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LASER PHOTOLYSIS INVESTIGATIONS OF LIGAND BINDING WITH MODELS OF THE ACTIVE SITE OF RESPIRATORY HEMOPROTEINS: KINETIC AND THERMODYNAMIC ASPECTS

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During the past 15 years, laser photolysis has been the method of choice for probing the complex reaction kinetics of respiratory proteins. In an attempt to determine the structural parameters which govern their reactivity, synthetic heme model compounds capable of simulating particular aspects of the reactivity of the active site of hemoproteins have been successively proposed. Laser photolysis of heme compounds merely induces a reversible photodissociation of one ligand at a time. This is equivalent to performing a fast concentration jump "in situ" and provides a powerful, fast and "clean" chemical relaxation technique. To gather association and dissociation rate constants of various ligands (O_2, CO, nitrogenous bases) special methods have been developed or adapted. The problem of comparing and classifying a large number of collected data has been greatly simplified by introducing a Linear Free Energy Relationships formalism. In the first part of this paper, some of the methods and concepts which have emerged from several years of investigations of heme proteins and heme models and which are of a sufficient generality to be useful in other fields of chemical kinetics are reviewed. In the second part of the paper we present the application of the preceding methods to a kinetic study of a series of heme models which were specifically designed to investigate the important problem of H-bonding as a stabilizing factor of the oxygenated heme model and hemoprotein complexes.

KEY WORDS: Laser photolysis, ligand binding, picket fence porphyrins, H-bonding, LFER, information theory.

ABBREVIATIONS: LFER: Linear Free Energy Relationship; BHP: Basket-Handles-Porphyrin; 1,2-Me_2Im, 1,2-dimethylimidazole.

INTRODUCTION

Kinetic studies of the reaction of oxygen and other ligands with iron(II) porphyrins are directly relevant to the understanding of the mechanism by which hemoglobin

and myoglobin respectively transport and store oxygen. During the past 15 years, flash and laser photolysis have been the methods of choice for probing the complex reaction kinetics of respiratory proteins. Despite the wealth of information collected, many questions still escape a direct investigation simply because the structural parameters of the proteins cannot be adjusted externally. The accumulation of rate constants values is, in itself, of limited interest. What really matters is the nature of those structural parameters which ultimately govern the reactivity. The need for a stepwise modelization has been at the origin of the design of heme model compounds capable of simulating particular aspects of the reactivity of the active site of hemoproteins. Kinetic investigations then permit to quantify, in terms of activation and binding free energy, the effect of a given structural parameter upon the reaction path.

There are of course a few minimum requirements that any realistic model must satisfy in order to reproduce the most pertinent features of the active site of hemoproteins. The latter consists essentially of a heme group (iron(II) protoporphyrin IX) embedded in the protein "pocket" and chelated by a histidine residue (the "proximal" histidine F8) (see Figure 1). On the other side of the heme group, the iron atom may bind small ligands such as oxygen or carbon monoxide reversibly. These ligands may be submitted to polar, hydrophobic or steric interactions with other residues of the protein (e.g. the "distal" histidine E7). The liganded heme is planar

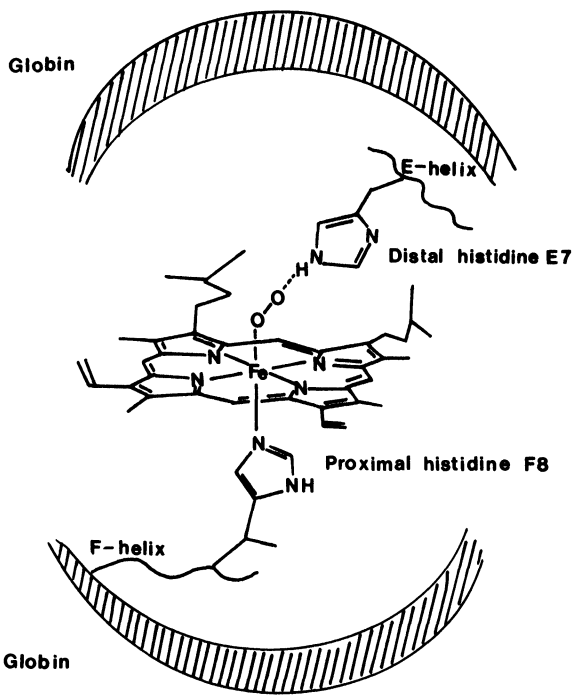


Figure 1 The oxygen binding site in hemoglobin or myoglobin. Note the bent geometry of the Fe-O-O bond and the possibility of H-bond with the distal histidine E7.

and the electronic configuration is low spin ($S = 0$). In the unliganded state, the iron atom is high spin ($S = 2$) and lies about 0.5 A out of the heme plane in the direction of the proximal histidine. Therefore heme model compounds must include a priori: (i) the pentacoordination of the heme, with possibilities for introducing constraints in the Fe-proximal base bond; (ii) a variable chemical environment of the binding site (e.g. to test distal polarity effects, H-bonding, etc.) and (iii) a possibility of adjusting the steric interference of the small ligand with the "heme pocket." The various molecular structures which have been successively proposed are described in review articles.[1,2] Only those compounds which exhibit remarkable properties in direct relation with the scope of the present work will be referenced at the most appropriate place in the paper.

Several important features of oxygen binding with hemoproteins have been suggested or supported by the study of heme-model compounds. Pauling first proposed the bent geometry of the iron-dioxygen bond shown in Figure 1.[3] Experimental support came first from the X-ray analysis of an oxygenated model compound, the "picket-fence porphyrin."[4] This geometry has since been indeed observed in oxymyoglobin[5] and oxyhemoglobin.[6] To account for cooperative oxygen binding by hemoglobin, Perutz[7,8] suggested that constraints exerted upon the proximal base might lead to reduced ligand affinities. This was indeed confirmed with heme models in which the proximal base was sterically hindered[9–12] or constrained.[13–14] The present work deals with a third question suggested in Figure 1: to what extent does the distal histidine contribute an additional stabilization by hydrogen bonding of the bent Fe-O_2 bond?

The experimental approach of the reactivity of heme-proteins and of heme models is essentially based on simple chemical relaxation principles. Many liganded hemeproteins as well as most hexacoordinated iron(II) porphyrin complexes undergo a reversible photodissociation which provides a convenient means for measuring ligand recombination ("on") rates. In case of a chemical system at equilibrium however, we shall show that dissociation ("off") rates are in fact the most relevant parameters to characterize a system. Special methods, like the photo-triggered ligand exchange technique, have been devised[15,16] and subsequently adapted to determine the dissociation rate constants of ligands from hemoproteins and hememodels.[17–19] Thus, laser photolysis of heme compounds, by merely causing in situ a sudden concentration change without inducing irreversible chemical transformations becomes a powerful, fast and "clean" chemical relaxation technique of wide applicability.

Very often, one is interested in the way the rate parameters of a particular reaction are changed within a series of closely related reactants. Not only does the task of comparing a large number of numerical values become increasingly difficult, but also the legitimacy of such comparisons is not always obvious. How closely related two molecules are (e.g. two porphyrin models) is not a simple matter of chemical intuition but should be decided on sound physical criteria. In a previous work[14] we have shown that Linear Free Energy Relationships (LFERs) provide a simple and adequate formalism to rationalize a large amount of data and to classify structurally related molecules into reacting families.

In Part I of this paper we review the concepts and methods which have emerged from several years of investigations of heme-model compounds but which happen to be of a sufficient generality to be useful also in other fields of chemical kinetics. In Section I-1 we review the general background and present the various coordination complexes of iron(II) porphyrins. In Section I-2 we describe the laser photolysis techniques which have been applied to their study. Section I-3 summarizes the LFER formalism. In Section I-4, we propose a connection between chemical kinetics and information theory which justifies the development of accurate methods for measuring dissociation rates. These concepts and techniques are applied in Part II of the paper to the specific problem of the stabilization of the oxygenated complex by hydrogen bonding in a series of newly synthesized heme-model compounds.

