Biochem. J. (1976) **159**, 371–376
Printed in Great Britain

The Kinetics and Mechanism of the Recombination Reaction between Apomyoglobin and Haemin

By PAUL A. ADAMS
Department of Chemistry, University of Rhodesia, P.O. Box MP 167, Salisbury, Rhodesia

(*Received* 13 *May* 1976)

A simple rapid-mixing technique is described which allows the recombination reaction between apomyoglobin and haemin to be studied within 0.3 s of the splitting of myoglobin by dilute HCl. Evidence is presented that indicates that the recombination process occurs between folded 'native' apomyoglobin and monomeric haemin. Postulation of a one (or more)-intermediate recombination process, as suggested by other studies, is not necessary to explain the results. The effect, on the kinetics and mechanism of recombination, of the time of exposure to acid pH of the split myoglobin solution was investigated. The effect of temperature on the recombination kinetics was also studied.

Kinetic studies on the rate and mechanism of the recombination of apohaemoprotein with haemin and its derivatives are of importance in determining the accessibility of the haem-binding site in such apoproteins. Since the pioneering studies of Hill & Holden (1926), two approaches to the study of the recombination process have been developed. The procedure most commonly used in the past two decades is that developed by Gibson & Antonini (1960), in which a stopped-flow technique is used to mix solutions of haemin (or derivatives) and apoprotein before observation of the recombination spectrophotometrically. The second method, used in a series of studies by Maehly (1955), involves splitting of the haemoprotein with acid (usually HCl) and initiating the recombination process by neutralization with base (usually NaOH). The recombination is again monitored by u.v./visible spectrophotometry, but no separation of the haem and apoprotein is involved.

Two serious criticisms can be levelled against the first procedure.

(1) The preparation of the isolated apoprotein involves reasonably prolonged exposure of the apoprotein to both acid pH and organic solvent. This would be expected to give apoprotein whose configuration was in an unfolded or 'denatured' state. This criticism is particularly valid if the preparative method used is the acid/acetone procedure. The modification by Yonetani (1967) of Teale's (1959) acid/butanone method is far more satisfactory, but does not appear to have been used in any major study on the recombination process.

(2) The molecular state of haemin in aqueous solution is almost certainly an aggregate of haemin molecules, the particle weights of the aggregates ranging up to 10^5 daltons. Since the molecular state of the haemin is not exactly defined, interpretation of kinetic results obtained with the biologically most relevant porphyrin is therefore rendered impossible. Under these conditions the usual approach has been to use derivatives such as carboxyhaem, which is considered to be monomeric in solution.

The method of Maehly (1955) suffers from the disadvantage that the time which elapses between the addition of acid and neutralization with base is of the order of tens of seconds. Therefore part of criticism (1) above applies equally to this procedure. In addition, the achievement of an accurately reproducible final recombination pH is technically very difficult if base is used for the neutralization. Since the recombination rate is markedly pH-dependent, the reproducibility of kinetic studies will be much affected.

In the light of these considerations, it is hardly surprising that difficulty has been experienced in obtaining reproducible quantitative kinetic data for the recombination process (Gibson & Antonini, 1960). However, results obtained for a variety of haemoproteins by using the stopped-flow method agree in several respects. The recombination appears multiphasic, the first step being (of necessity) the combination of unfolded apoprotein with porphyrin (rate constant approx. $10^8 M^{-1} \cdot s^{-1}$). This very rapid bimolecular step appears to be followed by one or more (Itagaki *et al.*, 1967) conformational refolding processes, giving ultimately 'native' haemoprotein.

The investigations of Asakura & Yonetani (1969) give qualitative support to the multiphasic nature of the recombination process for cytochrome *c* peroxidase. However, they were unable to analyse their recombination data in terms of either first- or second-order kinetics, and therefore concluded that the re-

combination mechanism was more complex than the simple one-intermediate scheme (Gibson & Antonini, 1960).

I have investigated the kinetics of haemin–apomyoglobin recombination by using a modification of the technique of Maehly (1955). In my method, the time elapsed between splitting and recombination of the haemoprotein can be shortened with practice to less than 0.3s, allowing the kinetics of recombination to be studied under conditions of varying exposure time to acid pH. The effect of temperature on the recombination kinetics has been studied and comparison with previous studies made.

