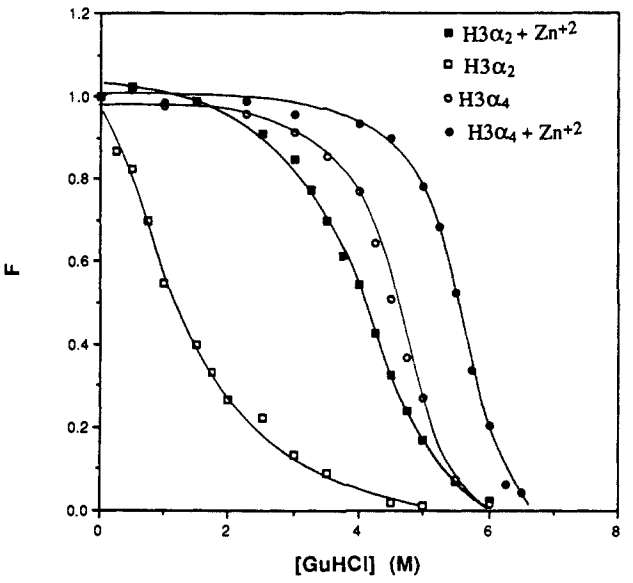

Figure 3. Fraction of initial ellipticity at 222 nm, (F), as a function guanidine hydrochloride concentration for 5.0 μM H3α_2 and H3α_4 in the presence and absence of 1.0 mM $ZnCl_2$.

Table I. Free Energy of Association of the Proteins in 3.0 M Guanidine Hydrochloride (5.0 μM in the Presence and Absence of 1.0 mM $ZnCl_2$)[a]

	midpoint (M)		ΔG_0 (kcal/mol)		
peptide	$-Zn^{+2}$	$+Zn^{+2}$	$-Zn^{+2}$	$+Zn^{+2}$	$\Delta\Delta G$
α_2	4.5	4.5	9.9	9.9	0.0
H2α_2	2.9	4.0	7.0	9.0	1.0
H3α_2	1.3	4.1	4.9	8.7	1.9
H3α_4	4.4	5.6	10.5	13.3	2.8

[a] ΔG_0 was calculated at various concentrations of guanidine hydrochloride as described previously[2] and the data for each peptide were extrapolated or interpolated to 3.0 M guanidine hydrochloride. This guanidine concentration was chosen to minimize errors associated with lengthy extrapolations. However, similar results were obtained when the data were extrapolated to 0 M guanidine hydrochloride. Midpoint refers to the concentration of guanidine·HCl at which the ellipticity at 222 nm was half that observed in its absence.

multiple, slowly exchanging complexes with similar stabilities are formed.

The conformational stability of all three peptides increased upon addition of Zn^{2+}. Figure 3 illustrates the variation in θ_{222} (a measure of helical content) of H3α_2 and H3α_4 as a function of guanidine hydrochloride concentration. In the absence of Zn^{2+}, the H3α_2 dimer is less stable than α_2 (Table I). This is consistent with our expectation that substitution of a polar His residue for Leu at position 7 of the helices (largely buried in models) would destabilize the folding of H3α_2. In the presence of 1.0 mM Zn^{2+} H3α_2 shows a large increase in stability, with a corresponding decrease in the free energy of dimerization of -1.9 kcal/(mol of bound Zn^{2+}) (Table I). In contrast, α_2 showed no change in stability in the presence of 1.0 mM Zn^{2+} (Table I), providing further evidence that the substituted His residues were essential for binding. In the absence of Zn^{2+}, the stability of H2α_2 was intermediate between H3α_2 and α_2, consistent with the fact that only one Leu was changed to His in H2α_2 (Table I). In the presence of Zn^{2+}, the protein showed increased stability, but the enhancement was about half that for H3α_2. Finally, H3α_4 unfolded at considerably higher guanidine concentrations (Figure 3) and showed an increase in stability of -2.8 kcal/mol in the presence of 1.0 mM Zn^{2+} (Table I).

These data establish the feasibility of designing metalloproteins and represent a significant step toward the de novo design of catalytically active proteins. As expected from previous work on monomeric helices bearing His residues separated by a single turn of α-helix,[6] H2α_2 bound Zn^{2+}, although the complex was not unique nor was the increase in stability as great as for H3α_2. Thus, it appears that all three His residues complex Zn^{2+} in H3α_2 and H3α_4. We are currently determining their specificity and affinity for other ions.

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Direct Evidence for an Equilibrium between Early Photolysis Intermediates of Rhodopsin

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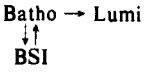
Recently we showed that bathorhodopsin (Batho) produced by photolysis of rhodopsin does not decay directly to lumirhodopsin (Lumi), but rather forms a blue-shifted intermediate (BSI), which then decays to Lumi.[1] BSI was first observed in artificial visual pigments,[2,3] and time-resolved spectral studies later confirmed the existence of this photolysis intermediate in native rhodopsin as well.[1] The temperature dependence[1] and linear dichroism[4] of the time-resolved spectra were shown to be best fit by a mechanism involving an equilibrium between Batho and BSI (mechanism b in Figure 1[5]). In this communication we present more direct evidence of this equilibrium mechanism that excludes the simple sequential path (mechanism a in Figure 1).