I. GENERAL CONSIDERATIONS

I-1. *Iron(II)-Porphyrin Complexes*

Similarly to the prosthetic group of oxygen-carrying hemoproteins, synthetic iron(II) porphyrins (P) contain a (d^6) Fe(II) ion which is strongly coordinated (intermediate spin, $S = 1$) to the four nitrogen atoms of a tetrapyrrole macrocycle. Two additional ligands may interact with the d_z^2 orbital which is directed perpendicularly to the heme plane. In the complexes of interest to us, a nitrogenous base (e.g. pyridine, imidazole, etc.), either intrinsically bound to the macrocyle or added as a free ligand, takes over the role of the "proximal" histidine of myoglobin or hemoglobin. The penta-coordinated species (high spin, $S = 2$) may then reversibly bind O_2 or CO axially, on the opposite, "distal" side of the heme plane to give a hexa-coordinated complex (low spin, $S = 0$).

Figure 2 shows the main species which are obtained when a nitrogenous base (B) plus another ligand (L = B′, O_2 or CO) are added; the kinetic and equilibrium constants are defined according to ref. 12 and 20 (see the legend of Figure 2). In order to model the active site of hemoproteins, the minimum requirement is to reproduce the pentacoordination of the ferrous ion. However, addition of a free base in solution leads directly to the hexa-coordinated complex BPB (hemochrome), because $K_B \ll K_B^B$. Pentacoordination may be enforced in several ways: first, using sterically encumbered bases such as 1,2-dimethylimidazole (1,2-Me_2Im) prevents the formation of the hemochrome; the same result is achieved when the porphyrin itself presents a great deal of steric hindrance on one face; another possibility is to synthesize models incorporating the axial base covalently attached to the porphyrin ring in such a way as to permit intramolecular coordination with the iron atom.[21–25] The carboxyhemochrome BPCO is by far the complex having the highest equilibrium constant and is therefore the dominant species even in presence of oxygen and of some excess B. The oxyhemochrome BPO_2 is generally unstable, due essentially to the presence of traces of water. It more or less readily oxidizes to BP(Fe III$^+$)OH^- (which may be reversibly reduced) or even worse irreversibly to μ-oxo-dimers BP(Fe III$^+$)-O-O-P(Fe III$^+$)B. Obviously the globin of hemoproteins efficiently protects the protoporphyrin IX from these highly undesirable side-reactions.

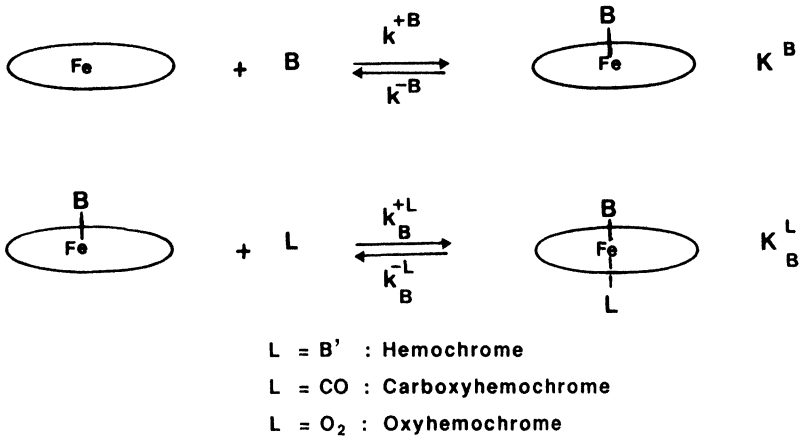


Figure 2 Main species which are obtained by adding a nitrogenous base (B) plus another ligand (L = B', O_2 or CO) to an iron(II) porphyrin. The equilibrium constants are defined according to reference 20; the superscript denotes the ligand exchanged, the subscript gives the ligand remaining through the reaction; the kinetic rate constants are defined in a same manner,[12] with an additional sign in the superscript to indicate whether the ligand is added (+) or lost (−).

To conclude this section, we show on Figure 3 two heme models which we have intensively investigated. These so-called "basket-handle" porphyrins[24,25] contain a nitrogenous base in their "proximal" handle (e.g. an imidazole as in Figure 3) and exist as pure pentacoordinated species in solution. The length of the proximal handle could be varied, so as to achieve various degrees of constraint upon the proximal base-iron bond. The handles were anchored to the macrocycle either using an ether- or an amide-connection. This changes the polarity at the vicinity of the distal ligand

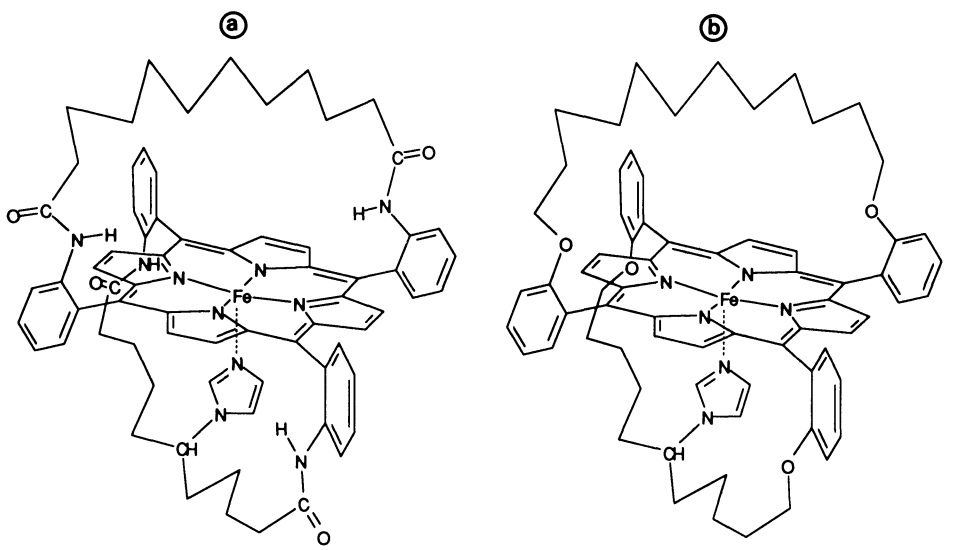


Figure 3 Amide (a) and ether (b) "Basket-Handle" porphyrins. The internally chelated base may be a pyridine or an imidazole.

and also modifies the degrees of freedom of the handles because of the rigidity of the planar amido-group. In addition, the distal handle considerably slows down autoxidation. μ-oxo-dimers formation is not observed with these compounds.

I-2. *Determination of Rate Constants using Laser Phototriggered Reactions*

The property of hexa-coordinated Fe(II)-porphyrin complexes to photodissociate has provided a powerful tool for investigating the dynamics of ligand binding. The first applications to hemoproteins already laid down the general principles.[16,26] These still conventional "flash"-photolytic studies were subsequently applied to the reactions of the first chelated heme-models.[12,17] The use of laser photolysis has permitted to investigate new and faster reactions[18] and to develop more extensively photo-triggered ligand exchange experiments.[19] The complexes which have been found to be photosensitive are principally the carboxyhemochromes BPCO, which photodissociates with a yield of approximately unity and the hemochromes BPB′ with a yield of only a few percent. In the few favourable circumstances when an oxyhemochrome BPO_2 was chemically stable enough to permit a direct photolytic study, the photodissociation yield seemed to be intermediate between the previous values.

