Accelerated Publications

Bradykinin and Its Gly^6 Analogue Are Substrates of Cyclophilin: A Fluorine-19 Magnetization Transfer Study

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ABSTRACT: Fluorine-19 magnetization transfer experiments have been used to determine the rates of cis/trans isomerization about the X–Pro^7 peptide bond in [*p*-fluoro-Phe^8]bradykinin (cis/trans ratio ~0.1) and its Gly^6 analogue (cis/trans ratio ~0.4). The measurements were carried out both prior to and after the addition of cyclophilin, which has recently been shown to have peptidyl-proline cis/trans isomerase activity and is the apparent target enzyme of the immunosuppressive agent cyclosporin A. Magnetization transfer measurements over the temperature range 40–75 °C in the absence of enzyme give activation energies of 22.8 and 23.0 kcal/mol for [*p*-fluoro-Phe^8]bradykinin and its Gly^6 analogue, respectively. The values for the uncatalyzed cis → trans rate constant, k_c, are determined by extrapolation to be 4.8×10^{-2} and 2.1×10^{-2} s^{-1} for the two peptides at 25 °C. The enzyme-catalyzed enhancement of the cis/trans interconversion rate was proportional to added cyclophilin concentration and was strongly sequence specific, with bradykinin a much better substrate than [Gly^6]bradykinin. At a peptide concentration of 2.2 mM, the catalytic activity expressed as k_c per micromolar cyclophilin was determined to be 1.2 s^{-1}/μM for [*p*-fluoro-Phe^8]bradykinin and 0.13 s^{-1}/μM for the Gly^6 analogue. The increased cis → trans interconversion rates were strongly inhibited by cyclosporin A and the 6-(methylalanine) derivative, which bind to cyclophilin, but not by the 1-(tetrahydrofurfuryl) derivative of cyclosporin that binds weakly.

The presence of proline residues in proteins and biologically active peptides introduces both conformational constraints and the potential for significant conformational heterogeneity as a result of the slow cis/trans isomerism of imide bonds (Stewart & Siddall, 1970). For short peptides, these characteristics frequently lead to the observation of significant levels of cis and trans conformers, with lifetimes in aqueous solution sufficient to permit observation of both conformers with NMR spectroscopy (Gerig, 1971; Thomas & Williams, 1972; Evans & Rabenstein, 1974; Grathwohl & Wuthrich, 1976a,b, 1981). Cis/trans isomerism has been proposed to have a number of biophysical consequences; in particular, it has been proposed that the cis/trans interconversion of peptide–proline imide bonds may be rate limiting in protein folding and unfolding kinetics (Brandts et al., 1975). Support for the conclusion that cis/trans isomerism is biochemically significant has recently been derived from the discovery that cyclophilin, the apparent target enzyme for the immunosuppressive drug cyclosporin A (Handschumacher et al., 1984; Harding et al., 1986; Koletsky et al., 1986), possesses peptidyl-proline cis/trans isomerase activity (Fischer et al., 1989b; Takahashi et al., 1989). Similar activity has been observed with an 11–12-kdalton protein (FKBP)[1] that specifically binds FK506, a new immunosuppressive agent, but not cyclosporin A (Sierkierka et al., 1989; Harding et al., 1989). The nature of the physiologically important substrates for the corresponding enzymes, cyclophilin and FKBP, has yet to be determined.

Despite the general occurrence of proline residues in biologically active peptides, little is known about the effects of cis/trans conformational heterogeneity on receptor recognition or interaction processes. For example, different isomers might exhibit differential affinities for different receptor classes. As a result of the homology between the Ser^6-Pro^7-Phe^8 sequence of the biologically active peptide bradykinin (sequence: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and the test peptides which have been used to study cyclophilin-catalyzed cis/trans isomerization (Fischer et al., 1984, 1989a,b; Takahashi et al., 1989; Harrison & Stein, 1990), it was of interest to determine whether this biologically active peptide is also a substrate for cyclophilin. We have previously noted that, despite a strong preference for the trans conformation of all X–Pro bonds in bradykinin, a large increase in the cis/trans ratio of the sixth peptide bond, in addition to a small decrease in activity, results from the substitution of a Gly^6 residue for the native Ser^6 (London et al., 1979). On this basis, we have suggested that the predominant trans conformer is most probably the biologically active form of the peptide and that a principle role of Ser^6 is to favor the proportion in the trans conformation. In order to evaluate the possibility that [Gly^6]bradykinin and bradykinin are substrates for cyclophilin, we have prepared two *p*-fluorophenylalanine analogues [Gly^6,*p*-fluoro-Phe^8]- and [*p*-fluoro-Phe^8]bradykinin, and conducted magnetization transfer studies as a function of added cyclophilin. The results demonstrate a significant, sequence-specific enhancement of

