aerated solutions, in agreement with the data in Figure 2.

For 11-*cis*-retinal, a possible complication is the higher amount of the 12-(*S*)-cis conformer (one estimate being ~60% in acetone),[26] which makes direct light absorption of this conformer with ensuing photochemistry of its own no longer negligibly small.

Additional Comments. So far, we have overlooked bimolecular association as a possible explanation for the observed concentration effects, even though such phenomena were discussed in the literature concerning retinal fluorescence properties.[27] The reasons for our preference for the involvement of isomeric triplets are the following.

Firstly, the concept of isomeric triplets with different chemical properties has been well documented in simple dienes and trienes.[18,19] Their involvement in pentaenes seems to be unavoidable. Secondly, the retinal triplets produced under selective sensitization at low retinal concentrations[7] have the same chemical properties (favoring formation of 9-cis rather than 13-cis) as those produced under non-selective sensitization and high retinal concentration, in agreement with identical cisoid triplets. Thirdly, examples of singlet exciplexes are well known, many with high fluorescence yields. Such was also the case for retinal dimer (zero fluorescence yield for retinal monomer).[27] The phenomena described here are not related because they involve the triplet state. Fourthly, the concept of dimeric association will be difficult to account for quantum yields greater than 2 as observed in the hindered isomers. Nevertheless, it will be desirable to obtain independent corroborative evidence to support the involvement of isomeric triplets, such as their direct detection by time-resolved resonance Raman spectroscopy. An ideal condition for favorable population of cisoid triplets could be the selective sensitization condition employed by Jensen et al.[7] Results from designed ring-fused retinoids (many already in the literature in visual pigment analogue studies)[28] that impede conformational interconversion will also be interesting.

Acknowledgment. The work was supported by a grant from the National Science Foundation (CHE-16500).

Registry No. *all-trans*-Retinal, 116-31-4; 13-*cis*-retinal, 472-86-6; 9-*cis*-retinal, 514-85-2; 7-*cis*-retinal, 24315-14-8; 11-*cis*-retinal, 564-87-4; 7,9-*dicis*-retinal, 56085-53-1; 7,13-*dicis*-retinal, 56085-54-2; 11,13-*dicis*-retinal, 564-88-5; 7,9,13-*tricis*-retinal, 56085-55-3; 9,13-*dicis*-retinal, 23790-80-9.

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(29) One referee notes that the determined valued of ~0.5 for 11-*cis*-retinal is not larger than that reported for intersystem crossing efficiency,[6] but the error limits for both numbers are greater than 10%, making a direct comparison unreliable.

Mechanistic Studies of Enzymic and Nonenzymic Prolyl Cis-Trans Isomerization

Richard K. Harrison and Ross L. Stein*

Contribution from the Department of Enzymology, R80N-A54, Merck Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065.
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Abstract: The cyclosporin A binding protein, cyclophilin (CyP), and the FK-506 binding protein, FKBP, catalyze the cis-to-trans isomerization of Xaa-Pro bonds in peptides. To probe the mechanism of these reactions and their nonenzymatic counterparts, we determined the following: (1) substrate specificities of CyP and FKBP; (2) dependencies of k_c/K_m on pH and solvent deuterium; (3) secondary deuterium isotope effects; and (4) temperature-dependencies. The results indicate that (1) for cis-to-trans isomerization of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA, values of k_c/K_m for the CyP-catalyzed reactions show little dependence on Xaa. In contrast, for FKBP, k_c/K_m displays a marked dependence on Xaa with a preference for hydrophobic residues. (2) For both enzymes, k_c/K_m is independent of both pH and isotopic composition of the solvent. (3) For the CyP-catalyzed cis-to-trans isomerization of Suc-Ala-Gly(L,L)-*cis*-Pro-Phe-pNA (L = H, D), ${}^{H}(k_c/K_m)/{}^{D}(k_c/K_m) = 1.13 \pm 0.01$ and is independent of temperature between 2 and 30 °C (2 °C, ${}^{H}k/{}^{D}k = 1.14 \pm 0.02$; 10 °C, ${}^{H}k/{}^{D}k = 1.13 \pm 0.01$; 30 °C, ${}^{H}k/{}^{D}k = 1.14 \pm 0.03$). A secondary deuterium isotope effect of 1.13 suggests that, in the transition state of this reaction, the Xaa-Pro bond is twisted out of planarity. This value is inconsistent with mechanisms involving nucleophilic catalysis. (4) Eyring plots of $\ln [(k_c/K_m)/T]$ vs $1/T$ for reactions of FKBP are linear, while Eyring plots for CyP catalysis are curved and display a maximum around 30 °C. The data for CyP were fit to a model involving a temperature-dependent, reversible isomerization of active to an inactive enzyme. This model is supported by the temperature-independence of the secondary deuterium isotope effect (see 3 above) and the independence of the equilibrium of the two enzyme forms on substrate structure. The activation parameters that were calculated from the Eyring plots indicated enthalpy-entropy compensation for both CyP and FKBP with the critical temperature, T_c, equal to 287 and 260 K, respectively. We interpret the compensation in terms of a simple mechanism in which stronger transition-state interactions between enzyme and substrate (more positive $\Delta H^{\ddagger}$) are accompanied by greater restrictions of translational and rotational freedom (more negative $\Delta S^{\ddagger}$).

Introduction

The cis-trans isomerization of Xaa-Pro bonds (Scheme I) is a reaction of biochemical interest due to its often rate-limiting role in protein folding. The central role that this reaction plays in biochemistry is further highlighted by the existence of an ubiquitous enzyme in nature that catalyzes this reaction. This enzyme, peptidyl prolyl cis-trans isomerase (PPI),[1] was first described in 1984;[2] it catalyzes prolyl isomerization in both peptides[2] and proteins.

(1) Abbreviations: CyP, cyclophilin; FKBP, FK506 binding protein; rhFKBP, recombinant human FK506 binding protein; PPI, peptidyl prolyl cis-trans isomerases; Suc, *N*-succinyl; pNA, *p*-nitroanilide; α-CT, α-chymotrypsin; DMA, *N*,*N*-dimethylacetamide.

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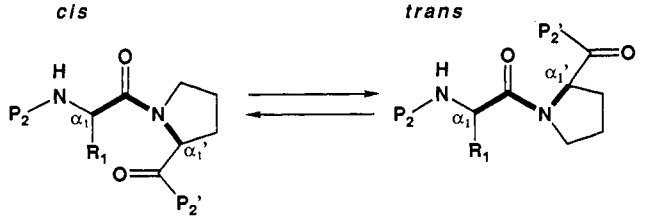
Scheme I. Prolyl Isomerization



Recent interest in PPI is sparked by the discovery that cyclophilin (CyP), the binding protein for the immunosuppressant drug cyclospiron A, is identical to PPI.[3,4] Remarkably, FKBP, the binding protein for another immunosuppressant, FK-506, also possesses PPI activity.[5,6] Furthermore, the PPI activities of CyP and FKBP are potently inhibited by their respective ligands with no cross-inhibition.[3-5,7,8] Given these discoveries, it became clear that a new generation of immunosuppressant drugs might be developed from potent inhibitors of the various PPIs and that the design of these inhibitors would be driven by mechanistic and structural studies.

