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The Combination of Carbon Monoxide–Haem with Apoperoxidase

BY CHARLES PHELPS AND ERALDO ANTONINI
Department of Biochemistry, University of Bristol, and Consiglio Nazionale delle Ricerche Centre for Molecular Biology, Istituto di Chimica Biologica, University of Rome, Italy

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1. Static titrations reveal an exact stoicheiometry between various haem derivatives and apoperoxidase prepared from one isoenzyme of the horseradish enzyme. 2. Carbon monoxide–protohaem reacts rapidly with apoperoxidase and the kinetics can be accounted for by a mechanism already applied to the reaction of carbon monoxide–haem derivatives with apomyoglobin and apohaemoglobin. 3. According to this mechanism a complex is formed first whose combination and dissociation velocity constants are 5×10^8 M^{-1}sec.$^{-1}$ and 10^3sec.$^{-1}$ at pH 9·1 and 20°. The complex is converted into carbon monoxide–haemoprotein in a first-order process with a rate constant of 235sec.$^{-1}$ for peroxidase and 364sec.$^{-1}$ for myoglobin at pH 9·1 and 20°. 4. The effects of pH and temperature were examined. The activation energy for the process of complex-isomerization is about 13kcal./mole. 5. The similarity in the kinetics of the reactions of carbon monoxide–haem with apoperoxidase and with apomyoglobin suggests structural similarities at the haem-binding sites of the two proteins.

Horseradish peroxidase contains a ferriprotoporphyrin IX prosthetic group functionally linked to a single polypeptide chain of molecular weight 40000. The reactivity of the haem of horseradish peroxidase is similar to that of the haemoproteins haemoglobin and myoglobin, which have been extensively studied (Rossi-Fanelli, Antonini & Caputo, 1964).

Theorell & Maehly (1950) showed that horseradish apoperoxidase, prepared by acid–acetone treatment of the enzyme, recombines slowly with haematin with a half-time of 7–10min.

This finding is in contradiction to the studies on the recombination of haem derivatives with other apohaemoproteins extensively investigated by Gibson & Antonini (1960, 1963). There it was shown that the physical and chemical nature of the haem derivatives themselves determines the rate of reaction with apoprotein, and that, when the predominantly monomeric carbon monoxide–haem was chosen, the rate of reaction between it and apohaemoglobin was not only very fast but also occurred in two stages, which could be analysed in terms of a minimum scheme:

$$\text{Haem} + \text{apoprotein} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{haem–protein complex} \xrightarrow{k_3} \text{haemoprotein}$$

It is the object of the present paper to report similar experiments on apoperoxidase that demonstrate that the combination with carbon monoxide–haem is indeed fast, with derived rate constants very similar to those of other haemoproteins.

The finding by Shannon, Kay & Lew (1966) that horseradish peroxidase exists in four groups of isoenzymes necessitates the purification of one group of these components, which has been studied in detail.

Parallel experiments have also been carried out with sperm-whale apomyoglobin to furnish a comparison between the two proteins. In most cases experiments were performed consecutively with each protein solution and the great degree of similarity of the results on the two systems supports the contention that the haem site on the two proteins may be very similar.

EXPERIMENTAL

Materials

Horseradish peroxidase. This was a commercial product (lot no. 6517238) obtained from C. F. Boehringer und Soehne

