Acknowledgment. This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. We thank Shin-Etsu Chemical Co. Ltd. for a gift of chlorosilanes.

Supplementary Material Available: Typical experimental procedures for the reactions of **1a** with CO and **9** with CO and spectral data for **5a,b**, **6a,b**, **12**, and **13** (3 pages). Ordering information is given on any current masthead page.

(13) The reaction of (1-*tert*-butylvinyl)lithium with CO in THF was relatively sluggish, presumably due to the proximate steric effect, and did not afford products similar to **5** and **6** but gave an enediol disilyl ether arising from intermolecular reaction with incorporation of two molecules of CO. It has been reported that the reaction of unsubstituted vinyllithium with CO gave polymeric product, see: Sawa, Y.; Miki, T.; Ryang, M.; Tsutsumi, S. *Technol. Rep. Osaka Univ.* **1963**, *13*, No. 561.

Confirmation of the Secondary Deuterium Isotope Effect for the Peptidyl Prolyl Cis–Trans Isomerase Activity of Cyclophilin by a Competitive, Double-Label Technique

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Cyclophilin, the binding protein for the immunosuppressive drug cyclosporin, has recently been shown to catalyze the cis–trans isomerization of proline imide bonds in peptides and proteins.[1,2] Since these initial reports, there has been intense interest in determining the link between the immunoregulatory and isomerase activity that this protein posses. The ability to create potent immunosuppressants based on cyclophilin's isomerase activity is an attractive target for the rational design of immunosuppressive drugs. Critical to rational design is an understanding of the mechanism of action of the isomerase activity. In attempts to definitively characterize the mechanism of this enzyme, two groups have determined the secondary β-deuterium (β-D) kinetic isotope effect (defined as the ratio of rate constants, k_H/k_D, and abbreviated as ^Dk) for the cis-to-trans isomerization of Suc-Ala-Gly-(L,L)-*cis*-Pro-Phe-pNA (L = H, D). While Fischer and his co-workers[3] report a β-D effect of 0.91 and claim formation of a tetrahedral intermediate in the transition state, we report an effect of 1.13[4] and claim catalysis by distortion. A clear distinction between the two is imperative if successful rational inhibitor design is to be realized.

To distinguish the two mechanisms, we have used a competitive double-label technique.[5] To apply this technique, isotopically substituted substrates are labeled with a second, distinct reporting group. In the present case, the ^{14}C- and ^{3}H-methyl esters of Suc-Ala-Gly-Pro-Phe-pNA and Suc-Ala-Gly-(D,D)-Pro-Phe-pNA, respectively, were used. The isotope effect is then calculated from the dependence of the $^{14}C/^{3}H$ ratio of a mixture of the substrates on the fraction of reaction.

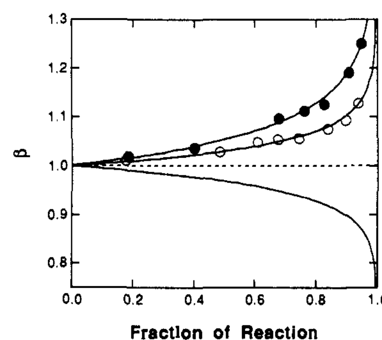

Figure 1. The dependence of β on the fraction of reaction for the nonenzymatic (open circles) and cyclophilin-catalyzed (filled circles) isomerization of MeOSuc-Ala-Gly-(L,L)-Pro-Phe-pNA. The lines through the data are the nonlinear least-squares fit to eq 3. The line in the bottom half of the figure was drawn according to eq 3 with $\alpha = 1.047$. Methyl esters of the peptide Suc-Ala-Gly-(L,L)-Pro-Phe-pNA (L = H, D; Bachem) were prepared by reaction with diazomethane and radiolabeled methyl iodide. Specific activities were as follows: $H_3{}^{14}CO$-Suc-Ala-Gly-Pro-Phe-pNA, 80 μCi/mg; ^3H_3CO-Suc-Ala-Gly-(D,D)-Pro-Phe-pNA, 108 μCi/mg. Cyclophilin from calf thymus was purified by the method of Harding et al.[7] and kindly supplied to us by Dr. John Siekierka (Immunology Department, Merck Research Laboratories). Determination of active-site concentration was reported elsewhere[6] and was 56 nM in these experiments. For isotope-effect determinations, 1.2 mg/mL solutions of each substrate in DMSO were mixed to a final volume of 300 μL so that $^{14}C/^{3}H$ was near 1. The 300-μL mixtures were then added to 10 mL of assay buffer (50 mM HEPES, pH 7.8) for a final substrate concentration of 70 μM. Values of β were determined as described in the text.

