bond in $^3Pt_2^*$ is considerably shorter[17] than in the ground-state $Pt_2(POP)_4^{4-}$, there has been a tendency to suggest that $^3Pt_2^*$ represents the rare case for which the partial molar volume ($\bar{V}$) of an excited state is smaller than that of the corresponding ground state. If this were so, one would expect the deactivation from $^3Pt_2^*$ would occur with an increase in volume resulting in a positive ΔV^*_d in contrast to the negative values observed. Thus it appears likely that, as we proposed previously,[9c] compensating increases in other bonds, most likely the Pt-P bonds, give a $^3Pt_2^*$ with a total ($\bar{V}$) somewhat larger than that of the ground state. This suggestion has been recently confirmed by photoacoustic calorimetry techniques,[18] which indicated that $\Delta\bar{V}$ upon excitation of aqueous $Pt_2(POP)_4^{4-}$ to $^3Pt_2^*$ is +0.5 cm^3 mol^{-1}.[18]

The quenching of $^3Pt_2^*$ by O_2 undoubtedly occurs by energy transfer.[2] Since the rate in methanol at ambient pressure (k_q = 3.3×10^9 L mol^{-1} s^{-1}) is within an order of magnitude of diffusion limits in methanol (1.1×10^{10} L mol^{-1} s^{-1}),[19] the positive ΔV^*_q for oxygen quenching (+2.8 ± 0.5 cm^3 mol^{-1}) may simply reflect pressure-induced changes in methanol viscosity. The decreased diffusion rate is predicted from the Stokes-Einstein equation $D = k_BT/(4\pi\eta r)$, where D is the diffusion coefficient, η is the solvent viscosity, and r is the radius of a spherical particle.

Despite the increase in viscosity engendered by increased pressure, the quenching rates between $^3Pt_2^*$ and the organic substrates are enhanced by higher pressure, in direct contrast to quenching by O_2 (Table II). If one considers that the measured ΔV^*_q for the quenching process may be partially offset by a positive contribution due to increased viscosity, then the pressure effect on k_q is qualitatively even larger. These results are consistent with a significant amount of organization in the transition state and a mechanism involving the intimate association between the H atom donor R-H and the $^3Pt_2^*$. Activation enthalpies and entropies determined by Harvey[12] from the temperature dependence of the quenching show that the rates are largely governed by the negative ΔS^* for the reactions. Activation enthalpy and entropy values measured were +2.5 kcal mol^{-1} and -33 eu for isopropyl alcohol, +2.4 kcal mol^{-1} and -19 eu for benzyl alcohol, and +1.6 kcal mol^{-1} and -26 eu for α-methylbenzyl alcohol. The substantially negative ΔS^*_q values are consistent with the negative ΔV^*_q values for the latter two substrates and point to a significant amount of organization in the formation of the transition state.

For allyl alcohol, k_q approaches the diffusion-control limit. Therefore the very small ΔV^*_q of about -1 cm^3 mol^{-1} may be interpreted as a cancellation of two opposing contributions, a positive ΔV^* caused by the increase in viscosity and a negative one from the increased organization of the quenching reaction, although an alternative quenching mechanism such as addition of the double bond to a Pt center may be dominant. The quenching of $^3Pt_2^*$ by cyclohexene is much slower than the diffusion-control limit, so any positive contribution to ΔV^* would be smaller, and coordination of the olefin moiety to the axial site of the platinum dimer may be responsible for the ΔV^*_q of -7.6 cm^3 mol^{-1}. The small ΔV^*_q determined for Bu_3SnH may simply reflect the bulkiness of this substrate. This is certainly consistent with the results for the benzylic quenchers; the sterically bulkier quenchers gave less negative ΔV^*_q values.

In summary, a positive ΔV^* was observed for $^3Pt_2^*$ quenching by O_2, which almost certainly occurs by energy transfer. This effect is the probable result of pressure-induced viscosity changes in the solution. In contrast, ΔV^* values determined for quenching by the hydrogen atom donors benzyl alcohol and benzyl methyl ether are substantially negative. This implies a transition state formed by an associative interaction between the benzylic H atom donor and a site on the excited-state complex $^3Pt_2^*$, presumably one of the metal atoms. Such a mechanism would also be consistent with the strongly negative ΔS^* values determined previously[12] for similar reactions of $^3Pt_2^*$. The pressure effects are attenuated for the faster quencher allyl alcohol. While a less negative ΔV^* would be a likely consequence of an earlier transition state for H atom transfer, it may also reflect the compensating pressure effect on solvent viscosity for these reactions which more closely approach the diffusion limit.