Experimental

Ferrimyoglobin

Ferrimyoglobin was purified from commercial sperm-whale myoglobin (98% by Fe analysis) by using the recrystallization procedure of Yonetani & Schleyer (1967). The purity index (E_{409}/E_{280}) of our preparation was 5.77 at pH5.0 and 23°C (cf. the value of 5.80 obtained by Yonetani & Schleyer, 1967). Stock solutions of the myoglobin were made up so as to give an E_{409} of approx. 0.50 when 0.0500± 0.0002ml was diluted to 3.00ml with phosphate buffer, pH7.40 (8.7mM-KH_2PO_4/31mM-Na_2HPO_4).

Instrumentation and methods

Spectrophotometric measurements were carried out in a Pye Unicam SP.1700 double-beam spectrophotometer with an AR25 linear recorder. The photometric accuracy of the system was checked with standard solutions of pure *p*-nitrophenol in 1mM-NaOH (A.R. grade). The observed E_{400} was 17991 ±40. The photomultiplier linearity was checked at 400nm by using the same solution in a variable-path-length cell. The response was found to be linear within 0.003 E_{400} unit in the range 0–1.0 E_{400} unit.

The splitting–recombination process was initiated by using the double-syringe apparatus shown in Fig. 1. The two 2ml plastic syringes are cemented together with epoxy resin and enclosed in a water-circulating jacket. The plastic tubes (2mm internal diam.), which replace the syringe needles, are taped together temporarily after each syringe is charged to facilitate positioning in the mouth of the reaction cuvette.

In a typical experiment, 0.0500ml of stock ferrimyoglobin solution was incubated in a 10mm-path-length cuvette in the constant-temperature cell holder of the spectrophotometer; 1.35ml of HCl (pH2.65–2.80) and 1.60ml of phosphate buffer, pH7.40 (17.3mM-KH_2PO_4/61mM-Na_2HPO_4), were kept at the cuvette temperature in the double-syringe device. Decomposition of the myoglobin into haem and apoprotein was achieved by rapid injection of acid into the cuvette, recombination being initiated by injection of buffer into the acid; both processes were followed at 409nm. The pH of the solution after injection was invariably between 7.32 and 7.36, the degree of splitting of the myoglobin being >95% after 0.4s exposure to pH2.75.

The mixing time of the sample-injection technique could be shortened with practice to approx. 0.05s. Mixing efficiency is maximized, and cavitation avoided, by injection into the angle of the cuvette corner with the tips of the plastic tubes about 3mm below the cuvette rim. Stray-light effects, caused by operating the spectrophotometer with the sample compartment open, must be as far as possible eliminated from the system. A shield is fitted over the entire sample compartment of the instrument, and the injection tubes are then inserted through a small hole directly above the sample cuvette. If the complete system is kept in a partially darkened room, stray light is less than 0.2% at an E_{400} of 1.5. Temperature control is by circulation of thermostatically controlled water from an external bath; control was measured directly to be better than ±0.05°C.

Fig. 2 shows a typical reaction trace obtained at 34.06°C, the time of exposure to acid being 1.1s. Fig. 3 illustrates the analysis of the trace shown in Fig. 2. Obviously the concentration of apoprotein

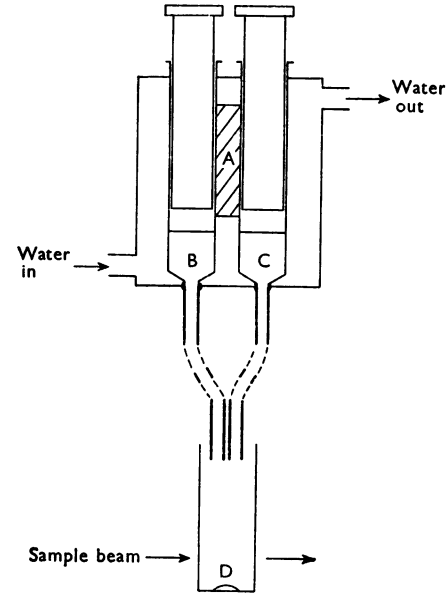

Fig. 1. *Schematic diagram of the double-syringe apparatus used to investigate the decomposition–recombination kinetics of myoglobin*

A, Perspex block joining the two syringes; B, dilute acid (pH2.65–2.80); C, buffer (pH7.40); D, myoglobin solution.