In this paper, we report results of our continuing investigation of the mechanisms of enzymic and nonenzymic prolyl isomerization. In particular, we wanted to determine temperature-dependencies of the kinetic constants and isotope effects that characterize these reactions. For the enzymic reactions, our preliminary investigation of substrate speficity suggests interesting mechanistic differences between CyP and FKBP.[8] However, we felt that before we could draw any real mechanistic insights from this data we would have to determine a complete thermodynamic profile (i.e., $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta G^{\ddagger}$) for these reactions, since enthalpy–entropy compensation could cause catalytic events to exhibit similar values of $\Delta G^{\ddagger}$ despite being controlled by entirely different thermodynamic forces (i.e., $\Delta H^{\ddagger}$ or $\Delta S^{\ddagger}$). We also hoped that the temperature-dependence of the secondary β-deuterium isotope for PPI-catalyzed cis-to-trans isomerization of Suc-Ala-Gly(L,L)-*cis*-Pro-Phe-pNA (L = H, D) would provide information about reaction steps that limit the rates of these reactions. Finally, a theme that runs throughout all of this work is that the chemistry of nonenzymic prolyl isomerization and the mechanisms of the nonezymic catalysis of this reaction dictates the catalytic strategy that is used by prolyl isomerases.

Materials and Methods

General. Buffer salts and deuterium oxide were from Sigma Chemical Co. Water was distilled and passed through a deionizer. Buffers for the solvent isotope effect studies were prepared as described previously.[9,10] Peptides that were used as potential inhibitors of FKBP were from Bachem or Sigma. FKBP (JURKAT human T-cell line) and CyP (calf thymus) were purified according to published procedures[5,7] and were provided to us by Dr. John Siekierka of the Department of Immunology Research, Merck Sharp & Dohme Research Laboratories.

Substrates. Substrates of structure Suc-Ala-Xaa-Pro-Phe-pNA, where Xaa is Gly, Ala, Val, Ile, Nle, Leu, Phe, Trp, His, Lys, and Glu, were prepared by Bachem, Switzerland. Stock solutions of substrates (2.2 mM) were prepared in DMSO and diluted into buffer to concentrations between 70 and 75 μM.

Kinetic Methods. Kinetic experiments were performed as previously described.[6]

Chromatographic Conditions. The chromatographic apparatus consisted of a Waters 680 controller, two Waters 510 isocratic pumps, a Hitachi 655-A autosampler, and a Kratos 757 variable wavelength detector. Solutions of peptides (0.7 mM) (Suc-Ala-Xaa-Pro-Phe-pNA, where Xaa = Gly, Ala, Val, Ile, Nle, Leu, Phe, Trp, His, Lys, and Glu) were made in 50 mM sodium phosphate buffer (pH 7.0) containing 30% CH_3CN (v/v) and injected onto a Whatman Partisil C_8 column (4.6 mm × 25 cm). The mobile phase was 50 mM sodium phosphate buffer (pH 7.0) containing 30% CH_3CN (v/v) and was run at a flow rate of 1.5 mL/min. The UV absorbance of the peptides in the mobile-phase effluent was recorded at 215 nm.

Table I. Specificities of the PPI Activities of Cyclophilin and FKBP toward Substrates of Structure Suc-Ala-Xaa-Pro-Phe-pNA[a]

Xaa	k_c/K_m (mM^{-1} s^{-1})[b] CyP	FKBP	Xaa	k_c/K_m (mM^{-1} s^{-1})[b] CyP	FKBP
Gly	1200	1.2	Phe	1400	620
Ala	3200	53	Trp	360	110
Val	3200	170	His	600	28
Ile		320	Lys	920	28
Nle		330	Glu	2100	0.6
Leu	2700	640			

[a] Reactions were conducted at pH 7.8 in buffered solutions containing 50 mM HEPES. Final concentrations in reaction solutions were [α-CT] = 74 μM and [Suc-Ala-Xaa-Pro-Phe-pNA] = 70 μM. Temperature = 10 °C. Xaa = Ala for CyP and Leu for FKBP. [b] Values of k_c/K_m were determined as described in the text.

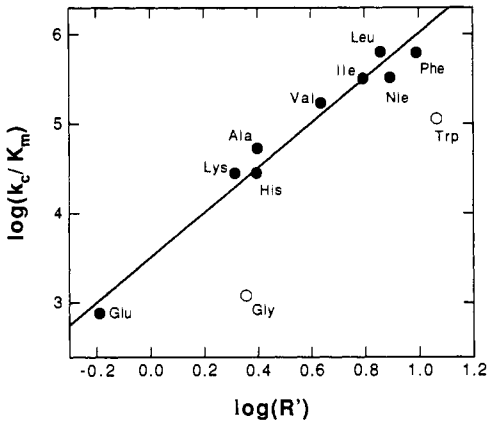


Figure 1. Dependence of k_c/K_m for the FKBP-catalyzed cis-to-trans isomerization of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA on the hydrophobicity of Xaa. Hydrophobicity was quantitated as R', the HPLC capacity factor.[12] Data for the outliers, Xaa = Gly and Trp, were not used in the linear regression analysis shown as the solid line.

Results

P_1 Substrate Specificity for CyP and FKBP. Values of k_c/K_m were determined as described previously[2,6,8] for CyP- and FKBP-catalyzed cis-to-trans isomerization of substrates of general structure, Suc-Ala-Xaa-*cis*-Pro-Phe-pNA, and are collected in Table I. These results indicate a marked difference in P_1 specificity between the two enzymes, with CyP having a broader specificity. Our results are for FKBP isolated from the JURKAT human T-cell line and are similar to those for recombinant human FKBP.[11]

It is interesting that k_c/K_m for both enzymes decreases when Xaa is Trp, suggesting that the S_1 pocket has a defined shape and size, especially for FKBP. There also appears to be at least a casual correlation between the hydrophobicity of the amino acid side chain and k_c/K_m. We attempted to quantitate this correlation by calculating HPLC capacity factors, R',[12] for each of the peptides. For a given substance, R' is equal to $(t_R - t_0)/t_0$ where t_R is the elution time of a retained substance (the peptide) and t_0 is the elution time of an unretained substance, in this case simply the solvent front. R' reflects the hydrophobicity of a substance: the larger the value of R' for a peptide, the more hydrophobic is the peptide. Thus, we anticipate that the logarithm of R', which

