Kinetics of Formation of the Peroxidatic Intermediate from Deuteroferriheme and Hydrogen Peroxide†

Peter Jones, Kenneth Prudhoe, Terrence Robson, and Henry C. Kelly*,‡

ABSTRACT: The pre-steady-state kinetics of the formation of the peroxidatic intermediate, obtained from the reaction of deuteroferriheme [chloro(dihydrogen 3,7,12,17-tetramethyl-2-18-porphinedipropionato(2^-))iron(III)] and hydrogen peroxide, have been studied at varying concentrations of heme at pH 7.75 and 8.5 at 25°, utilizing stopped-flow spectrophotometry. A Michaelis–Menten mechanism is indicated, as previously suggested by D. Portsmouth and E. A. Beal ((1971), *Eur. J. Biochem. 19,* 479), however pseudo-first-order rate constants, obtained utilizing excess hydrogen peroxide, increase with decreasing stoichiometric concentration of ferriheme. Consideration of the dimerization equilibria in deuteroferriheme solutions (K. Prudhoe, (1971), Ph.D. Thesis, University of Newcastle-upon-Tyne; P. Jones *et al.* (1974), *J. Chem. Soc., Dalton Trans.*, 911) results in a correlation of such effects with the fraction, α, of total ferriheme present in monomeric form, and indicates that it is deuteroferriheme monomer that is the active form of this catalyst in its conversion to the peroxidatic intermediate.

Iron–porphyrin complexes constitute the prosthetic groups in a number of biological catalysts which include various oxidase enzymes. An example is the iron(III) complex protoferriheme [chloro(dihydrogen 3,7,12,17-tetramethyl-8,13-divinyl-2,18-porphinedipropionato(2^-))iron(III)] which is in the active site for the catalytic decomposition of hydrogen peroxide by the enzyme catalase (EC 1.11.1.6). Many such complexes, including protoferriheme and deuteroferriheme (where the two vinyl substituents in protoporphyrin IX have been replaced by hydrogen atoms) are themselves catalysts for the decomposition of H_2O_2 (von Euler and Josephson, 1927; Kremer, 1965a,b; Brown *et al.*, 1968; Brown and Jones, 1968a).

In recent years, Brown *et al.* (1970a,b) have studied the kinetics of action of such complexes on hydrogen peroxide decomposition as part of a larger investigation of the nature and function of iron-centered catalysts. Their results indicate a qualitative similarity in the modes of action of the ferrihemes with other iron(III) species from the hexaaquoiron(III) ion to catalase itself, and suggest that the relatively high activity of catalase is due to the ability of this enzyme to utilize, as substrate, molecular hydrogen peroxide (its natural form at physiological pH) in contrast to the simpler iron(III) systems which primarily employ the conjugate base of H_2O_2, *i.e.*, the HO_2^- ion.

Critical to a study of this catalytic activity of ferrihemes has been the elucidation of aspects of their structure, particularly regarding their dimerization equilibria in aqueous solution (Brown *et al.*, 1970c; Jones *et al.*, 1974). Recent investigations of Jones *et al.* (1973a) have shown that it is only the monomeric form of deuteroferriheme that contributes significantly to the activity of this catalyst which, at pH values above the pK_a of H_2O_2, approaches the activity of bacterial catalase. The corresponding monomeric species is also predominantly responsible for the activity of protoferriheme, although with this complex, a measurable activity of dimer is exhibited with a pH dependence further consistent with the hypothesis that the substrate employed is, indeed, hydroperoxide anion (Jones *et al.*, 1973a).

Studies of the kinetics of ferriheme catalysis of H_2O_2 decomposition indicate a modified Michaelis–Menten mechanism (von Euler and Josephson, 1927) involving the formation of a steady-state concentration of intermediate species (Kremer, 1965a; review by Brown *et al.*, 1970b) analogous to catalase compound I (Chance, 1947) and which, like catalase compound I (Chance *et al.*, 1952), exhibit peroxidatic activity (Kremer, 1965b; Portsmouth and Beal, 1971; Robson, 1973). At high relative concentrations of H_2O_2 significant oxidative cleavage of heme occurs to form bile pigment (Falk, 1964). Kinetic studies of such destructive oxidation have been made by Brown and Jones (1968a) on protoferriheme and by Jones *et al.* (1973b) on deuteroferriheme, where, through the use of stopped-flow spectrophotometry, it was demonstrated that deuteroferriheme monomer is the reactive species.