For a first-order reaction, where

$$[S]_t = [S]_0 \exp(-kt) \quad (1)$$

we can define k_H and k_D as the rate constants for the protio and deuterio substrates, and if we let $\alpha = k_D/k_H$, the following equation holds:

$$\frac{[S_H]_t/[S_H]_0}{[S_D]_t/[S_D]_0} = \frac{\exp(-k_H t)}{\exp(-\alpha k_H t)} \quad (2)$$

If we now define f, the fraction of reaction, as $1 - \exp(-kt)$, and a term β as $([S_H]/[S_D])_0/([S]_H/[S_D])_t$, eq 2 can be rewritten as

$$\beta = \frac{(1-f)^{\alpha}}{(1-f)} = (1-f)^{\alpha-1} \quad (3)$$

A plot of β vs f will increase exponentially from 1.0 for a normal kinetic isotope effect ($\alpha < 1$) and decrease exponentially from 1.0 for an inverse kinetic isotope effect ($\alpha > 1$). In the double-label experiments of this study, β is equal to $(^{14}C/^{3}H)_0/(^{14}C/^{3}H)_t$ for the substrate.

Isotope-effect determinations were performed at 4 °C in a chymotrypsin-coupled assay described elsewhere.[4] In a typical determination, the reaction was initiated by addition of chymotrypsin ($[CT]_0$ = 70 μM) to a thermally equilibrated solution of substrate ($[S]_0$ = 70 μM) and enzyme ($[PPI]_0$ = 56 nM). At 30-s intervals, 150-μL aliquots of the reaction mixture were withdrawn and added to 100-μL aliquots of a 0.7 mM solution of α_1-proteinase inhibitor (Sigma A-9024), and the resultant solution was stored at room temperature until chromatographed. Substrate and products were completely separated by HPLC. $^{14}C/^{3}H$ ratios for unreacted substrate were determined by liquid scintillation counting of 2.0-mL samples of the appropriate column fraction. Finally, β values were calculated from $^{14}C/^{3}H$ ratios for the unreacted substrate at several times throughout the course of the reaction.

Figure 1 is a plot of β as a function of f. The filled circles correspond to reaction in the presence of cyclophilin. An observed isotope effect, $^D(k_{obsd})$, of 1.080 ± 0.002 is obtained from a nonlinear least-squares fit of the data to eq 3. In a second de-

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termination, a value of 1.055 ± 0.001 was obtained (data not shown) and demonstrates the reproducibility of this technique. The open circles are for an uncatalyzed reaction and provide $^{D}(k_u)$ = 1.044 ± 0.001.

$^{D}(k_{obsd})$ reflects the isotope effects for both the enzyme-catalyzed and uncatalyzed reactions and can be expressed as $^{D}(k_{obsd}) = {}^{D}(k_E)C_E + {}^{D}(k_u)C_u$, where C_E and C_u are fractional contributions to rate limitation of the observed reaction by the enzyme-catalyzed and uncatalyzed reactions.[7] In the present case, $C_u = k_u/k_{obsd}$ and, by definition, $C_E = 1 - C_u$.[7] Rate constants were determined spectrophotometrically[4] and are k_{obsd} = 0.0119 s^{-1} and k_u = 0.00421 s^{-1}. Using $^{D}(k_u)$ = 1.044 and $^{D}(k_{obsd})$ = 1.081, we calculate $^{D}(k_E)$ = 1.106 ± 0.016.[8]

The solid line in the lower half of Figure 1 was drawn according to eq 3 with α = 1.047 [$^{D}(k_{obsd})$ = 0.955] and models the situation claimed by Fischer[3] where of $^{D}(k_u)$ = 1.05 and $^{D}(k_E)$ = 0.91. This further illustrates that our data cannot be fit to a model incorporating an inverse value for $^{D}(k_E)$.

In summary, the β-deuterium isotope effect of 1.10 that we determined in this study confirms our earlier finding of 1.13[4] and does not lend support to any mechanism involving nucleophilic catalysis. Mechanisms for this enzyme will likely employ some form of catalysis by distortion.[4]

Acknowledgment. We gratefully acknowledge Dr. Jeff Hermes (Biophysics Department, Merck Research Laboratories) for suggesting this experiment.