G.m.b.H., Mannheim, Germany. Before purification this material was characterized by an E_{400}/E_{280} ratio 0·32 in 0·1 M-phosphate buffer, pH 7·0. A 1 g. portion of this material was dissolved in a small volume (5 ml.) of 5 mM-sodium acetate buffer, pH 4·4, and dialysed against a large excess of the same buffer for 24 hr. The solution was then layered on a column (3 cm. × 30 cm.) of CM-cellulose that had been previously equilibrated with the same buffer. The protein was then separated by the method developed by Shannon *et al.* (1966). The small quantity of A isoenzymes was washed through in the first buffer. A linear gradient, formed by mixing 5 mM-acetate buffer with 0·1 M-acetate buffer, pH 4·4, eluted a major fraction of B isoenzyme. This was collected and dialysed against saturated $(NH_4)_2SO_4$ solution, pH 7·0. The precipitated peroxidase was centrifuged, dissolved in a small volume of 5 mM-tris–HCl buffer, pH 8·4, dialysed overnight against the same buffer and chromatographed on a small column (2 cm. × 15 cm.) of DEAE-cellulose. The peroxidase was eluted with the same buffer, dialysed against saturated $(NH_4)_2SO_4$ solution, pH 7·0, centrifuged, dissolved in water and exhaustively dialysed for 36 hr. against four changes of water. The final solution gave an E_{400}/E_{280} ratio 3·01 in 0·1 M-phosphate buffer, pH 7·0, and was freeze-dried, yielding 35–45 mg. When assayed by the purpurogallin procedure, the enzyme gave values in the range 850–975 units/mg. of enzyme. The purification of the enzyme was performed at 0–4°.

Horseradish apoperoxidase. This was prepared by treating a salt-free solution of peroxidase (1–2%) with 20–30 vol. of acid–acetone (3 ml. of 2 M-HCl/l. of acetone) at −15°. The precipitate was centrifuged at −15°, taken up in a minimum amount of cold water and re-treated with acid–acetone. The centrifuged precipitate was washed once with cold acetone, dissolved in water, and dialysed for 6–7 hr. against water and then against 1·5 mM-$NaHCO_3$ for 20 hr. This was followed by 20 mM-sodium phosphate buffer, pH 7·0, for 24 hr. and lastly many changes of water before the protein solution was freeze-dried. A 0·1% solution showed a Soret-band absorption of less than 0·03. It possessed no catalytic activity as measured by the purpurogallin assay.

Sperm-whale myoglobin. This was purchased from Seravac Laboratories Ltd., Maidenhead, Berks.

Apomyoglobin. This was prepared as described above for horseradish apoperoxidase. A 0·1% solution showed a Soret-band absorption of less than 0·02.

Haematin. Haematin solutions were prepared by dissolving crystalline protohaem in 10 mM-NaOH to give concentrations about 0·1 mM. This solution was further diluted with buffer as required for the experiments.

Carbon monoxide–haem. CO–haem solutions were prepared by reducing the necessary volume of stock haematin solution with $Na_2S_2O_4$ (0·5 mg./ml.) and diluting with deoxygenated buffer that had been equilibrated with a suitable partial pressure (0·5–1 atm.) of CO.

Buffer solutions. These were either 0·2 M-sodium phosphate buffer, pH 7·0, or 50 mM-sodium borate buffer, pH 9·1.

Reagents. These were of analytical quality where possible, and the water used was double-glass-distilled.

Methods

Stopped-flow kinetic determinations were carried out with the Gibson–Durrum apparatus (Gibson & Milnes, 1964) with a 2 cm. observation tube. Band widths were 1–2 nm., and the dead-time of the instrument was 3·4 msec.

Kinetic difference spectra were measured on the same instrument. CO–haem solutions of 1·6 μM were mixed with a 1·9 μM-protein solution and the spectral wavelength was varied at 4 nm. intervals over the range 404–432 nm.

Spectra were measured with a Beckman model DK 1 or a Cary model 11 recording spectrophotometer. In static titrations of apoprotein with haem derivatives difference spectra were recorded by adding 5 μl. increments of haem solution from an Agla micrometer-drive syringe (Burroughs Wellcome and Co.) to both 3 ml. of protein solution and 3 ml. of buffer solution.

Flash-photolysis experiments were performed in the apparatus described by Antonini, Chiancone & Brunori (1967) with either 100 J or 300 J energy flashes of 100 and 300 μsec. duration respectively. The recombination of the photodissociated CO was followed at 435 nm. both for peroxidase and for myoglobin.

Peroxidase activity was measured by the purpurogallin assay procedure developed by the Sigma Chemical Co., St Louis, Mo., U.S.A. This method in our hands was not reproducible to any great accuracy, but furnished comparative values for the reactivation of the enzyme by haematin.