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Steric Effects on Geminate Recombinations

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Abstract: Steric effects on the binding of isonitrile ligands to iron(II) porphyrins were investigated by picosecond flash photolysis. Two different types of steric effects were distinguished and characterized: (1) steric restrictions to porphyrin planarity and (2) blocking of the pathway for ligand approach. Heme planarity was restricted by coordinating 1,2-dimethylimidazole trans to the ligand binding site being investigated. Blocking of the binding site was explored by using adamantane heme 6,6-cyclophane, in which the adamantane moiety forms a "cap" over the binding site. Results of picosecond kinetic measurements demonstrate that the first effect, heme nonplanarity or "trans strain", influences the bond-making step, whereas the second effect, ligand blocking, involves a conformational preequilibrium prior to bond making. Relevance of these findings for contact pair recombination, in general, and for heme protein ligation, in particular, are discussed.

How chemical bonds are formed is a fundamental question that has attracted interest for decades and is being actively investigated today. Since the bond making itself is very fast, inferences drawn from mixing reagents can only be indirect. Consequently, "perturbation methods" are popular, in which one investigates the reforming of bonds following homolysis or heterolysis.

Geminate recombination of free radicals after homolytic photolysis or thermolysis was first considered[1] for simple diatomics

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like I_2. It has become a fundamental concept used to explain product yields, rearrangements, and viscosity effects in organic chemistry.[2] Equations 1 and 2 describe such observations.

$$\mathrm{R{-}R} \longrightarrow [\mathrm{R\bullet\ \bullet R}] \longrightarrow \mathrm{R{-}R} \quad (1)$$

$$[\mathrm{R\bullet\ \bullet R}] \longrightarrow 2\mathrm{R}\bullet \quad (2)$$

Similar effects should be expected for Lewis acid–base heterolysis and for inorganic as well as organic reactions. Some examples have been reported. For example, evidence for cage effects in the photosolvolysis of transition-metal coordination compounds was presented more than a decade ago.[3,4] These reactions may be described by eq 3. Noyes[5] distinguished primary and secondary

$$\mathrm{M \leftarrow L \rightleftarrows [M\ :L] \rightarrow M + :L} \quad (3)$$

geminate recombination of radicals. Later investigations[6] that focused on ionic heterolysis processes and sought to explain rearrangement products and special salt effects required such an elaboration to a four-state system but used the descriptive terms "contact ion pair" (or "intimate ion pair") and "solvent-separated ion pair", as illustrated by eq 4.

$$\mathrm{RX \rightleftarrows [R^+\ X^-]_c \rightleftarrows [R^+ \parallel X^-]_s \rightarrow R^+ + X^-} \quad (4)$$

The advance of flash photolysis and kinetic spectroscopy that accompanied the development of nanosecond, picosecond, and femtosecond[7] lasers now permits the direct observation in ordinary liquid solution[8] of "geminate pairs" or "cage partners" that previously were inferred from reaction products and the effects of scavengers or viscosity changes or, at best, were detected in rigid matrices at low temperatures. This has made it possible to distinguish the actual bond-making process in the primary recombination from the formation of encounter pairs, which contributes to secondary geminate recombination just as to bulk recombination. With this distinction experimentally available, it is possible to explore[9] the same kind of specific structural and medium effects upon the actual bond making as were formerly investigated for the overall reaction by using slower methods.

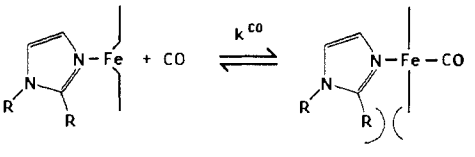
Meanwhile, geminate recombination has reappeared recently in other contexts. In particular, low-temperature studies of heme protein–CO complexes revealed multistep processes that were interpreted in terms of a sequence of forms of the iron–CO geminate pair.[10] The pertinence of such low-temperature investigations to physiology at ambient conditions has been discussed.[11] More recently, geminate recombination at ambient conditions has been used to explain transients observed in both picosecond[12-14] and nanosecond[15-18] measurements. A survey[19,20] of a number of protein variants and a variety of ligands, along with parallel studies on simpler model hemes,[21] brought together the picosecond and nanosecond measurements and revealed that a fast geminate recombination occurs in picoseconds in both proteins and model hemes, while a slow geminate recombination process occurs over hundreds of nanoseconds only in the protein systems. Three orders of magnitude separate the time scales, and little recombination, if any, was observed at intervening times. Consequently, we proposed[19] as a working hypothesis that the fast process was essentially identical with the previously described "contact pair" recombination, and the slow process was a "protein-separated" process, analogous in some ways to the earlier concept of recombination by a solvent-separated pair. We propose eq 5 for geminate recombination in heme proteins as the minimal approximation useful for studies of chemical mechanism. We

$$\mathrm{MbL \rightleftarrows [Mb\ L]_c \rightleftarrows [Mb \parallel L]_p \rightleftarrows Mb + L} \quad (5)$$