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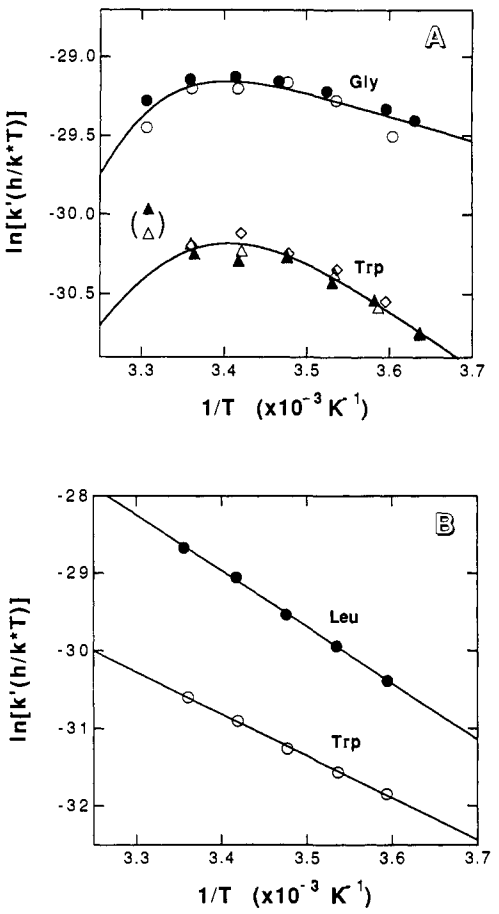

Figure 2. Eyring plots for the PPI-catalyzed cis-to-trans isomerization of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA. A: CyP catalysis. Different symbols represent results from independent kinetic experiments. Theoretical lines were drawn according to nonlinear least-squares analysis of the data according to eq 3 (see Table III for best fit parameters). B: FKBP catalysis. Theoretical lines were drawn from linear least-squares analysis of data (see Table III for best fit parameters).

is proportional to the free energy of transfer from the aqueous to the hydrophobic phase, might correlate with the logarithm of k_c/K_m. This correlation is shown in Figure 1 and, when the outliers Xaa = Gly and Trp are excluded, is quite good (slope = 3.5 ± 0.1; y-intercept = 2.5 ± 0.2).

Lack of Inhibition of FKBP by Proline-Containing Peptides. Several proline-containing peptides were tested as inhibitors of FKBP. These peptides included the following: *Ala-Pro*, *Ala-Pro*-Gly, *Ala-Pro*-Phe, Ala-*Ala-Pro*-Ala, Ala-*Ala-Pro*-Ala-Ala, Asp-Ser-*Asp-Pro*-Arg, Arg-Tyr-*Leu-Pro*-Thr, pyroGlu-His-Trp-Ser-Tyr-Gly-Trp-*Leu-Pro*-GlyNH$_2$, Phe-Asn-*Leu-Pro*-Leu-Gly-Asn-Tyr-*Lys-Pro*-Lys, *Arg-Pro-Pro*-Gly-Phe-*Ser-Pro*-Phe-Arg, and pyro*Glu-Pro*-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-MetNH$_2$. Although several of these peptides contained Leu at P_1 and therefore fulfilled the enzyme's specificity requirement at this position, no inhibition was seen at concentrations of peptide as high as 5 mM. This translates into a 0.5 mM concentration for the *cis*-Pro form of the peptide. If we now make the conservative assumption that we would have been able to detect no less than 20% inhibition, we can calculate that $K_{i,cis} \geq 2$ mM and $K_{i,trans} \geq 20$ mM. Since these peptides are, in fact, alternate substrate inhibitors, we can also state that $K_{m,cis} = K_{i,cis} \geq 2$ mM and $K_{m,trans} = K_{i,trans} \geq 20$ mM. This estimate is in fair agreement with the $K_{m,cis}$ value of about 1 mM that was recently reported by Rich's group.[13]

Dependence of k_c/K_m on pH and Solvent Deuterium for Catalysis by FKBP. As we reported previously for CyP,[6] k_c/K_m is independent of pH between 5.5 and 9 (data not shown) and exhibits a slightly inverse solvent isotope effect: $^{DOD}(k_c/K_m) = 0.92 \pm 0.09$.

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Table II. Activation Parameters for the Cis-to-Trans Prolyl Isomerization of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA[a]

Xaa	$\Delta H^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (eu)
Gly	18.6	−2.91
Ala	20.7	4.98
Leu	21.4	6.87
Trp	19.1	−2.59

[a] Reactions were conducted at pH 7.8 in buffered solutions containing 50 mM HEPES. Final concentrations in reaction solutions were [α-CT] = 74 μM and [Suc-Ala-Xaa-Pro-Phe-pNA] = 70 μM. Parameters were calculated as described in the text.

Table III. Thermodynamic Parameters for the PPI-Catalyzed Cis-to-Trans Isomerization of Suc-Ala-Xaa-Pro-Phe-pNA[a]

PPI	Xaa	$\Delta H^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (eu)	$\Delta G^{\ddagger\,b}$ (kcal/mol)	ΔH (kcal/mol)	ΔS (eu)
CyP	Gly	3.2	−47	17	36	120
CyP	Ala	4.3	−41	16	45	145
CyP	Trp	7.5	−34	17	27	90
CyP	Leu	7.9	−29	16	44	140
FKBP	Trp	11	−25	18		
FKBP	Ala	12	−21	18		
FKBP	Leu	15	−8.6	17		

[a] Reactions were conducted at pH 7.8 in buffered solutions containing 50 mM HEPES. Final concentrations in reaction solutions were [α-CT] = 74 μM and [Suc-Ala-Xaa-Pro-Phe-pNA] = 70 μM. Parameters were based on values of k_c/K_m that were determined as described in the text. Analysis of Eyring plots was according to the model of Scheme II and eq 3. [b] Calculated with T = 283 K.

Dependence of k_c/K_m on Temperature for CyP and FKBP. Values of k_c/K_m were determined at seven temperatures between 2 and 30 °C for CyP- and FKBP-catalyzed isomerizations. The results of these experiments are summarized in Figure 2 as Eyring plots of ln $[k'(h/k^*T)]$ vs $1/T$, where h is Planck's constant, k^* is Boltzmann's constant, T is expressed in degrees Kelvin, and k' is a pseudo-first-order rate constant obtained by multiplying k_c/K_m by a standard-state enzyme concentration of 10^{-6} M. In ideal cases, where linear Eyring plots are observed, the enthalpy of activation can be obtained from the slope, $-\Delta H^\ddagger/R$, and the entropy of activation obtained from the y-intercept, $\Delta S^\ddagger/R$, where R is the gas constant.

For nonenzymic isomerization, the Eyring plots are linear (data not shown) and provide the activation parameters that are summarized in Table II. The activation parameters for these reactions show no obvious dependence on the P_1 residue and provide average values: $\Delta H^\ddagger$ = 20 ± 1 kcal/mol and $\Delta S^\ddagger$ = 1 ± 5 eu ($-T\Delta S^\ddagger$ = −0.3 ± 1.5 kcal/mol; T = 283 K). These values are similar to the activation parameters for isomerization of other Pro-containing peptides (see Table V).

For the FKBP-catalyzed cis-to-trans isomerizations of Suc-Ala-Xaa-*cis*-Pro-Phe-pNA, where Xaa = Trp, Ala, and Leu, the Eyring plots are also linear (Figure 2B). The activation parameters for these reactions are summarized in Table III. In contrast, the Eyring plots for reactions of CyP (Figure 2A) are nonlinear. The simplest mechanism that can account for this nonlinearity is shown in Scheme II in which the enzyme exists in two interconvertible forms, E and E′, and only E is enzymatically active. The observed k_c/K_m for this mechanism is given:

$$\left(\frac{k_c}{K_m}\right)_{obsd} = \frac{k_1}{1+K} \qquad (1)$$

where k_1 is k_c/K_m for the reaction of substrate with E_1 and K is the equilibrium constant defined by $[E_2]/[E_1]$.