RESULTS

Static titration of apoperoxidase with various haem derivatives. The results of titrating a solution of apoperoxidase with (*a*) ferric haematin and (*b*) ferrohaem in 0·1 M-phosphate buffer, pH 7·0, at 20° are shown in Fig. 1. The protein solutions were as indicated in the legend. It is evident from inspection of the curves that, whereas ferrohaem titrates exactly, the ferric derivative shows no clear stoicheiometric end point, though graphical extrapolation can produce an expected equivalence point. The spectra obtained after titration were characteristic of the two reconstituted peroxidase proteins, and assay of the reconstituted ferric enzyme showed that 77–82% of the activity was regained after such titrations, as judged by purpurogallin production.

A similar experiment with carbon monoxide–haem gave a clear end-point titration.

Combination of carbon monoxide–haem with apoperoxidase. Since ferrohaem derivatives gave more satisfactory static titrations, the kinetics of combination of apoperoxidase with the better authenticated carbon monoxide–haem were examined next. The sharp spectrum, high extinction coefficient and the 1 : 1 stoicheiometry for iron and carbon monoxide reported by Hill (1926) suggest that this compound may be predominantly monomeric or a monomer–dimer mixture with a reasonable concentration of monomer, so that it would reflect more nearly the true kinetics of haem derivatives in combining with apoprotein. Equilibrium studies by Shack & Clark (1947) have suggested that ferric haematin in aqueous buffer solutions is polydisperse with particle weights representing micelles of up to 100 haematin molecules.

Fig. 2 reveals that the reaction with carbon

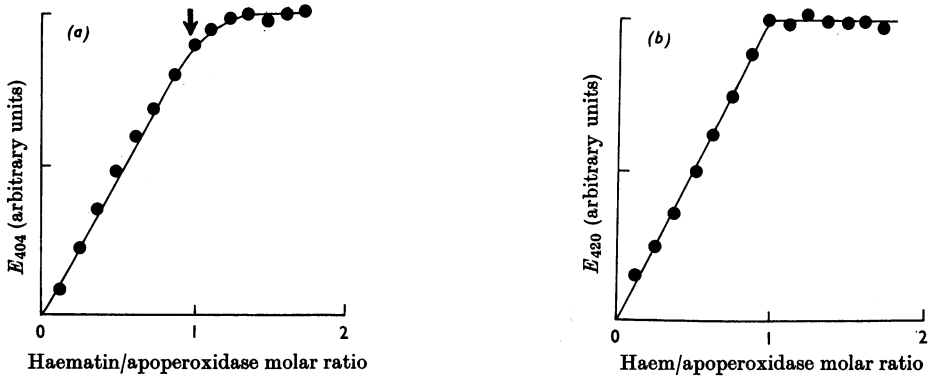

Fig. 1. Static titrations of apoperoxidase with (*a*) haematin and (*b*) ferrohaem. In (*a*) 1 ml. of 3·8 μM-apoperoxidase in the experimental cuvette was titrated with 52·4 μM-haematin in 0·1 M-phosphate buffer, pH 7·0, which was added to both experimental and reference cuvettes. The latter contained 0·1 M-phosphate buffer, pH 7·0, at 20°. In (*b*) the conditions were the same except that 0·5 mg. of $Na_2S_2O_4$ was added to both experimental and blank cuvettes before titration. Some crumbs of $Na_2S_2O_4$ were added to a stock solution of deoxygenated haematin.