know that (5) is not the complete story of everything that happens after photolysis of heme proteins. There are fast processes occurring in the first few picoseconds that are not addressed explicitly in eq 5. These are said[22] to reveal steps involved in creating the ground-state five-coordinate species, which proceeds in parallel with some amount of recombination from excited electronic states. There is also evidence[18,23] that the nanosecond recombination from the protein-separated species is more complicated than can be explained fully with eq 5, at least in some cases. Tetrameric hemoglobin capable of allosteric behavior exhibits additional conformational changes that are not considered. Since the intermediates in eq 5 show spectral signatures that can often be correlated with those of previously known model systems (such as stable five-coordinate species), we have continued our study in order to distinguish the electronic, structural, and solvent-induced effects upon geminate recombination in these systems.

In this paper, we treat two kinds of steric effects, which we will call "strain" and "blocking". Such steric effects were previously investigated for the overall combination reactions; here we undertake to clarify the extent to which they affect bond making itself within the caged pair. We report measurements on simple, well-defined heme model systems and then comment briefly on the pertinence to proteins.

It is established already that the overall rates of ligation of a heme system decrease if the fifth ligand (trans to the binding site; "proximal" in the language of heme proteins) is bulky and presents steric strain to the planar heme structure.[24-27] An example is 2-*R*-imidazole. The overall rate constant k_{on} for bimolecular combination with CO is much slower for R = CH_3 than for R = H. This effect here is called a trans strain effect. It was referred to by H. C. Brown[28] as "B strain" or "back strain".



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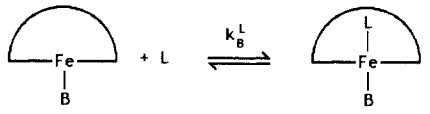
A second and quite distinct steric effect results from placing a large group directly in the path of the incoming ligand ("distal" to the binding site, in the language of heme proteins). This is well-illustrated when cyclophane heme compounds are studied.[29-34] The overall rate constant k_{on} can be reduced as much as 10^6-fold by this effect, which we call here the blocking effect.



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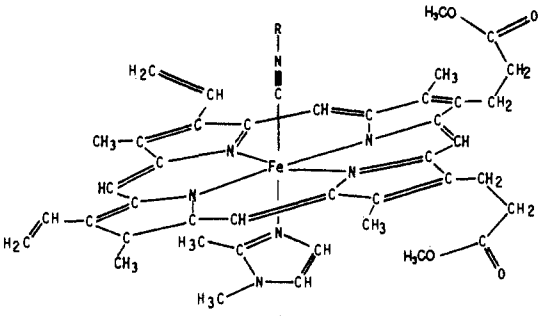
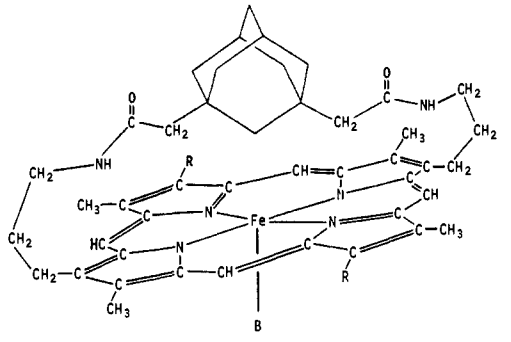
It is of some interest to determine where in the ligation process these effects are realized and how they change with details of the structure. In order to investigate this, we have studied the picosecond, nanosecond, and microsecond kinetics of ligation of isocyanides to both the back-strained protoheme dimethyl ester–1,2-dimethylimidazole complex **1** and the blocked adamantane heme 6,6-cyclophane–dicyclohexylimidazole complex **2**.



1



2

R = $CH_2CH_2COOCH_2Ph$

Experimental Procedures

Materials. Protoheme dimethyl ester (PHDME),[31] monochelated protoheme (MCPH),[31] and adamantane heme (1,3-adamantane-3,13-porphyrin-6,6-cyclophane)[30] were obtained from earlier studies. The alkyl isocyanides were also prepared by literature procedures[35] and were kept under argon below 0 °C to prevent degradation. Toluene was distilled from CaH_2 and stored over 4 Å molecular sieve. 1-Methylimidazole (1MeIm) (Aldrich) was distilled from sodium under reduced pressure (20 Torr). 1,2-Dimethylimidazole (DMI) (Aldrich) was used as received, with appropriate precautions taken on account of its hygroscopic properties. 1,5-Dicyclohexylimidazole (DCI)[33] was used with the cyclophane heme because its bulk greatly reduces the binding affinity on the distal (capped) side.