The rates of ligand binding to Fe(II) porphyrins span several orders of magnitude. The combination rate of the sixth ("distal") ligand are in the range of 10^6–10^7 $Mole^{-1}.sec^{-1}$ (k_B^{+CO}), 10^7–10^8 $Mole^{-1}.sec^{-1}$ ($k_B^{+O_2}$), 10^8–10^9 $Mole^{-1}.sec^{-1}$ (k_B^{+B}) and require fast relaxation techniques to be investigated. For instance, the photodissociation of hemochromes had escaped observation by flash-lamp photolysis since it combines the difficulties of a low yield and a fast, submicrosecond, rebinding rate.[18] The dissociation rates of hexacoordinated complexes may extend from about 10^4 sec^{-1} (some $k_B^{-O_2}$) down to about 10^{-3} sec^{-1} (k_B^{-CO}). In view of the importance of dissociation rates, special techniques have been developed to take care of their measurement.

The simplest method to determine the kinetic parameters from photolysis experiments consists to record the recombination reaction after photodeligation; the relevant kinetic scheme is:

$$\mathrm{BPL} \underset{k_B^{-L}}{\overset{h\nu;\ k_B^{+L}}{\rightleftharpoons}} \mathrm{BP} + L$$

Scheme I

It is well known that, provided the conditions of pseudo-first order ($[L] \gg [P],[B]$) are satisfied, the recombination reaction is exponential and its rate k_r^L is given by:

$$k_r^L = k_B^{+L} \cdot [L] + k_B^{-L} \tag{1}$$

The association and dissociation rate constants are thus obtained directly from the slope and the intercept of the linear plots of k_r^L against $[L]$. Carbon monoxide complexes, which are indefinitely stable and have a high photodissociation yield, could be, in principle, investigated by this technique. Since CO is the ligand which is lost in the photoreaction, k_B^{+CO} and k_B^{-CO} determine the relaxation rate; however,

these CO derivatives are very stable complexes with dissociation rate constants which are always negligible compared to the product $k_B^{+CO} \cdot [CO]$; therefore the plot goes through the origin and the dissociation rate cannot be determined in this way. However, if the equilibrium constant can be measured independently by performing a static spectrophotometric titration of the CO complex, then k_B^{-CO} is simply calculated as k_B^{+CO}/K_B^{CO}.

Although the quantum yields are lower for hemochromes and oxyhemochromes, the direct rebinding technique can be applied also to these compounds which lose respectively a base and an oxygen molecule in the photodissociation process. In the case of oxyhemochromes, a specific difficulty arises from the irreversible auto-oxidation into iron(III) μ-oxo-dimers which occurs whenever the porphyrin does not possess an efficient steric protection on both faces; the oxidation-time of most oxygenated complexes of simple iron(II) porphyrins is often so short that a direct study is impracticable.

In principle, the simplest way of measuring independently the low dissociation rate of a ligand L is to mix rapidly a solution of the associated complex BPL with a second solution containing only a very strong ligand (e.g. CO or NO) in a high concentration. The pentacoordinated BP species which are formed at each spontaneous dissociation of L are then immediately and irreversibly trapped by CO. The rate of disappearance of the initial species is then limited by k_B^{-L} :

$$\mathrm{BPL} + \mathrm{CO} \overset{k_B^{-L}}{\rightleftharpoons} \mathrm{BP} + L + \mathrm{CO} \rightleftharpoons \mathrm{BPCO} + L$$

Scheme II

Instead of mixing, which is a slow process, an elegant solution is offered by the technique of photo-triggered ligand replacement which has been originally proposed[15,16] for investigating heme proteins. The relevant kinetic scheme is nothing but the preceding one in reverse order:

$$\mathrm{BPCO} + L \overset{h\nu,\ k_B^{+CO}}{\rightleftharpoons} \mathrm{BP} + L + \mathrm{CO} \underset{k_B^{-L}}{\overset{k_B^{+L}}{\rightleftharpoons}} \mathrm{BPL} + \mathrm{CO}$$

Scheme III

For all usual ligands (except NO) BPCO is by far the dominant species. Photodissociation leads to the penta-coordinated BP species, and L and CO compete for rebinding. By choosing adequate ligand concentrations, it is generally possible to adjust the reaction rates such that L recombines faster than CO with BP. As a consequence, the reaction presents an initial and rapid (from about 100 *ns* to a few μs, depending on conditions) step corresponding to the transient formation of a certain amount of BPL. But BPL is out of equilibrium under the experimental conditions. At a rate which depends on k_B^{-L} and on the various ligand concentrations, L spontaneously dissociates (see section I-4) so that ligand replacement may progressively take place, restoring the initial carboxyhemochrome via BP. The rate constant k_f^L of BPL formation is mainly governed by k_B^{+L} and the rate constant k_{ech}

of the slow ligand exchange phase by k_B^{-L}. The exact expressions of these relaxation constants are:

$$k_f^L - k_r^{CO} = k_B^{+L} \cdot [L] + k_B^{-L} \tag{2}$$

and

$$(k_{ECH})^{-1} = \frac{1}{k_B^{-L}} \cdot \frac{k_f^L}{k_r^{CO}} \tag{3}$$

k_r^{CO} is the relaxation rate for the recombination of CO with BP in the absence of oxygen, which can be determined independently by performing a direct rebinding experiment according to Scheme I. Figure 4 shows the time evolution of the oxyhemochrome concentration in a real experiment described in Part II.

At the time the method was first developed for studying hemoproteins, photolysis was performed using conventional flash photolysis techniques. The time resolution was insufficient to observe the fast initial step of oxygen binding; $k_B^{-O_2}$ was determined using an approximation to Eq. (3) and an extrapolation procedure in which the exchange time was plotted against the concentration ratio $[O_2]/[CO]$.[16] The method worked well with the strong oxygen binding hemoproteins. When transposed to the study of heme models, which are not as good as proteins for binding O_2, this approximation was found to be insufficient. Formulas 2 and 3 have been rederived[19] for the most general case. The method of photo-triggered competitive rebinding has now been applied not only to oxyhemochromes, but also for studying

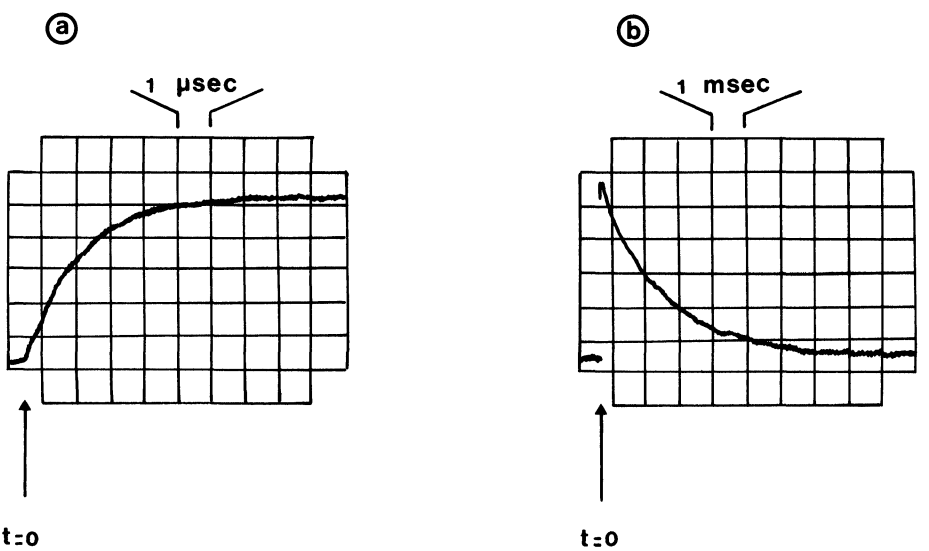


Figure 4 Competitive rebinding between O_2 and CO with compound 7-1,2-Me_2Im. Toluene, 25 C; [CO] = 4.3×10^{-3}M; $[O_2] = 2.1 \times 10^{-3}$M; [1,2-$Me_2$Im] = 2.4×10^{-3}M; absorbance scale: 0.01/division. Trace (a) fast absorbance change ΔA due to the transient formation of the oxyhemochrome. The fact that $\Delta A(0) = O$ shows that the monitoring wavelength is isosbestic for BPCO and BP. Trace (b) slow replacement of oxygen by CO to give back the initial carboxyhemochrome.