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[1] Abbreviations: Abu, α-aminobutyric acid; BK, bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg); CsA, cyclosporin A; cyclophilin, also called peptidyl-proline cis/trans isomerase; FKBP, FK506 binding protein; Glt, glutaryl; pNA, 4-nitroanilide; 6-MeAla-CsA, 6-(*N*-methyl-L-alanine) derivative of cyclosporin A; 1-THF-CsA, 1-(tetrahydrofurfuryl) derivative of cyclosporin A as described by Durette et al. (1988); MeLeu, *N*-methylleucine; Sar, sarcosine; SDS, sodium dodecyl sulfate.

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the cis/trans isomerization rate in both peptides that is strongly inhibited by cyclosporin A.

Materials and Methods

The [Gly^6,*p*-fluoro-Phe^8]- and [*p*-fluoro-Phe^8]bradykinin derivatives were synthesized by solid-phase methods (Stewart & Young, 1984) and purified by countercurrent distribution methods as previously described (Cann et al., 1976, 1990). The major isoform of calf thymus cyclophilin was purified to homogeneity as previously described (Harding et al., 1986). Fluorine-19 NMR studies were carried out at 339.7 MHz on an NT-360 with a 5-mm ^{1}H NMR probe retuned to the ^{19}F resonance frequency or on a GE GN 500-MHz spectrometer operating at 470 MHz with a 5-mm ^{19}F probe obtained from GE. The higher field was found to be most critical for studies of the [*p*-fluoro-Phe^8]bradykinin due to the small chemical shift difference of 0.185 ppm between cis and trans ^{19}F resonances. Additionally, the use of two spectrometers allowed simultaneous measurements to be carried out on two different peptides, thus minimizing any possible differences caused by the relative instability of the enzyme. T_1 values determined at the higher field were approximately 20% shorter (Table I), apparently due to field-dependent chemical shift anisotropy (CSA) contributions (Hull & Sykes, 1975), but it was assumed that the cis $\leftrightarrow$ trans interconversion rate was field independent. Since we are dealing with two-site exchange and the ^{19}F NMR spectra of the peptides consist of only two lines, the pulse sequence of Robinson et al. (1985) is ideally suited to the inversion transfer experiment since it avoids the need for selective pulses. The sequence used was

$$90°_x\text{–}\tau_1\text{–}90°_x\text{–}\tau_2\text{–}90°_\phi\text{–acquire} \quad (1)$$

where $\tau_1 = 1/(2|\delta_c - \delta_t|)$, τ_2 is the variable delay during which transfer of magnetization occurs, and the carrier frequency of the rf is set at the position of the resonance (cis or trans) which is to be selectively inverted. The phase ϕ of the final 90° pulse and the receiver are cycled through x, y, $-x$, $-y$. Nine to 13 delays were used per study. For the more difficult bradykinin studies, the cis or the trans resonance was initially inverted in separate studies, and the results were fit simultaneously by use of a single set of rate constants. For the simple case of interconversion between two (cis and trans) peptide bond conformations described by

$$\text{cis} \underset{k_t}{\overset{k_c}{\rightleftharpoons}} \text{trans} \quad (2)$$

the magnetization transfer equations (Baine et al., 1981; Led & Gesmar, 1982) can be expressed as

$$M_c(t) = M_c(\infty) + C_1 \exp(\lambda_1 t) + C_2 \exp(\lambda_2 t) \quad (3)$$

$$M_t(t) = M_t(\infty) + C_3 \exp(\lambda_1 t) + C_4 \exp(\lambda_2 t) \quad (4)$$

where

$$\lambda_{1,2} = \{-(k_{1c} + k_{1t}) \pm [(k_{1c} - k_{1t})^2 + 4k_c k_t]^{1/2}\}/2 \quad (5)$$