Equation 1 can now be expressed in the pseudo-thermodynamic terms allowed by transition-state theory:

$$k' = \frac{(k^*T/h)e^{-\Delta G^\ddagger/RT}}{e^{-\Delta G/RT}+1} \qquad (2)$$

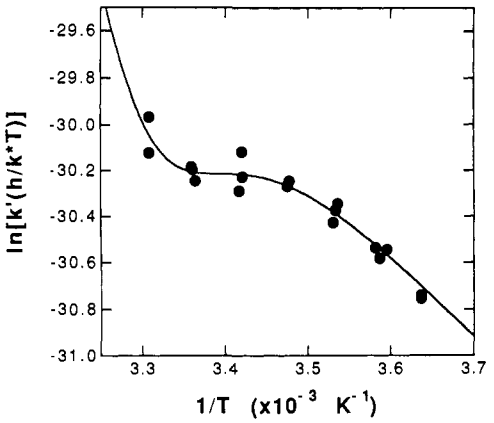
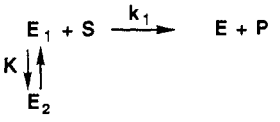


Figure 3. Temperature-dependence for the CyP-catalyzed isomerization of Suc-Ala-Trp-Pro-Phe-pNA. Theoretical line was drawn according to the model of Scheme II (see text for details).

Scheme II. Enzyme Mechanism Involving Conversion of Two Enzyme Forms



where $k' = (k_c/K_m)_{obsd}\,[E]_{standard\ state}$ ($[E]_{standard\ state} = 10^{-6}$ M) and ΔG^* and ΔG refer to $k_1[E]_{standard\ state}$ and K, respectively.

Equation 2 can be expanded and rearranged as shown in eq 3. This expression is suitable for plotting as an Eyring plot.

$$\ln\left[k\left(\frac{h}{k^*T}\right)\right] = \ln\left[\frac{\exp\left(\frac{\Delta H^*}{RT} + \frac{\Delta S^*}{R}\right)}{\exp\left(\frac{-\Delta H}{RT} + \frac{\Delta S}{R}\right) + 1}\right] \quad (3)$$

When the data sets for the reactions of CyP are fit to eq 3 by nonlinear least squares, the best fit parameters of Table III are obtained. Values of ΔH and ΔS for the four compounds are very similar and, at 10 °C, yield nearly identical K values of 2.0, 3.9, 1.5, and 4.4 for Xaa = Gly, Ala, Trp, and Leu, respectively. This supports the mechanism of Scheme II since, according to this mechanism, K should not depend on structural features of the substrate.

In fitting the data for Suc-Ala-Trp-Pro-Phe-pNA to eq 3, the two data points at the highest temperature were not used. However, we believe that these data points are accurate reflections of the kinetic situation at higher temperatures since they reflect two independent experiments. The upward departure of k_c/K_m at high temperature requires that E_2 be enzymatically active. Thus, we must modify the mechanism of Scheme II to include a new term, k_2, as shown in Scheme III. The rate equation becomes

$$\left(\frac{k_c}{K_m}\right)_{obsd} = \frac{1}{1+K}(k_1 + Kk_2) \quad (4)$$

When the data sets for reaction of CyP with Suc-Ala-Trp-Pro-Phe-pNA are fit to the thermodynamic form of eq 4 with ΔH^*_1 and ΔS^*_1 constrained to 7.5 kcal/mol and −34 eu, respectively, the best fit parameters that we obtain are as follows: ΔH = 29 ± 3 kcal/mol, ΔS = 98 ± 13 eu, ΔH^*_2 = 35 ± 9 kcal/mol, and ΔS^*_2 = 55 ± 30 eu. These parameters were used to draw the theoretical line through the data in Figure 3.

Temperature-Dependence of the Secondary Deuterium Isotope Effect for CyP Catalysis. The secondary deuterium isotope effect was determined to be independent of temperature for the CyP-catalyzed cis-to-trans isomerization of Suc-Ala-Gly(L,L)-*cis*-Pro-Phe-pNA (L = H, D). For 2, 10, and 30 °C, $^D(k_c/K_m)$ is 1.14 ± 0.02, 1.13 ± 0.01, and 1.14 ± 0.03, respectively. The temperature-independence of the isotope effect is consistent with the mechanisms of Schemes II and III in which the curvature observed in the Eyring plots is explained in terms of multiple forms of free enzyme. However, the temperature-independence of the isotope effect is not consistent with mechanisms involving a change in the rate-limiting step, which would be anticipated to be accompanied with a temperature-sensitive secondary deuterium isotope effect.

Scheme III. Complete Mechanism

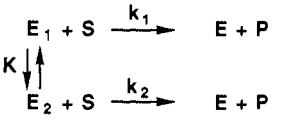


Table IV. Nonenzymic Rotation about Amide Bonds

parameter	Suc-Ala-*Ala-Pro*-Phe-pNA[a]	*N,N*-dimethylacetamide[b,c]
K_{eq}[d,e]	0.1	
$k_{c/t}$ (s^{-1})[e]	9.4×10^{-3}	9.1×10^{-3} [c]
$k_{t/c}$ (s^{-1})[e]	9.4×10^{-2}	
activation parameters	ΔH^* = 20.7 kcal/mol $-T\Delta S^*$ = −1.4 kcal/mol[e] ΔG^* = 19.3 kcal/mol[e]	ΔH^* = 19.1 kcal/mol[b] $-T\Delta S^*$ = 0.2 kcal/mol[b,e] ΔG^* = 19.3 kcal/mol[b,e]
pH-dependence	none, pH 5.5–9.0	pH 1.8, k = 0.051 s^{-1} pH 7.0, k = 6.7 s^{-1} [c]
solvent-dependence[c]		water, k = 0.025 s^{-1} acetone, k = 0.33 s^{-1} cyclohexane, k = 1.5 s^{-1}
solvent deuterium isotope effect	1.07 ± 0.01	
secondary deuterium isotope effect	1.05 ± 0.02[f]	1.10 ± 0.05[c]

[a] Reference 6. [b] Reference 15. [c] Reference 14. [d] K_{eq} = [cis]/[trans] = $k_{t/c}/k_{c/t}$. [e] 283 K. [f] Determined with Suc-Ala-Gly(L,L)-Pro-Phe-pNA (L = H, D).

Discussion

Prolyl cis–trans isomerization presents mechanistic enzymologists a rare opportunity to explore an enzyme-catalyzed reaction that proceeds at convenient nonenzymic rates. This situation allows us to apply the same mechanistic probes to both the enzymic and nonenzymic reactions and thus permits a direct evaluation of the enzyme's catalytic power in terms of stabilization of the nonenzymic transition state. Given this, we will first discuss results relating to nonenzymic prolyl cis–trans isomerization and then our work on the enzyme-catalyzed reaction for both CyP and FKBP. Finally, we will present a mechanism that accounts for catalysis by CyP and FKBP that allows us to rationalize the catalytic power that is possessed by these enzymes.

