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Interaction of cyclosporin A and two cyclosporin analogs with cyclophilin: relationship between structure and binding

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Abstract

The immunosuppressant drug cyclosporin A exists as various conformers in water. Up to 1 h is needed to reach maximum complex formation after mixing the drug with its receptor, cyclophilin, or an antibody, indicating that only a fraction of the conformers in aqueous solution adopts a conformation suitable for binding. In the present study we compare the binding behavior of cyclosporin to that of two analogs, using a biosensor instrument (BIAcore, Pharmacia). The amount of complex formation was measured as a function of time after adding the peptides to cyclophilin. The equilibrium affinity constants of cyclophilin for these analogs have been measured. The slow binding of cyclosporin to cyclophilin compared to the instant binding of the cyclosporin analogs supports the hypothesis that cyclophilin recognizes a well defined conformation of cyclosporin that exists in water prior to binding.

1. Introduction

Cyclosporin A (CS) is a widely known cyclic undecapeptide which is used as an immunosuppressive drug, under the trade name Sandimmune®. A major binding protein for CS is the cytoplasmic protein cyclophilin (CYP) [1], an enzyme with peptidyl prolyl *cis–trans* isomerase or rotamase activity [2,3]. CS blocks the T-cell activation that occurs in response to antigen binding to the T-cell by forming a complex with CYP and calcineurin, a serine–threonine phosphatase [4], and thus inhibits the hydrolysis of phosphates of the cytosolic nucleic factor of activated T-cell (NFATc), a step which is necessary to start IL2 transcription [5].

Much attention has been focused on structural studies of CS since the knowledge of its active conformation has great implications for drug design. The CS conformation in the crystalline state was determined by X-ray crystallography [6] and its conformation in organic solvents by nuclear magnetic resonance (NMR) spectroscopy [7]. The conformation of CS in a complex with CYP [8–11] or with the Fab fragment of the antibody [12,13] is completely different from that in the crystal or in apolar solvents. In particular, no intramolecular hydrogen bonds are observed in the CYP- or antibody-bound conformation and thus the amide groups of the peptide link-

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ages are available for hydrogen bonding to protein or to water. Another striking difference is that the peptide bond between MeLeu 9 and MeLeu 10 is *trans* in the CS–CYP complex and *cis* in crystal or apolar solvents.

Due to its poor solubility and to its multiple conformations in water, the stereochemistry of these conformations of CS in water or buffers can be only investigated with difficulty by NMR spectroscopy, which requires high peptide concentrations in the mg/ml range. However, previous studies of CS in polar solvents like DMSO indicated that the peptide adopts multiple conformational states [14]. The conformation of the active form of the drug is thus difficult to establish. It was initially believed that only the 9,10-*cis* conformer exists in free form in solution, and that the 9,10-*trans* conformer is induced by binding to CYP [14–16]. The observation that the conformation of CS bound to a Fab fragment is similar to that bound to CYP suggested that the *cis–trans* isomerisation at the 9,10 peptide bond takes place easily in an aqueous environment [12]. Kinetic and spectroscopic observations showed that the *trans* conformer of CS, which predominates in tetrahydrofuran (THF) in presence of lithium chloride (LiCl) [7,17], inhibits the CYP rotamase activity, while the *cis* conformer, which is observed in THF alone [7,17], has no biological activity [18]. The same authors also showed a time dependent inhibition of rotamase activity, which may be interpreted as a slow interconversion between the two conformers. In a recent study using the Pharmacia BIAcore™ biosensor instrument, the amount of CS–CYP complex formed was measured as a function of temperature and time either adding CS to CYP or CS to an antibody [19]. Up to 1 h was needed to reach maximum CS–protein complex formation. This observation confirmed the slow binding of CS to these proteins which could be interpreted as a slow conversion of various conformers occurring in solution into the 9,10-*trans* conformer, which then binds to the protein.

In a recent NMR study [20] it has been shown that a substituent in position 3 of cyclosporin stabilizes a single conformation in either DMSO or water compared to multiple conformations of CS in these polar solvents. It has also been shown that this unique conformation in the case of the water soluble [D-MeSer^3-D-Ser-(O-Gly)^8]CS is almost identical to that of CS complexed to CYP. In the present study, the capacity of [D-MeSer^3-D-Ser-(O-Gly)^8]CS and of the structurally related (D-MeSer^3)CS (which has about the same water solubility as CS) to bind to CYP was measured by a biosensor technique [19]. The chemical structures of the two analogs and of CS are shown in Fig. 1. The two analogs, which have a conformation in water similar to

