Kinetics and Mechanism of Isomerization of Cyclosporin A

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The kinetics of isomerization of cyclosporin A to isocyclosporin A were studied in various nonaqueous solvents as a function of temperature and added methanesulfonic acid. The rate of isomerization was found to be acid-catalyzed over the acid concentration range studied. The choice of organic solvent significantly altered the rate of isomerization. For a series of alcohols, the rate was enhanced with increasing dielectric constant of the media, however, this correlation did not hold upon introduction of the dipolar aprotic solvent, tetrahydrofuran. Conversion of cyclosporin A to isocyclosporin A in tetrahydrofuran was found to contain diminished side reactions as compared to alcoholic solvents. The rate of conversion of isocyclosporin A to cyclosporin A was determined in aqueous buffers as a function of pH, buffer concentration, and temperature. The rates of conversion were extremely rapid compared to the forward reaction. Based on the pH dependencies of dilute solution reactivities, isocyclosporin A displayed a kinetically generated pK_a value of 6.9 for the secondary amine moiety. From pH 8 to pH 10 the pH–rate profile plot is linear, with a slope approximately equal to unity, indicating apparent hydroxide ion catalysis. The break in pH–rate profile suggests a change in the rate-determining step upon protonation of isocyclosporin A. The rate of isomerization in plasma was comparable with that found in a pH 7.4 buffer solution, indicating that plasma proteins do not significantly alter the isomerization kinetics of isocyclosporin A to cyclosporin A.

KEY WORDS: cyclosporin A; isomerization; *N,O*-acyl migration; isocyclosporin A; cyclic peptide, peptide bond cleavage.

INTRODUCTION

Design of highly constrained cyclic peptides resistant to enzymatic degradation has led to development of biologically active peptide analogues with improved oral bioavailability (1–3). Understanding the nonenzymatic stability of cyclic peptides under various conditions is essential for their development as pharmaceutical products. A variety of chemical reactions such as deamidation, oxidation, β-elimination, and proteolysis is known to affect linear peptides (4). However, little work has been done in examining the chemical reactivities of cyclic peptides.

Proteolysis at the X-Ser and X-Thre bond in proteins has been studied by many investigators (5–7). Recent work on the kinetics of degradation of a linear decapeptide, RS 26306, in aqueous solution demonstrated that under slightly acidic conditions (pH 4–6) serine-catalyzed peptide bond hydrolysis becomes an important degradation pathway (8). It was suggested that the rearrangement involves *N,O*-acyl migration that converts the usual N-peptide bond into an O-peptide bond. The O-peptide is not isolated because it was postulated to undergo further hydrolysis resulting in fragmentation of the peptide.

The rate of rearrangement is dependent on such factors as acidity, temperature, and the adopted solution conformation of the peptide. Cyclization imparts constraint and, consequently, reduces the number of possible solution conformers, which in turn may retard or enhance the rate and the extent of acyl migration.

Cyclosporin A (CsA; Fig. 1) is a hydrophobic cyclic undecapeptide that is widely used as an immunosuppressive agent in organ and bone marrow transplantation. This paper focuses on the chemical stability of cyclosporin A, specifically spontaneous cleavage of the peptide bond at the amino acid residue containing the β-hydroxyl functionality (MeBmt). Rüegger and co-workers (9) showed that acid treatment of CsA in the absence of H_2O effected an *N,O*-acyl migration of the methyl valine moiety to the first residue to furnish isocyclosporin A (isoCsA). In this study, the rate of conversion of CsA to isoCsA was determined in various nonaqueous solvents as a function of temperature and added methanesulfonic acid concentration. The rate of conversion of isoCsA back to CsA was also determined in aqueous buffers as a function of pH, buffer concentration, and temperature. Degradation of CsA and isoCsA under aqueous acidic conditions is currently under investigation.

MATERIALS AND METHODS

Materials

Cyclosporin A was obtained from Sandoz (Hanover, N.J.) and isocyclosporin A (isoCsA) was synthesized by the procedure described by Rüegger and co-workers (9). Methanol, ethanol, 2-propanol and tetrahydrofuran were all of HPLC grade and were dried and freshly distilled. Methanesulfonic acid was dried azeotropically using benzene. The water was deionized and glass distilled (Mega-Pure System Model mp-1, Corning). All other chemicals were of reagent grade and used without further purification.