Since a peroxidatic intermediate is formed prior to, and subsequently involved in, both H_2O_2 decomposition and destructive oxidation, we were interested in studying the kinetics of its formation with particular curiosity as to the role of ferriheme dimerization on this process. A study of the formation of the peroxidatic intermediate which is obtained from deuteroferriheme and H_2O_2 has been reported by Portsmouth and Beal (1971) whose results are consistent with a Michaelis–Menten mechanism. Their kinetic parameters, however, were obtained on the assumption that the catalyst concentration was that of total ferriheme and, since their study was conducted at a single ferriheme concentration (5 μM), it gives no information on the question of whether dimerization equilibria play an important role. Preliminary studies by Prudhoe (1971), involving the effect of changes in total deuteroferriheme concentration on rate, suggest deuteroferriheme monomer to be the active form of the catalyst for the formation of the peroxidatic intermediate. In this paper, we wish to present results which substantiate this conclusion.

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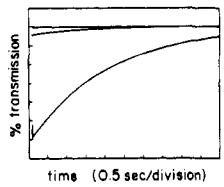

FIGURE 1: Stopped-flow trace. [T] = 4.64×10^{-6} M, $[H_2O_2]_0 = 1.87 \times 10^{-4}$ M, pH 8.5, μ = 0.1 M, t = 25°, Vertical scale corresponds to 6.25% transmission per division. Total increase in transmission from $t_0 \rightarrow t_\infty$ = 36.6%.

Experimental Section

Materials. All reagents were of Analar (Analytical Reagent) grade, and the water employed was triply distilled. Deuteroferriheme was prepared by a previously described procedure involving treatment of protoferriheme with resorcinol (Falk, 1964). Stock solutions containing 10^{-3}–10^{-4} M ferriheme were prepared by dissolving accurately weighed samples in 1–3 ml of 0.1 N NaOH and diluting with appropriate buffer solutions to the required volumes. In all cases, the capacity of buffer exceeded the concentration of alkali used to solubilize the iron-porphyrin complex. Samples of these stock solutions were then diluted with the same buffer to give solutions of appropriate concentration for stopped-flow studies (10^{-5}–10^{-6} M in deuteroferriheme with NaCl added to give an ionic strength, μ = 0.2 M). Hydrogen peroxide (unstabilized, 85% by weight) was obtained from Interox Chemicals, Ltd. A 0.4 M stock solution was prepared, chilled and used to prepare, by dilution, solutions of 10^{-5}–0.008 M H_2O_2 for kinetic studies. Fresh preparations were obtained each day that such experiments were to be undertaken. The concentration of the 0.4 M stock solution was monitored regularly *via* a conventional iodometric analysis (Brown and Jones, 1968a). Phosphate (KH_2PO_4–Na_2HPO_4) and carbonate ($NaHCO_3$–Na_2CO_3) buffers were employed for studies at pH 7.75 and 8.50, respectively.

Kinetic Studies. A Durrum-Gibson D-110 stopped-flow spectrophotometer was employed in conjunction with a Tektronix oscilloscope. Calibration consisted of filling the two syringes with respectively the appropriate deuteroferriheme solution and distilled water, and bringing them to temperature equilibrium following which the base line on the oscilloscope trace was set at 100% transmission for the stopped-flow mixture at the appropriate wavelength. For the heme–H_2O_2 study, the appropriate hydrogen peroxide solution was substituted for the water used in calibration. Since conversion of deuteroferriheme to the peroxidatic intermediate results in a decrease in optical density of the solution, the progress of the reaction was followed by observing increases in transmission with time for the stopped-flow mixture. The change in transmission at 384 nm was examined for studies of solutions containing 1×10^{-6} M initial deuteroferriheme at pH 7.75. All other experiments were carried out at λ 377 nm which is an isosbestic point for monomeric and dimeric deuteroferriheme (Robson, 1973). Two types of experiments were conducted.