the binding of nitrogenous bases[18] and water[28] with pentacoordinated porphyrin complexes (L = B or L = H20 in Scheme III). It presents several advantages: (i) easy measurement of slow dissociation rates; (ii) the photodissociation yield of BPCO is practically unity, this means that larger signals are obtained than if hemochromes or oxyhemochromes were flashed directly; (iii) the species of interest need not be stable. Although the exchange step is a relatively slow process (from a few milliseconds to several hundred milliseconds), it is faster than auto-oxidation of the oxygenated complex for example. Thus, many experiments can be successively performed without any significant porphyrin degradation; (iv) in its complete form,[19] the method takes advantage of the jump in time resolution due to the advent of laser techniques, and permits, in one single experiment, to obtain information both on the combination and dissociation rates. Although the methods described above have been developed specifically for the study of porphyrin complexes, they have a sufficient degree of generality to be easily transposed to other chemical systems.

I-3. *Linear Free Energy Relationships*

Once the rate parameters and equilibrium constant of a given reaction (e.g., oxygen binding to a porphyrin) have been collected for a number N of molecules, one is at first faced with the unexpected problem of finding a means for comparing $3N$ numerical values which usually span several orders of magnitude. A $\log k^{+/-}$ versus $\log k$ plot is then very convenient. But its main advantage is to eventually reveal the existence of a linear free energy relationship. Consider Figure 5 in which the representative points of three molecules undergoing the same reaction have been plotted. It is at once evident that, compared to molecule 1, molecule 2 achieves a higher affinity by mainly decreasing the "off" rate. In contrast, molecule 3, despite an equal affinity, exhibits different rate parameters than molecule 2. Thus, the plot displays visually in a simple way the general trends found in comparing molecules with each other from the point of view of a given reaction. If a straight line is drawn through the corresponding points of molecules 1 and 2, the line relative to k^+ and the line relative to k^- will converge in a common point of the $\log k^{+/-}$ axis and their slope substract to unity. This result is immediately derived by writing the equations of the lines as:

$$\log k^+ = \alpha \cdot \log K + \log k_o^+ \tag{4}$$

and

$$\log k^- = \beta \cdot \log K + \log k_o^- \tag{5}$$

They are simultaneously satisfied if:

$$\alpha - \beta = 1 \quad \text{and} \quad k_o^+ = k_o^- \tag{6}$$

More than often, several molecules of a series, and not just two of them, are found to have their representative points lying on one line (with more or less scatter). In Part II of the paper we give a typical example. Such a correlation is certainly not coincidental; it has its origin in the existence of a Linear Free Energy Relationship (LFER) which appears when only smooth changes in the free energy of products,

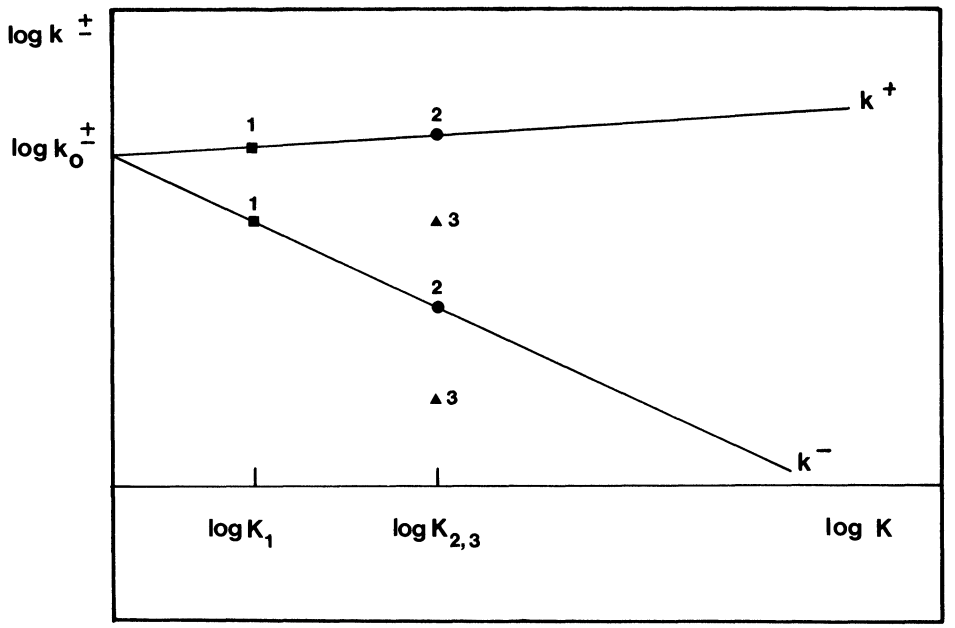


Figure 5 Comparing rate parameters and equilibrium constants using a double logarithmic plot (explanations in the text).

reactants and transition state are occurring upon going from one molecule to another.

LFERs can be traced back to Broenstedt relations; Hammett's correlations, also well known in organic chemistry, are another example.[29] The possibility of using LFERs to characterize the reactivity of hemoproteins was suggested some years ago (see reference 31 and references therein). In the simple form that we shall now describe, the LFER formalism has proved an efficient tool for classifying a large number of heme-model compounds into only a few distinct reaction-families. Instead of performing tedious pairwise comparisons, which are even often questionable, we suggested that LFERs should be used to find out those molecules which are objectively comparable.[14] LFERs have also been applied recently to classify the effects of aminoacid substitutions on an enzymatic reaction in engineered proteins.[32]

It is not difficult to find the physical significance of a log–log linear relation between rate and equilibrium constant, like the one suggested above. In terms of Gibb's free energy, the simplest reaction leading from Reactants (R) to Products (P) can be described by the diagram of Figure 6. The equilibrium constant is given by:

$$K = \exp(-(G_p = G_R)/RT) \tag{7}$$

In the transition-state theory the rate constants are expressed by:

