous phase.

In a typical extraction, ferrimyoglobin solution containing approx. 5mg of protein/ml was cooled in ice/water; dilute HCl (1M) was added to adjust the pH to 3.5, and the weakly acid protein solution immediately shaken (1 min) with an equal volume of ice-cold diethyl ketone. The resulting emulsion was

broken by centrifugation (5000rev./min) at 0°C for 1min, and the upper ketone layer removed. The aqueous phase was again extracted with an equal volume of diethyl ketone at 0°C, and after centrifugation the aqueous layer was dialysed against 5 litres of deionized water containing 100mg of $NaHCO_3$ at 0–4°C. After further dialysis against deionized water (3×5 litres), the apomyoglobin solution was stored at 0–4°C, at which temperature it is stable for at least 2 weeks.

The mildness of this haem-extraction procedure is apparent on extended dialysis of the apoprotein preparation, virtually no precipitation of denatured apoprotein occurring if the technique described is used. This is in contrast with both the acid/acetone and the acid/butanone techniques, where considerable amounts of denatured protein are deposited on exhaustive dialysis of the apoprotein solution.

Two additional observations are of relevance. First, attempted extraction of haem with higher ketones (such as methyl *n*-pentyl ketone) with very low (<1%) solubility in water is not effective. Extraction of haem by these ketones is so slow as to make the procedure impractical; obviously some slight mutual solubility is necessary in order to facilitate transport of haem across the water/ketone interface. Secondly, haem is completely removed from the protein at pH 3.5, in agreement with the observations of Yonetani (1967), but in marked contrast with the observations of Breslow (1964), who found haem removal from myoglobin to be incomplete above pH1.5. I think that the latter observation is almost certainly due to the presence in commercial myoglobin samples of small amounts of a contaminating haemoprotein(s) that is less easily split than is myoglobin. Fig. 1(*a*) shows the extraction procedure carried out on a commercial myoglobin sample oxidized with $K_3Fe(CN)_6$ and dialysed, but not recrystallized; Fig. 1(*b*) shows extraction of the same sample after two $(NH_4)_2SO_4$ recrystallizations. In the first case there is a residual haemoprotein present (to the extent of approx. 5%) which is not extracted by diethyl ketone at pH2.6. In the second case, extraction of haem is 98.5% complete after one ketone extraction and 99.7% complete after two extractions at pH3.5. No attempt has been made to identify the contaminating haemoprotein(s), although from the data presented by Yonetani (1967) it appears not to be either haemoglobin or catalase.

Absorption coefficient of apomyoglobin at 280nm

The absorption coefficient of apomyoglobin was determined on four separate apomyoglobin preparations containing between 1.5 and 4mg of apoprotein/ml. Protein was determined by the biuret method (Gornall *et al.*, 1949) with twice-recrystallized bovine serum albumin as standard, duplicate protein determinations being carried out in all cases. The absorption coefficient at 280nm was

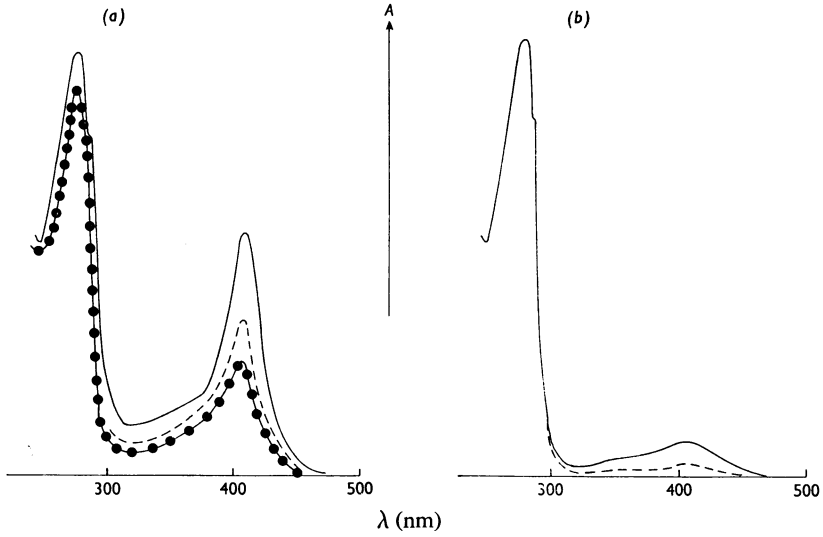

Fig. 1. *Comparison of diethyl ketone extraction of unpurified and purified sperm-whale ferrimyoglobin*
(*a*) Extraction of haem from unpurified commercial myoglobin sample ($A_{409}/A_{280} = 4.71$) at pH 2.60. ——, One extraction with diethyl ketone; – – – –, two extractions with diethyl ketone; ●–●, six extractions with diethyl ketone. (*b*) Extraction of haem from twice-recrystallized ferrimyoglobin ($A_{409}/A_{280} = 5.41$) at pH 3.50. ——, One extraction with diethyl ketone; – – – –, two extractions with diethyl ketone.