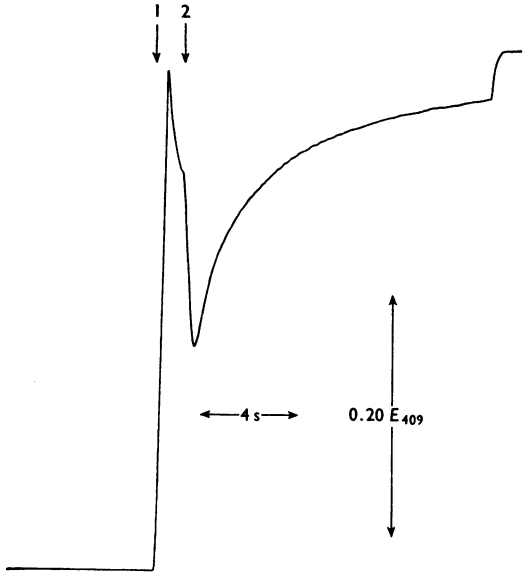

Fig. 2. *Typical reaction trace obtained by using the double-syringe technique for ferrimyoglobin decomposition–recombination kinetics*

Stock ferrimyoglobin solution (0.0500ml) was split with 1.35ml of HCl (pH2.79) (arrow 1), recombination being initiated by injection of 1.65ml of phosphate buffer (pH7.40) (arrow 2); the reaction temperature was 34.06°C. After 1.1s exposure to pH2.79 the myoglobin was 97.6% split. $E_\infty = 0.505$.

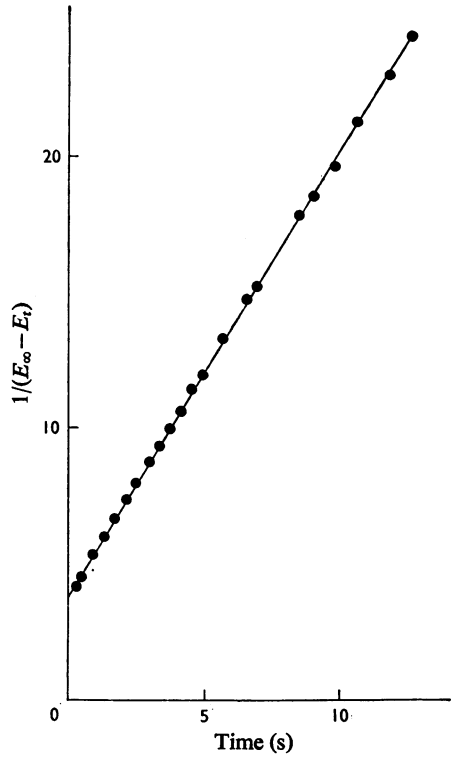

Fig. 3. *Kinetic analysis of the trace shown in Fig.* 2

It was assumed that the recombination process follows simple second-order kinetics. The second-order recombination rate constant (k_{+2}) evaluated from the plot has a value of $1.741 \times 10^5 M^{-1} \cdot s^{-1}$.

and haem produced on splitting must be the same; therefore if the recombination reaction involves the simple combination of monomeric haemin with apoprotein to give native myoglobin directly, the process should follow second-order kinetics. Under these conditions a plot of $(E_\infty - E_t)^{-1}$ against time (t) should be a straight line. As shown in Fig. 3 the recombination is very precisely second-order to >85% reaction. (Kinetic systems obeying a particular rate law to 80% reaction are usually regarded as obeying that law over the whole reaction range. Deviations occurring above this arbitrary extent of reaction are as likely to be caused by small errors in the estimation of E_∞ values, as by genuine deviations from the observed rate law.) Second-order rate constants for the recombination process were calculated by use of a measured extinction-coefficient difference of $1.060 \times 10^5 M^{-1} \cdot cm^{-1}$ between native and split myoglobin solutions at 409nm.