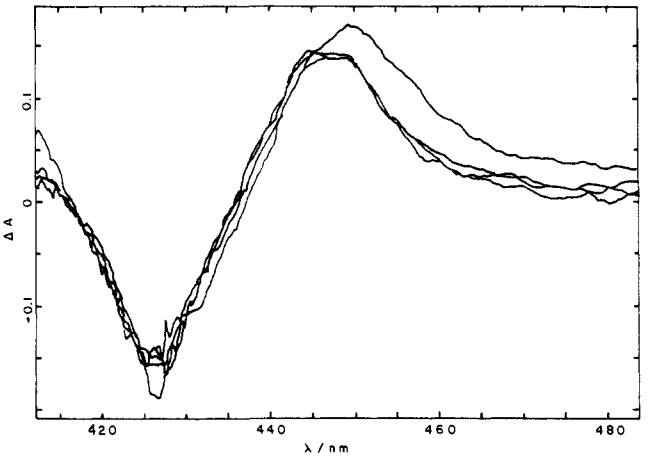


Figure 1. Picosecond transient absorption difference spectra for (1,2-dimethylimidazole)protoheme–dimethyl ester–CNMe in DMF, [DMI] = 2.3 M and [MeNC] = 4×10^{-3} M. Spectra recorded at 0 (top trace from 445 to 480 nm, lowest at 426 nm), 10, 67, and 500 ps. Relaxation to the ground-state, five-coordinate species within the contact pair is complete in less than 10 ps. No subsequent spectral changes attributable to geminate recombination are observed.

Preparation of Solutions. PHDME was dissolved in a minimum of CH_2Cl_2 and added to 4 mL of an 80/20 toluene/1 MeIm (v/v) solution until the absorbance of the Soret maximum was about 0.7 in a 2-mm cell. The concentration of the heme was approximately 1×10^{-5} M. The solution was then transferred to a tonometer where the oxygen was removed by several freeze–pump–thaw cycles. Argon was added, and the sample was then reduced from Fe^{3+} to Fe^{2+} by using either Zn/amalgam or by addition of about 1 μL of saturated solution of sodium dithionite-/18-crown-6/methanol.[36] When the reduction was carried out with Zn/amalgam, the solution was stirred overnight to ensure complete reduction. The reduced solutions were cannulated into a degassed 2-mm fused silica cuvette (Precision) for kinetic studies. The various isocyanides were injected into the cuvette by syringe to a final ligand concentration of $1-4 \times 10^{-3}$ M.

The adamantane heme samples were prepared in a fashion similar to the PHDME samples, except that the isocyanides were added prior to reduction and DCI was used in place of 1MeIm as the proximal base. The solvent was toluene with 0.4 M DCI added.

The MCPH samples in *N,N*-dimethylformamide (DMF) were prepared in a fashion similar to the PHDME samples. The DMF was used as received, from a freshly opened bottle. No external proximal ligand was added.

Instrumental Methods. The picosecond kinetic measurements were carried out by using an apparatus described elsewhere.[19] Briefly, a colliding-pulse mode-locked ring dye laser generates 70 fs pulses at 628 nm. These are amplified at 10 Hz in a four-stage dye amplifier pumped by a Nd-YAG laser. Frequency doubling generates 50 μJ pulses for photolysis, while the residual fundamental wavelength traverses a variable-length delay line before being focused into a continuum-generating cell to produce probe light in the Soret region. Photolysis and probe beams are reduced to about 1-mm diameter and carefully overlapped in a 2-mm path-length fused silica cell. The transmitted probe continuum is dispersed in a small spectrograph, and the spectrum was recorded with a SIT vidicon. A reference spectrum is recorded simultaneously. The cell is translated to minimize artifacts that might arise from irreversible photolysis. From 200 to 1000 laser shots are summed at each time delay. Overall time resolution is better than 1 ps.

The apparatus used to measure the overall "on" rates was also described previously.[19] An excimer-pumped dye laser generates photolysis pulses of about 2 mJ at 540 nm. The continuous probe light passes through monochromators before and after sample. The photomultiplier output is digitized with a Biomation 805 or a Biomation 6500 transient recorder. Each digital record is transfered to a small microcomputer for accumulation. Typically, a kinetic measurement sums 200 laser shots at 1-Hz repetition rate. Bimolecular combination rates are determined from the slope of a plot of the observed combination rate vs ligand

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concentration, over a range in which ligand concentration is in sufficient excess to assure pseudo-first-order kinetics.

Conventional UV-visible spectra were recorded before and after each experiment by using a Kontron 810 spectrophotometer to guard against sample degradation.

Results and Discussion

There is evidence from ultrafast Raman spectroscopy[22,37] as well as these picosecond transient absorption studies that the contact pair recombination of a heme (at least after a few picoseconds) involves the ground-state five-coordinate heme. We will provide further analysis elsewhere. Meanwhile, we assume it to be the case for the following considerations.