In one series, the time scale was adjusted to allow observation of the reaction from the time of mixing, t_0, to the attainment of a "steady-state" transmission corresponding to t_∞. This "infinity value" of the transmission was determined by retriggering the signal to the oscilloscope and scanning repetitively until a reproducible horizontal trace was obtained. Such reproducible traces were possible provided the

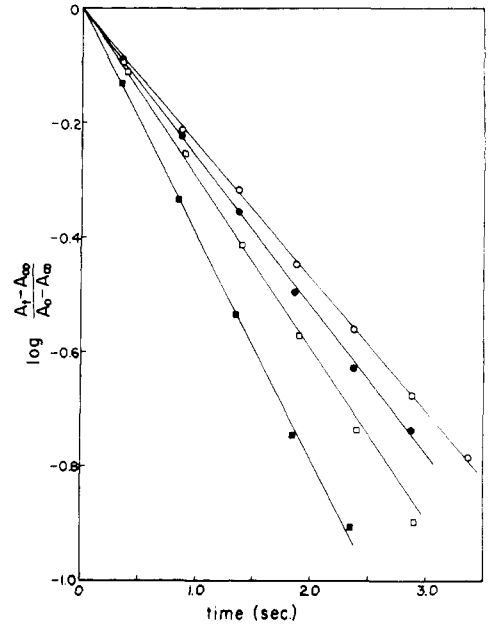

FIGURE 2: Plot of log $[(A_t - A_\infty)/(A_0 - A_\infty)]$ *vs.* time for formation of the peroxidatic intermediate from deuteroferriheme and H_2O_2, also showing variation of k_{obsd} with stoichiometric concentration of heme at pH 8.5, μ = 0.1 M, t = 25° (Table II). $[H_2O_2]_0 = 1.87 \times 10^{-4}$ M. [T] $\times 10^{-6}$ (M): (○) 4.64; (●) 3.71; (□) 2.98; (■) 1.48.

H_2O_2 concentration was not sufficiently high to cause, within the selected time scale, significant destructive oxidation of heme, a reaction which results in the continued increase in transmission of the solution beyond that corresponding to formation of the peroxidatic intermediate (Jones *et al.*, 1973b). In a second series of experiments, "initial rate" studies were made in which only a small percentage of the reaction was followed immediately after mixing of the solutions. For both types of study, repeated mixings were made to ensure reproducible oscilloscopic traces following which Polaroid photographs were obtained of the selected stored traces.

Results and Discussion

A typical trace of the change in transmission accompanying the formation of the peroxidatic intermediate from deuteroferriheme and H_2O_2 is shown in Figure 1. From calculated absorbances at given time intervals linear plots of log $(A_t - A_\infty)$ *vs.* time are obtained generally over 2–3 half-lives (Figure 2). Thus, under conditions where the initial H_2O_2 concentration, $[H_2O_2]_0$, exceeds the initial stoichiometric deuteroferriheme by factors of about 10–500, the reaction is first order in deuteroferriheme. At a given initial concentration of heme, the variation of the observed first-order rate constant, k_{obsd}, with $[H_2O_2]_0$ is that predicted for a Michaelis–Menten pathway showing a linear dependence on $[H_2O_2]_0$ at relatively low substrate concentrations and approaching saturation at higher values (Figure 3). Accurate measurements of k_{obsd} near its limiting value are restricted, however, by destructive oxidation of the heme. Attempts were made to estimate k_{obsd} values at relatively high initial concentrations of H_2O_2, however, from initial rate measurements and by extrapolation of data obtained from the correlation of such initial rate data with values of k_{obsd} determined at sufficiently low $[H_2O_2]_0$, that the heme "destruction" reaction was negligible.

In discussing this approach, the stoichiometric mechanism depicted in (1) is assumed, following Portsmouth and

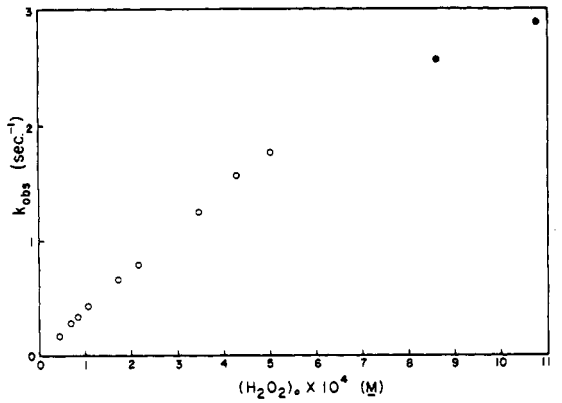

FIGURE 3: Variation of pseudo-first-order rate constant, k_{obsd}, with $[H_2O_2]_0$: [T] = 3.85 × 10^{-6} M, pH 7.75, μ = 0.1 M, t = 25°. (○) Values obtained from slope of log $[(A_t - A_\infty)/(A_0 - A_\infty)]$ *vs.* time data; (●) values obtained from $(\Delta A/\Delta t)_i$ data.