$$k_{1c} = k_c + 1/T_{1c};\ k_{1t} = k_t + 1/T_{1t} \quad (6)$$

$$C_1 = \{(\lambda_2 + k_{1c}) \times [M_c(\infty) - M_c(0)] - k_t[M_t(\infty) - M_t(0)]\}/(\lambda_1 - \lambda_2)$$

$$C_2 = \{-(\lambda_1 + k_{1c}) \times [M_c(\infty) - M_c(0)] + k_t[M_t(\infty) - M_t(0)]\}/(\lambda_1 - \lambda_2)$$

$$C_3 = \{-k_c[M_c(\infty) - M_c(0)] - (\lambda_1 + k_{1c}) \times [M_t(\infty) - M_t(0)]\}/(\lambda_1 - \lambda_2)$$

$$C_4 = \{k_c[M_c(\infty) - M_c(0)] + (\lambda_2 + k_{1c}) \times [M_t(\infty) - M_t(0)]\}/(\lambda_1 - \lambda_2)$$

In the above equations, $M_c(t)$ and $M_t(t)$ measure the time-dependent integrals of cis and trans resonances, T_{1c} and T_{1t} are the cis and trans spin–lattice relaxation times, $M_c(\infty)$, $M_c(0)$, $M_t(\infty)$, and $M_t(0)$ correspond to the cis and trans resonances in the fully relaxed state and after the initial (specific) peak inversion. $M_c(\infty)$ and $M_t(\infty)$ were determined directly from the fully relaxed spectra and a normalization condition. For most studies, the independently measured T_{1c} and T_{1t} values were used and the rate constant, k_c, and initial magnetization values varied to yield an optimal fit as judged by minimization of the square of the difference between theory and experiment [k_t is set by the requirement that $M_c(\infty)k_c = M_t(\infty)k_t$]. For the temperature-dependent studies, the T_{1c} and T_{1t} values were also allowed to vary.

All studies were run at 25 °C unless otherwise stated. Peptides were dissolved in a buffer containing 0.2 M NaCl, 5 mM 2-mercaptoethanol, 20 mM sodium phosphate, pH 7.0, and 25% D_2O used for the lock. Aliquots of a stock solution of cyclophilin (1 mg/mL) were added directly to 0.3-mL solutions initially containing 2.6 mM peptide in buffer.

Results

The substitution of a glycine residue for Ser^6 of bradykinin significantly increases the cis/trans ratio about the sixth peptide bond from ~0.1 to ~0.4 (London et al., 1979). The physical basis for this effect has been analyzed in detail on the basis of chemical shift, coupling constant, and relaxation data of model peptides and results primarily from an unfavorable interaction between the Phe^8 side chain and the Gly^6 carbonyl oxygen in the trans configuration (Anteunis et al., 1981). The introduction of a fluorine reporter group on Phe^8 of bradykinin, which has only a minimal effect on peptide activity (Fisher et al., 1977), provides a convenient and sensitive means of monitoring the cis/trans ratio (Cann et al., 1990) as well as interconversion kinetics. Additionally, although previous ^{13}C NMR studies indicated the presence of small amounts of cis peptidyl–proline bonds in bradykinin (London et al., 1978), the use of a ^{19}F label on Phe^8 allows for the first time an accurate determination of the cis/trans ratio corresponding to the sixth peptide bond of bradykinin itself. The ^{19}F resonances in bradykinin show a considerably smaller chemical shift difference of 0.18 ppm compared with 0.62 ppm in the Gly^6 analogue and a cis/trans ratio of 0.13, similar to the value of 0.15 observed in the C-terminal fragment, Ser-Pro-Phe-Arg (London et al., 1978).

Fluorine-19 spin–lattice relaxation times for both peptides are summarized in Table I. From these data, several conclusions may be drawn. The T_1 values for both conformations of both peptides decrease with increasing magnetic field strength. In addition, a small (~6%) ^{19}F{^{1}H} NOE was observed for both peptides at 11.75 T. These observations are consistent with relaxation contributions arising from both dipolar and chemical shift anisotropy (CSA) mechanisms, as is observed in larger proteins containing fluoro aromatic amino acids (Hull & Sykes, 1975). The T_1 values of the cis conformers are significantly shorter than those of the trans. In the case of the [Gly^6,*p*-fluoro-Phe^8]bradykinin, the shorter T_1 value of the cis conformer is consistent with the stronger intramolecular interaction between the Phe^8 phenyl ring and the Gly^6 residue analyzed in detail previously for model peptides (Anteunis et al., 1981). The T_1 values for the cis and trans conformations of [*p*-fluoro-Phe^8]bradykinin determined in the presence of enzyme are more similar, due to the interconversion between the two conformations which tends to average the spin–lattice relaxation behavior of the two forms. Addition of cyclophilin plus the inhibitor 6-MeAla-CsA, which blocks the active site of the enzyme, results in a minimal perturbation

