REVERSIBLE OXYGENATION OF PROTOHEME-IMIDAZOLE COMPLEX IN AQUEOUS SOLUTION (1,2)

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SUMMARY

Solutions of protohemin in aqueous buffer containing imidazole were reduced and exposed to carbon monoxide forming the carbon monoxide-imidazole complex similar to that in carboxyhemoglobin. This complex is stable for long periods in the presence of low pressures of oxygen and thus the standard flash photolysis methods can be used to determine rates of combination of the heme-imidazole complex with oxygen. Combination rates for both carbon monoxide and oxygen are faster than any on rates for hemoglobin and oxygen dissociation rates are also faster. But the equilibrium constant for binding of this isolated site is larger than that for hemoglobin.

The reversible oxygen binding in heme proteins and the factors influencing the rates and equilibria in these processes have been the subject of intense investigation for almost a century (3). In these investigations the desire to study oxygenation of the isolated heme or heme-imidazole complex under physiological conditions is expressed but such a study has not been accomplished until now. Because the isolated heme-imidazole complex is immediately oxidized to hemin when exposed to oxygen in aqueous solutions such a study has been considered out of reach (3b).

We recently reported equilibria for reversible oxygenation of simple heme compounds in organic solvents (4) which indicated that such compounds were much more stable towards oxidation than had been thought (4a,b). However, simple hemes oxidize faster in aqueous solution (3,4) making the usual equilibria difficult to study in aqueous environment. This difficulty has now been overcome by taking advantage of the fact (5) that dipyridine hemochromes or heme-base-CO complexes are more stable to oxidation than are free hemes.

When simple hemes are placed in a dilute aqueous imidazole solution and treated with carbon monoxide, the resulting visible spectrum indicates formation of the monomeric imidazole-heme-CO complex which is stable toward

oxidation at low pressures of oxygen for up to one hour at room temperature. This solution can be used in the flash photolysis kinetic study similar to that described by Gibson (6).

We report a method by which heme oxygenation can be studied in any environment and show that isolated protoheme-imidazole complex is kinetically similar to some of the steps in hemoglobin oxygenation.

METHODS

Solutions of about 3×10^{-6} M protohemin (Sigma) in aqueous phosphate buffer at pH = 7.3, containing 10^{-3} to 10^{-1} M imidazole (Aldrich) and 2% cetyltrimethylammonium bromide (Sigma) were carefully degassed in a special cell-tonometer. This cell consisted of a clear pyrex square cell 1 × 1 cm by 5 cm, attached to an apparatus having a total volume of about 100 ml, which was equipped with a three-way stopcock. A 1 ml space above the stopcock was terminated by a septum fitting where gases could be quantitatively admitted with gas tight syringes. After dissolved oxygen was removed the hemin was reduced with a stoichiometrical amount of sodium dithionite and a know quantity of carbon monoxide introduced. The visible spectrum of this solution was essentially identical to that of a similar concentration of carboxymyoglobin.

The cell was placed in a flash photolysis apparatus similar to that described by Gibson (6). Solutions were first flashed at various concentrations of carbon monoxide, then at one concentration of carbon monoxide and various concentrations of oxygen by the methods outlined by Gibson *et al.* (6). The relaxation curves were cleanly first order in absorption (7,8). Incomplete reduction in some experiments did not affect the kinetics. The presence of some hemin eliminates the possibility that we are studying oxidation rather than oxygenation, because re-reduction cannot be occurring. The observed rates are shown in Table 1.

Solutions which contained 0.01 M or more of imidazole were stable in the indicated carbon monoxide-oxygen atmospheres for more than half an hour.

Table 1

RATES OF ABSORPTION CHANGES UPON FLASHING SOLUTIONS OF PROTOHEME IN AQUEOUS PHOSPHATE BUFFER pH = 7.3, CONTAINING IMIDAZOLE AND 2% CTAB AT 22°.[a]

Conc. Imidazole M	Pressures (Torr) CO	O_2	Conc. ($M \times 10^5$) CO	O_2	$1'_{obs}$ (sec^{-1})	k'_{obs} (sec^{-1})	R (sec^{-1})
0.001	12.5		1.6		1510		
0.01	12.5		1.6		410		
	29		3.7		890		
	29	25	3.7	4.5			76.96
	29	66.5	3.7	12			41.22
	29	150	3.7	27			18.06
0.02	21		2.8		200		
	54		7.0		490		
	54	16.6	7.0	3.0			103.9
	54	58	7.0	10		4500	43.99
	54	99.7	7.0	18		7200	28.31
	54	166	7.0	30			21

[a]Solubilities of CO and O_2 were taken as those in water. See reference 3c.