Kinetic Procedures (CsA to IsoCsA)

Dilute solutions of CsA ($1–3 \times 10^{-5}$ *M*) were prepared in various organic solvents (methanol, ethanol, 2-propanol, and tetrahydrofuran) containing variable concentrations of methanesulfonic acid. At appropriate time intervals, samples were withdrawn and diluted with HPLC mobile phase before analysis by HPLC (see Analytical Procedure, below). Pseudo-first-order rate constants for the apparent conversion reaction of CsA to isoCsA were obtained by following the disappearance of CsA for at least three half-lives.

Kinetic Procedure (IsoCsA to CsA)

The pH of aqueous buffer solutions was adjusted at the experimental temperatures using a Corning pH meter which was standardized at the experimental temperature with NBS buffer solutions. The ionic strength of the solutions was adjusted to 0.15 with KCl.

Stock solutions of isoCsA were prepared in acetonitrile.

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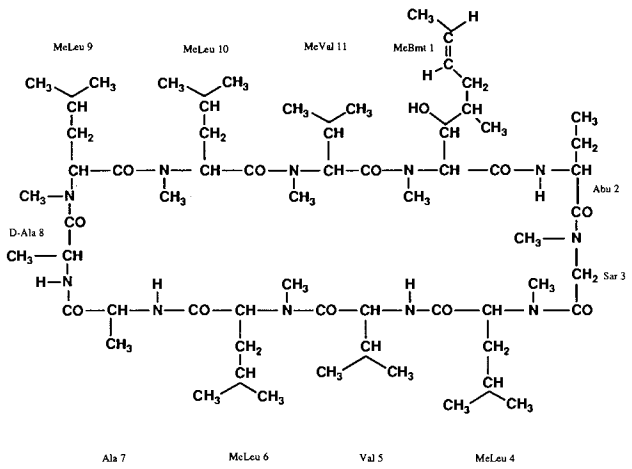


Fig. 1. Chemical structure of cyclosporin A (CsA) with the standard numeration of the amino acid residues. MeBmt, (4*R*)-4-[(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine; Abu, L-α-amino-butyric acid; MeLeu, *N*-methylleucine; MeVal, *N*-methylvaline.

A 1-ml aliquot of stock solution was used to prepare 100-ml dilute solutions of isoCsA ($4–6 \times 10^{-6}$ *M*) in the pH-adjusted buffer solutions. At appropriate time intervals, samples were withdrawn and analyzed. The interconversion kinetics of isoCsA to CsA were studied at 37 ± 0.2°C over a pH range of 6–10 at three or four buffer concentrations ranging from 0.01 to 0.06 *M*. Pseudo-first-order rate constants for the apparent conversion of isoCsA to CsA were obtained using the same protocol as above.

The rate of conversion of isoCsA to CsA in plasma was studied by adding a 100-μl aliquot of isoCsA in dimethyl sulfoxide to 5 ml of plasma. Samples were withdrawn at appropriate time intervals, mixed with a 2× volume of acetonitrile, centrifuged, and then analyzed using the method described under Analytical Procedure.

Determination of the Dissociation Constant of IsoCsA

The apparent ionization constant of isoCsA in aqueous solution was estimated by curve fitting the pH–rate profile. Due to the minimal aqueous solubility and lack of appropriate chromophoric properties of isoCsA, no attempt was made to determine the pK_a of isoCsA independently.