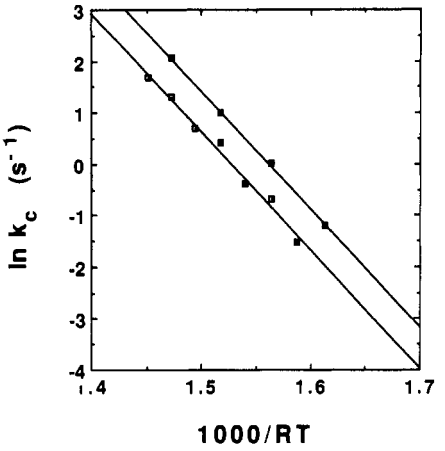

FIGURE 1: Arrhenius plots of the cis → trans rate constant, ln k_c, as a function of $1000/RT$ for [Gly^6,*p*-fluoro-Phe^8]bradykinin (□) and [*p*-fluoro-Phe^8]bradykinin (■). Studies were carried out with 2.2 mM peptide in 20 mM sodium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 0.2 M NaCl buffer containing 25% D_2O for the lock.

of the T_1 values of the free peptides, as expected. These T_1 values ultimately determine the range of interconversion rates which is accessible to measurement by magnetization transfer (Led & Gesmar, 1982).

Subsequent to a selective inversion of the trans resonance of fluorinated bradykinin or its Gly^6 analogue in the absence of enzyme, no significant magnetization transfer could be observed at 25 °C, indicating that the isomerization rate at this temperature is considerably slower than $1/T_1$. In order to determine the uncatalyzed cis/trans isomerization rates, temperature-dependent magnetization transfer studies were carried out over the range from 40 to 70 °C. Values for k_c, the cis → trans kinetic rate constant, were determined by fitting the data essentially as described by Led and Gesmar (1982) and then plotted as a function of $1000/RT$ and fit to the relation $k_c = k_o e^{-E_A/RT}$ (Figure 1). The Arrhenius plots yielded values of $k_o = 1.8 \times 10^{15}$ s^{-1} and $E_A = 23.0$ kcal/mol for [Gly^6,*p*-fluoro-Phe^8]bradykinin and of $k_o = 3.14 \times 10^{15}$ s^{-1} and $E_A = 22.8$ kcal/mol for [*p*-fluoro-Phe^8]bradykinin. From these results, the uncatalyzed cis → trans rate constants at 25 °C are determined to be 2.1×10^{-2} and 4.8×10^{-2} s^{-1}, for the two peptides. The activation energies obtained are in

Table I: Fluorine-19 Spin–Lattice Relaxation Times of Bradykinin Derivatives

peptide	T_1 (s)[a] 8.5 T	11.75 T
[*p*-fluoro-Phe^8]BK[b]		
trans	0.98 ± 0.01	0.78 ± 0.01
cis	0.91 ± 0.05	0.70 ± 0.02
[Gly^6,*p*-fluoro-Phe^8]BK[b]		
trans	0.98 ± 0.02	0.79 ± 0.01
cis	0.77 ± 0.02	0.66 ± 0.01
[*p*-fluoro-Phe^8]BK + cyclophilin[c]		
trans		0.76 ± 0.04
cis		0.72 ± 0.02
[*p*-fluoro-Phe^8]BK + cyclophilin + 6-MeAla-CsA[d]		
trans		0.78 ± 0.01
cis		0.71 ± 0.03
[Gly^6,*p*-fluoro-Phe^8]BK + cyclophilin + 6-MeAla-CsA[e]		
trans	1.02 ± 0.01	
cis	0.90 ± 0.01	

[a] T_1 values corresponding to the field strengths indicated. [b] [Peptide] = 2.6 mM. [c] [Cyclophilin] = 7.4 μM. [d] Concentrations as in entry two of Table II. [e] Concentrations as in last entry of Table II.