Nonenzymic Prolyl Isomerization. The principal data that forms the basis of our mechanistic hypothesis for nonenzymic prolyl cis–trans isomerization are summarized in Table IV, where we tabulate results of our mechanistic experiments with Suc-Ala-Ala-Pro-Phe-pNA along with literature studies with the simple amide, *N,N*-dimethylacetamide.[14,15] As we will see, C–N rotation in DMA is mechanistically analogous to prolyl isomerization.

The single most revealing mechanistic parameter is the secondary β-deuterium isotope effect. For cis-to-trans prolyl isomerization of Suc-Ala-Gly-Pro-Phe-pNA, the isotope effect for the two hydrogens of glycine is 1.05 ± 0.02,[6,16,17] while for C–N rotation in DMA the isotope effect for the three hydrogens of the acetyl moiety is 1.10 ± 0.05. Significantly, the effect for two deuteriums in DMA can be calculated to be 1.05. These isotope effects indicate a transition state in which the force field associated with the β-hydrogens is weakened relative to the force field of the reactant state. In the framework of the hyperconjugation model of β-deuterium isotope effects, these isotope effects suggest that the hyperconjugation between the β-hydrogens and the carbonyl

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Table V. Activation Parameters for Prolyl Cis-to-Trans Isomerization

	peptide	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
1	Suc-Ala-Leu-Pro-Phe-pNA[a]	21.3	6.56
2	Suc-Ala-Ala-Pro-Phe-pNA[a]	20.2	3.14
3	Gly-Gly-Pro-Ala[b]	19.5	0.90
4	*N,N*-dimethylacetamide[c]	19.1	-0.80
5	Suc-Ala-Trp-Pro-Phe-pNA[a]	19.1	-2.62
6	Suc-Ala-Gly-Pro-Phe-pNA[a]	18.6	-3.04
7	Gly-Pro[d]	20.0	0.0
8	Ala-Pro[e]	18.9	-7.0
9	Val-Pro[e]	18.8	-8.1
10	His-Pro[f]	18.9	-8.9
11	Gly-Gly-Lys-Phe-Pro[g]	16.1	-18.9

[a] This work. [b] Reference 34. [c] Reference 14. [d] Reference 36. [e] Reference 37. [f] Reference 38. [g] Reference 39.

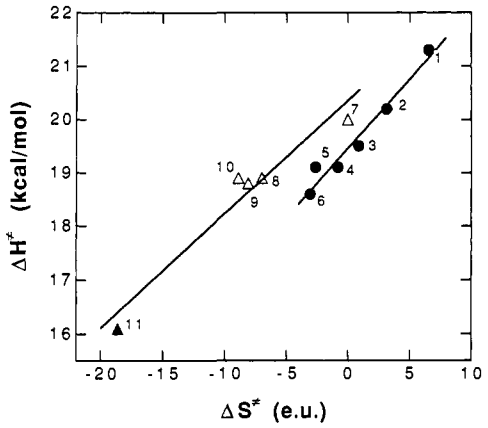
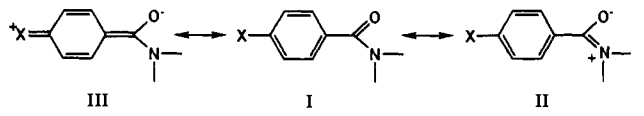


Figure 4. Enthalpy–entropy compensation for nonenzymic prolyl isomerization (see Table V for literature references) for proline-containing oligopeptides (●), dipeptide (△), and Gly-Gly-Lys-Phe-Pro (▲). Key to structures: 1, Suc-Ala-Leu-Pro-Phe-pNA; 2, Suc-Ala-Ala-Pro-Phe-pNA; 3, Gly-Gly-Pro-Ala; 4, *N,N*-dimethylacetamide; 5, Suc-Ala-Trp-Pro-Phe-pNA; 6, Suc-Ala-Gly-Pro-Phe-pNA; 7, Gly-Pro; 8, Ala-Pro; 9, Val-Pro; 10, His-Pro; 11, Gly-Gly-Lys-Phe-Pro. Linear regression analysis of the data for compounds 1–6 yields a slope, or critical temperature, T_c, of 260 ± 21 K, while analysis of the data for compounds 7–11 yields T_c = 209 ± 27 K.

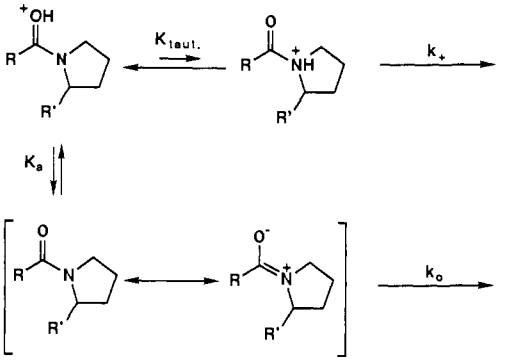
group is enhanced in the transition state relative to the reactant state. This situation would be obtained if the transition state were characterized by partial rotation about the C–N bond. Partial rotation would destroy the double bond character of the C–N bond, enhance the double bond and "ketone" character of the carbonyl, and thus enhance the hyperconjugative interaction between the β-hydrogens and the carbonyl group. These isotope effects allow us to eliminate mechanisms involving nucleophilic participation by solvent since isomerization by any of these mechanisms would involve the intermediacy of a tetrahedral adduct and a transition state that would be characterized by a decreased ability of the β-hydrogens to hyperconjugate. Mechanisms of this sort generate inverse isotope effects (i.e., $k_H/k_D < 1$) and are the rule in acyl addition and amide and ester hydrolyses.

A mechanism involving partial bond rotation in the transition state with no solvent participation is also supported by the rest of the data of Table IV. First, the rotational barrier for both cis-to-trans prolyl isomerization and C–N bond rotation in DMA is entirely enthalpic. An entropy of activation near zero is consistent with a unimolecular reaction and a transition state with no solvent participation and little solvent reorganization. The generality of these activation parameters is illustrated in Table V where we summarize the activation parameters for cis-to-trans isomerizations in a number of peptides. While the data clearly indicate that all of these reactions are enthalpically driven, they also suggest that there may be a mechanistic difference between isomerization of proline-containing oligopeptides and dipeptides since for dipeptides the $\Delta S^{\ddagger}$ values are more negative than for

Scheme IV. Resonance Forms for 4-Substituted *N,N*-Dimethylbenzamides



Scheme V. Mechanism for Acid-Catalyzed Prolyl Isomerization



the oligopeptides. That this phenomenon may be related to intramolecular hydrogen bonding or ionic interactions is supported by the very negative value of $\Delta S^{\ddagger}$ for isomerization of Gly-Gly-Lys-Phe-Pro. The mechanistic difference between the di- and oligopeptides is graphically illustrated in the enthalpy–entropy compensation plot[18] of Figure 4. For the oligopeptides, the slope of correlation, or the critical temperature T_c,[18] is 260 ± 21 K, while for the dipeptides T_c = 209 ± 27 K. These results support the notion that these reactions may fall into two classes, depending on the position of the proline residue.