Analytical Procedure

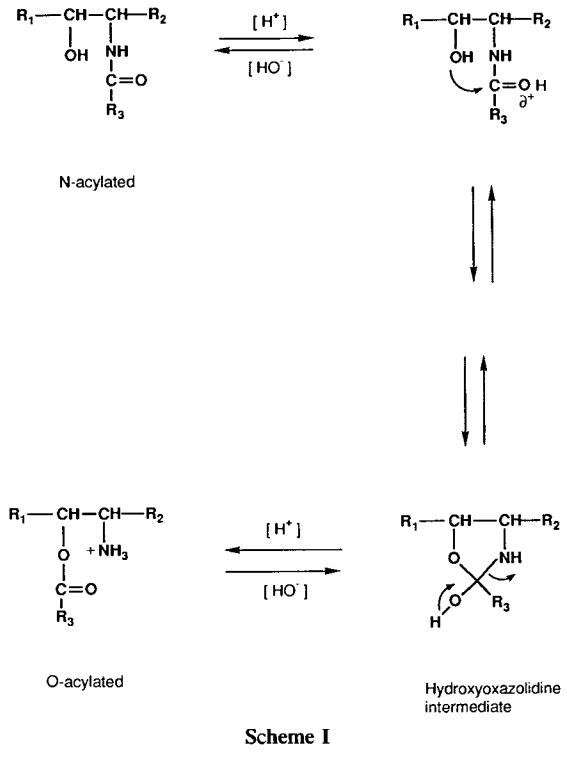
High-performance liquid chromatography (HPLC) was performed using a system consisting of a Shimadzu SPD-6A variable-wavelength detector operating at 200 nm; Shimadzu LC-6A pumps, a Shimadzu SIL-6B auto injector, and a Shimadzu CR601 integrator for peak processing. The HPLC studies were conducted using a reverse-phase analytical column C8 (15 cm × 3.9 mm) with a mean particle diameter of 5 μm. All the analyses were performed under isocratic condition at 70°C to minimize the peak broadening associated with CsA analysis (10). This also improved the peak shape for isoCsA. Flow rate was set at 1.5 ml/min. The mobile phase contained 27 parts 10 m*M* phosphate buffer (pH 4.6), 46 parts acetonitrile, and 27 parts methanol. Retention volumes for CsA and isoCsA were 12.1 and 15.3 ml, respectively. Calibration curves were constructed from linear plots of peak height versus concentration. The plasma kinetic study was conducted using a procedure identical to that above except the mobile phase contained 33 parts 10 m*M* phosphate buffer (pH 4.6), 47 parts acetonitrile, and 20 parts methanol. Retention volumes for CsA and isoCsA were 23.7 and 36.3 ml, respectively.

RESULTS AND DISCUSSION

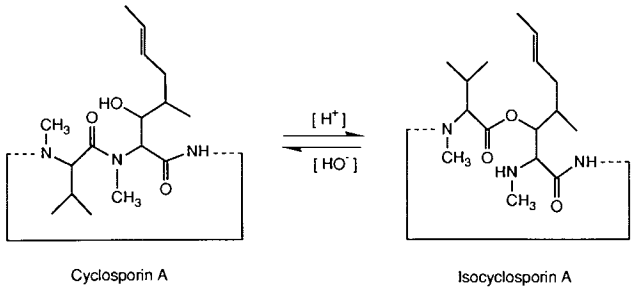
Conversion of CsA to IsoCsA

The *N,O*-acyl migration phenomenon, first studied by Bergmann *et al.* (11), has been subjected to extensive investigation using various model compounds such as acylated amino alcohols and amino alcohol amino acids, serine and threonine (12). Duesnuelles and Casals (13) were able to demonstrate that the amino groups of serine and threonine were among the first to be liberated during mild acid hydrolysis of proteins. The proposed mechanism of *N,O*-acyl migration, represented in Scheme I, involves the formation of a hydroxyoxazolidine intermediate followed by its breakdown to afford the O-acylated compound. The shift is readily reversible and equilibrium is dependent on the acidity of the media.

The kinetics of CsA conversion to isoCsA in organic solvents were studied in dilute solution as a function of added methanesulfonic acid, temperature, and the choice of organic solvent. Under the experimental conditions, the isomerization of CsA to isoCsA was found to be the predominant pathway (Scheme II) leading to the loss of CsA. A representative plot of the disappearance of CsA and appearance of isoCsA is shown in Fig. 2. The interconversion of CsA to isoCsA followed apparent first-order kinetics for at



Scheme I



Scheme II

least three half-lives and the reaction appeared to be complete. The yield of isoCsA was estimated to be about 80%.