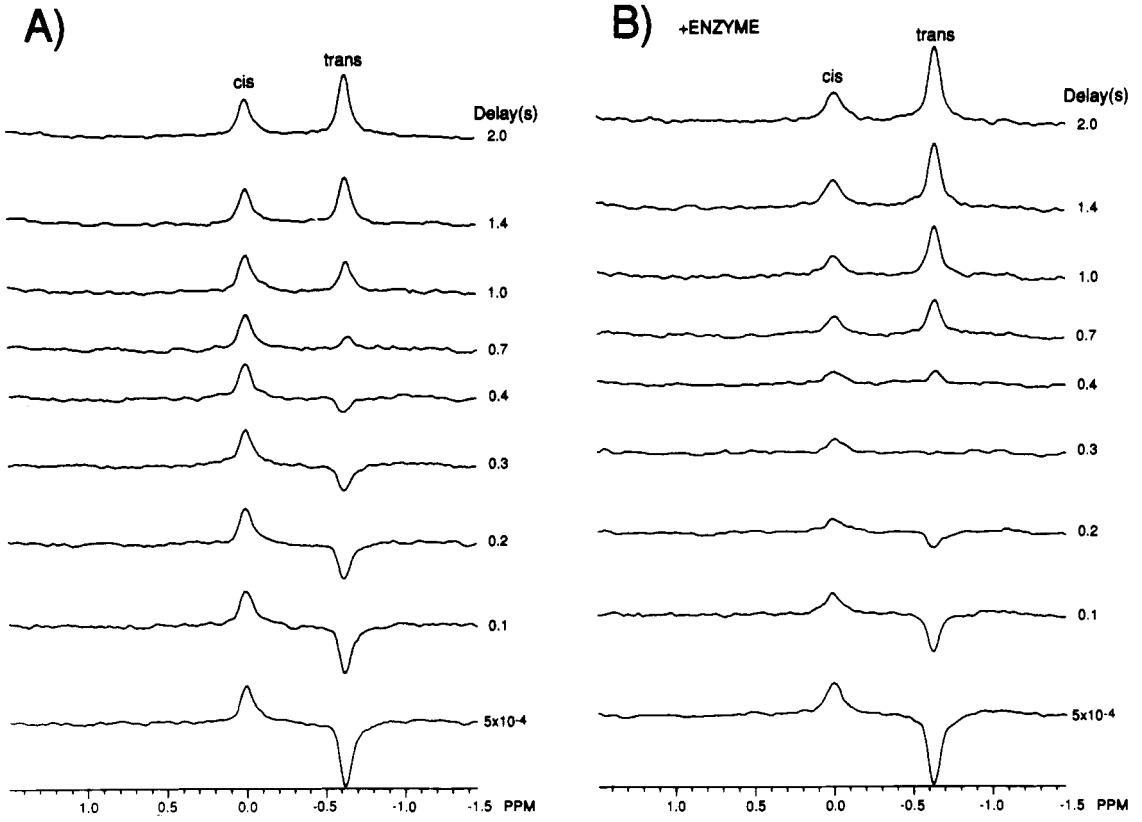

FIGURE 2: Inversion transfer experiment in which the trans resonance of [Gly^6,*p*-fluoro-Phe^8]bradykinin is initially inverted: (A) no enzyme added; (B) same as (A) plus 25.5 μM cyclophilin. Experiments were run at 25 °C, 8.5 T, in a buffer containing 0.2 M NaCl, 5 mM 2-mercaptoethanol, and 20 mM sodium phosphate, pH 7.0, and 25% D_2O was used for the lock. The effects of the cyclophilin on the rate of magnetization transfer are most clearly observed by comparing the spectra corresponding to the 0.3-s delay. Thus, the effect of the more rapid cis/trans isomerism in (B) is to partially average out the positive cis and negative trans resonances observed in (A).