Results

Effect of acid-exposure time on the recombination process

By using the technique discussed in the Experimental section, the effect of time of exposure of the myoglobin solution to acid pH on the rate and kinetics of the recombination process was investigated. A typical series of second-order plots, obtained at 32°C at a splitting pH of 2.75, is shown in Fig. 4. These show qualitatively that a decrease in recombination rate occurs with increasing time of exposure of the solution of split myoglobin to acid pH. The second-order rate constants, calculated from the results shown in Fig. 4, are plotted against the exposure time to acid pH in Fig. 5, which shows that the observed rate-constant decrease is biphasic. First, there is a very large, rapid decrease in the rate constant, followed by a slow, apparently zero-order decrease. By using a curve-stripping procedure the initial rapid decrease can be shown to be kinetically first order (inset of Fig. 5); this result was found at all temperatures studied.

In the initial first-order-decrease phase, the recombination kinetics were observed to be very accurately second order (over at least 80% recombination). However, as the exposure time was increased into the slow zero-order-decrease phase, increasing

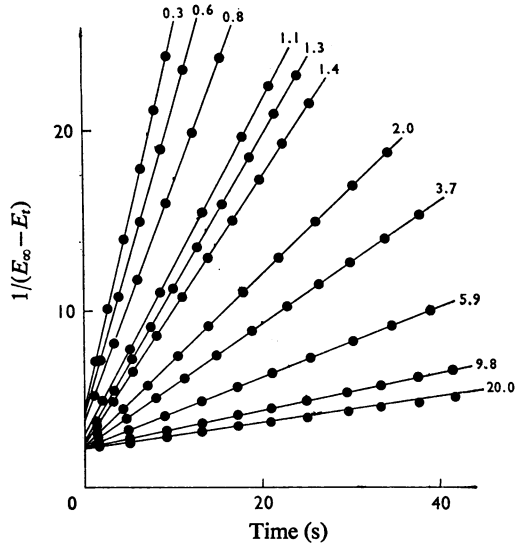

Fig. 4. *Typical series of experimental results obtained at 31.90°C with a splitting pH of 2.75 and recombination pH of 7.40*

The numbers appended against each rate curve represent the time (in s) the solution was exposed to pH2.75 before recombination was initiated.

deviation from second-order kinetics became apparent. At 25°C after 20s exposure to pH2.73, the recombination is accurately second order for the first 65%; after 75s exposure, however, only the first 47% is accurately second order. The deviations observed invariably reflected a slowing of the reaction below the expected second-order rate.

Limiting second-order recombination rate constants were calculated by extrapolation of the first-order plots (inset of Fig. 5) to zero time, and addition of the zero-order-phase rate constant extrapolated to zero time (broken line in Fig. 5). The second-order rate constant, extrapolated from the zero-order-decrease phase, was found to be virtually temperature-independent, the mean value for 11 temperatures being $3.1 \times 10^4 M^{-1} \cdot s^{-1}$. Arrhenius plots for the rate-constant–temperature data at 11 temperatures are shown in Figs. 6 and 7. Activation and pseudo-thermodynamic activation parameters, evaluated from a least-squares analysis of these plots, are shown in Table 1.

Discussion

The biphasic variation in the second-order recombination rate constant, demonstrated in this study, does not appear to have been observed in previous studies. The reason for this is almost certainly that in previous studies (Maehly, 1955) neutralization of the split haemoprotein solution could not be initiated

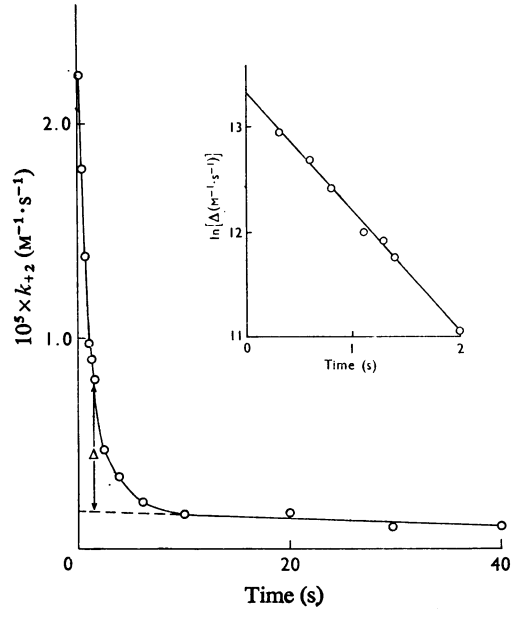

Fig. 5. *Plot of the second-order recombination rate constants derived from Fig. 4, against the time of exposure of the myoglobin solution to pH2.75*