Beal (1971), and a modification of the terminology of Jones *et al.* (1973b) is employed where [T] denotes initial stoichiometric ferriheme concentration including monomeric and dimeric species, [F], free total ferriheme at time t, [I], the concentration of the Michaelis–Menten complex, and

$$F + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} I' \qquad (1)$$

[I′], that of the peroxidatic intermediate. Thus, [T] = [F] + [I] + [I′]. Letting A_t denote the absorbancy at unit path length for the solution at time t, and ϵ_i the molar extinction coefficient for component i, acknowledging $\epsilon_{H_2O_2} = 0$ at 377 nm

$$A_t = \epsilon_F[F] + \epsilon_I[I] + \epsilon_{I'}[I'] \qquad (2)$$

$$A_t = \epsilon_F([T] - [I] - [I']) + \epsilon_I[I] + \epsilon_{I'}[I'] \qquad (3)$$

$$A_t = \epsilon_F[T] - \epsilon_F[I] + \epsilon_I[I] + (\epsilon_{I'} - \epsilon_F)[I'] \qquad (4)$$

Assuming that a steady-state concentration of I is formed very rapidly, the rate of change of optical density is related to the reaction velocity, $V \equiv d[I']/dt$, as follows

$$dA_t/dt = (\epsilon_{I'} - \epsilon_F)d[I']/dt \qquad (5)$$

With the time scale adjusted for rapid scan of the first few per cent reaction, the increase in transmission is essentially linear with time (Figure 4). Thus $\Delta A/\Delta t$ over a short

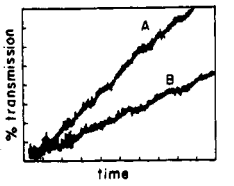

FIGURE 4: Initial rate stopped-flow traces. [T] = 4.64 × 10^{-6} M, $[H_2O_2]_0$ = 7.46 × 10^{-4} M, pH 8.5, μ = 0.1 M, t = 25°. Vertical scale corresponds to 1.25% transmission per division. Time scale: A, 20 msec/division; B, 10 msec/division.

time interval at the beginning of reaction, $(\Delta A/\Delta t)_i$, is a reasonable measure of dA/dt and, therefore, is a parameter proportional to the initial rate, V_i. In solutions containing H_2O_2 at relative concentrations sufficiently high to give pseudo-first-order kinetics for the formation of I′, but sufficiently low that destructive oxidation of the heme is negligible relative to I′ formation, *i.e.*, where reliable transmission values at t_∞ can be obtained to allow direct determination of k_{obsd} from slopes of lines as depicted in Figure 2, a linear relation between $(\Delta A/\Delta t)_i$ and k_{obsd} is found for a given initial value of [T] at a given pH and temperature. Thus, for [T] = 3.85 × 10^{-6} M, pH 7.75, μ = 0.1 M, and t = 25°, correlation of $(\Delta A_t/\Delta t)_i$ with k_{obsd} for nine points (including the origin) gave

$$-(\Delta A_t/\Delta t)_i = 0.167k_{obsd} \quad (r = 0.999) \qquad (6)$$

In solutions in which the ratio $[H_2O_2]_0/[T]$ is about 200–400, destructive oxidation occurs at a rate which, though slow compared to the formation of I′, is significant enough to prevent attainment of reliable t_∞ transmission values. If it is presumed that in the early stages of such reactions, this "destruction" of heme has not proceeded to a significant extent, then $(\Delta A/\Delta t)_i$ values obtained for such solutions should reflect the same proportionality to k_{obsd} and estimation of k_{obsd} values should be possible by extrapolation of the $(\Delta A/\Delta t)_i$, k_{obsd} data to the aappropriate measured $(\Delta A/\Delta t)_i$ value. Acknowledging limitations in the extent to which such extrapolation is justified, we find where extrapolation is carried to obtain k_{obsd} values which are larger by no more than a factor of about 2 than those conveniently measured from log $(A_t - A_\infty)$ *vs.* time data, such k_{obsd} values are consistent with the established pattern of Michaelis–Menten kinetics for the formation of the peroxidatic intermediate. The k_{obsd} values so obtained in a typical