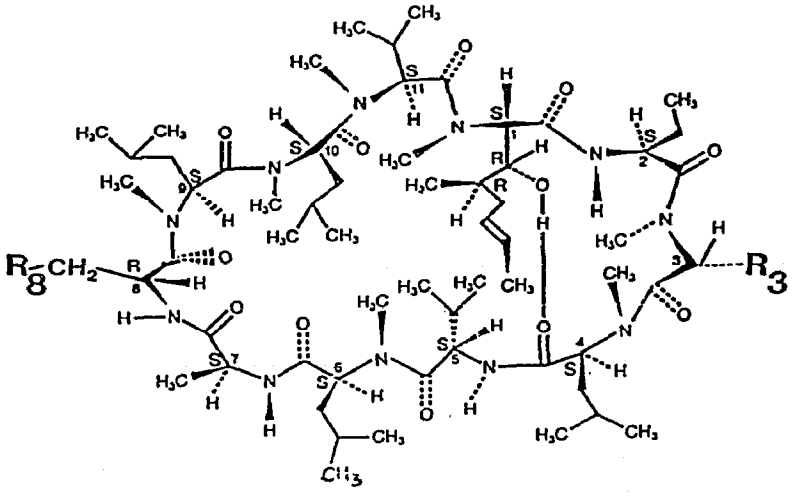

Fig. 1. Chemical structure of CS and CS analogs. $R_3 = H$, $R_8 = H$: cyclosporin A (CS); $R_3 = CH_2OH$, $R_8 = H$: (D-MeSer^3)CS [26]; $R_3 = CH_2OH$, $R_8 = OCOCH_2NH_2.HCl$: [D-MeSer^3-D-Ser-(O-Gly)^8]CS hydrochloride.

the conformation of CS complexed with CYP were found to bind instantly to CYP, compared to CS which binds slowly. This is in agreement with the hypothesis that CYP selectively binds to a conformer of CS that already exists in water.

2. Experimental

2.1. Equipment and reagents

The BIAcore instrument and reagents for interaction analysis were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). The reagents include the CM5 sensor chips, surfactant P20, N-hydroxysuccinimide (NHS), N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and 1 *M* ethanolamine-hydrochloride pH 8.5.

CS and CS analogs are described in ref. 20. D-Lys^{8}-CS/BSA conjugate (BSA/CS) [21] and CYP were kindly provided by Drs. V.F.J. Quesniaux, M. Schreier and M. Zurini (Sandoz, Basel).

2.2. Immobilization of BSA–CS conjugate and monitoring of changes in CYP–CS interaction with time

The BIAcore instrument allows quantitative analysis of molecular interaction in real time. This instrument uses surface plasmon resonance (SPR), a quantum mechanical phenomenon that allows the detection of changes in the refractive index close to the surface of a thin gold film placed on a glass support (sensor chip). As the refractive index is directly correlated to the concentration of material in the medium, the system can detect the binding between a molecule immobilized on the sensor chip and a ligand introduced in a flow passing over the surface. Changes in the concentration of molecules is expressed in resonance units (RU). A signal of 1000 RU corresponds approximately to a surface concentration change of 1 ng/mm^2. A carboxylated dextran layer is attached to the gold surface, so that interactions occur in this hydrophilic matrix [22,23].

A BSA/CS conjugate was coupled through primary amino groups to the dextran matrix of a CM5 sensor chip as described previously [19]. For that purpose the carboxyl groups of the dextran matrix were first activated to N-hydroxysuccinimide esters. Next the BSA/CS at 7.5 μg/ml in 10 m*M* formate buffer (pH 3) as described [19], was injected. To block the unreacted groups, 35 μl of ethanolamine were injected. After washing the surface with 15 μl of 10% acetonitrile in 0.1 *M* NaOH, the surface was ready for use. Under the chosen conditions 3000 to 5000 RU of BSA/CS conjugate were immobilized.

For binding experiments lyophilized powder of CS was dissolved at a concentration of 1 mg/ml (833 μM) in either dimethyl sulfoxide (DMSO), or tetrahydrofuran (THF) or THF containing 0.47 *M* of lithium chloride (LiCl). Solutions of CS (100 n*M*) in HBS buffer pH 7.4 (10 m*M* Hepes, 150 m*M* NaCl, 3.4 m*M* EDTA, 0.05% surfactant P20) were prepared from these 1 mg/ml (833 μM) stock solutions. Analogs were dissolved in DMSO. The amount of free CYP present in the peptide–CYP mixtures was followed over time by injecting the mixtures repeatedly on a BSA/CS immobilized surface. Aliquots of 20 μl were injected at a flow-rate of 10 μl/min, followed by 4 μl of 0.1 *M* HCl to regenerate the surface. The CYP response was read 10 s before regeneration. Due to the washing procedures between these steps, readings were obtained every 7 min, starting 5 min after adding the peptides to CYP. The CYP concentration was 50 n*M* and peptide concentrations were chosen so that approximately 50% of the protein remained free for binding to BSA/CS (CS = 100 n*M*; CS analogs around 50 n*M*).