The biphasic nature of the curve is clearly demonstrated. The inset illustrates the fact that the initial rapid decrease in the second-order rate obeys first-order kinetics. $\ln \Delta$ (Δ evaluated as indicated in the main Figure) is plotted against the time of exposure to pH2.75.

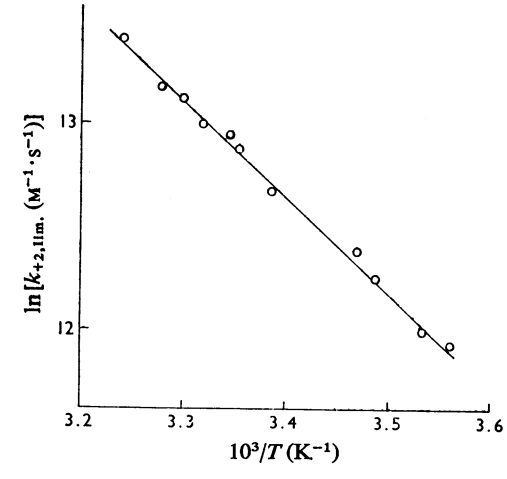

Fig. 6. *Arrhenius plot of the limiting second-order rate constants for the recombination of apomyoglobin with haemin*

The limiting rate constants are evaluated as described in the text, and shown in Fig. 5.

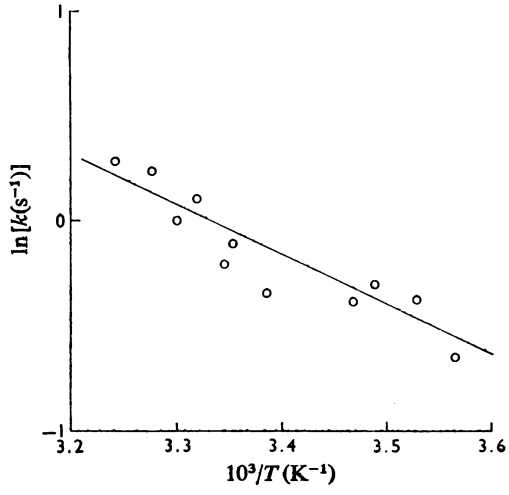

Fig. 7. *Arrhenius plot of the first-order rate constant characterizing the rapid-decay phase of the second-order recombination rate constant*

The first-order rate constant is evaluated from the slope of the first-order plot (inset, Fig. 5).

before the rapid initial fall in the recombination rate was complete.

The slow zero-order decrease in the second-order recombination rate constant can almost certainly be ascribed to aggregation of the haemin in acid solution. Changes on a similar time-scale have been noted in the spectra of split horseradish peroxidase (Maehly, 1952) and have been interpreted in terms of aggregation of the iron porphyrin. This interpretation is supported by the observation that, as the exposure time to acid pH is increased, a parallel increasing deviation from accurate second-order recombination kinetics is observed. The limiting second-order recombination rate constants for this slow-decrease phase (extrapolated to zero time as shown by the broken line in Fig. 5) are in excellent agreement with the limiting value of approx. 5×10^4 M$^{-1}\cdot$s^{-1} proposed by Gibson & Antonini (1960) for the recombination of aggregated haemin with globin. The values obtained in the present study were virtually temperature-independent, and were in the range 2.7×10^4–3.6×10^4 M$^{-1}\cdot$s^{-1}. Preliminary studies using added apomyoglobin (prepared as detailed by Yonetani, 1967) or very weakly buffered (pH9.2) solution of haemin showed that in this zero-order phase the rate of recombination was unaffected by added apoprotein, but increased by addition of haemin. Again this indicates aggregation of the haemin as the cause of this slow-decrease phase.