$$k^+ = X \cdot \exp(-(G_T - G_R)/RT) \tag{8}$$

and

$$k^- = X \cdot \exp(-(G_T - G_P)/RT) \tag{9}$$

where X is some universal factor (usually taken as $k.T/h$ in Eyring's theory of

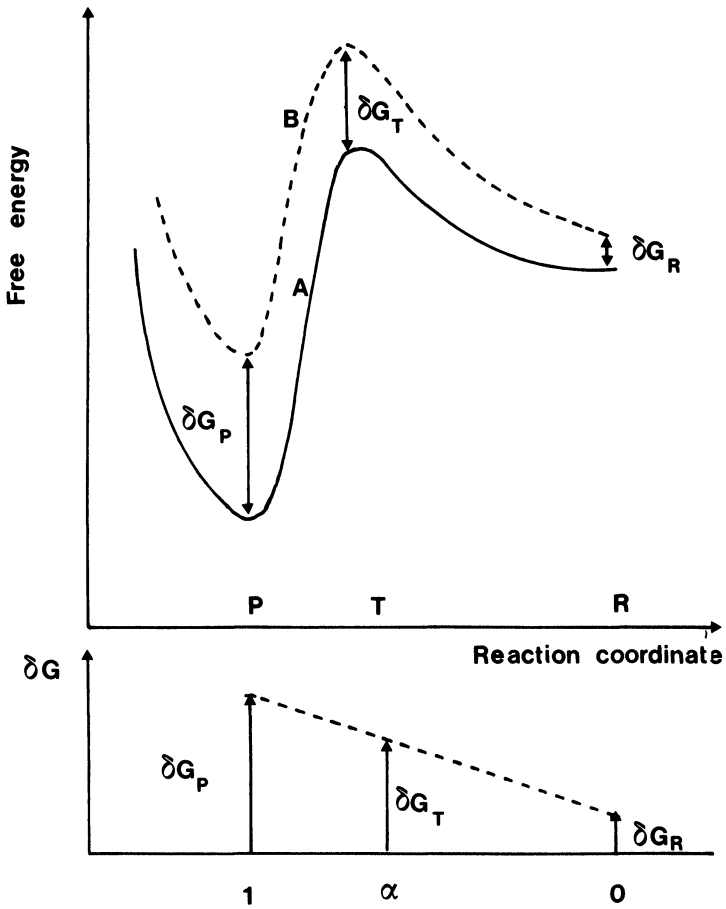


Figure 6 Top: Differences in the free energy diagram of two molecules A and B undergoing the same reaction. Bottom: definition of the α-character of the transition state from the correlated changes in the free energy of the reactants (R), products (P) and transition state (T) upon going from molecule A to molecule B. The limiting values $\alpha = 1$, $\delta G_T = \delta G_P$ and $\alpha = 0$, $\delta G_T = \delta G_R$ correspond to the extreme situations where the transition state is purely "product-" or "reactant-" like respectively.

absolute reaction rates). In Eqs (7)–(9), the Free energy is taken respectively in the potential wells of reactants (G_R), products (G_P) and at the top of the energy barrier, i.e., at the transition state (G_T). Therefore, in the empirical log–log representation, one is actually comparing free energy differences. Expressing the rate constants in Eqs (4)–(5) in terms of Eqs (7)–(9), and differentiating one obtains:

$$\delta G_T = \alpha\, \delta G_P + (1 - \alpha)\, \delta G_R \tag{10}$$

Equation (10) means that when several molecules are linearly correlated in the log $k^{+/-}$ versus log K plot, then the change in the free energy at the transition state upon going from one molecule to another is a linear interpolation of the corresponding

changes in the free energy of the products and of the reactants (see Figure 6). This is therefore a very particular property which objectively suggests a strong thermodynamic parentage in the series of molecules. Alternatively, using Eq. (10) as an hypothesis, the existence of linear log k/log K plot can be deduced.[29,31] However, we find that the present approach, which is based on observation, is less artificial. Usually, the reaction coordinate is parametrized such that $\alpha = 1$ at P and $\alpha = 0$ at R. The α-value therefore characterizes the nature of the transition state somewhere between these two extremes. The two limiting situations have been indeed observed in a few cases.[14] In general, however, intermediate α-values are found (see Part II).

I-4. *Information Theory and Chemical Kinetics*

Reaction rate constants are essentially transition probabilities per unit time interval. Thus, chemical kinetics in a sense provide a probabilistic description of the dynamics of chemical systems. Information theory deals with situations where the outcome of an experiment is uncertain, i.e., is described by a probability distribution. It is therefore natural to expect some connection to exist between both fields. In this section we present an elementary discussion of a possible link. This will prompt us to examine into detail some simple but fundamental questions about the significance of the rate parameters which are the subject of investigations by chemical relaxation techniques.

Ligand binding to heme models is a particular example of the elementary equilibrium reactions:

$$D + L \rightleftarrows A \quad \text{with} \quad K = k^+/k^- = \frac{[A]}{[D][L]} \tag{11}$$

where $[A]$ and $[D]$ are the respective concentrations of the associated and dissociated forms of a complex with a ligand L, k^+ and k^- the forward and backward reaction rate constants, and K the equilibrium constant. Conditions are usually chosen such that either $[L] \gg [A]$, $[D]$ or $[L]$ = constant. In this "pseudo" first-order approximation, the proportions a and d of species A and D at equilibrium are given by:

$$d = \frac{1}{K \cdot [L] + 1} \quad \text{and} \quad a = \frac{K \cdot [L]}{K \cdot [L] + 1} \tag{12}$$

Thus the knowledge of K (e.g. after a titration) permits to calculate the probability to find an associated complex by selecting at random a molecule out of the reaction mixture. This, however, constitutes a very limited description of the system since identical K-values can be obtained by an infinite number of combinations of the rate parameters $k+$ and $k-$. In a dynamic steady-state the average proportion of each species remains constant in time but individual molecules are permanently associating and dissociating. A ligand remains bound only for a limited period of time, after which the complex dissociates. Similarly, a molecule like, say a bare porphyrin, in presence of a concentration of free ligand, does not remain indefinitely unliganded. Intuitively, we consider that a system is dynamically stable when it undergoes only a few transitions per unit time in either direction. Thus, we may tentatively quantify a

dynamic equilibrium by the average number of transitions observed per unit of time. For the simple case considered in Eq. (12), a fraction d of molecules will give rise to $d \cdot k^+ \cdot [L]$ forward transitions and a fraction a to $a \cdot k-$ backward transitions per second. (For a two-states model, the equilibrium condition implies that both numbers are equal). The average number of transitions observed in one second is therefore:

$$W = d \cdot k^+ \cdot [L] + a \cdot k^- \tag{13}$$

Using Eq. (12), it can be seen that the intrinsic lifetime of the associated complex becomes the limiting step as the ligand concentration increases. In this limit, $W = 2 \cdot k^-$, meaning that the rate of ligand exchange is at most equal to k^- and that a ligand is immediately replaced by another one to maintain the equilibrium condition. These simple considerations are in agreement with intuition which suggests that the "off" rate might be a more informative estimator than K of the properties of systems like the one considered here.

The above results have been obtained using a naive picture and a simple system. It is possible to recast them into the more general framework of information theory. The objective "measure" of our uncertainty about the occurrence of any one among i events is Shannon's entropy:[33]

$$H = -\sum_i P_i \cdot \log P_i \tag{14}$$

in which P_i is the probability of the i-th event.

The entropy vanishes when the outcome of an event is certain (one of the probabilities equals unity, no uncertainty is left) and goes through a maximum in the situation of greatest uncertainty, i.e., when all P's are equal. For continuous probabilities, Shannon has also shown that the discrete P's must be replaced by the probability density function $P(x)$ such that the probability of a favourable event is equal to $P(x) \cdot \mathrm{d}x$ when the variable value is comprised between x and $x + \mathrm{d}x$. The sum in Eq. (14) then becomes an integral over x.

According to the preceding discussion, there is an uncertainty associated with the observation of a transition from any state of a system in dynamic equilibrium and therefore an associated entropy in the sense of information theory. How complicated the system may be, it is always possible to define the lifetime or, alternatively, the overall rate of disappearance of a given species as a combination of elementary rate constants. Let $N(i)$ be the equilibrium proportion of species i and $k(i)$ its rate of disappearance. The probability that a transition starts from state i within a time interval $\mathrm{d}t$ is equal to $N(i) \cdot k(i) \cdot \mathrm{d}t$. Hence, the probability to observe a transition from any species during the same interval $\mathrm{d}t$ is the sum of all these terms:

$$W\,\mathrm{d}t = \sum_i N(i) \cdot k(i)\,\mathrm{d}t \tag{15}$$

This is a constant probability per unit time interval which, like all first-order processes, leads to an exponential decrease for the global probability $P(t) = \exp(-W \cdot t)$ that *no transition has occurred at time t*. From this follows that the

probability to observe *exactly one transition between times t and t + dt must be equal to P(t) × W · dt. i.e.:*

$$dP\ (t,\ t + \mathrm{d}t) = (W) \cdot \exp\ (-W \cdot t) \cdot \mathrm{d}t \tag{16}$$

which is the required probability density function. The classical result of the calculation is then:

$$H = \log e/W \tag{17}$$

In the practical case it is generally possible to choose conditions such that one species strongly dominates (e.g., using large [*L*] values). For this species *s*, $N(s) = 1$ and $W = k(s)$. Equation (17) then reduces to

$$H = \log e - \log k(s) \tag{18}$$

(The constant term is unimportant, it simply results from the choice of the base of the logarithms used). This shows that the natural measure of the uncertainty about the occurrence of a transition in a system at equilibrium is ultimately related to the "off" rate constant of the dominant species. The smaller the "off" rate, the longer the lifetime and the larger the entropy. A large uncertainty about the exact time at which the complex will dissociate means that the ligand is engaged in a strong bond. In the example shown in Figure 5, compounds 2 and 3 have the same equilibrium constant but molecule 3 is clearly more stable than molecule 2 in the liganded state since $k_3^- \ll k_2^-$.