Strain. Consider first the effects of heme nonplanarity, which involves trans strain. Figure 1 shows picosecond transient difference spectra for the photolysis of the PHDME–DMI–MeNC complex **1**. The DMI coordinated opposite the ligand binding site of interest pulls the iron out of plane to produce a strained heme that was characterized previously[24-26] and proposed as a model for T-state proteins. In the difference spectra, the prominent decrease in absorption at 426 nm marks the disappearance of the six-coordinate liganded species upon photolysis. The five-coordinate deliganded species is expected to show a transient increase in absorption centered near 445 nm, and that appears also in Figure 1. There may be a slight recovery of absorption (decrease in bleaching) in the six-coordinate region over the first few picoseconds, which is accompanied by a decrease in transient absorption over a broad band centered near 460 nm. Petrich and Martin[22] have interpreted spectral changes occurring in the first few picoseconds as evidence for such an "excited-state recombination". However, this effect, if present at all, is quite small, and there is no further recombination to form the ligated species over the subsequent tens to hundreds of picoseconds. This failure of the geminate pair to recombine was confirmed for two different solvents, toluene and DMF. The lack of recombination from the ground state of the five-coordinate contact pair for the strained system is in striking contrast to the previously reported[21] unstrained 1MeIm complex, which showed substantial recombination from the contact pair; a total of 66% recombination to form the six-coordinate ground state with an observed rate of 1.1×10^{11} s^{-1}.

Strained and unstrained systems differ also in their overall, bimolecular combination rates. Bimolecular combination rates were measured by flash photolysis for the combination of MeNC (at concentrations from 5×10^{-4}–2×10^{-2} M) with strained DMI–PHDME in toluene ([DMI] = 0.1 M]). The bimolecular "on" rate was $k_{on} = 1.4 \times 10^7$ M^{-1} s^{-1}, which is to be compared with the published[38] value for the unstrained analogue of $k_{on} = 1.4 \times 10^8$ M^{-1} s^{-1}, a factor of ten greater. The bimolecular kinetics were also measured by using DMF as solvent. For unstrained MCPH–MeNC in DMF, $k_{on} = 2.7 \times 10^7$ M^{-1} s^{-1}; for strained DMI–PHDME–MeNC in DMF, $k_{on} = 1.2 \times 10^6$ M^{-1} s^{-1}. This represents a decrease in k_{on} by a ratio of about 20-fold when DMI is substituted for 1MeIm in order to induce trans strain. Similar strain effects on carbon monoxide bimolecular binding rates have been reported.[24] The fact that overall rates are lower in DMF for both strained and unstrained systems suggests that formation of the contact pair must be slower in DMF either because DMF may coordinate weakly with the iron or because the energy cost of desolvating the ligand is greater in DMF.

This change in geminate recombination efficiency in the presence of trans strain is well understood in terms of the three state model of eq 8. The simplest and quite plausible explanation

$$\mathrm{HmL} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\mathrm{Hm\ \ L}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{Hm + L} \qquad (8)$$

for the absence of contact pair return in the strained system is

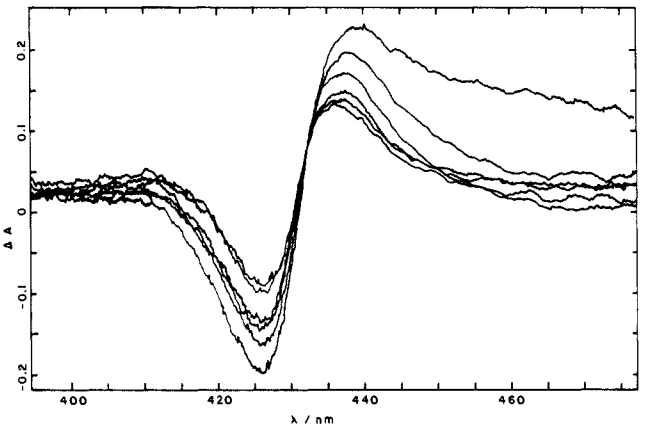

Figure 2. Picosecond transient absorption difference spectra for (dicyclohexylimidazole)adamantane 6,6-heme cyclophane–CNBu^t in toluene, [DCI] = 0.25 M and [Bu^{t}NC] = 5×10^{-3} M. Spectra recorded at times of 0, 2, 5, 10, 17, and 62 ps. Absorbance at 438 nm decreases with increasing time. The spectral change at 460 nm is over quickly as "excited-state" processes are completed. The decrease at 440 nm marks the disappearance of the five-coordinate ground-state species; and the increase at 426 nm marks the reappearance of the six-coordinate heme, both occurring over 20 ps within the contact geminate pair.