found to be 15570 $M^{-1} \cdot cm^{-1}$ ($\pm$436 $M^{-1} \cdot cm^{-1}$), in good agreement with the values of 15900 $M^{-1} \cdot cm^{-1}$ ($\pm$900 $M^{-1} \cdot cm^{-1}$) (Harrison & Blout, 1965) and 14900 $M^{-1} \cdot cm^{-1}$ (Gibson, 1964), but in poor agreement with the value of 10900 $M^{-1} \cdot cm^{-1}$ proposed by Dickinson (1976).

Haemin

Haemin was obtained from BDH Chemicals, Poole, Dorset, U.K. (Fe 8.6%, N 8.45–8.75%) and was used without further purification. Alkaline haematin solutions were made up in 0.1 M-NaOH and were used within 4h. Although alkaline haematin solutions contain a considerable proportion of dimer (see, e.g., Gallagher & Elliot, 1968), the solution concentration was calculated on the basis of monomeric haematin; kinetic evidence presented below suggests that the rate of dissociation of dimeric haematin is considerably more rapid than the recombination studied. Gibson (1964) has shown that the rate of dissociation of porphyrin dimers in aqueous solution is very much more rapid than the rate of recombination of porphyrin monomers and globin, and Asakura & Yonetani (1969) consider that freshly prepared alkaline porphyrin solutions contain dimeric molecules in a readily dissociable state. It is therefore reasonable to assume that the rate of the dimer/monomer interconversion is too rapid to interfere with the recombination processes studied here.

Instrumentation and methods

The formation of ferrimyoglobin was followed at 409nm by using a Unicam SP. 500 Series 2 single-beam spectrophotometer, the 10mV output of which was connected to a Philips PM8100 chart recorder. The system response time was determined at 510ms for a 0–90% deflexion; this was approximately twice as fast as the fastest initial rate measured in this study. The system was calibrated in a similar manner to that described previously (Adams, 1976).

Recombination reactions were initiated by using the single-syringe technique described previously (Adams & Swart, 1977). Apomyoglobin solution (1.6ml) was incubated in the constant-temperature cell holder of the spectrophotometer for 5–10min; recombination was initiated by injection of 1.4ml of buffer plus alkaline haematin (0.0050–0.0300ml of stock solution) into the cuvette as described previously (Adams, 1976; Adams & Swart, 1977). The haematin was maintained at pH13 until the instant of recombination, as shown diagrammatically in Fig. 2. All experiments were carried out at 21°C. Buffer solutions were 80mM-sodium/potassium phosphate, pH7.2, the addition of 0.030ml of 0.1 M-NaOH to 3ml of buffer caused a pH change of less than 0.05pH unit.

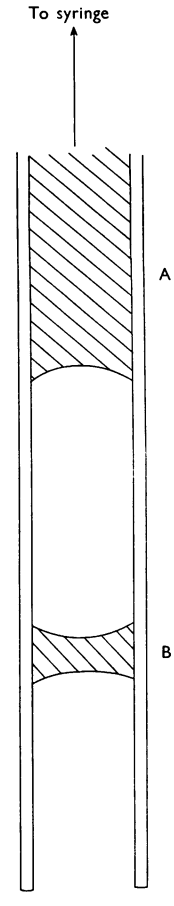

Fig. 2. *Illustration of the technique used to maintain the alkaline haematin solution at pH 13 until the instant of recombination with apomyoglobin*

Buffer (A) is withdrawn approx. 4cm from the mouth of the injection tube and the interior of the 2mm tube dried with a rolled facial tissue. Alkaline haematin solution (0.0050–0.0300ml) is deposited approx. 2cm from the mouth of the tube (B) by using a g.l.c. micro-syringe. The haematin solution is held in position (without spreading) by surface forces, until the moment of injection.

Results

Fig. 3 shows a typical reaction trace obtained by using the technique described. Recombination reactions were carried out under conditions such that [apoprotein] $\simeq$ [haem], or under conditions where the ratio [apoprotein]/[haem] varied between 0.33 and 3. In this way the kinetics could be critically examined to determine the true reaction order.