The mechanism that we have advanced for prolyl isomerization is also supported by the observation that isomerization rate constants are independent of pH in the range 5–9 and that the solvent deuterium isotope effect for the reaction is equal to 1. These results indicate that the reactions proceed with no general-acid/general-base catalysis and, as discussed above, no involvement of solvent. That this reaction has a nonpolar transition state relative to the reactant state is indicated by the observation that the rate constant for C–N bond rotation in DMA is accelerated in nonpolar solvents.[14]

This mechanism is also supported by a study of substituent effects on the barrier height in amide bond rotations for a series of 4-substituted *N,N*-dimethylbenzamides.[19] The data can be correlated with σ_p values[20] and yield a ρ value of −1.14 ± 0.06 (n = 7, r^2 = 0.986). The negative ρ value indicates that electron-donating substituents accelerate the reaction. This can be rationalized in the context of Scheme IV, where resonance forms for these substrates are shown. The rotational barrier about the C–N bond is decreased as resonance forms I and III predominate. If R is electron-donating, these resonance forms will contribute more to the structure of the amide than will II and C–N rotation will therefore be accelerated.

The picture that emerges for the transition state for amide bond rotation at neutral pH is one characterized by partial rotation about the bond, with no nucleophilic participation by solvent and no significant solvent reorganization. Since in the transition state polar resonance structures for the amide bond are no longer possible, the transition state is less polar than the reactant. However, at extremes of pH, this mechanism changes. At acid pH, both cis–trans prolyl isomerization[21,22] and C–N bond rotation

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Table VI. Probes of Mechanism for Cis-to-Trans Prolyl Isomerization Catalyzed by Cyclophilin and FK-506 Binding Protein

mechanistic probe	cyclophilin-catalyzed	FKBP-catalyzed
steady-state kinetics	Suc-Ala-Ala-Pro-Phe-pNA	Suc-Ala-Leu-Pro-Phe-pNA
	$(k_c/K_m)_{c/t}$ = 3.2 μM^{-1} s^{-1}	$(k_c/K_m)_{c/t}$ = 0.64 μM^{-1} s^{-1}
	$(k_c)_{c/t} \geq 6400$ s^{-1}	$(k_c)_{c/t} \geq 1300$ s^{-1}
	$(K_m)_{c/t} \geq 2$ mM	$(K_m)_{c/t} \geq 2$ mM
P_1 substrate specificity	broad	narrow; preference for hydrophobic residues
pH-dependence	none	none
temperature-dependence	$\Delta H^{\ddagger}$ = 4.3 kcal/mol	$\Delta H^{\ddagger}$ = 14.5 kcal/mol
	$-T\Delta S^{\ddagger}$ = 12.3 kcal/mol	$-T\Delta S^{\ddagger}$ = 2.6 kcal/mol
	curved Eyring plot	
solvent isotope effect	0.98 ± 0.08	0.92 ± 0.09
secondary isotope effect	1.13 ± 0.01 (direct determination)	
	1.11 ± 0.02 (competitive determination)	

in DMA are accelerated.[15] For example, at 298 K, the free energy barrier to rotation in DMA decreases from 19.3 at pH 7.0 to 16.4 kcal/mol at pH 1.8,[15] which translates to a 130-fold increase in rate constant from 0.051 to 6.7 s^{-1}. Although the carbonyl oxygen is thought to be the predominant site of protonation in acid,[23,24] the mechanism for acid-catalyzed C–N bond rotation must involve the relatively rare N-protonated species[25] in which rotation becomes freer due to the loss of double bond character that the C–N bond experiences upon N-protonation. This is illustrated in Scheme V, where K_a is the acid dissociation constant for the amide protonated on the oxygen, $K_{taut.}$ is the equilibrium constant for the tautomerization of the protonated amide and equals $[NH^+]/[OH^+]$, and K_{NH^+} is the acid dissociation constant for the amide protonated on the nitrogen and equals $K_a/K_{taut.}$. Typical values of K_a range from 1 to 10^2 M ($-2 < pK_a < 0$), and we can assume that $K_{taut.}$ is less than 0.1. Thus, K_{NH^+} will probably be greater than 10^2 M ($pK_{NH^+} < -2$).

Now, if we take amide rotation in *N,N*-dimethylacetamide as an example, we can assume that k_0 equals 0.05 s^{-1}, which is the observed rate constant at neutral pH. The knowledge that at a pH of 1.8 k_{obsd} equals 6.7 s^{-1} allows us to calculate k_+/K_{NH^+} equal to 420 M^{-1} s^{-1} (see eq 5). Assuming that K_{NH^+} is greater than

$$k_{obsd} = \frac{k_+}{1 + \frac{K_{NH^+}}{[H^+]}} + k_0 \qquad (5)$$

10^2 M, we see that k_+ will be greater than or equal to 42 000 s^{-1}. Thus, $k_+/k_0 \geq 10^6$, which represents the rate acceleration from acid catalysis. This will become important when we discuss the origins of enzymic catalytic power for PPI.

At alkaline pH, an interesting situation occurs. The activation parameters for C–N rotation in DMA at pH 11.8 are as follows:[15] $\Delta G^{\ddagger}$ = 19.0 kcal/mol, $\Delta H^{\ddagger}$ = 16.3 kcal, and $-T\Delta S^{\ddagger}$ = 2.7 kcal/mol. The large contribution to $\Delta G^{\ddagger}$ that is made from the entropy term may indicate nucleophilic participation of hydroxyl, leading to the formation of a tetrahedral adduct. At the stage of this tetrahedral species, the C–N bond has lost all double bond character and rotation will be energetically more favorable. This may be reflected in the low $\Delta H^{\ddagger}$ value.

Enzyme-Catalyzed Prolyl Isomerization. Our studies of CyP- and FKBP-catalyzed isomerization of substrates of general structure Suc-Ala-Xaa-Pro-Phe-pNA are summarized in Table VI. These results will form the basis for the discussion in this section.

As we saw with nonenzymatic prolyl isomerization, the most compelling mechanistic information for the enzymic reaction comes from the secondary deuterium isotope effect determinations. In earlier publications from this laboratory, we report that the isotope effect for the Cyp-catalyzed cis-to-trans isomerization of Suc-Ala-Gly(L,L)-*cis*-Pro-Phe-pNA (L = H, D) is 1.12.[6,17] More recently, the isotope effect for the FKBP-catalyzed reaction was found to be 1.12 ± 0.02 (personal communication, Dr. David Livingston, Vertex, Inc.).[26] Isotope effects of this magnitude rule out mechanisms involving nucleophilic catalysis[16] but favor catalysis by distortion in which the enzyme induces strain or distortion in the substrate.[6] This can be the result of geometric, desolvation, or electrostatic destabilization but is dependent upon the binding energy between enzyme and substrate since such destabilization that is induced in the substrate will only result in rate enhancement if it is "paid" for by binding energy.