Table I. Geminate and Bulk Rate Constants for the Reaction of Alkyl Isocyanides with Model Hemes and Sperm Whale Myoglobin at 20 °C[a]

compound[b]	k_{-1}[c] (s^{-1})	k_2[e] (s^{-1})	k_{-2}[d] (M^{-1} s^{-1})	k_{on} (M^{-1} s^{-1})
adamantane–CNMe	3.3×10^{10}	5.3×10^{10}	5.0×10^5	1.9×10^5[e]
adamantane–CNBu^t	5.5×10^{10}	6.3×10^{10}	4.5×10^5	2.1×10^5[e]
PHDME–CNMe	7.5×10^{10}	3.8×10^{10}	3.0×10^8	2.0×10^8[f]
PHDME–CNBu^t	7.6×10^{10}	4.2×10^{10}	2.2×10^8	1.4×10^8[f]
SWMb–CNMe	1.3×10^{10}	4.0×10^{10}	[g]	[g]
SWMb/CNBu^t	2.6×10^{10}	8.0×10^9	[g]	[g]

[a] Calculated from the observed picosecond decay rate, $k_{gem} = k_{-1} + k_2$, and the observed fractional escape from the contact pair measured at a few hundred picoseconds, $\Gamma = k_2/(k_{-1} + k_2)$.[19] [b] Solvent composition: PHDME, 80/20 toluene/1 MeIm (v/v); adamantane, toluene with 0.25–0.4 M DCI SWMb, bis-tris buffer, ph 7. [c] Precision: ±25%. PHDME data from ref 21; SWMb data from ref 19. [d] Derived from $k_{on} = k_{-2}k_{-1}/(k_{-1} + k_2)$. [e] Precision: ±10%. [f] From ref 38. [g] Entry not pertinent, because the protein-separated pair intervenes.

a marked reduction in k_{-1}. Such a change in k_{-1} is also consistent with measurements of the overall combination rates observed for the two species. The effective overall rate constant for combination is calculated from eq 8 to be $k_{on} = k_{-2}k_{-1}/(k_{-1} + k_2)$. Any change between strained and unstrained systems should depend almost exclusively upon k_{-1}. Since the same ligand is involved in the strained and unstrained cases, k_2 and k_{-2} should be almost unchanged. A decrease in k_{-1} by a factor of 10 or 20 would reduce the fraction of geminate return from the 0.66 measured for the unstrained system to about 0.03–0.06 in the strained system. Such a miniscule amount of recombination is below the detection limit of picosecond measurements, in agreement with our observations.

These results on geminate binding in strained heme provide strong evidence that steric effects that change heme planarity affect specifically the bond-forming step. As has been noted previously,[24-26] they also affect the bond-breaking transition state.

Blocking. Consider next the effect of blocking access to the ligand binding site. Table I collects together rate constants for the adamantane cyclophane heme measured in this study along with previously recorded data for unblocked analogues. Results are shown for both methyl isocyanide and the bulkier *tert*-butyl isocyanide as ligands. Also shown for comparison are rate constants for bond forming and bond breaking of the same ligands with sperm whale myoglobin. The picosecond rates come from the analysis of transient spectra, a typical example of which is shown in Figure 2. The overall combination rates come from measuring bimolecular association following nanosecond flash

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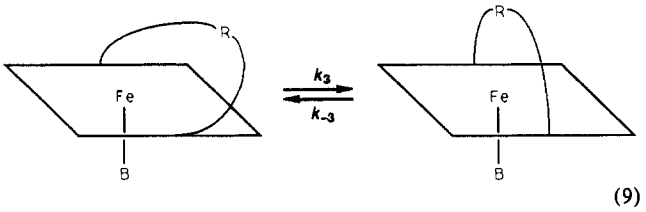
photolysis of solutions having ligand concentrations ranging from 10^{-3} to 0.2 M. Kinetic traces were recorded to observe both the recovery of the six-coordinate species and the disappearance of the five-coordinate transient.

The data in Table I show that blocking reduces the overall bimolecular k_{on} by a factor of 1000, and yet the geminate bond-formation rate constant k_{-1} is reduced by a factor near 2. The discrepancy must be attributed, within the model described by eq 8, to a large reduction in k_{-2}, which is the rate constant that characterizes the formation of the encounter complex. It is especially noteworthy that in spite of the large reduction in k_{on} there is no steric discrimination on the basis of the size of the entering ligand. All this constitutes a direct observation on the picosecond time scale of the predictions for the contact pair that were stated clearly some time ago, following a study of the variation of equilibrium constants and overall kinetic rate constants for a variety of model systems.[33]

Blocking in cyclophane hemes is related to, but certainly not identical with, the classical frontal strain effect. F-strain effects on metal ligation have been discussed in terms of a "cone angle" that describes van der Waals contacts for an entering ligand.[39] Blocking in cyclophane hemes differs in important details from situations that can be described by "cones" of access. According to the crystal structure,[30] the adamantane group in **2** is held directly over the ring in the unliganded system preventing any binding at all. The only way binding can occur is through a conformational change that opens this "gate". NMR studies have been interpreted as suggesting that the adamantane group flips back and forth at an unknown rate between two conformations in which it has moved over to the edge of the porphyrin.[33]



(9)

Molecular models indicate that this would "open the gate". The very similar bond making rate constants shown in Table I for k_{-1}, independent of ligand size for *t*-BuNC and MeNC, suggest that the adamantane heme has either a "fully open gate" or a "fully closed gate".