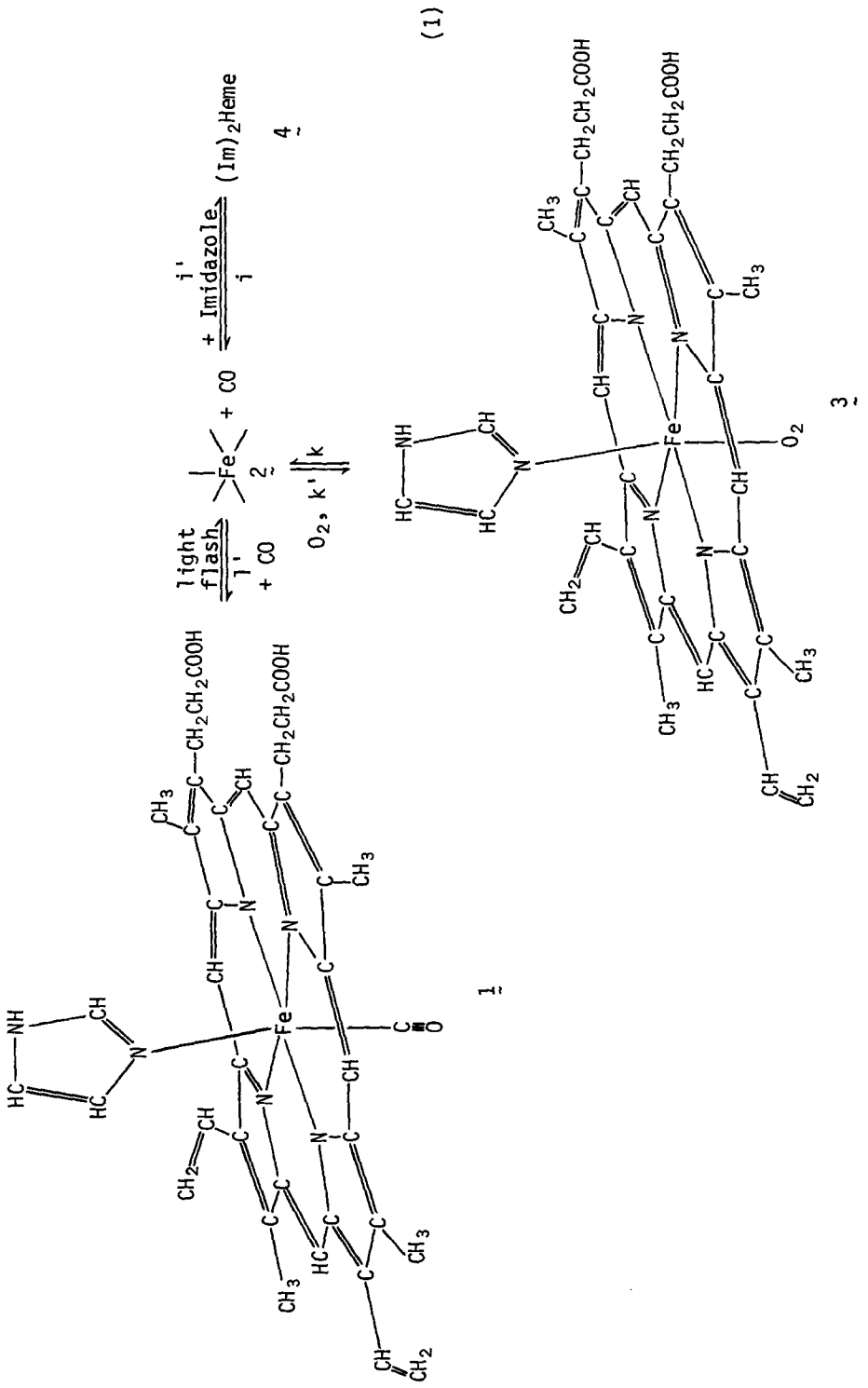
However, at 0.001 M imidazole, the addition of oxygen caused rather rapid oxidation which was accelerated during each flash.

DISCUSSION AND CONCLUSIONS

The reaction studied is shown in equation 1. Except for the problem of conversion to $\underset{\sim}{4}$ discussed above, the compound $\underset{\sim}{1}$ represents the active site of carboxyhemoglobin obtained by replacing the globin attached to the proximal imidazole by a hydrogen atom. At a steady small concentration of $\underset{\sim}{2}$ the return to $\underset{\sim}{1}$ after flashing is given by

$$\frac{d\underset{\sim}{1}}{dt} = \frac{i\underset{\sim}{4} + k\underset{\sim}{3}}{1 + \frac{k'O_2}{1'CO} + \frac{i'Im}{1'CO}} \qquad (2)$$

Under conditions where the concentration of $\underset{\sim}{4}$ is small and i'Im << d'CO this equation becomes equation 3, that employed in the determination of k, k' and I' in myoglobin by Gibson (3c,6).


light flash
1'
+ CO
O2, k'
k
2
+ CO
i'
+ Imidazole
i
(Im)2Heme
4
(1)
1
3
Fe
O2
CO

$$\frac{1}{R} = \frac{1}{k} + \frac{k'O_2}{kl'CO} \tag{3}$$

where

$$\frac{d\underset{\sim}{1}}{dt} = R(\underset{\sim}{3}) \tag{4}$$

Rate constants derived from equation 3 and Table 1 are shown in Table 2. Although we do not quite achieve these conditions for the determination of l' or k' because l' changes by a factor of 4 with an imidazole concentration change from 10^{-3} to 10^{-2} M, the slow decay in the presence of oxygen is almost independent of imidazole concentration near 10^{-2} M. Thus our k' and l' are probably somewhat inaccurate whereas the k value is probably accurate because, in addition to the independence of the slow rate on imidazole concentrations, it is also independent of CO and O_2 solubilities.