As can be seen in Fig. 3, the kinetics of isomerization of CsA to isoCsA were found to be acid-catalyzed over the acid concentration range studied. This is consistent with the mechanism proposed in Scheme I. The data in Fig. 3 show that the reaction is apparent first order in hydrogen ion up to 0.02 *M* methanesulfonic acid. Above 0.02 *M* some curvature was observed. The mechanistic implication of the curvature is unknown at this time.

It has been our and other investigators' intention to improve the sensitivity of the bioanalytical procedure of CsA by first converting it to isoCsA. IsoCsA can then be further derivatized with fluorogenic agents (14). The change in reaction rate in various solvents is likely associated with the difference in solvation energies of the reactant and the transition state (15). The influence of solvent on the isomerization kinetics of the CsA-to-isoCsA conversion described by the Kirkwood-Onsager function (16) is shown in Fig. 4. For a series of alcohols, the rate of isomerization was enhanced with increasing dielectric constant of the medium. This is consistent with the general proposed mechanism of *N,O*-acyl migration in which a nonelectrolyte reactant forms a transition state with an ionic character (Scheme I). However, such a correlation did not hold upon introduction of the

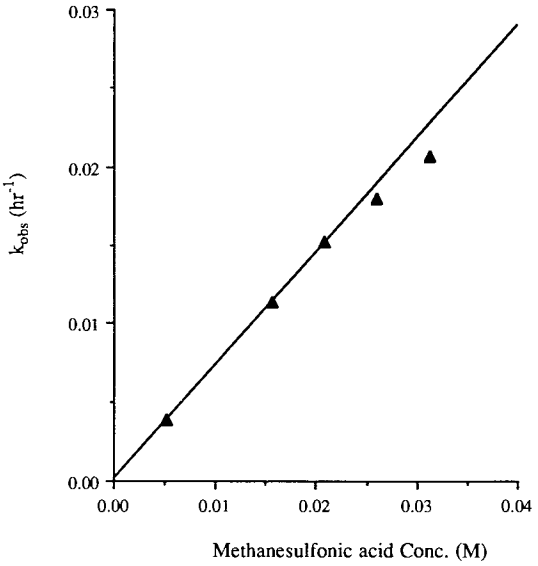


Fig. 3. Dependence of the apparent first-order rate constants of isomerization of cyclosporin A on methanesulfonic acid concentration in methanol at 37°C.

dipolar aprotic solvents, tetrahydrofuran (THF). This may be attributed to the fact that the Kirkwood-Onsager function describes only the bulk properties of the solvent (based on dielectric constant) and tends to ignore specific solvent–solute interactions as well as other solvent properties such as acidity and hydrogen bonding capability (17). Based on the chromatographic data, the conversion of CsA to isoCsA in THF was found to contain diminished side reactions as compared to the alcoholic solvents. Further studies are in progress to determine whether an improvement in yield of isoCsA is achieved.

Conversion of IsoCsA to CsA

The kinetics of isoCsA-to-CsA conversion were studied

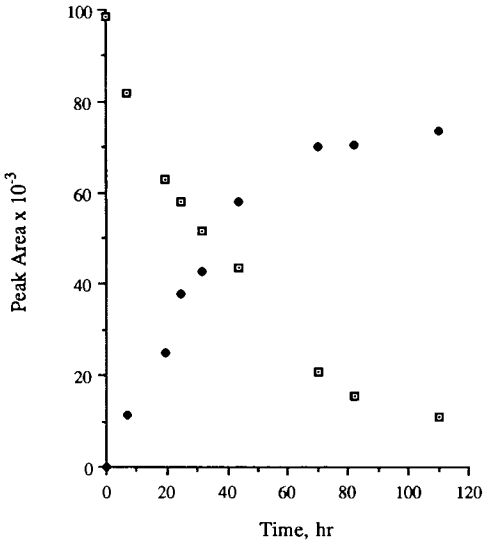


Fig. 2. Plot of disappearance of cyclosporin A (□) and appearance of isocyclosporin A (♦) in 0.026 *M* methanesulfonic acid solution in methanol at 37°C.