Aggregation of the haem cannot, however, account for the rapid first-order decrease in the rate constant observed at short times of acid exposure. If aggregation were an explanation, the decrease would not be

Table 1. *Activation parameters, calculated from Arrhenius plots of the rate constants characterizing the recombination of apomyoglobin and monomeric haemin, and the rapid-decrease phase in the recombination rate constant*

Stage studied	Recombination (from Fig. 6)	First-order decrease (from Fig. 7)
Frequency factor (s^{-1})	2.54×10^{12}	1.89×10^3
Activation energy (kJ)	38.9 ± 0.96	18.8 ± 3.8
ΔH^* (kJ)	36.4	16.3
ΔS^* (J·degree^{-1}·mol^{-1})	−34.7	−209.7

expected to exhibit first-order kinetics; further, the recombination would not be expected to be very precisely second order over the whole recombination process. The initial rapid fall in the second-order rate constant must therefore be interpreted in terms of some change in the apoprotein. This explanation is supported by preliminary studies, which indicate that in this phase of the recombination-rate-constant decrease both added apoprotein and haemin increase the recombination rate.

It is as well at this stage of the discussion to note that the decrease in the rate constant is not related to the degree of splitting of the myoglobin. Although the splitting curve shows a similarity to Fig. 5, the myoglobin is completely split in less than 1 s at the pH used in the present study. In addition the rate constants for the recombination are calculated in terms of M^{-1}, and are thus independent of the concentrations of apomyoglobin and haem that are recombined.

Before speculation as to the possible nature of changes in the apoprotein in the initial rapid-decrease phase of the recombination rate constant, the physical meaning of the second-order recombination rate constant extrapolated from data in this rapid-decrease phase will be discussed.

At zero time of exposure to acid, no aggregation of haemin, or change in the apoprotein, can have occurred in the split solution. The extrapolated recombination rate constant at zero exposure time must therefore represent the rate of recombination of monomeric haemin with native, folded apomyoglobin. This interpretation is supported by further considerations.

Gibson & Antonini (1960) found a recombination rate constant for apomyoglobin and carboxyhaem of approx. 1.4×10^7 M$^{-1}\cdot$s^{-1} at 20°C (this value has been interpolated to pH7.4 from their data at pH7.1 and 9.2). The value interpolated to 20°C from data in the present study is approx. 2.7×10^5 M$^{-1}\cdot$s^{-1} at pH7.40. Gibson & Antonini (1960) interpreted their results in terms of a diffusion-controlled reaction between monomeric porphyrin and a site freely accessible from all angles of the hemisphere of approach. The apoprotein is therefore regarded as being in an unfolded configuration. If, however, the apo-

myoglobin were in the folded form the recombination rate constant would be lower, since the approach of the haemin is now sterically constrained. To a first approximation, the ratio of the rate constants for recombination involving unfolded and folded apomyoglobin will be in the ratio of half the surface area of the folded protein to the area of the binding site on the surface of the protein. It is well known that the haem molecule fits 'edgeways' into a cleft in the surface of myoglobin (see, e.g., Kendrew *et al.*, 1961), and it is therefore possible to make a rough estimate of a hypothetical recombination rate for monomeric haemin and folded apoprotein by using the results of Gibson & Antonini (1960) for the recombination of carboxyhaem and unfolded apoprotein.

It will be assumed that the area of the binding cleft of myoglobin is equal to the area of a haemin molecule edge-on. The haemin molecule will be assumed to be a square of edge 1.5nm and thickness 0.25nm; the required 'cleft' area is thus $0.375\,nm^2$. Taking myoglobin to be a sphere of radius 2.0nm the hemisphere surface area is calculated as $25.13\,nm^2$. The hypothetical second-order recombination rate constant for monomeric haemin and folded apomyoglobin is then given as $1.4\times10^7\times0.375/25.13$, i.e. $2.1\times10^5\,M^{-1}\cdot s^{-1}$. The agreement with the experimental value of $2.7\times10^5\,M^{-1}\cdot s^{-1}$ is, considering the crudity of the model, good. The calculated value is relatively insensitive to changes in the molecular dimensions used, the values given above being merely rough estimates made from crystallographic and structural data.