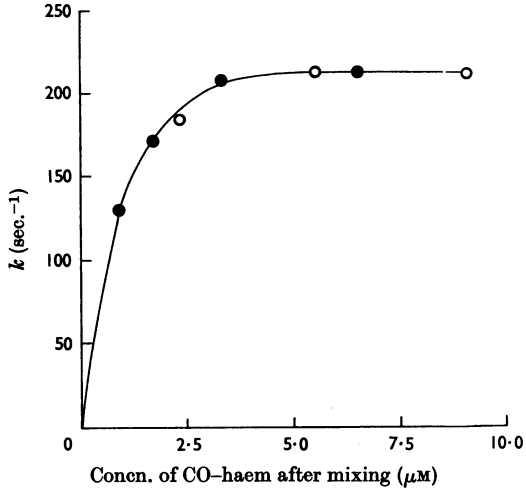

Fig. 2. Reaction of CO–haem with apoperoxidase in 0·01 M-phosphate buffer, pH 7·1, at 20°. The ordinate expresses the pseudo-first-order rate constant and the abscissa shows the concentration of CO–haem. ○, 2·0 μM-Apoperoxidase; ●, 3·7 μM-apoperoxidase.

monoxide–haem is fast. The results of independent sets of experiments with two different apoperoxidase preparations are described in terms of the initial pseudo-first-order rate constant plotted against the final carbon monoxide–haem concentration. The rate constant was determined from the initial slope of the curve of the logarithm of the ratio of the extinction at zero time to that at times (t) up to 20 msec., against t, and was corrected for the reaction that occurred in the dead-time of the instrument (3·4 msec.). The choice of such a rate constant follows implicitly from the treatment developed by Gibson & Antonini (1960).

In combination experiments with either apoperoxidase or apomyoglobin and carbon monoxide–haem, the plot of log (E_0/E_t) against t showed that each curve had an initial fast component and also a slow process. Since the rate constant for the slow component was variable, depending, apparently, only on the particular preparation and age of parent haematin used, it was attributed to reactions dependent on physical state of carbon monoxide–haem in solution.

Effect of varying the wavelength of the observation beam. In one set of experiments the wavelength was varied over the range 404–432 nm. at 4 nm. intervals and the results are shown in Fig. 3(*a*), where the total extinction change occurring in the reaction after mixing haem solution at 1·6 μM and protein solution at 1·9 μM is plotted against wavelength. Fig. 3(*b*) shows the absorption spectra of carbon monoxide–haem and of carbon monoxide–ferroperoxidase. It is apparent from a comparison with Fig. 3(*a*) that the kinetic difference spectra can best be interpreted as a disappearance of carbon monoxide–haem ($\lambda_{max.}$ 406·5 nm.) and a simultaneous appearance of carbon monoxide–ferroperoxidase ($\lambda_{max.}$ 422 nm.).

Effect of pH *and temperature.* No detailed investigation of these parameters was made, but experiments were carried out at pH 9·1 at 20° and 33·5° and compared with the results obtained at 20° in 0·1 M-phosphate buffer, pH 7·0. From these it appears that the observed rate is not significantly changed at the higher pH, and the temperature coefficient corresponds to an activation energy of 12–13 kcal./mole.