Still one might expect that even with a fully open gate, the structure is somewhat strained with bulky *t*-BuNC bound. This expectation is supported by the finding[30] that the methyl groups on the bound *t*-BuNC have different chemical shifts in the NMR spectrum. Furthermore, the overall dissociation rate constant (inferred from k_{on} and previously measured[30] equilibrium constants) for *t*-BuNC increases to 7 s^{-1} in the adamantane heme from about 1 s^{-1} in the unblocked MCPH analogue. However, these are modest changes compared to the great change in k_{on}.

The fact that the rate constants for bond formation k_{-1} and the efficiency for geminate return are changed little for either of two quite different sized ligands upon introduction of the blocking adamantane group provides strong evidence that the picosecond intermediate, the geminate pair, is a contact pair in which little or no translation or rotation of the ligand has occurred. If the conformation of the adamantane cap were to move to its thermodynamically most stable state, similar to its position in the crystal structure, after photolysis and before geminate return, and this were to account entirely for the factor of 1000 change in the k_{on} rates, then the k_{-1} rate constants would be reduced by 1000, and geminate return would become too small to measure. Since that does not happen, it is clear that the geminate return is faster than the conformational changes that lead to blocking. Consequently, the overall reaction scheme can be represented as a four-state system by combining eqs 8 and 9:

$$\text{Ad*L} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [\text{Ad*}\ \ \text{L}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Ad*} + \text{L} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \text{Ad} + \text{L} \quad (10)$$

In this scheme, Ad* is the open state, which may or may not be ligated, while Ad is the closed state, which is never ligated.

The rate of ligation for the overall binding reaction of the four-state scheme would be given in full by

$$k_{obs} = k_{-3}\ \text{Ad} \times \frac{k_{-2}\text{L}}{k_{-2}\text{L} + k_3} \times \frac{k_{-1}}{k_{-1} + k_2} \quad (11)$$

Since k_{-1} and k_2 refer to the contact pair in which the ligand is essentially in van der Waals contact with iron, the finding that k_{-1} and k_2 both resemble the values for unhindered (unblocked) heme systems is not surprising.

One additional prediction of the model is that at concentrations of L sufficiently high that $k_{-2}\text{L} \gg k_3$ one should observe overall combinations rates that are independent of ligand concentration. That is, formation of the open conformation should become rate-limiting. We have not achieved that limit within the range of concentrations that were accessible, [L] < 0.25 M. (At high concentrations the ligand begins to compete for the proximal binding site.) Consequently, we cannot at this time specify k_{-2} in mechanism 10 and compare it to the analogous process in an unhindered binding reaction. What has been measured and reported in Table I is an "effective" $k'_{-2} = k_{-2}k_{-3}/k_3 = 5 \times 10^5\ M^{-1}\ s^{-1}$, compared to a value of $k_{-2} = 2 \times 10^8\ M^{-1}\ s^{-1}$ for the simple unhindered system. The 1000-fold steric effect, therefore, occurs in the assembly of the contact pair. Under our conditions, this appears to be predominately in the factor k_{-3}/k_3, which characterizes the conformational equilibrium. One would expect k_{-2} for Ad* to be somewhat less than in an unhindered system. However, if such an effect on k_{-2} were large, then one ought to find a large difference between MeNC and *t*-BuNC, contrary to fact.

Recent low-temperature kinetic studies of overall carbon monoxide recombination to other hindered cyclophane compounds by Tetreau and co-workers[40] have revealed two processes, in striking contrast to the single process they observed for unhindered systems. It is possible that their low-temperature result corresponds to a situation in which the conformational change characterized by k_{-3} has become rate-limiting.

Relevance to Heme Proteins. Isolating the contact geminate pair by means of ultrafast flash photolysis and systematically investigating its properties provides increased confidence in models developed earlier to explain correlations between protein functional behavior and the equilibrium and overall kinetic properties of simple model compounds that are constructed to illustrate one aspect at a time of protein control mechanisms.

That trans strain, illustrated by DMI–PHDME, is the dominant feature distinguishing R and T conformations in tetrameric hemoglobin was argued earlier[24,25,31] and related to a large prior literature. It is useful to point out additional arguments that have been made recently[41] based on static and time-resolved Raman spectra, which offer quite direct evidence about chemical bonding. The present study confirms that these trans, or proximal, effects do involve the bond-forming process itself.