II. STABILIZATION OF OXYGENATED HEME MODELS BY INTERNAL HYDROGEN BONDING

The results of the previous sections suggest that a decrease of the dissociation rate following a small structural perturbation might be indicative of the presence of an additional stabilizing factor in the associated state. This simple assumption was at the origin of our interest in the question of the possible existence of a hydrogen bond between the dioxygen molecule and the distal (E7) histidine. Neutron diffraction revealed indeed an oxygen-histidine hydrogen bond in oxymyoglobin[34] and a similar bond was suggested by X-ray studies of oxyhemoglobin.[6] At about the same time we were investigating pyridine-chelated BHPs. Whereas the oxygen association rate constant was only 20% larger in the amide-BHP, its disociation rate was smaller by a factor of ten compared to the ether-BHP.[35] Accordingly the longer intrinsic "life-time" of the oxygenated complex must correspond to a stronger bond. We proposed that H-bonding with the amide protons might be responsible for the increased stability, adding about 5 kJ.mole^{-1} to the free energy of binding in the oxygenated complex. The conclusion, suggested by the kinetic study, was soon confirmed by an NMR investigation comparing oxygen and carbon monoxide complexes in both series.[36] In contrast, a re-examination of the structural data of the oxygenated "picket-fence" porphyrin *I* indicates only weak hydrogen bonding,[37] in spite of the presence of four amide groups (Figure 7). Although the protons still point toward the

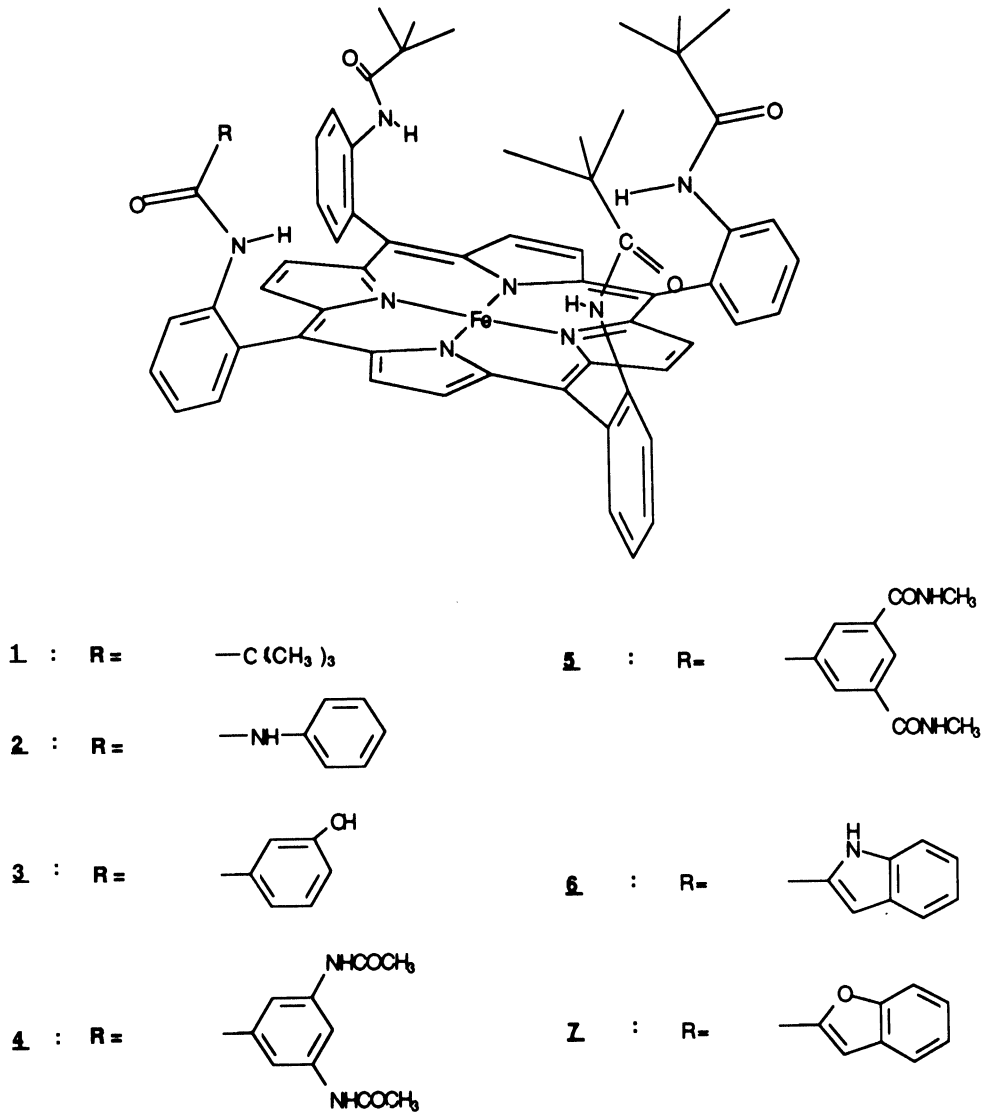


Figure 7 "Picket Fence" derivatives *1–7* studied in this work.

terminal oxygen atom, they are at a longer distance than in a-BHPs, because of the bulk of the tert butyl groups which tend to keep the flexible pickets away from the centre.

To model hydrogen bonding more precisely, new porphyrin derivatives *2–7* (Figure 7) have been designed. Here, substitution of one of the tert butyl groups in a picket is expected to modulate the strength of hydrogen interaction. This may occur in two ways, either by direct interaction with the newly introduced OH or HN group

or, more indirectly, by altering the steric constraints of the picket superstructure. As an application of the methods described in Section I-2 above, we now report new kinetic data concerning compounds *2–7*.

II-1. *Materials and Methods*

Compound *1* was prepared according to reference.[38] The synthesis and characterization of derivatives *2–7* will be published elsewhere.[39,40] The procedures used for preparing the various porphyrin complexes were as previously described.[41] Iron(III) porphyrins were reduced to the Fe(II) forms by sodium dithionite in wet toluene under anaerobic conditions. The penta-coordinated species were obtained by addition of a deaerated solution of 1,2-dimethylimidazole (1,2-Me_2Im).

The hexa-coordinated complexes were prepared by bubbling CO/O_2 gas mixtures into the sample. Final gas equilibration and measurements were performed in a thermostated gas-tight cell at 298 *K*. The gas solubilities in toluene at 298 K were taken as 5.3×10^{-3} M · atm^{-1} (O_2) and 7.2×10^{-3} M · atm^{-1} (CO).[42] Extraneous ligand concentrations were always at least ten times the porphyrin concentration so that bimolecular reactions gave rise to simple pseudo-first order kinetics.

Laser flash photolysis was performed using the apparatus already described.[18] In brief, we use the second harmonic (532 nm) of a Q-switched *N*d/YAG laser (QUANTEL). The laser pulse (20 ns) was always much shorter than the fastest kinetics observed in this work. The rebinding kinetics were recorded using either a pulsed Xenon arc (75 *W*) or a continuously running *W* quartz-iodine lamp (100 W). The time constant of the detection system could be adjusted from 10 nsec to several msec, with an average signal to noise ratio of about 50. The transients were displayed on a Tektronix 7834 Storage oscilloscope.