TABLE I: Kinetic Data for the Formation of the Peroxidatic Intermediate from the Reaction of Deuteroferriheme with H_2O_2.[a]

$[H_2O_2]_0 \times 10^3$ (M)	$1/[H_2O_2]_0 \times 10^{-3}$ (M^{-1})	k_{obsd} (sec^{-1})	$1/k_{obsd}$ (sec)	k_m (sec^{-1})	$1/k_m$ (sec)
0.0431	23.2	0.173	5.78	0.836	1.20
0.0690	14.5	0.289	3.46	1.40	0.716
0.0860	11.6	0.333	3.00	1.61	0.622
0.108	9.26	0.430	2.33	2.08	0.481
0.172	5.81	0.662	1.51	3.20	0.317
0.216	4.63	0.781	1.28	3.77	0.265
0.345	2.90	1.25	0.801	6.03	0.166
0.431	2.32	1.57	0.639	7.57	0.132
0.502	1.99	1.78	0.562	8.59	0.116
0.860	1.16	(2.57)	(0.389)	(12.4)	(0.0805)
1.08	0.926	(2.89)	(0.346)	(14.0)	(0.0716)

[a] [T] = 3.85 × 10^{-6} M, pH 7.75, μ = 0.1 M, t = 25°.

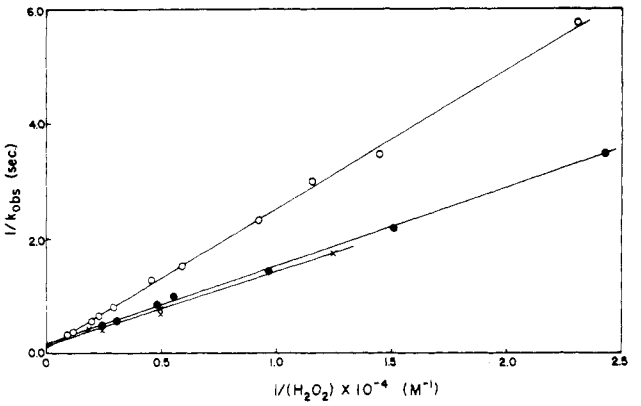

FIGURE 5: Reciprocal (Lineweaver-Burk) plot of $1/k_{obsd}$ *vs.* $1/[H_2O_2]_0$; pH 7.75, μ = 0.1 M, t = 25°: (○) [T] = 3.85×10^{-6} M, (●) [T] = 1.54×10^{-6} M, (×) [T] = 1.0×10^{-6} M. Lines represent linear regression analysis for each series.

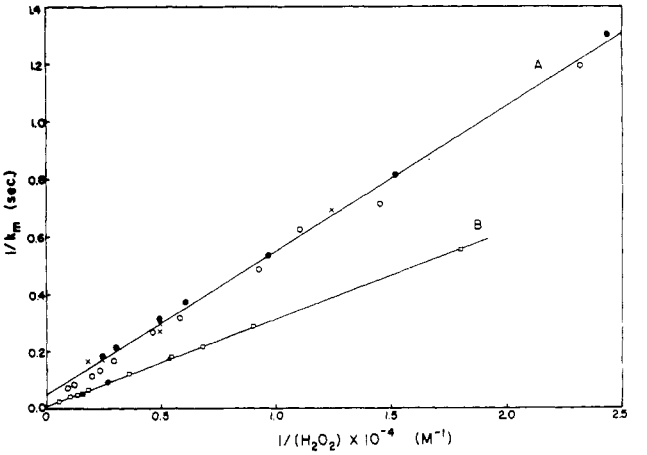

FIGURE 6: Reciprocal plot of $1/k_m$ *vs.* $1/[H_2O_2]_0$. (A) pH 7.75, μ = 0.1 M, t = 25°. Points correspond to values of [T] in Figure 5. Line represents linear regression analysis of composite data for the three designated values of [T]. (B) pH 8.5, μ = 0.1 M, t = 25°; (□) [T] = 3.71×10^{-6} M. Line represents linear regression analysis for 10 points; ■, +, ▲ correspond to $1/k_m$ (av) *vs.* $1/[H_2O_2]_0$ where $1/k_m$ (av) denotes the reciprocal of the average k_m value for four initial deuteroferriheme concentrations at each of the three $[H_2O_2]_0$ values in Table II.

study are parenthesized in Table I, and characteristically noted in Figure 3.