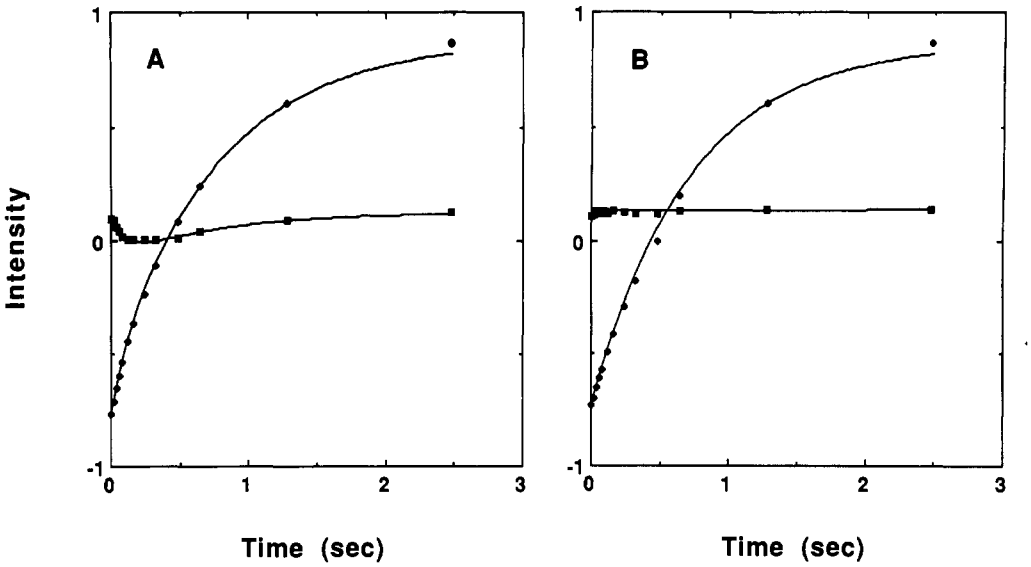

FIGURE 3: Effect of inhibitor on magnetization transfer kinetics of bradykinin. Smooth curves represent fitted eqs 3 and 4. (A) Time-dependent magnetization of the cis (■) and trans (◆) resonances of 2.2 mM [*p*-fluoro-Phe^8]bradykinin in buffer containing 7.4 μM enzyme; (B) same as (A) after the addition of 6-MeAla-CsA to a final concentration of 20 μM.

good agreement with those reported in the literature for model X-Pro peptides (Cheng & Bovey, 1977; Roques et al., 1977), although the rates at 25 °C are at least 1 order of magnitude faster than those reported for most dipeptides (Grathwohl & Wuthrich, 1981; Lin & Brandts, 1979a,b).

Although no magnetization transfer was observable at 25 °C in the absence of enzyme (Figure 2A), the addition of micromolar concentrations of cyclophilin led to rate enhancements sufficient to allow easily measured transfer at this temperature (Figure 2B). Cis → trans isomerization rate constants k_c were extracted from the data as above. Plots of k_c as a function of added enzyme concentration were reasonably linear. At peptide concentrations of 2 mM, the catalytic activity expressed as k_c per micromolar enzyme was determined to be 1.2 s^{-1} μM^{-1} for [*p*-fluoro-Phe^8]bradykinin and 0.13 s^{-1} μM^{-1} for [Gly^6,*p*-fluoro-Phe^8]bradykinin. Following the addition of cyclosporin A or its 6-(*N*-methyl-L-alanine) analogue (Durette et al., 1988) at a 3:1 molar excess over cyclophilin, the rate of magnetization transfer was reduced to essentially unobservable levels (Figure 3). The corresponding 1-(tetrahydrofurfuryl) derivative of cyclosporin A (Durette et al., 1988), which has only 1% the affinity of cyclosporin A for cyclophilin, was very much less active (Table II).

Table II: Effect of Cyclosporin A Analogues on k_c Value[a]

substrate	conditions	k_c (s^{-1})
[*p*-fluoro-Phe^8]BK	7.43 μM cyclophilin	7.2–8.6
[*p*-fluoro-Phe^8]BK	7.3 μM cyclophilin + 20 μM 6-MeAla-CsA	0.2[b]
[*p*-fluoro-Phe^8]BK	7.3 μM cyclophilin + 20 μM CsA	0.1[b]
[*p*-fluoro-Phe^8]BK	7.3 μM cyclophilin + 20 μM 1-THF-CsA[c]	4.5
[Gly^6,*p*-fluoro-Phe^8]BK	25.5 μM cyclophilin	3.1
[Gly^6,*p*-fluoro-Phe^8]BK	24.2 μM cyclophilin + 30 μM 6-MeAla-CsA	0.2[b]

[a] Other conditions as in Figure 2. [b] Rates are not significantly different from zero. [c] 1-THA-CsA = (1-(tetrahydrofurfuryl) derivative of cyclosporin A.