In principle, the rate constants k_B^{+1} and $k_B^{-O_2}$ can be obtained from the kinetics of equilibrium relaxation according to the techniques described in Section I-2 above. The time evolution of the various species can be followed from the transient absorbance changes. Typical oscillograms in an experiment of competitive rebinding are shown in Figure 4. Direct rebinding experiments involve only two species, BP and BPL (Scheme I). Since BPL is the dominant species at equilibrium, the initial absorbance is given by $D(0) = (\epsilon_{BPL}) \cdot [BPL(0)]$; ($\epsilon_x$ denotes the molar extinction coefficient of species X).

At a time *t* after the laser perturbation, the absorbance change is $\Delta D(t) = (\epsilon_{BP} - \epsilon_{BPL}) \cdot [BP(t)]$. Thus, the absorbance change is proportional to the concentration of the photolysis product and can be monitored at any wavelength where $\epsilon_{BP} - \epsilon_{BPL} =/= 0$.

In competitive rebinding experiments, three species are involved. The absorbance change is now given by $\Delta D(t) = (\epsilon_{BP} - \epsilon_{BPCO}) \cdot [BP(t)] + (\epsilon_{BPO_2} - \epsilon_{BPCO}) \cdot [BPO2(t)]$. Proportionality to the concentration of one species can only be achieved at the isosbestic wavelengths for the other species. Therefore, monitoring the concentration of the oxygenated complex according to Eq. (2) and (3) requires to determine the isosbestic wavelengths for BP and BPCO. Since photodissociation corresponds to the transformation BPCO $\longrightarrow$ BP, they can be determined

accurately kinetically as the wavelengths for which there is no initial absorbance change immediately following the laser pulse (see trace a in Figure 4).

Applying Eqs (1)–(3) implies that schemes I and III provide a complete description of all kinetic processes which occur after photolysis. However, if the base concentration is too low, photodissociation of the gaseous ligand may lead to rapid base elimination;[12] in contrast, at very high base concentration the transient formation of a hemochrome can be faster than CO or O_2 rebinding even when the binding constant K_B^B is very low, as is the case with the encumbered 1,2-Me_2Im. Both processes may spoil the kinetics and lead to erroneous rates of ligand rebinding. These difficulties were overcome by the usual procedure:[11] the base concentration was varied systematically over four orders of magnitude (10^{-4} to 1 M); it has been always possible to find an appropriate concentration range within which the rebinding rate of O_2 and CO remained independent of the base concentration, indicating that no process other than I and III was occurring (Figure 8).

II-2. *Results and Discussion*

In presence of a large excess of 1,2- Me_2Im, the oxyhemochromes of porphyrins *1–7* were stable enough towards oxidation to allow a direct study by laser photolysis. The

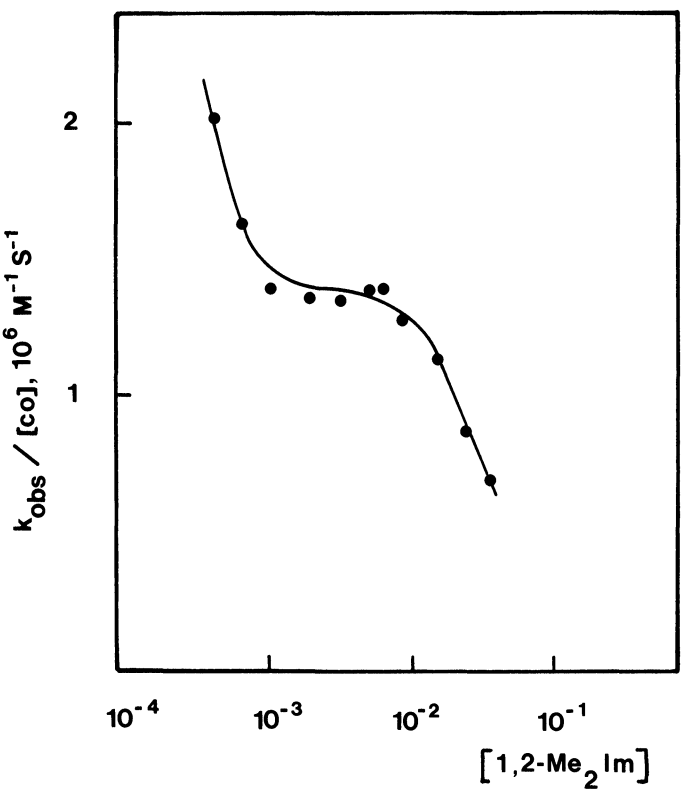
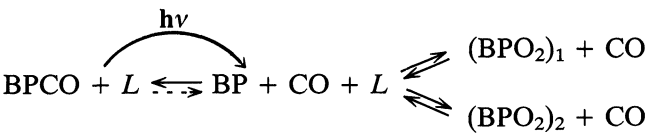


Figure 8 Apparent second order rate constant versus base concentration for CO rebinding with compound *3*-(1,2-Me_2Im) in toluene, 25 C with [CO] = 7.2×10^{-3} M.

slope and the intercept of the linear plots of $k_r^{O_2}$ against $[O_2]$ should be equal to $k_B^{+O_2}$ and $k_B^{-O_2}$ respectively (Eq. (1)). However $k_B^{-O_2}$ was generally too small compared to the product $k_B^{+O_2} \cdot [O_2]$ to be determined accurately in this way. We performed an independent determination of these kinetic rate constants using the competitive rebinding technique described by Scheme III. Whenever reliable values could be obtained using both methods, they were found to be in good agreement.

Equations (1), (2) and (3) assume that all kinetics are exponential. This was indeed found to be the case for compounds *1,2,5,6* and *7*, like for all other porphyrins previously studied. However, an unusual observation was made with compounds *3* and *4*: the slow exchange kinetics (k_{ech}) could be accurately described only by a sum of two exponentials. The relative amplitude of both compounds, and the two values of $k_B^{-O_2}$ which were calculated according to Eq. (3) remained unchanged when the base, CO and O_2 concentrations were varied by factors of 3–5, 5 and 10 respectively. These observations imply the presence of two distinct oxygenated species dissociating at different rates.

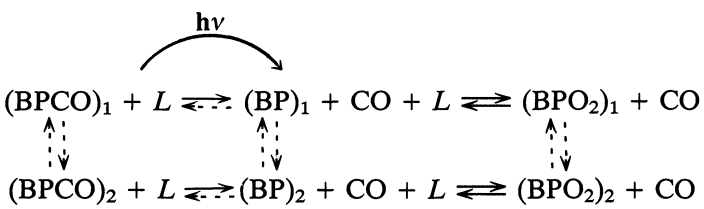
Several hypotheses can be formulated to account for these unexpected findings. The first possibility to be considered is the existence of two isomeric oxygenated complexes, while all other forms of the porphyrin would present only one isomer. This could be the case for instance if there were two inequivalent orientations of the H-bonding group or even of oxygen itself within the final complex. The relevant kinetic scheme is:



Scheme IV

In our present experimental conditions, the relaxation rates for the equilibria between the pentacoordinated species and the oxyhemochromes are several orders of magnitude faster than the CO recombination rate. Therefore, the two isomeric complexes transiently populated after laser photolysis would quickly equilibrate via the common pentacoordinated species BP before CO recombination could occur. Since they cannot be distinguished spectrosopically, they would appear as one single species and decay according to monoexponential kinetics; therefore, this hypothesis does not fit the data and cannot be retained.