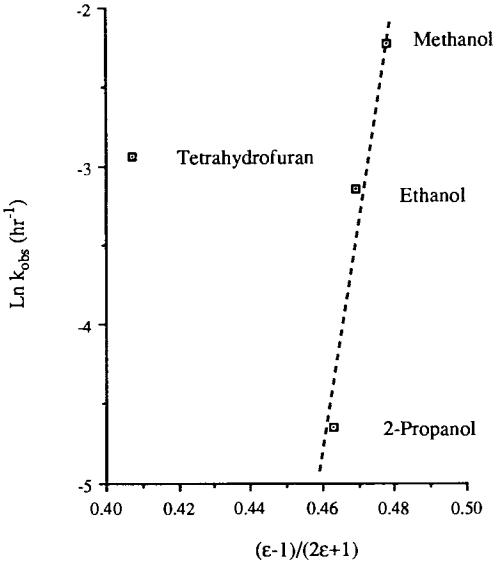


Fig. 4. Correlation between ln k_{obs} and the Kirkwood-Onsager function for isomerization of cyclosporin A to isocyclosporin A at 50°C (where ε is the bulk dielectric constant).

in dilute aqueous solution as a function of pH, temperature, and buffer concentration. Under the experimental conditions utilized, isomerization of isoCsA to CsA was found to be the predominant and apparent reaction pathway. A representative plot of the disappearance of isoCsA and appearance of CsA is shown in Fig. 5. The complete conversion of isoCsA to CsA followed apparent first-order kinetics for at least two to three half-lives in the pH range 6–10.

Figure 6 represents a partial pH–rate profile for isomerization at 37°C, where the rate constant, k_{obs}, is extrapolated to zero buffer concentration. From pH 8 to pH 10 the plot is linear, with a slope approximately equal to unity, indicating apparent hydroxide ion catalysis. At pH values below 8 the pH–rate profile can be described by two kinetically indistinguishable processes, an apparent base-catalyzed isomerization of the protonated isoCsA and spontaneous isomerization of the corresponding free amine. Since the conjugated acid may be considered to be a nonreactive species, it was assumed that the pH–rate profile could be described by Eq. (1).

$$k_{obs} = k_O f_A + k_{OH}[HO^-]f_A \quad (1)$$

where f_A (Ka/[Ka + H^+]) is the fraction of isoCsA in its free-base form, K_a is the apparent dissociation constant for the protonation form of isoCsA, and k_{OH} and k_O are the microscopic second-order rate constant for specific base-catalyzed and spontaneous first-order rate constant for the isomerization of isoCsA to CsA, respectively. The kinetic rate constant and dissociation constant parameters (±SEM) used to generate the theoretical profile (Fig. 6) are the following: k_O = 1.4 (±0.3) × 10^{-3} min^{-1}, k_{OH} = 3.4 (±0.4) × 10^2 min^{-1} M^{-1}, and K_a = 1.3 (±0.5) × 10^{-7} (pK_a = 6.9).

It was not possible to measure the apparent pK_a of isoCsA by other means because of the limited aqueous solubility and lack of appropriate chromophoric properties. The kinetically generated pK_a value of 6.9 is reasonable for an aliphatic amine of the type seen in isoCsA (18).

Based on the pH dependencies of dilute solution reac-

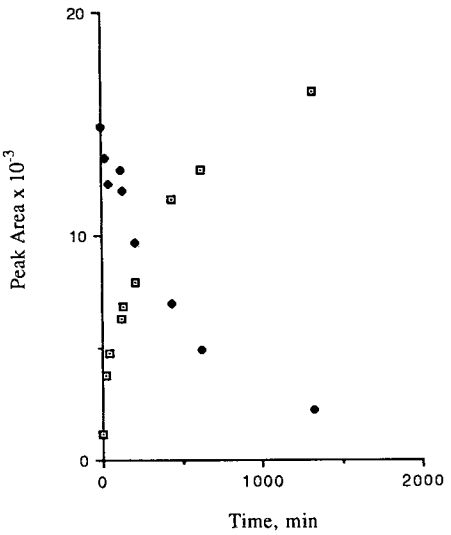


Fig. 5. Plot of disappearance of isocyclosporin A (♦) and appearance of cyclosporin A (⊡) at 37°C in 0.04 *M* aqueous Tris buffer (pH 8.0 and μ = 0.15 with KCl).