Fig. 4 shows a representative series of results which are analysed for both first- and second-order

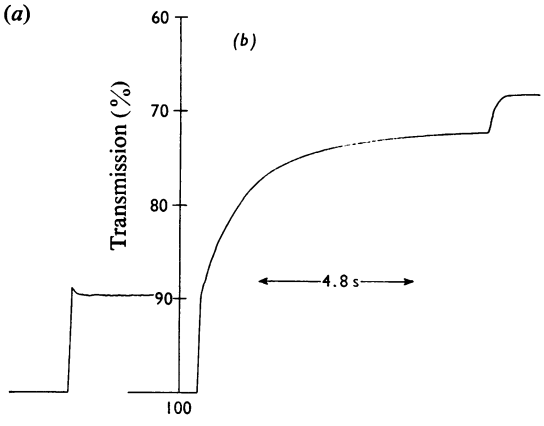

Fig. 3. *Typical reaction traces obtained by using the experimental technique described in the text and Fig. 2* (*a*) Alkaline haematin (0.0100ml; 0.2645mM) injected to a final volume of 3.01ml with buffer. (*b*) As in (*a*), but injected into apomyoglobin of final concentration 0.8961μM.

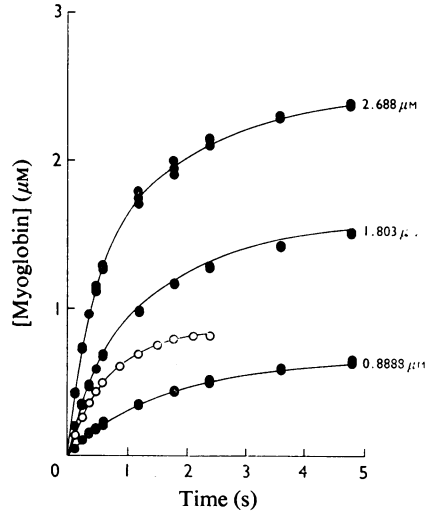

Fig. 4. *Progress curves for the formation of ferrimyoglobin from apomyoglobin and alkaline haematin*
Results at equimolar haematin/apoprotein concentrations (shown on Figure) are given; three repeat experiments were carried out at each concentration. O, Example of a non-equal concentration experiment, [haematin] = 0.8842μM, [apomyoglobin] = 2.681μM. The solid lines were calculated by using a mean second-order recombination rate constant of 5.8×10^5 M$^{-1}\cdot$s^{-1} at 21°C (see Fig. 5*b*).

kinetic dependence in Figs. 5(*a*) and 5(*b*); the solid lines in Fig. 4 were calculated on the basis of a mean second-order recombination rate constant of $5.8\times10^5\pm0.41\times10^5$ M$^{-1}\cdot$s^{-1}, evaluated from the linear second-order plots at eight equimolar apoprotein/haem concentrations in the range 0.5–3μM. If the rate of the conversion of haematin dimer into haematin monomer were rate-limiting in the present study, the rate of haemoprotein formation would have reflected this process, i.e. first-order kinetics would have been observed. Since the observed kinetics are accurately second-order, dimer→monomer dissociation is definitely not rate-limiting.

Discussion

The results presented here demonstrate unequivocally that the recombination observed is a single-stage second-order process with a rate constant at 21°C of 5.8×10^5 M$^{-1}\cdot$s^{-1}. No evidence of a genuine multistage recombination process is observed; the kinetic plots show quite clearly that the absorbance at zero time is (to within±5%) that observed when alkaline haematin alone is injected into the reaction buffer (see Fig. 3). Deviation from precise second-order kinetics is observed at extents of reaction greater than 70%; however, this can be attributed to a combination of aggregation of the haemin at pH7.2 and slight errors in the estimation of absorption at infinite time. The observed recombination rate constant is in good agreement with the value of 3.3×10^5 M$^{-1}\cdot$s^{-1} interpolated from the results of a previous paper (Adams, 1976), but is considerably lower than the value of 1.1×10^7 M$^{-1}\cdot$s^{-1} obtained by Gibson & Antonini (1960) at pH7.1 and 20°C.

A particularly stringent test of the second-order recombination kinetics is provided by results obtained when recombination is initiated between non-equal concentrations of apoprotein and haem. Fig. 4 shows an example of such an experiment; the solid line is calculated by using the mean second-order rate constant given above. In six such experiments, with the ratio of apoprotein to haem concentrations between 0.33 and 3, good agreement between the experimental and calculated haemoprotein-formation curves was observed up to 75% recombination, by using a second-order kinetic model.