Discussion

From these data, it can be concluded that both bradykinin and its Gly^6 analogue are substrates of the enzyme cyclophilin. The high specificity characteristic of an enzyme-catalyzed reaction is evident from the comparison of the kinetics observed for the two peptides. It cannot be determined at this point whether the different activities toward the two peptides are a consequence of differences in k_{cat} and/or K_m, and further studies aimed at determining both parameters are in progress. Although the K_m for bradykinin has yet to be determined, studies with other peptides suggest that in general K_m values are in the millimolar range (Fischer et al., 1989b; Harrison & Stein, 1990). Assuming that the bradykinin and [Gly^6]-bradykinin concentrations used in the studies are well below the K_m values, the k_{cat}/K_m values determined are similar to the value of 2 s^{-1} μM^{-1} reported for the test peptide Suc-Ala-Ala-Pro-Phe-pNA under somewhat different conditions (pH 7.8, 10 °C; Harrison & Stein, 1990). The lower activity of the Gly^6-substituted bradykinin is analogous to the lower activity observed for Glt-Ala-Gly-Pro-Phe-pNA compared with Glt-Ala-Ala-Pro-Phe-pNA (Fischer et al., 1989a). These results suggest a preference for substrates in which the proline residue is preceded by an L-amino acid. The observation that only cyclosporin structures that bind tightly to cyclophilin completely inhibit activity is also consistent with the expected enzymatic specificity of the reaction and demonstrates that catalysis requires the interaction of bradykinin with the active site of the enzyme.

In comparison with previous spectrophotometric assays of cyclophilin activity, the NMR approach offers several advantages, particularly the fact that the cis ↔ trans interconversion is directly monitored, in comparison with assays based on the conformational selectivity of chymotrypsin cleavage which only monitors the conformational equilibrium indirectly. Further, the degradation of cyclophilin or of other peptides present in competitive inhibition studies by the chymotrypsin or inhibition of chymotrypsin action could significantly alter the results of the assay. In principle, these kinetic measurements could be done with ^1H NMR by use of selective inversion transfer, saturation transfer, or two-dimensional exchange spectroscopy. However, unless the ^1H spectrum of the peptide has two appropriate resolved lines, quantitative determination of the rate constant becomes difficult and time consuming. No such convenient and diagnostically useful pairs of cis and trans resonances have yet been identified in the proton spectrum of bradykinin, and of course, the existence of three proline residues introduces the possibility of eight

separate isomers to distinguish.

Although the structural homology between bradykinin or its Gly^6 analogue and cyclosporin A is not overwhelming, a structural analogy can be drawn between the Ser^6(Gly^6)-Pro^7-Phe^8-Arg^9 sequence of the former and the Abu^2-Sar^3-MeLeu^4-Val^5 sequence of the latter, which form a β-turn as part of an antiparallel β-sheet involving residues 1–6 of cyclosporin A (Loosli et al., 1985; Wenger, 1985). Although the solution structure of bradykinin appears to be largely disordered, involving rapid interconversion among a broad set of conformations (London et al., 1978; Denys et al., 1982), some evidence exists indicating that this portion of the peptide spends part of the time as a β-turn [Cann et al., 1983; London et al. (1990) and references cited therein]. Recent CD (Cann et al., 1986) and NMR studies (Cann et al., 1990; Lee et al., 1990) show that the tendency of the C-terminal Ser-Pro-Phe-Arg sequence to form a β-turn is greatly enhanced in the presence of detergents such as SDS. Also of interest in this context is the recent proposal by Fischer (1989) that the cis/trans isomerization catalyzed by cyclophilin is actually a means of forming β-turns in proteins which can serve as nucleation points for protein folding. Hence, the catalytic activity observed here for bradykinin appears to be consistent with an affinity of the enzyme for β-turn structures.

Given the predominant cytosolic location of the enzyme and the fact that bradykinin presumably interacts with receptors on the outer surface of the cell, a direct involvement of the enzyme in the physiologic functioning of bradykinin seems unlikely. Nevertheless, some cyclophilin activity has been found to be associated with membranes (R. R. Handschumacher, unpublished data). From the standpoint of bradykinin activity, it is noteworthy that all of the bradykinin antagonists which have recently been developed (Vavrek & Stewart, 1985; Steranka et al., 1989) contain a D-Phe^7 for Pro^7 substitution, and hence will strongly favor the trans peptide bond conformation.

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