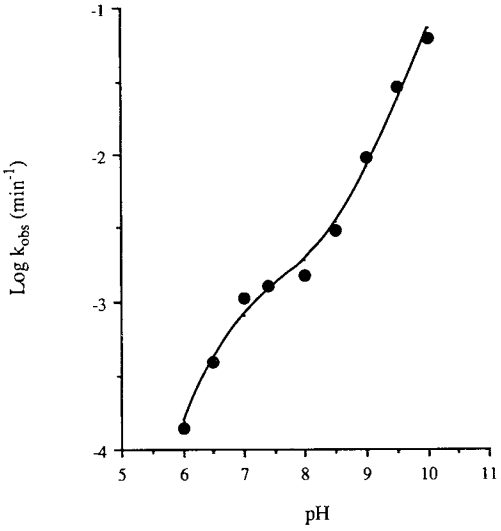
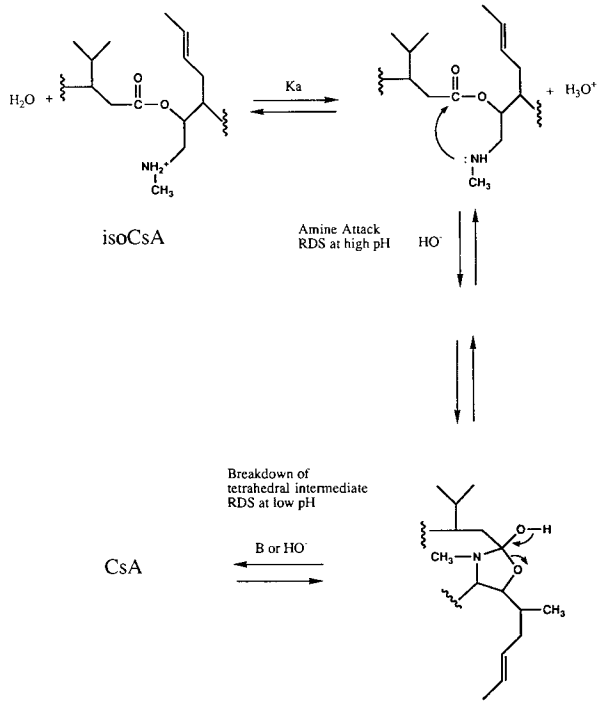


Fig. 6. Partial pH–rate profile for the isomerization of isocyclosporin A to cyclosporin A at 37°C (μ = 0.15). The solid line represents the theoretical profile based on Eq. (1) and values of 1.4 × 10^{-3} for k_O, 3.4 × 10^2 min^{-1} M^{-1} for k_{OH}, and 1.3 × 10^{-7} for K_a.

tivities of isoCsA, the ionization of secondary amine moiety had a significant effect on the apparent rate constant. The break in the curve suggests a change in the rate-determining step upon ionization of isoCsA. Aminolysis of model esters with a poor alcoholic leaving group has revealed that at high pH values amine attack is the rate-determining step since alkoxide is a far better leaving group than the amine anion (19), whereas amine expulsion is preferred to alcohol expulsion at lower pH values in which the breakdown of tetrahedral intermediate becomes the rate-determining step (Scheme III). The amine attack and breakdown of tetrahe-



Scheme III

dral intermediate steps are both subject to pronounced general base catalysis in these model compounds, although catalysis is less significant for the latter step than the former (20). As shown in Fig. 7, slight buffer catalysis was observed for the isomerization of isoCsA to CsA at lower pH values (6.0–7.4). The magnitude of buffer catalysis was enhanced by increasing the pH of solution for phosphate buffer consistent with a general base-catalyzed mechanism, possibly breakdown of the tetrahedral intermediate. At pH values above the pK_a, there was no significant catalytic effect on the rate-determining step by the buffers employed. The depressed or lack of dependence of the apparent rate constant for isomerization of isoCsA to CsA on the total buffer concentration may be attributed to the sterically hindered reactive site imposed by the rigid cyclic structure of CsA.