The experiments reported here, when taken in conjunction with previous studies (e.g. Asakura & Yonetani, 1969; Gibson & Antonini, 1960; Itagaki *et al.*, 1967) seem to suggest that the mechanism of haem/apoprotein recombination depends to a considerable extent on the conditions used in the preparation of the apoprotein. This is to some extent supported by the observation that the recombination between apoprotein prepared by the acid/acetone

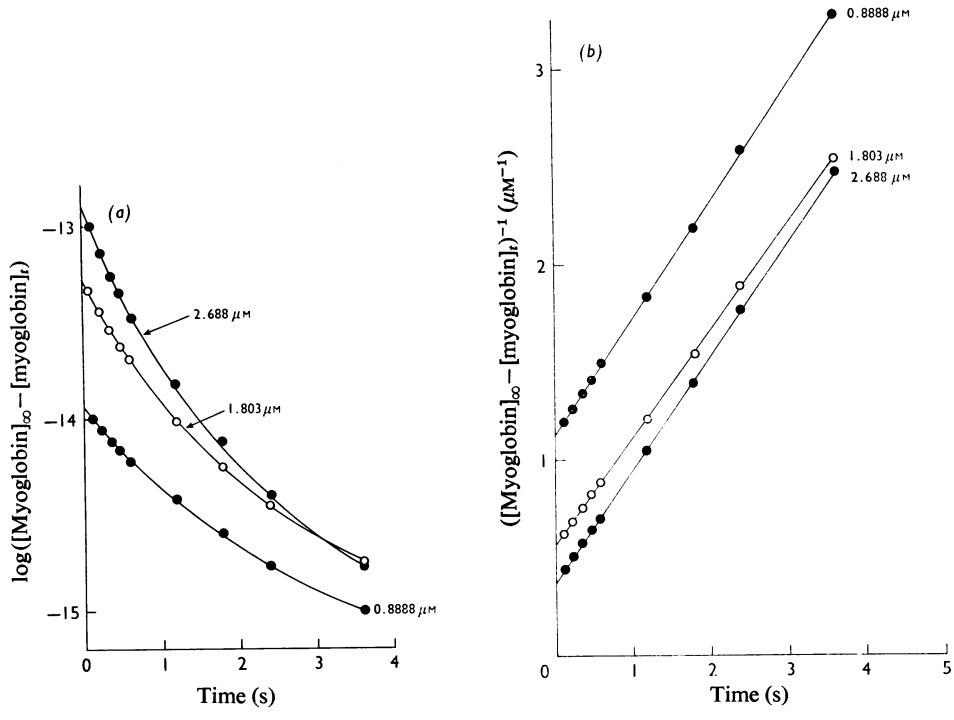

Fig. 5. *Elucidation of the kinetics of the recombination process for equimolar concentrations of apomyoglobin and alkaline haematin*

(*a*) First-order kinetic plot of the equimolar concentration kinetic data shown in Fig. 4. Data points shown are mean values for the three kinetic runs at each concentration. (*b*) Second-order kinetic plots for the same data as in (*a*). Rate constants calculated for the three concentrations are: 5.96, 5.52 and $5.84 \times 10^5 M^{-1} \cdot s^{-1}$.

method of Rossi-Fanelli *et al.* (1958) and alkaline haematin was too rapid to follow quantitatively by the experimental technique used here. The invariance of the absorption coefficient for the apoprotein prepared by different techniques suggests, however, that no gross conformational differences exist between apoprotein molecules prepared by the different techniques. The results of Gibson & Antonini (1960) on the rate of recombination between carboxyhaem and alkali-denatured globin appear to support the view that the apoprotein prepared by the acid/acetone technique is not in a totally unfolded form, since the rate of recombination is very much greater for alkali-denatured apoprotein than for untreated apoprotein. One can therefore postulate that very mild preparative methods (such as that used in the present study) will give rise to a folded apoprotein that will recombine with haem in a single-stage second-order process. The more extreme the conditions used in the preparation of the apoprotein (with regard to both pH and exposure to organic solvent), the more unfolded the apoprotein which will result; such preparations would be expected to show multistage non-second-order recombination kinetics. In addition, as the apoprotein becomes more folded so the second-order recombination rate could be expected to fall progressively below that calculated for a diffusion-controlled process with free access of haem to the binding site on the apoprotein (approx. $10^8 M^{-1} \cdot s^{-1}$). This argument is in agreement with my observations.

Rossi-Fanelli & Antonini (1960) have demonstrated that free interchange of haem occurs between haemoprotein molecules at room temperature, physiological pH and in aqueous solution. It is a reasonable assumption that this process also occurs in the intra- and extra-cellular environment in living systems. It must be assumed that these interchange reactions occur without the unfolding of apoprotein, since the investigations described here show that a single-stage recombination process is quite feasible. It is unlikely that significant aggregation of haem occurs in such processes *in vivo*, since the concentration of free haem at any given time is considerably less than the lowest concentration studied here (Banerjee, 1962). Therefore it seems likely that the combination of apoprotein and haem *in vivo* proceeds via a simple second-order mechanism.

I am indebted to Mrs. C. Adams for carrying out the protein determinations mentioned in this work.

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