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Potential for Water Catalysis in Flavin-Mediated Hydroxylation. A Theoretical Study

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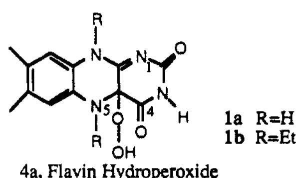
One of the most important biochemical oxygen atom transfer reactions involves catalysis by flavoenzymes.[1-6] The flavin coenzyme functions as an electron conduit between a variety of substrates. These tricyclic isoalloxazine moieties are among the more versatile of the redox cofactors in biochemistry. When molecular oxygen is the reducible substrate for dihydroflavin reoxidation, the highly cited flavin 4a-hydroperoxide (**1a**) has been



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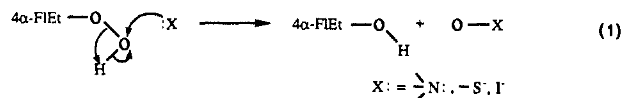
implicated as the key intermediate that serves as either the oxygen donor or its immediate precursor. The generally accepted mechanism for oxygen transfer is attack by the substrate on the distal oxygen with a direct nucleophilic displacement of the β-peroxy oxygen (eq 1).[1c] Model enzyme **1b** is intermediate in



reactivity between a peracid and an alkyl hydrogen peroxide.[7] In discussions of this mechanism to date, no provision has been made for the energy requirements of the 1,2-hydrogen shift. In the following paper in this issue[8] we established the potential energy surface for oxygen donation from H_2O_2 to amines and alkenes in the gas phase to be almost entirely dominated by the energetic requirements (56.0 kcal/mol)[9] for the 1,2-hydrogen migration to afford water oxide. We now describe the remarkable catalytic effect of one and two molecules of water on the transition state for the oxidation of ammonia.[9,10]

Although the barriers (Table I) for a concerted S_N2 displacement by ammonia on hydrogen peroxide are surprisingly high (51.4 kcal/mol), a direct displacement of OH^- from H_2O_2 without a 1,2-hydrogen shift would afford OH^- and H_3NOH^+ that is 198.2 kcal/mol (HF/6-31G*) above the energy of the reactants in the gas phase. Solvation of a hydroxide ion by one water molecule affording bihydroxide ion ($H_3O_2^-$) is accompanied by only 35.2 kcal/mol (HF/6-31G*) of stabilization. Consequently, the oxidation of NH_3 must be accompanied by a formal 1,2-hydrogen migration that is complete prior to N–O bond making in the transition state. The overall oxygen transfer is therefore very likely a two-step process involving S_N2 attack by ammonia on water oxide.[8] By analogy, a comparable reaction occurring in a flavin-mediated oxygen atom donation in the absence of solvent participation must also involve a similar hydrogen shift to the α-oxygen prior to displacement of the pseudobase (4a-FlOH). Secondly, the transition structure for this type of concerted reaction also has a complete octet of electrons around the so-called "electrophilic" oxygen. Given the observed half-life for native **1a** (~2.5 ms),[2b] such a process involving a simple 1,2-hydrogen migration (eq 1) would exhibit an energy barrier that is far in excess of that of an oxygen donor that is capable of achieving aromatic hydroxylation.

The origin of the barrier for a 1,2-hydrogen shift across an O–O bond may be attributed in part to the fact that the rearrangement is a four-electron process involving a filled-in-plane σ-type O–O bond at the migration terminus that has pseudo-π^* symmetry[11] and the concerted process is formally forbidden on the basis of symmetry arguments. The barrier for formation of water oxide is 56.0 kcal/mol[8] while a concerted rearrangement of H_2O_2 hydrogen bonded to one molecule of water (TS-1) still exhibits a barrier of 44.1 kcal/mol (MP4SDTQ/6-31G*//HF/6-31G*) from the $H_2O_2 \cdot H_2O$ complex. In principle, a water molecule serving as a catalyst can circumvent this problem in an alkyl hydrogen peroxide like **1a** by accepting a proton from the distal peroxide oxygen and transferring one of its hydrogens to the

(7) The N[5]-ethylflavin **1b** is about a 10^3-fold better oxygen donor than *m*-chloroperbenzoic acid but 10^3-10^6-fold less reactive than ROOH.[2a]

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