The method is therefore somewhat limited by the requirement of low concentrations of imidazole to keep the kinetics simple and higher concentrations of imidazole to prevent oxidation of the heme (9).

Although the oxygen on rates change with concentration, indicating some competition between imidazole and oxygen (and possibly some reaction of four

Table 2

RATE CONSTANTS FOR REACTION OF PROTOHEME-IMIDAZOLE COMPLEX WITH O_2 AND CO

Conc. Imidazole M	l' (M^{-1} sec^{-1})[a]	k' (M^{-1} sec^{-1})	k (sec^{-1})	k'/k M^{-1}
0.001	9.3×10^7			
0.01	2.5×10^7	5.5×10^7[a]	310[b]	1.8×10^5
0.02	7.1×10^6	4.1×10^7[a] (3.8×10^7)[c]	420[b]	1×10^5

[a]Observed as a first-order decay. $l' = l'_{obs}/[CO]$, $k' = k'_{obs}/[O_2]$. [b]Obtained from the intercept of equation 3. [c]Obtained from the slope of equation 3.

coordinate heme), the rates at low imidazole concentration are probably near those for reaction of five coordinate iron with oxygen. The results at 0.01 M imidazole, $l' = 2 \times 10^7\ M^{-1}\ sec^{-1}$, $k' \cong 6 \times 10^7\ M^{-1}\ sec^{-1}$, and $k \cong 300$, are similar to one of the steps in hemoglobin oxygenation. The equilibrium constant $K \cong 2 \times 10^5\ M^{-1}$ is somewhat smaller than the K for myoglobin (3c) but larger than that for hemoglobin.

A significant aspect of this communication is the recognition that carbon monoxide protects simple heme compounds against oxidation even in aqueous solution and that (under some conditions) during the time between flashing off carbon monoxide and the occurrence of the processes shown in equation 1, oxidation does not occur. This makes it now feasible to study the oxygenation of protoheme and other hemes in practically any environment. A large variety of studies of oxygenation of protoheme in the presence of peptides, proteins, bases, amino acids, detergents, organic solvents, etc. can be immediately envisioned.

When these possibilities are combined with the observation (8) that the slower oxidation

$$\text{Im-Heme}^{II} + O_2 \xrightarrow{k_{oxid}} \text{Heme}^{III} \qquad (5)$$

can be followed by stopped flow methods (10) under the conditions used here, then it becomes clear that this approach makes possible the simultaneous studies of oxygenation and oxidation of heme compounds in various environments. The factors which determine whether a heme protein will oxygenate or oxidize can therefore be determined.

We are now using these methods to study both protoheme and synthetic model heme compounds.

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REFERENCES

1. This research was supported by the National Institutes of Health, Grant HL 13581.
2. Presented at the Meeting of the American Chemical Society, Atlantic City, New Jersey, Sept. 10, 1974.
3. a. Antonini, E. and M. Brunori, "Hemoglobin and Myoglobin in Their Reactions with Ligands," North Holland Publishing Co., Amsterdam, 1971, p. 153. b. p. 348. c. p. 221.
4. a. Chang, C. K. and T. G. Traylor, J. Amer. Chem. Soc., 95, 8477 (1973). b. Chang, C. K. and T. G. Traylor, J. Amer. Chem. Soc., 95, 5810 (1974). c. Brinigar, W. S. and C. K. Chang, J. Amer. Chem. Soc., 96, 5595 (1974). d. Brinigar, W. S., C. K. Chang, J. Geibel, and T. G. Traylor, J. Amer. Chem. Soc., 96, 5597 (1974). e. Chang, C. K. and T. G. Traylor, Proc. Nat. Acad. Sci. USA, in press.
5. Wang, J. W., "Oxygenases," O. Hayaishi, Ed., Academic Press, New York, 1962, p. 508. Although carbonmonoxy pyridine heme was said to be stable toward oxygen in benzene, it was said to oxidize rapidly in water (p. 503) when exposed to air.
6. Gibson, Q. H., J. Physiol., 134, 112 (1956).
7. Rate constants for sperm whale myoglobin, determined with this apparatus, were in agreement with previous studies (3c,6).
8. The absorbance changes during flashing correspond to the changes from carbonmonoxy to deoxy to oxy to carbonmonoxy derivatives previously determined for hemes (4). An intermediate of a related heme has also been identified in aqueous solution by flash spectroscopy (4e).
9. The kinetic complexities inherent in the studies of mixtures of bases of iron complexes and oxygen are completely overcome by covalently attaching the heme to the base as we have done in our heme protein model compounds (4a,b). The kinetic behavior of some of these in solution is exactly that of myoglobin except for the somewhat different values of rate constants.
10. Kao, H. W. and J. W. Wang, Biochem., 4, 342 (1965).