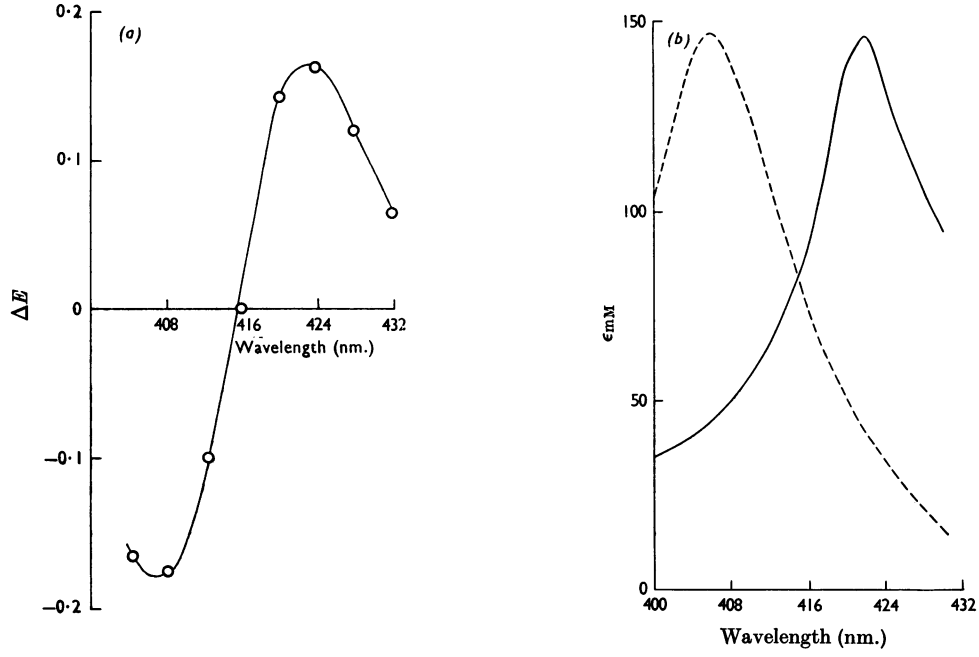

Fig. 3. (*a*) Effect of varying the wavelength of observation on the total change in extinction after combination of CO–haem with apoperoxidase. 1·6 μM-CO–haem was mixed with 1·9 μM-apoperoxidase in 0·1 M-phosphate buffer, pH 7·0, at 20°. A 2 cm. light-path was used. (*b*) Absolute absorption spectra of CO–haem (– – – –) and CO–ferroperoxidase (——) in 0·1 M-phosphate buffer, pH 7·0.

Experiments with flash photolysis. It was hoped that corroborative results on the species formed in the fast reaction between carbon monoxide–haem and apoperoxidase would be obtained by rapidly mixing solutions of the two reactants, photodissociating the carbon monoxide from the newly formed species at various times after mixing and then looking at the recombination of carbon monoxide with the haem prosthetic group.

In one series of experiments in 0·1 M-phosphate buffer, pH 7·0, at 20° 20 μM-carbon monoxide–haem was mixed with 20 μM-apomyoglobin in a 1 mm. cell and a 300 J flash was fired about 10 msec. after mixing. The resultant trace showed that the species formed by photodissociation had a rate of recombination with carbon monoxide similar to that of myoglobin (Smith & Gibson, 1959).

With similar concentrations of apoperoxidase and carbon monoxide–haem under identical conditions, the trace revealed some heterogeneity. A fast-reacting species was formed that possessed the same rate of recombination as that of myoglobin and could have been due similarly to haemochromogen (Smith, 1959), and a slow component that possessed about the same rate of recombination as that of carbon monoxide–ferroperoxidase (Kertesz, Antonini, Brunori, Wyman & Zito, 1965). To clarify this situation, an experiment was devised in which three identical solutions of apoperoxidase were each titrated with a different amount of carbon monoxide–haem so as to give solutions that were each with respect to the proteins (*a*) half titrated with haem, (*b*) equivalently titrated and (*c*) had a twofold molar excess of carbon monoxide–haem over apoprotein. These solutions were then subjected to flash photolysis and the kinetics of recombination of carbon monoxide with peroxidase at 435 nm. were followed. The results are shown in Fig. 4, where the extinction changes are recorded against time for each of the three solutions. The combination velocity constant calculated for the slow component from the pseudo-first-order rate constant and the carbon monoxide concentration is $4{\cdot}9 \times 10^3$ M^{-1}sec.$^{-1}$ at 20° in borate buffer, pH 9·1, which compares with the value of $4{\cdot}5 \times 10^3$ M^{-1}sec.$^{-1}$ obtained by Kertesz *et al.* (1965) under the same conditions and confirms the suggestion that the identity of the material produced by mixing carbon monoxide–haem with apoperoxidase is indeed carbon monoxide–ferroperoxidase.