A point of mechanistic interest is the difference in magnitude of the isotope effects for the nonenzymatic and enzyme-catalyzed reactions which are 1.05 and 1.12, respectively. Since the reactant state is the same for both reactions, that is, free, uncomplexed substrate, the difference in isotope effect must signal a difference in transition-state structure. The larger isotope effect for the enzyme-catalyzed reaction indicates that the transition-state force field associated with the hydrogens of the glycine is loose relative to the nonenzymic reaction. This translates into greater hyperconjugative delocalization of β-CH electrons in the transition state for the enzymic reaction. One explanation for this involves a model in which aqueous solvation impedes hyperconjugation of the β-CH electrons into the carbonyl group. Loss of solvation upon binding of the substrate in a hydrophobic enzyme active site would then increase hyperconjugation. In a purely aqueous environment, the transition state would still be solvated and the loss of hyperconjugation would not be as great as in the enzymic case. Solvation isotope effects such as this have been observed on transfer of carbonyl compounds from water to hydrophobic solvents.[27] In these cases, normal β-deuterium isotope effects on the order of 1–2% per deuterium were measured. The investigators suggest that these effects originate from a smaller hyperconjugative release of β-CH electrons into the carbonyl center in water than in organic solvents. For prolyl isomerase, transfer of substrate to a hydrophobic active site will not only explain our large, normal isotope effects but also help to account for how the enzyme effects catalysis, since, as we pointed out above, this will stabilize the apolar transition state and thus lead to rate enhancement.[14,28]

A matter of particular interest is the difference in substrate specificity between CyP and FKBP. The pertinent data are in Table I where we have summarized values of k_c/K_m for the CyP- and FKBP-catalyzed prolyl cis-to-trans isomerization of substrates of general structure Suc-Ala-Xaa-*cis*-Pro-Phe-pNA. We see that, while k_c/K_m for CyP-catalyzed isomerization shows little dependence on Xaa, k_c/K_m values for FKBP-catalyzed isomerization display a marked dependence on Xaa and vary over 3 orders of magnitude with a preference for large, hydrophobic residues. One of the goals of this study is to understand the mechanistic origins of this specificity difference.

As we just stated, values of k_c/K_m for reactions of FKBP increase with increasing hydrophobicity of the P_1 residue (see Figure 1). When $\Delta G^{\ddagger}$ values for these reactions are dissected into $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values (see Table III), we see that these reactions proceed with large values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$. In contrast, k_c/K_m values for reactions catalyzed by CyP have no significant dependence on the P_1 residue, and these reactions proceed with more

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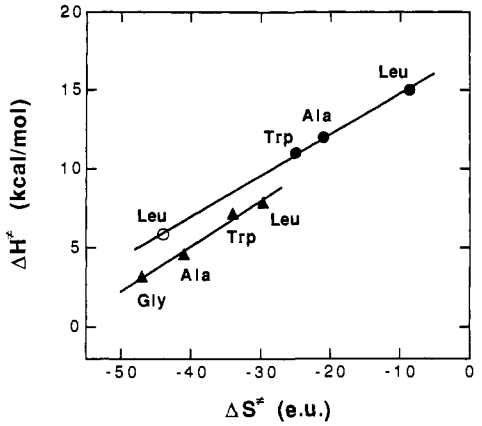


Figure 5. Enthalpy–entropy compensation for PPI catalysis for FKBP (●), rhFKBP (○),[11] and CyP (▲). Data taken from Table III.

negative values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$.

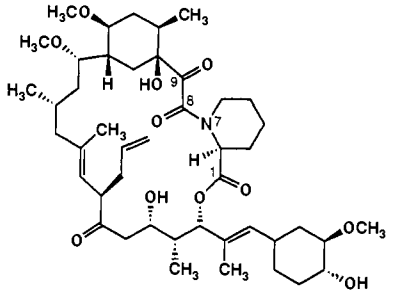
Now, if we assume that the active sites of these enzymes have a hydrophobic pocket at S_1 as well as discrete subsites for substrate amino acids, we can explain these results by assigning different levels of importance to these different modes of interaction for the two enzymes. To account for the P_1 specificity of FKBP, we not only assume a more prominent role for P_1–S_1 interactions but also that these interactions are characterized by dehydration of the Michaelis complex, E:S, as it proceeds to the transition state, $[E:S]^{\ddagger}$. What we are suggesting here is that in E:S the P_1 residue is not yet buried in S_1 and that the active site and the substrate are still at least partially solvated. As E:S proceeds to $[E:S]^{\ddagger}$, the P_1 residue becomes buried in the S_1 pocket and the residual water of solvation is expelled from the active site. This scenario can reasonably account for the large values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ that we observe for reactions of FKBP, since the formation of hydrophobic contacts between apolar groups in aqueous solution is known to be accompanied by positive enthalpy and entropy changes.[29]

Likewise, to account for the lack of P_1 specificity for CyP, we assume that subsite interactions play a more prominent role than do P_1–S_1 interactions. Thus, the P_1–S_1 hydrophobic interactions that dominate the thermodynamic parameters for FKBP have a smaller role for this enzyme.

Another point of mechanistic interest is the enthalpy–entropy compensation that we observe for reactions of both CyP and FKBP (Figure 5). This compensation even includes a reaction that is catalyzed by rhFKBP.[11] The molecular origins of enthalpy–entropy compensation in enzyme catalysis are unclear.[18,30-32] The simplest explanation that one can advance involves a situation in which stronger transition-state interactions between enzyme and substrate, which will manifest themselves in lower $\Delta H^{\ddagger}$ values, are accompanied by greater restrictions of translational and rotational freedom, which will manifest themselves in more negative $\Delta S^{\ddagger}$ values.[30,32] That there are two separate correlations for the two enzymes is consistent with the mechanistic and specificity differences that we described above.

Origin of Catalytic Power. One goal of mechanistic enzymology is to understand the origins of enzyme catalytic power. We noted earlier in this paper that prolyl cis–trans isomerization proceeds at reaction rates that are conveniently measured and, therefore, can be subjected to detailed mechanistic study. In this final section, we compare enzymic and nonenzymic prolyl isomerization with a view toward probing the mechanistic origins of the catalytic power of these enzymes. Since we have shown that the transition states for enzymic and nonenzymic prolyl isomerization have similar structures, addressing this issue for prolyl isomerase should be easier relative to enzymes whose reactions proceed through transition states that bear questionable similarity to the transition state of the nonenzymatic reaction. As detailed above, both spontaneous and PPI-catalyzed prolyl isomerization proceed through transition states characterized by partial rotation about the amide linkage with no catalytic proton bridging to solvent or the enzyme surface.

Chart I. Structure of FK-506



Our starting point is catalytic efficiency, which we define as the ratio of first-order rate constants for the decomposition of the Michaelis complex, $k_{c,c/t}$, to uncatalyzed prolyl cis-to-trans isomerization, $k_{c/t}$:

$$\frac{k_{c,c/t}}{k_{c/t}} \geq 6 \times 10^5$$

The task we are faced with here is to determine what molecular events are responsible for this value.