The significance of k_{obsd} is shown from examination of the consequences of (1). Setting $V = k_3[I]$ and assuming a steady-state concentration of [I]

$$V \equiv \frac{d[I']}{dt} = \frac{k_1 k_3 [F][H_2O_2]}{k_2 + k_3} \quad (7)$$

It follows that

$$V = \frac{k_3([T] - [I'])[H_2O_2]}{K_M + [H_2O_2]} \quad (8)$$

and

$$\frac{-\ln([T] - [I'])}{[T]} = \frac{k_3[H_2O_2]}{K_M + [H_2O_2]} t \quad (9)$$

If the optical density at t_∞ is now assumed to represent that for the solution containing a steady-state concentration of peroxidatic intermediate, $[I']_{ss}$, the extinction at unit path length for the solution at various stages can be described as follows. For the absorbance of the solution in the initial steady state of the Michaelis-Menten complex, $[I]_{ss}$

$$A_0 = \epsilon_F'[T] \quad (10)$$

where ϵ_F' denotes extinction of free ferriheme plus that of I. After time t,

$$A_t = \epsilon_F'([F] + [I]_{ss}) + \epsilon_{I'}[I'] \quad (11)$$

and upon attainment of the steady-state concentration of I'

$$A_\infty = \epsilon_F'([F]_{ss} + [I]_{ss}) + \epsilon_{I'}[I]_{ss} \quad (12)$$

Substituting ([T] − [I']) for ([F] + $[I]_{ss}$), it follows that

$$\frac{A_t - A_\infty}{A_0 - A_\infty} = \frac{[I']_{ss} - [I']}{[I']_{ss}} \quad (13)$$

Thus, k_{obsd}, which is determined from plots of log ($A_t - A_\infty$) *vs.* time, can be equated to $k_3[H_2O_2]_0/(K_M + [H_2O_2]_0)$ for the case where $[I']_{ss}$ = [T], *i.e.*, where the transmission infinity value corresponds to conversion of all heme to the peroxidatic intermediate.

The question of the role of monomer-dimer equilibria in deuteroferriheme on the kinetics of formation of the peroxidatic intermediate is examined through studies in carbonate buffer (pH 8.5) and phosphate buffer (pH 7.75). Table II and Figure 2 show the effect on k_{obsd} of changes in the stoichiometric concentration of deuteroferriheme at given initial concentrations of H_2O_2 at pH 8.5 and 25°. If the active form of the catalyst is accurately represented by total heme, k_{obsd} should be independent of [T] at a given value of $[H_2O_2]_0$. It is clear, however, that k_{obsd} increases with decreasing [T], and since the dissociation of ferriheme dimer increases with increasing dilution (see below) the data are suggestive that monomeric ferriheme indeed may be the active catalytic species.

From spectrophotometric studies, Prudhoe (1971) has determined the fraction, α, of total deuteroferriheme present as monomer as a function of stoichiometric ferriheme, temperature, and pH in various buffer systems. Such determinations employ the relation $K = K_{obsd}[H^+] = 3.4 \times 10^{-2}$ for the equilibria $2M \rightleftharpoons D + H^+$ at 25 ° and μ = 0.1 where M and D denote monomer and dimer, respectively, K represents the equilibrium constant for dimerization, and K_{obsd}, the ratio $[D]/[M]^2$ at a given acidity. Since $\alpha = [M]/[T]$ and $[D] = \frac{1}{2}([T] - [M])$ it follows that

$$K_{obsd} = \frac{1 - \alpha}{2\alpha^2[T]} \quad (14)$$

Thus, α can be calculated readily for given values of [T] and pH through a quadratic equation with only positive values having physical significance. Values of α so calculat-

TABLE II: Dependence of k_{obsd} and k_m on Stoichiometric Concentration of Deuteroferriheme at pH = 8.5, t = 25°.[a]

		$[H_2O_2]_0 \times 10^4$ (M)					
		1.87		3.73		7.54	
$[T] \times 10^6$ (M)	α	k_{obsd}	k_m	k_{obsd}	k_m	k_{obsd}	k_m
1.48	0.162	0.921	5.69	1.68	10.4	2.84	17.5
2.97	0.117	0.676	5.78	1.34	11.5	2.46	21.0
3.71	0.106	0.595	5.61	1.11	10.5	2.22	20.9
4.64	0.095	0.537	5.65	1.07	11.3	2.16	22.7

[a] k_{obsd} and k_m in sec^{-1}.