The observed energies of activation, $E_{a_{obs}}$ (19.9 ± 0.5 kcal/mol), for the conversion of isoCsA to CsA at pH 7.4, 0.02 *M* phosphate buffer was obtained from the temperature dependence (25, 29, 37, and 50°C) of the apparent rate constants (Table I). The data can be described by Eq. (2).

$$\ln k_{obs}\,(\text{min}^{-1}) = 26.5 - 9997/T \qquad (r = 1.00) \qquad (2)$$

The above equation was not corrected for the dissociation constant of water or the amine moiety, hence, $E_{a_{obs}}$ represents the contributions of the enthalpy of dissociation of water and the enthalpy of ionization of amine as well as the energy of activation for the reaction.

CsA is known to bind with a high affinity to a variety of proteins. Weber and co-workers (21) have presented NMR evidence displaying aqueous solution conformation of CsA upon complexation with cyclophilin. The NMR data indicate that the complexation with cyclophilin introduced a substantial change in the conformation of CsA for which all peptide bonds are in the *trans* form. It was found that all elements of secondary structure are lost and that intramolecular hydrogen bonds are disrupted, which, consequently, renders all the polar groups of CsA exposed to the solvent environment.

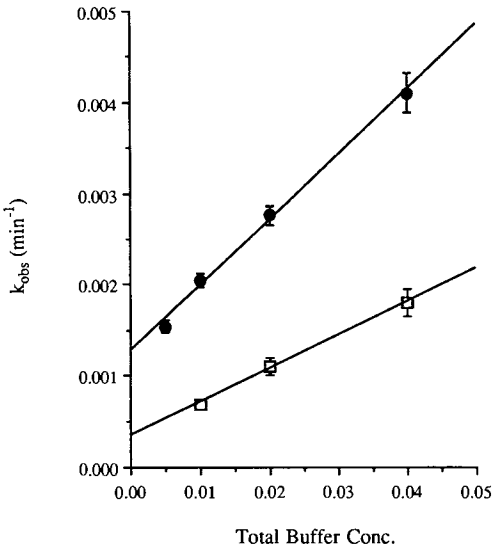


Fig. 7. Phosphate buffer concentration dependence of the apparent first-order rate constants for isomerization of isocyclosporin A to cyclosporin A at pH 7.4 (●) and 6.5 (□) (37°C and μ = 0.15).

Table I. Temperature Dependence of the Apparent Rate Constant for the Isomerization of Isocyclosporin A to Cyclosporin A at pH 7.4, 0.02 *M* Phosphate Buffer (μ = 0.15)

Temperature (°C)	k_{obs} (min^{-1} ± SE) × 10^{-3}
25	0.67 ± 0.04
29	1.05 ± 0.05
37	2.55 ± 0.04
50	8.96 ± 0.01

Based on this study, the rate of isomerization of isoCsA to CsA was determined in plasma at 37°C to measure what effect the plasma protein binding exerts on the interconversion kinetics. The objective is to determine whether a change, if any, in the conformation of isoCsA induced by plasma protein binding affects the observed kinetics. The rate and extent of isomerization in plasma (k_{obs} = 9.5 × 10^{-4} min^{-1}) are comparable with those found in a pH 7.4 buffer solution, indicating that plasma proteins do not significantly alter the isomerization kinetics of isoCsA to CsA. Based on the chromatographic data, no alternative degradation products were observed in plasma. However, the effect of complexation of isoCsA with other endogenous components, such as cyclophilin, on the rate of isomerization is yet to be determined.

In summary, the isomerization kinetics of CsA to isoCsA in nonaqueous solution were found to be acid-catalyzed and affected by the choice of organic solvent. The rates of isomerization of isoCsA to CsA in aqueous buffers as compared to the forward reaction were extremely rapid, apparently hydroxide ion catalyzed, and unaffected by the plasma protein binding.

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