Clues to the origin of the catalytic power of prolyl isomerases[33] can be found in examining the recently reported three-dimensional structure of the complex of FKBP and FK-506[34] in light of our proposed mechanism. Central to our understanding of how FKBP might catalyze cis–trans isomerization is the way in which this protein is revealed to bind FK506 at the pipecolic amide (C-1–C-8) and keto (C-9) moieties (Chart I). The X-ray structure shows that this structural unit is buried in the hydrophobic, active site cavity of FKBP with the pipecolinyl ring buttressed up against the indole ring of Trp^{59} and the oxygen of the amide carbonyl of C-8 hydrogen bonded to the ring OH of Tyr^{82}. Of greatest significance is the disposition of the C-9 carbonyl. In solution, the C-8 and C-9 carbonyls are orthogonal, and examination of the structure shows that this is maintained in the complex. In this complex, there are no hydrogen bonds to the C-9 keto oxygen, but rather this oxygen finds itself buried in a unique structural unit composed of the three hydrophobic rings of the conserved amino acids Phe^{99}, Tyr^{26}, and Phe^{36}.

This binding configuration immediately suggests a mechanism for catalysis.[33] According to this mechanism, when a proline-containing peptide, R-Xaa-Pro-R′, binds to FKBP, the amide oxygen of Xaa is inserted into the same hydrophobic hole that the keto oxygen of C-9 finds itself. Transfer from aqueous solution to this very hydrophobic environment destabilizes amide resonance structures in which the oxygen is negatively charged and favors resonance structures in which the amide carbonyl is more ketone-like. This is consistent with the observations that the rate constant for C–N bond rotation in DMA is accelerated in nonpolar solvents.[14] At the same time, the nitrogen atom of the proline ring, N-7, loses sp^2 character and resembles a cyclic tertiary amine. The picture is complete when Tyr^{59} donates a hydrogen bond to N-7, thereby enforcing the developing sp^3 character of this atom. This is equivalent to the observed acid catalysis for both cis–trans

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prolyl isomerization[21,22] and C–N bond rotation in DMA.[15] With these interactions, the bond between Xaa and Pro will have lost resonance stabilization and double bond character. The energy barrier to rotation about this bond will be much lower than rotation about an amide bond and, thus, FKBP will have effected catalysis.

This mechanistic hypothesis has value in that is explains the perplexing observation noted above that the secondary deuterium isotope effect for enzymic prolyl isomerization (k_H/k_D = 1.13 for cyclophilin[6,17] and k_H/k_D = 1.12 for FKBP, Dr. D. Livingston, Vertex Inc., personal communication) is unaccountably larger than the isotope effect for nonenzymic isomerization (k_H/k_D = 1.05[6,16,17]). The hydrophobic environment in which the amide bond finds itself in the enzyme active site will stabilize a transition state with less polar character than the transition state for reaction in solution. As we discussed above, this will magnify the enzymic isotope effect.[27]

Registry No. CyP, 95076-93-0; Suc-Ala-Gly-Pro-Phe-pNA, 128802-77-7; Suc-Ala-Ala-Pro-Phe-pNA, 70967-97-4; Suc-Ala-Val-Pro-Phe-pNA, 95192-38-4; Suc-Ala-Ile-Pro-Phe-pNA, 128802-79-9; Suc-Ala-Nle-Pro-Phe-pNA, 128802-72-2; Suc-Ala-Leu-Pro-Phe-pNA, 128802-78-8; Suc-Ala-Phe-Pro-Phe-pNA, 128802-73-3; Suc-Ala-Trp-Pro-Phe-pNA, 128822-32-2; Suc-Ala-His-Pro-Phe-pNA, 128802-75-5; Suc-Ala-Lys-Pro-Phe-pNA, 128802-74-4; Suc-Ala-Glu-Pro-Phe-pNA, 128802-76-6.

Stereospecific Synthesis of Aryl β-Glucosides: An Application to the Synthesis of a Prototype Corresponding to the Aryloxy Carbohydrate Domain of Vancomycin

Russell G. Dushin and Samuel J. Danishefsky*

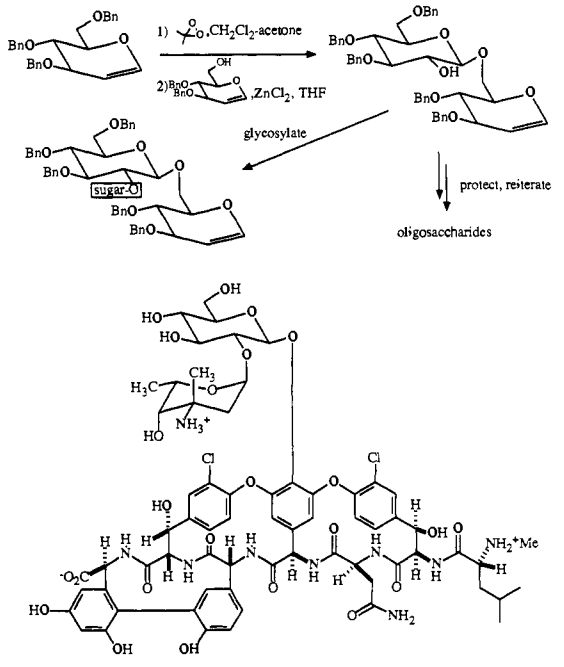
Contribution from the Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut 06525. *Received July 15, 1991*

Abstract: The reaction of 3,4,6-tri-O-benzyl-D-glucal (**2**) with 3,3-dimethyldioxirane gives rise to the 2α,3α-oxirane with high stereoselection. Reaction of this compound with various phenols under alkaline conditions affords aryl 2α-hydroxy-β-glycosides (**3a–d**). Oxidative coupling (I^+) of these compounds with glycals followed by deiodination gives rise to aryloxydisaccharides (**6a** and **6b**). In addition, proton-mediated coupling of **3a** and **3b** with vancosamine-derived glycal **8** affords **10a** and **10b**, which simulate the aryloxy carbohydrate domain of vancomycin.

Previous reports from these laboratories described the synthesis of various β-glycosides from the Lewis acid catalyzed addition of aliphatic alcohols to 1,2-anhydrosugars[1,2] (Scheme I). The anhydrosugars employed were readily prepared in situ from the stereoselective oxidation of the appropriate glycals using 3,3-dimethyldioxirane.[3] The simple two-step procedure provided high yields of β-glycosides bearing a unique hydroxyl group at C-2. Through the use of glycals as glycosyl acceptors, the method lends itself to ready reiteration and thereby provides rapid access to relatively complex oligosaccharides. Noteworthy is the fact that the method is ideally suited for the synthesis of complex glycosides in which the C-2 position is glycosylated (see Scheme I).

We had hoped to extend this chemistry to the synthesis of aryl β-glucosides. A focusing goal of the investigation was that of assembling a realistic prototype of the aryloxy carbohydrate domain of vancomycin (**1**).[4] In this naturally occurring glycopeptide antibiotic, the central phenolic ring of the peptide-based aglycone is attached through a β-linkage to a glucosyl residue which is, in turn, attached through a (2–1)-α-linkage to the 3-aminosugar, vancosamine. Given the remarkable advances which have been

Scheme I



recently registered by the Evans school[5] toward the syntheses of the complex aglycone sector of vancomycin and related com-

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