TABLE III: Kinetic Parameters Derived from Reciprocal Plot Data (Figures 5 and 6).

pH	$[T] \times 10^6$ (M)	$(1/k_3)$ (sec)	$(K_M/k_3 \times 10^4)$ (M sec)	r^a	No. of points
	(a) $1/k_{obsd}$ *vs.* $1/[H_2O_2]$ (data for pH 7.75 in Figure 5)				
7.75	1.00	0.114	1.31	0.995	5
7.75	1.54	0.163	1.36	0.999	7
7.75	3.85	0.101	2.42	0.999	11
8.50	3.71	0.0870	2.88	0.999	10
	(b) $1/k_m$ *vs.* $1/[H_2O_2]$ (data in Figure 6)				
7.75	1.00	0.0483	0.554	0.995	5
7.75	1.54	0.0608	0.505	0.999	7
7.75	3.85	0.0214	0.502	0.999	11
7.75	Composite	0.0245	0.507	0.996	23
8.50	3.71	0.0093	0.306	0.999	10

[a] r denotes correlation coefficient.

ed for four stoichiometric ferriheme concentrations are listed in Table II, along with values of another rate parameter, k_m, obtained by division of k_{obsd} by α for each ferriheme solution. The reproducibility of k_m values for four different deuteroferriheme solutions at each of three initial peroxide concentrations support the contention that monomeric heme is the catalytic agent for formation of the peroxidatic intermediate, and that when pseudo-first-order kinetics prevail, the expression $V = k_{obsd}[T]$ is appropriately replaced by $V = k_m[M]_0$ where k_m denotes the pseudo-first-order rate constant for the conversion of monomer to the peroxidatic intermediate, and $[M]_0$ is the stoichiometric concentration of monomeric heme. The stoichiometric mechanism for formation of I′, is therefore rewritten[1]

$$M + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} I \xrightarrow{k_3} I' \qquad (15)$$

and the velocity

$$V = \frac{k_3[M_0][H_2O_2]}{K_M + [H_2O_2]} \qquad (16)$$

Further support of this conclusion is reflected in a comparison of reciprocal plot data of Figure 5 with that of Figure 6 for studies in phosphate buffer at pH 7.75. Linear plots of $1/k_{obsd}$ *vs* $1/[H_2O_2]_0$ display a dependence on initial stoichiometric concentration of deuteroferriheme. Utilizing values of α at the respective values of [T] at pH 7.75 and 25° permits conversion of k_{obsd} to k_m and the corresponding plot of $1/k_m$ *vs.* $1/[H_2O_2]_0$ shows, within experimental error, independence of [T].[2]

Table III lists the slopes and intercepts obtained from a linear-regression analysis of data depicted in Figure 5, and corresponding data, which include studies at pH 8.5, shown in Figure 6. It would seem that the results strongly support the proposition that monomeric deuteroferriheme is the active form of this catalyst for the formation of the peroxidatic intermediate.

Kinetic parameters derived (Table IIIb and Figure 6) from composite analysis of data at pH 7.75 give $k_3 = 40.8$ sec^{-1} and $K_M = 2.07 \times 10^{-3}$ M, and at pH 8.5, $k_3 = 108$ sec^{-1} and $K_M = 3.29 \times 10^{-3}$ M. The increase in rate of formation of I′ with increasing basicity is not unexpected in view of the pH dependence of catalytic activity for peroxide decomposition. More extensive study of the pH, as well as temperature, dependence of this reaction is in progress.

Acknowledgment

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[1] Regarding the question of "complete" conversion of heme to I′, at this time, the assumptions of Portsmouth and Beal (1971) are followed as reflected in (1) and consequently in (15). Attainment of monomer-dimer equilibria is rapid compared to the formation of I′ (Jones *et al.*, 1974). For a given kinetic study, the integrated plot gives greatest weight to the early stages of reaction where relatively small changes in α occur with diminishing concentration of heme. This fact, plus the limited sensitivity of such plots, could readily account for their apparent linearity as [F] decreases with accompanying changes in the monomer–dimer ratio.