When the haem/protein ratio was varied from 1:1 to 2:1, the proportion of fast component increased from 15% to 45%. The increasing amount

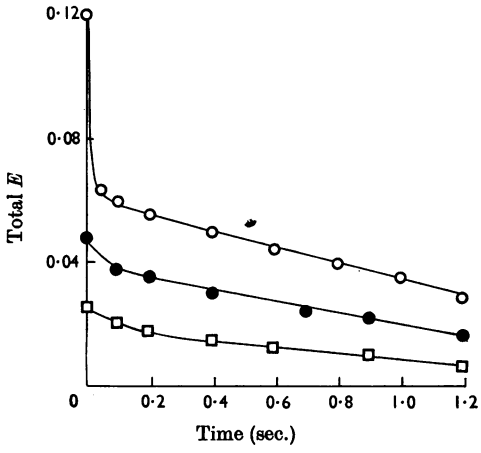

Fig. 4. Extinction changes after flash photolysis in solutions of recombined CO–ferroperoxidase as a function of time, in 50mM-borate buffer, pH 9·1, at 20°. The protein concentration throughout was 5μM after mixing. The light-path was 1cm. and the observation wavelength 435nm.; 300J flashes were used. CO–haem concentrations were: ○, 10μM; ●, 5μM; □, 2·5μM.

of fast component (second-order rate constant $8{\cdot}7 \times 10^5 M^{-1}sec.^{-1}$) seen as the carbon monoxide–haem/apoperoxidase ratio increases suggests that it represents non-specifically bound carbon monoxide–haem. The static titrations of apoperoxidase with carbon monoxide–haem were performed by difference spectroscopy between the solution of protein and the buffer solution by adding the haem to both cuvettes, as described above. It is proposed that the non-specifically bound haem has the same spectrum as free haem and thus could not be differentiated in these static experiments.

DISCUSSION

Nature of the reconstituted product. The results obtained in this work show that the combination of carbon monoxide–haem with apoperoxidase has a fast component with a half-time of reaction of about 5–10msec., but that the reaction is sometimes heterogeneous. The degree of heterogeneity depends on the preparation of haem and on the derivative chosen, as well as on the carbon monoxide–haem/protein ratio. In ascribing the slow reaction to non-specific binding of the haem by the protein, it is possible to invoke the fact that apohaemoglobin and apomyoglobin both show the same effects in reconstitution experiments.

The clearest indication of the heterogeneity of the bound haem is shown by the flash-photolysis experiments. The non-specifically bound haem, which increases as the haem/protein ratio increases, gives rates similar to that expected for haemochromogens, and the characteristically slow combination reaction between carbon monoxide and peroxidase leaves very little doubt that the product initially formed in the reconstitution experiments is indeed carbon monoxide–ferroperoxidase.

Mechanism of the reaction between carbon monoxide–haem and apoperoxidase. Gibson & Antonini (1960) have suggested a mechanism involving a first step in which the complex is reversibly formed between carbon monoxide–haem and apoprotein, which then, in the second step, is bound firmly and irreversibly to the protein. These postulates can be formulated as follows.

Let the concentration of apoprotein be [A], equal to concentration of carbon monoxide–haem, and let the concentration of complex be [X] with rate constants k_1 for formation and k_2 for breakdown. Let k_3 be the isomerization constant of the complex, i.e. the rate of formation of carbon monoxide–ferroperoxidase from the complex.

Then:

$$\frac{d[A]}{dt} = k_2[X] - k_1[A]^2 \quad (1)$$

and

$$\frac{d[X]}{dt} = k_1[A]^2 - (k_2 + k_3)[X] \quad (2)$$

Then:

$$\frac{d[A]}{dt} + \frac{d[X]}{dt} = -k_3[X] \quad (3)$$

If we assume that $k_1 + k_2 \gg k_3$, then at equilibrium, when $d[A]/dt = d[X]/dt$:

$$k_2[X] = k_1[A]^2 \quad (4)$$

This on differentiation yields:

$$\frac{2k_1[A]}{k_2} \cdot \frac{d[A]}{d[X]} = 1 \quad (5)$$

Multiplying eqn. (5) by $d[X]/dt$ and substituting in eqn. (3) gives:

$$\frac{d[A]}{dt} + \frac{2k_1}{k_2}[A] \cdot \frac{d[A]}{dt} = -k_3[X]$$

but from eqn. (4):

$$[X] = \frac{k_1}{k_2}[A]^2$$

Then:

$$\frac{d[A]}{dt} = \frac{-k_3[A]^2}{2[A] + k_2/k_1}$$

The pseudo-first-order velocity constant, r, is given by:

$$r = \frac{-1}{[A]} \cdot \frac{d[A]}{dt}$$

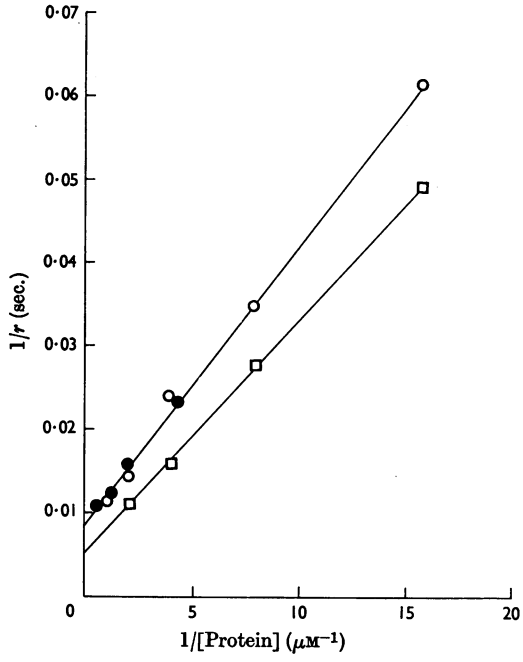

Fig. 5. Relation between the reciprocal of the observed first-order velocity constant, measured over the time-interval 4–10msec. after mixing, and the reciprocal of protein concentration added. ○ and ● refer to two different apoperoxidase preparations in 50mM-borate buffer, pH 9·1, at 20°; □ refers to a preparation of apomyoglobin under identical conditions.

So that:

$$\frac{1}{r}=\frac{2[A]+k_2/k_1}{k_3[A]}$$

So a plot of $1/r$ versus $1/[A]$ will give an intercept equal to $2/k_3$ and a slope k_2/k_1k_3 from which k_2/k_1 may be determined.

In Fig. 5 a plot of $1/r$ against $1/[A]$ shows that a good straight line is obtained for both apoperoxidase and apomyoglobin and leads to values of k_3 for apoperoxidase combination with carbon monoxide–haem of 235sec.$^{-1}$ and a k_2/k_1 ratio 0·775μM. The values for the same constants for apomyoglobin combination are 364sec.$^{-1}$ and 0·98μM. These experiments were done at 20° in 50mM-borate buffer, pH 9·1.

Thus apoperoxidase, apomyoglobin and apohaemoglobin all appear to combine with carbon monoxide–haem with similar rates, and presumably with the same mechanism. The observation by Theorell & Maehly (1950) that offered a contradiction to the general pattern can now be explained in terms of the anomalous physical state of haematin that was used in those experiments. The use of carbon monoxide–haem in the investigation outlined above has technical advantages already mentioned, both in terms of good spectrophotometric parameters and a predominantly monomeric molecule.

The rate of the first step of the reaction, k_1, can only be roughly gauged, as was shown by Gibson & Antonini (1960) for haemoglobin recombination. These workers suggested that a value of 5×10^8M^{-1}sec.$^{-1}$ would not be greatly in error. From the present work it seems necessary to assume a similar rate constant to within an order of magnitude. k_2 would then have a value in the range 5×10^2–10^3sec.$^{-1}$.

Thus the very rapid first step is within the range expected of a diffusion-controlled reaction, as shown by Alberty & Hammes (1958).

It is difficult for the reconstitution both of myoglobin and of peroxidase to ascribe the reaction steps to specific structures. A plausible explanation is that the first rapid phase reflects the binding of the haem iron to a site on the protein, a step associated with a large spectral change. This would be followed by processes involving the stabilization of the haem–protein linkage by interactions between the porphyrin molecule and contiguous parts of the protein, this event being associated with only minor spectral changes. This process is only slightly reversible under normal circumstances.

Just as the similarity of the haem-binding site in haemoglobin and myoglobin is reflected in essentially the same kinetics for haem binding, it is tempting to suggest that the general features of the haem–protein interactions are not dissimilar in peroxidase.

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