Figure 1 shows the time evolution of the Batho, BSI, and Lumi species implied by a sequential decay mechanism, with equilibrium, following rhodopsin photolysis. While the kinetics of the Batho and BSI concentrations will depend somewhat on the equilibrium constant in mechanism b, spectral overlap of different intermediates makes detecting such subtle kinetic differences after excitation with a single laser pulse difficult. A more practical way to discriminate between mechanisms a and b is to remove part of the Batho, formed by an initial laser excitation of rhodopsin, with a second laser pulse that is absorbed only by Batho and to then monitor the subsequent kinetics at a wavelength only absorbed by Batho. Thus, also shown in Figure 1 are the Batho time evolutions, assuming either mechanism a or b, that would occur following photolysis of this intermeiate by a second light pulse coming 93 ns after the initial photolysis pulse. For mechanism a, photolysis of Batho would result in a reduction of Batho concentration followed by continued exponential decay. However, if an equilibrium exists between Batho and BSI, the kinetics of Batho decay would differ. Waiting for 93 ns after the initial rhodopsin excitation allows a significant amount of BSI to form. If an equilibrium mechanism is valid, this BSI will back-react to form more Batho after the second photolysis pulse. Thus, after Batho photolysis there will be an initial rise in Batho concentration followed by an exponential decay. The difference in kinetics following double excitation provides a means to discriminate between these two mechanisms.

In the experiment reported here excitation of rhodopsin by a 0.5-mJ, 7-ns pulse of 532-nm light formed Batho, which was detected by using absorbance measured at 620 nm.[6] After 93

(1) Hug, S. J.; Lewis, J. W.; Einterz, C. M.; Thorgeirsson, T. E.; Kliger, D. S. *Biochemistry* **1990**, *29*, 1475-1485.
(2) Albeck, A.; Friedman, N.; Ottolenghi, M.; Sheves, M.; Einterz, C. M.; Hug, S. J.; Lewis, J. W.; Kliger, D. S. *Biophys. J.* **1989**, *55*, 233-241.
(3) Einterz, C. M.; Hug, S. J.; Lewis, J. W.; Kliger, D. S. *Biochemistry* **1990**, *29*, 1485-1491.
(4) Lewis, J. W.; Einterz, C. M.; Hug, S. J.; Kliger, D. S. *Biophys. J.* **1989**, *56*, 1101-1111.
(5) An alternative mechanism



had been shown to be inconsistent with the time-dependent spectral data.[1]

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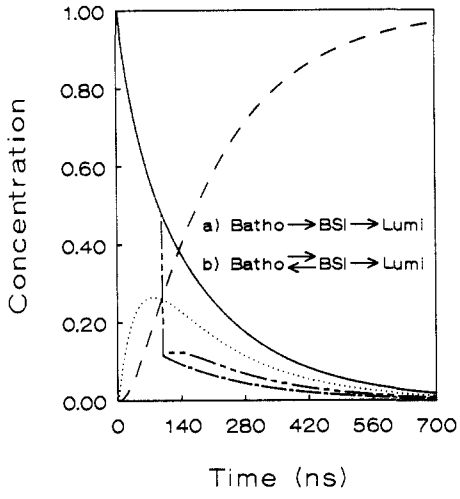

Figure 1. Concentrations of Batho (—), BSI (---), and Lumi (--) predicted by the equilibrium mechanism b. If 75% of the Batho remaining after 93 ns is removed, curves (-·-) and (-···-) describe the resulting Batho concentration predicted by the sequential (a) and equilibrium (b) mechanisms, respectively.

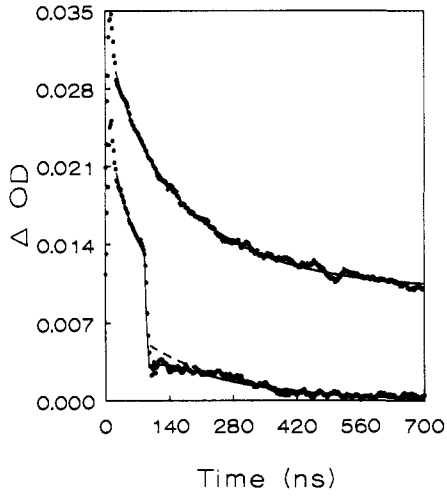

Figure 2. Optical density changes observed at 620 nm. The upper data points (offset 0.01 OD for clarity) show the result of 532-nm photolysis by itself. The line shows the fit using the equilibrium mechanism a with apparent lifetimes of 40 and 190 ns with $K_{eq} = 1.4$. The lower data points are the result of 532-nm photolysis at $t = 0$ followed by 605-nm photolysis 93 ± 3 ns later. (The result of photolysis by 605-nm light alone has been subtracted in order to remove a scattered actinic light artifact.) Using the equilibrium mechanism and assuming 76% photolysis of Batho produces the solid line fit to the data. The dashed line shows the fit assuming the simple sequential mechanism a and 60% photolysis of Batho. This percent was determined by fitting the data starting 93 ns after 605-nm photolysis to an exponential with lifetime 190 ns.