Blocking in cyclophane hemes is relevant to understanding distal side steric effects, according to which amino acid side groups in the heme pocket hinder ligand binding by blocking the binding site, perhaps even to such an extent as to "bend" the iron–ligand bond. Our previous picosecond kinetic studies showed that contact-pair return in myoglobin,[19] which is a hindered protein having blocking groups near the binding site, is very similar to that found in simple, flat, unhindered hemes.[21] The present study shows that the same contact pair return also characterizes blocked cyclophane heme models. As summarized in Table I, all three categories show little variation (no more than factors of two or three) in the

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bond-making step itself based on the size of the ligand.

Previous studies showed[26,23] that large distal side steric effects in proteins and in heme cyclophanes do not appreciably change dissociation rates k_1 for either CO or RNC. We now find that bond formation k_{-1} is also unaffected. These conclusions differ only slightly, but significantly, from the interpretation of steric effects based upon crystal structures and low-temperature kinetic studies.[10] The steric effect in adamantane heme is related to the steric effect in converting the protein-separated geminate pair to the contact pair. This process requires moving the adamantane moiety in the model and moving the distal imidazole (or other blocking group) in myoglobin. Although we suggest that the largest steric effect in heme proteins is on entry into the protein (assembly of the protein-separated pair [Hm ‖ L] from distinct-solvated species), we find evidence for further steric effects in the process which converts the protein-separated pair to the contact pair [Hm L]. This interpretation is consistent with the general four-state model of heme protein ligation proposed by Frauenfelder et al.[10] as well as the trajectory calculations of Karplus and Case.[42]

Previous studies demonstrated that the steric effects on binding dioxygen and carbon monoxide to adamantane heme cyclophane and other cyclophanes are essentially identical as judged by changes in association rates.[27,29] Recent studies of the kinetics of dioxygen and carbon monoxide binding to mutants of myoglobin in which the distal blocking group was changed[43] also demonstrate the same effects for each mutation, within a factor of about two, on rates of binding the two diatomic ligands. These similarities in model and myoglobin behavior add further evidence that there is a "local steric effect" operating near the iron in myoglobin. This steric effect could differentiate ligands on the basis of their size or shape, since they must fit into the contact pair space, but not on the basis of the angle of the Fe–X–Y bound state, because the steric effect operates before the bonding step. We, therefore, conclude that distal steric effects are identical for the diatomic ligands CO, NO, and O_2 in all the steps leading to ligation. The current results, taken together with previous studies, therefore, provide strong evidence that CO and O_2 are differentiated in their binding to model compounds and to heme proteins by polar effects (or hydrogen bonding effects) on dioxygen dissociation rates.

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Conclusion

Subpicosecond laser flash photolysis of two very different types of sterically hindered heme model compounds and measurement of geminate recombination from the resulting heme ligand contact pairs allow us to draw the following conclusions about steric effects on the geminate recombination process and on overall ligand binding: trans strain, which distorts heme planarity, reduces geminate recombination by an amount comparable to changes in overall combination rates, whereas blocking (frontal) steric effects, which do not alter heme planarity, have little effect on geminate return. Since blocking does exert tremendous influence on the overall combination rate, we conclude that blocking affects the assembly of the contact pair rather than bond making. Ligands of different size are not distinguished much in the geminate return (bond-forming) process; size discrimination occurs in the assembly of the contact pair. These conclusions should apply broadly to all metalloporphyrins and heme complexes; we conjecture that they will apply to some other metal complexes, particularly those that have limited fluxional mobility, and may be pertinent to free-radical geminate processes as well.

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Mechanism of Ligand Binding to Hemes and Hemoproteins. A High-Pressure Study

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Abstract: The effect of pressure on the recombination kinetics of small ligands binding to sperm whale myoglobin, protoheme dimethyl ester, and monochelated protoheme was studied with use of laser flash photolysis. The volumes of activation observed indicate that in both the protein and the models bond formation is the rate-determining step only for carbon monoxide, while for oxygen, isocyanides, and 1-methylimidazole almost no bond formation occurs in the transition state of the observed reaction. The effect of pressure on the escape of carbon monoxide, oxygen, and methyl isocyanide from the heme pocket of sperm whale myoglobin was also investigated. The volume increase observed for all ligands during this process is attributed to a "gatelike" conformational change in the protein. The results are discussed in terms of the previously proposed three- and four-state reaction schemes for model hemes and myoglobin, respectively.

Introduction

The mechanistic understanding of the binding of small neutral molecules to ferrous hemes and hemoproteins has attracted significant attention in recent years.[1-7] Model complexes are usually employed to improve our understanding of the reactions of the corresponding proteins. The application of picosecond and nanosecond laser flash photolysis techniques[8] has suggested that in

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