As another possibility, the porphyrin itself might exist as two distinct conformational or chemical isomers:



Scheme V

In this scheme the overall oxygen replacement by carbon monoxide would follow complicated kinetics and lead to apparent oxygen "off-rates" which might be expected to depend on the $[O_2]/[CO]$ concentration ratio. Since no change was observed upon increasing this ratio by a factor of 50, there can be no isomerization on the time scale of the experiment (about 10 msec) neither in the oxy, carboxy nor in the five-coordinated states of the porphyrin. Since the chemical characterization does not give any evidence for the presence of more than one permanent species, this second hypothesis could apply only to conformational isomers with an interconversion rate slower than 10 s^{-1}.

The most likely possibility remains the existence of two isomeric penta and hexacoordinated complexes due to unsymmetrical ligand binding on both faces of the porphyrin to give $\left\lfloor \frac{B}{L} \right\rfloor$ and $\left\lfloor \frac{L}{B} \right\rfloor$ (L = CO or O_2, B = 1,2-Me_2Im). This might seem somewhat surprising since X-ray crystal structure determinations[38] of "Picket-Fence" porphyrin *1* have shown that the gaseous ligand binds on the encumbered side, and the base on the unprotected face. However, four equilibrium constants are involved in determining the concentration ratio of both species. Their relative values may well vary within the series *1–7*. Since we were able to observe the formation of the hemochrome with porphyrins *1–7* using 1-methylimidazole as a ligand, there would not appear to be any particular steric difficulty to base binding on the picket side of the porphyrin. Whereas the association rate constants for the binding of a small ligand such as CO and O_2 on either face may be of a comparable order of magnitude, the dissociation rates would critically depend on any eventual stabilization of the complex by hydrogen bonding. Although there is no definitive evidence for the formation of the two oxyhemochromes $\left\lfloor \frac{B}{L} \right\rfloor$ and $\left\lfloor \frac{L}{B} \right\rfloor$ for compounds *3* and *4*, we believe that this hypothesis is the most reasonable one fitting the data.

The results are summarized in *Table 1*. Small differences in the kinetic values cannot be reasonably analyzed in terms of structural changes, since the conformation of the pickets in compounds *1–7* remains presently unknown. In contrast, L.F.E.R.s provide an objective means for comparing reactivity within the whole series of related molecules. Figure 9 shows that a significative correlation exists for O_2

Table 1 Kinetic rate parameters and equilibrium constants for CO and O_2 binding with "Picket-fence" derivatives *1–7*. Temperature: 25°C; Solvent: toluene; Base: B = 1,2-Me_2Im

Compound	$10^{-5}\ k_B^{+CO}\ (M^{-1}s^{-1})$	$10^{-7}\ k_B^{+O_2}\ (M^{-1}s^{-1})$	$10^{-4}\ k_B^{-O_2}\ (s^{-1})$	$10^{-3}\ K_B^{O_2}\ (M^{-1})$
1	15.9[a]	21.6[a]	5.6[a]	3.9[a]
	14.6[b]	18.2[b]	4.6[b]	4.0[b]
2	3.9	3.8	0.1	34.5
3	13.2	18.9	0.5 (77%)[c]	37.8
			4.1 (23%)[c]	4.6
4	12.8	11.0	3.1 (65%)[c]	3.5
			15.4 (35%)[c]	0.7
5	16.7	14.9	11.0	1.4
6	15.5	24.0	2.4	10.0
7	14.0	27.0	4.3	6.3

[a] This work.
[b] Calculated from ref. 11, after correction for the different values adopted in both works for CO and O_2 solubilities.
[c] Weight of each "component" when a biphasic exchange was observed.

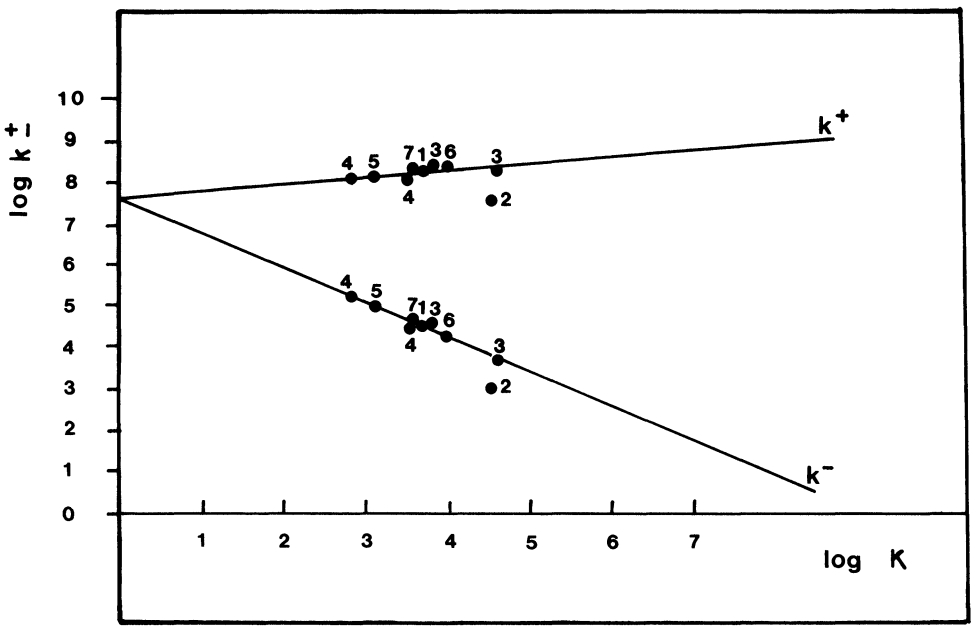


Figure 9 Linear Free Energy Relationship between rate and equilibrium constants for O_2 rebinding with the "Picket-Fence" derivatives *1–7*. Model *2* clearly deviates from the correlation and was not included in the calculation of the regression lines. Least-square fits: $k_B^{+O_2}$: slope = 0.17, intercept = 7.64; $k_B^{-O_2}$: slope = −0.83, intercept = 7.63.

binding. All compounds, except compound *2*, behave as one reactive family which is homogeneous from a thermodynamic point of view. Note that the slopes of both regression lines indeed substract to unity as discussed in Section I-3. Moreover, the correlation is quite indistinguishable from that previously found with BHPs with $\alpha = 0.17$. This value indicates that the transition state is almost "reactant-like." Such a behaviour, which is expected for compounds presenting a small "peripheral" steric hindrance, is consistent with the experimental findings described above and argue against too severe a steric hindrance above the Fe-Ligand axis. The fairly wide range of values covered by the equilibrium constants results mainly from changes in the dissociation rate constants. Only compounds *2* and *3* show a substantial decrease in $k_B^{-O_2}$ as compared to "Picket-Fence" porphyrin *1* (*Table 1*). This effect may be attributed to hydrogen bond stabilization of oxygen with a proton of the substituted picket.

A further interest of the L.F.E.R. shown in Figure 9 is to emphasize the specific behaviour of compound *2* which, despite having the same equilibrium constant as *3*, exhibits rate parameters that are both decreased by a factor of 5. This implies that the structural differences between compounds *2* and *3* affect only the free energy of the transition state. Since H-bond effects are manifest only in the energy of the final complex,[14] the destabilization of the transition state in *2* must have its origin mainly in steric effects. In this specific case, the substituted phenylurea picket might adopt a

conformation which projects a substituent deeper into the oxygen binding pocket than the other compounds.

The present considerations are based on kinetic arguments and cannot possibly answer all questions. Further investigations are now required to understand the results in terms of molecular structure and may be forthcoming from NMR, X-ray crystallography and molecular mechanic calculations. The power of the LFER formalism is its ability to tell us immediately which molecules, from a series, are indeed comparable from the point of view of their reactivity, to quantify the character of the transition state and finally to sort out those which present an exceptional behaviour.

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