± 3 ns, a 5-mJ, 7-ns pulse of 605-nm light was used to photolyze the remaining Batho. Samples[7] contained 66% glycerol to reduce rotational diffusion and thus increase efficiency of Batho photolysis.

As shown in Figure 2 (top) the data for 532-nm photolysis by itself is extremely well fit by the parameters previously determined from global analysis of spectra taken at different times following rhodopsin photolysis.[1,4] The predictions of the two mechanisms for the two-laser experiment differ significantly. The superior agreement of the kinetic data with the prediction of the equilibrium mechanism demonstrates the existence of back-flow from BSI to Batho, supporting the conclusion that had previously been based on indirect evidence. This raises the question of what structural features in the chromophore/protein pocket environment could be responsible for such a reversible reaction. The answer to this question will require considerably more structural information to be obtained on the new BSI intermediate.

Acknowledgment. This research was supported by Grant EY00983 from the National Eye Institute of the National Institutes of Health.

(6) Einterz, C. M.; Lewis, J. W.; Kliger, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 3699-3703. Pulses were produced by the second harmonic of a Nd-YAG laser (532 nm) and a dye laser pumped by a second Nd-YAG laser (605 nm). Both were vertically polarized and the probe beam was polarized at the magic angle (54.7°) to avoid kinetic artifacts due to rotational diffusion. The small optical density changes observed make the rotational diffusion effects on absorbance changes negligible under these conditions.

(7) Detergent suspensions of bovine rhodopsin in 2% octyl β-D-glucoside were prepared as in ref 1. The concentration here was 1 mg of rhodopsin/mL.

The Reactions of CF with Aromatics

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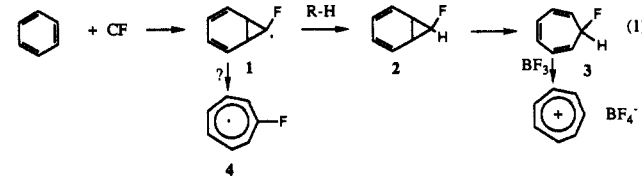
The reaction of C atoms with fluorocarbons gives the monovalent carbon species CF, which can be trapped by addition to alkenes to give a fluorocyclopropyl radical.[1] Subsequent abstraction of hydrogen produces a fluorocyclopropane.[1] We now report evidence that reaction of CF with aromatic compounds gives 7-fluoronorcaradienyl radicals which show some interesting reactions.

When arc-generated carbon and CF_4 are condensed with benzene at 77 K, the predominant fluorine-containing product is fluorobenzene. However, when CF is reacted with benzene in the presence of isobutane as a hydrogen donor and the product treated with BF_3, tropylium fluoroborate is also a product (eq 1, $C_7H_7^+BF4^-$:$C_6H_5F = 5.7$).[2] This product is consistent with the addition of CF to the aromatic ring to generate a 7-fluoronorcaradienyl radical, **1**, which abstracts hydrogen to give 7-fluoronorcaradiene (**2**),[3a] which then rapidly ring opens to 7-fluorocycloheptatriene (**3**). Subsequent reaction of **3** with BF_3 generates the tropylium fluoroborate.



An interesting question in this system is whether **1** undergoes electrocyclic ring opening to the fluorotropyl radical (**4**). In the reaction of CF with alkenes to give fluorocyclopropyl radicals, we have not observed ring opening to allyl radicals.[1] This electrocyclic ring opening is symmetry forbidden and is generally not observed for cyclopropyl radicals in the absence of a large thermodynamic driving force.[4] However, the ring opening of norcaradienyl radicals should have a large thermodynamic driving force and will not have the symmetry-imposed barrier present in the cyclopropyl radicals. Despite these considerations, several studies of benzonorcaradienyl radicals show that they require high

(1) Rahman, M.; McKee, M. L.; Shevlin, P. B. *J. Am. Chem. Soc.* **1986**, *108*, 6296.

(2) In a typical reaction, volatile organics (15 mmol each) are mixed on a vacuum line and cocondensed with arc-generated C vapor at 77 K. Products are generally characterized by ^{19}F NMR at 376 MHz. Although product yields calculated from the amount of C lost by the graphite rods are on the order of 1%, they are in fact higher than this as much of the carbon is lost from the rods in large chunks.

(3) (a) A consideration of bond dissociation energies[3b] indicates that this abstraction should be exothermic by 9 kcal/mol. (b) Smart, B. E. In *Molecular Structure and Energetics*; Liebman, J. F., Greenberg, A., Eds.; VCH Publishers: New York, 1986; Vol. 3, pp 141-191.

(4) Sustmann, S.; Ruchardt, C. *Chem. Ber.* **1975**, *108*, 3043.

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