The Arrhenius activation energy for the process under discussion is somewhat lower than the value obtained by Gibson & Antonini (1960), but is still rather high for a diffusion-controlled process. In addition, the activation entropy, although negative, is not of sufficient magnitude to suggest severe steric constraint in the formation of the transition state. The model proposed would be expected to give rise to a large negative $\Delta S^{\ddagger}$, and an activation energy of approx. 20kJ.

The cause of the decrease in the second-order recombination rate constant, observed during the first few seconds exposure to acid pH, is more difficult to ascertain. It is well known from crystallographic studies (Watson, 1966) that the interaction of haemin with apomyoglobin leads to a stabilization of the haemoprotein as a whole via hydrophobic interactions in the interior of the binding 'cleft'. As an obvious corollary it would be expected, and is in fact observed (see Gibson & Antonini, 1966), that the stability of the apoprotein would be considerably less than the stability of the native haemoprotein. Destabilization on removal of the haem presumably arises from two mutually related causes. First the rupture of the stabilizing interactive hydrophobic bonds between the haem and the globin, and secondly the exposure, via the now vacant binding 'cleft', of the hydrophobic interior of the globin to the polar aqueous solvent environment. Progressive breakdown of the hydrated globular apoprotein will then occur, to give a denatured random-coil configuration of the type postulated as the initial reactive form in the recombination studies of Gibson & Antonini (1960).

Two possible explanations for the rapid decrease in the second-order recombination rate constant, observed in the first few seconds' exposure to acid pH, can now be advanced.

The decrease could reflect steric blocking of the binding 'cleft' to the returning haemin molecule, by solvent molecules which have diffused into the cleft. This is supported by two considerations. First, such a diffusive process would probably obey pseudo-first-order kinetics, explaining the kinetics observed for the rate constant decrease. Secondly, the Arrhenius activation energy observed for the process is of the order of 19kJ, a value normally associated with diffusion-controlled processes. Arguing against this explanation is the relative slowness of the observed decrease, although this could be partially due to relatively severe steric limitations inherent in the proposed scheme. It is of interest that the activation entropy for this stage (see Table 1) suggests that a considerable degree of ordering occurs in the formation of the activated complex for the process.

Alternatively, the decrease could reflect reaction between the unfolding globular apomyoglobin and aggregating haemin. However, it is difficult to see how such a process could give rise to a first-order rate law for the decrease in recombination rate constant. In addition, the studies of Gibson & Antonini (1960) suggest that the unfolded globin reacts considerably more rapidly than does folded apoprotein, not, as observed here, more slowly.

References

Asakura, T. & Yonetani, T. (1969) *J. Biol. Chem.* **244**, 537–544

Gibson, Q. H. & Antonini, E. (1960) *Biochem. J.* **77**, 328–341

Gibson, Q. H. & Antonini, E. (1966) in *Hemes and Hemoproteins* (Chance, B., Yonetani, T. & Estabrook, R., eds.), pp. 67–78, Academic Press, New York

Hill, R. & Holden, H. F. (1926) *Biochem. J.* **20**, 1326–1339

Itagaki, E., Palmer, G. & Hager, L. P. (1967) *J. Biol. Chem.* **242**, 2272–2277

Kendrew, J. C., Watson, H. C., Strandberg, B. E. & Dickerson, R. E. (1961) *Nature (London)* **190**, 666–670

Maehly, A. C. (1952) *Biochim. Biophys. Acta* **8**, 1–17

Maehly, A. C. (1955) *Arch. Biochem. Biophys.* **56**, 507–524

Teale, F. W. J. (1959) *Biochim. Biophys. Acta* **35**, 543

Watson, H. C. (1966) in *Hemes and Hemoproteins* (Chance, B., Yonetani, T. & Estabrook, R., eds.), pp. 63–66, Academic Press, New York

Yonetani, T. (1967) *J. Biol. Chem.* **242**, 5008–5013

Yonetani, T. & Schleyer, H. (1967) *J. Biol. Chem.* **242**, 1974–1979