[2] Since previous studies (Jones *et al.*, 1973a) have shown that, under certain conditions a specific effect of the Na_2HPO–KH_2PO_4 buffer system can lead to relatively high catalytic activities in the deuteroferriheme catalyzed decomposition of H_2O_2, values of α utilized for such buffers were obtained by interpolation of the data of Prudhoe (1971) rather than by calculation from (14).

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Characterization of the Surface Protein Components in Adipocyte Plasma Membranes†

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ABSTRACT: Plasma membranes isolated from rat adipocytes were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gels stained with Coomassie Blue indicated the presence of more than 20 protein components with an apparent molecular weight range of 178,000–15,000. Staining with Schiff reagent further indicated the presence of two major glycoproteins of molecular weight 100,000 and 81,000 and several minor components. The lactoperoxidase-catalyzed iodination technique was used to determine which membrane components were located on the surface of the intact cell and to monitor changes in exposure of surface components in the presence of hormones and concanavalin A. Analysis of polyacrylamide gels of the membranes isolated from labeled cells indicated that four membrane components were labeled and that two of these components corresponded in electrophoretic mobility to the major glycoprotein bands. The pattern of incorporation of iodine was shown to depend on the nature of the buffer in which the adipocytes were suspended. Iodination was also carried out on the particulate fraction derived from homogenized cells, on the isolated plasma membrane, and on the lipid extracted plasma membrane. The analysis of the labeling patterns suggested that several membrane components were buried and only became available for iodination upon membrane perturbation. Radioactive label was also shown to be associated with a low molecular weight membrane component which could possibly be lipid or glycolipid. Iodination of intact adipocytes appeared to have no effect on the biological integrity of these cells as assayed by the conversion of glucose to carbon dioxide. Incubation of isolated adipocytes with glucagon, insulin, or concanavalin A had significant effects on the quantitative distribution of radioactivity incorporated into the membrane proteins.

The relationship of cell surface topology to the functional properties of animal cells has been extensively investigated in many systems (Mehrishi, 1972). A necessary aspect of this research is the characterization of these complex assemblies. The spatial arrangement of the components of surface membranes has been studied using a variety of chemical and enzymatic procedures (Berg, 1969; Steck *et al.*, 1971; Bretscher, 1971a; Rifkin *et al.*, 1972). The lactoperoxidase-catalyzed iodination of tyrosine in various proteins (Marchalonis, 1969) has been used to selectively label the protein components on the external surface of plasma membranes in various cell systems. The technique has been successfully applied to erythrocytes (Phillips and Morrison, 1970, 1971a,b; Hubbard and Cohn, 1972), lymphocytes (Marchalonis *et al.*, 1971), platelets (Phillips, 1972; Nachman *et al.*, 1973), mouse fibroblasts (L cells) (Poduslo *et al.*, 1972), *Micrococcus lysodeikticus* (Salton *et al.*, 1972), and to microsomal (Kreibich *et al.*, 1974) and mitochondrial membranes (Huber and Morrison, 1973) from rat liver.

The regulation of adipose cell metabolism has been extensively examined (Jeanrenaud and Hepp, 1970; Avruch *et al.*, 1972). The plasma membrane obtained from these cells has been shown to be the site of action of several hormones and to contain the hormone responsive adenylate cyclase system (Cuatrecasas, 1969, 1972; Birnbaumer *et al.*, 1969; Birnbaumer and Rodbell, 1969; Laudat *et al.*, 1972).

A study of the surface topology of the adipocyte plasma membrane should contribute significantly to the understanding of its regulatory functions. This report presents the results of a characterization of the protein and glycoprotein components of these membranes. Lactoperoxidase-catalyzed iodinations of intact adipose cells and plasma membrane preparations have been used to reveal the disposition of components on the membrane surface. The effect of hormones and the plant lectin concanavalin A on the exposure of surface components has also been investigated. While this manuscript was in preparation, a paper by Czech and Lynn (1973b) appeared in which is reported the iodination of intact adipocytes and isolated plasma membranes. The relevant portions of our report generally support the major conclusions of the aforementioned paper.

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