2.3. Equilibrium affinity measurements

The procedure for equilibrium affinity measurements has been described previously [19]. Constant analog concentrations were mixed with increasing amounts of CYP and preincubated for 1 h at room temperature. The CYP–CS mixture was then injected on a BSA/CS surface. The free protein bound to the immobilized CS. The

response level in RUs was expressed as free protein concentration, using a calibration curve established with known CYP concentrations on the same surface. Data were analysed using Scatchard plots.

3. Results and discussion

3.1. Monitoring variations in the amount of complex formed as a function of time

CS and analog solutions in Hepes buffer were prepared from 1 mg/ml stock solutions in DMSO. The level of free CYP was measured every 7 min following addition of the peptides in Hepes buffer to CYP, by repeated injection of the mixture on a sensor surface containing immobilized CS. The amount (in RU) of free protein present in the mixture which bound to the immobilized CS was recorded. Fig. 2 shows a comparison of the responses obtained with CS (Fig. 2A), (D-MeSer^3)CS (Fig. 2B) and [D-MeSer^3-D-Ser-(O-Gly)^8]CS (Fig. 2C). When CS initially dissolved in DMSO (Fig. 2A) was mixed with CYP just before the first injection (black triangles), the level of free CYP decreased during the first half hour following the addition of CS to CYP, indicating that the amount of complex formed in solution increased with time [19]. This behavior was not observed with analogs (D-MeSer^3)CS (Fig. 2B) and [D-MeSer^3-D-Ser-(O-Gly)^8]CS (Fig. 2C), which showed a stable response over time. Controls corresponding to CYP in the absence of peptide (black squares) or after 2 h preincubation (open squares) were stable. These two analogs which have a single conformation in water almost identical to the CS conformation in the CYP–CS complex bind instantly to CYP. These results strongly support the hypothesis that the conformation of CS, which binds to CYP, already exists at least in small amounts in water and is not formed by contact with the protein. In the case of CS there are several conformations in water. One of them, presumably representing the 9,10-*trans* conformer, interacts with CYP, which, as observed, is a time-dependent process.

The behavior of CS dissolved in THF or in THF/LiCl was also monitored (Fig. 3). These results are in good agreement with earlier observations on CS dissolved in trifluoroethanol (TFE) or TFE/LiCl [19]. Kofron *et al.* [18] observed differences in rotamase inhibition capacity of CS initially dissolved in THF (mainly stabilising the *cis* form) or in THF/LiCl (mainly stabilising the *trans* form). The difference is very small in our case. Thus substantial changes in the

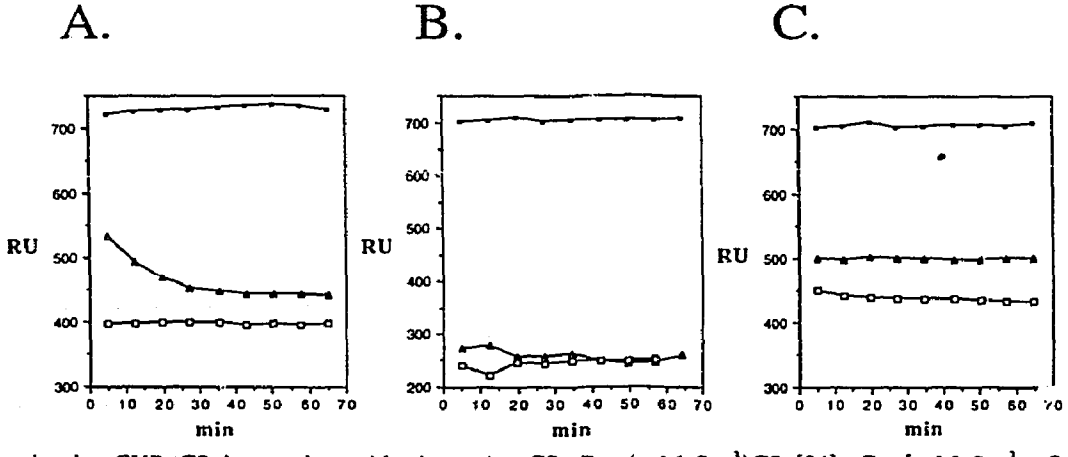

Fig. 2. Changes in the CYP–CS interaction with time. A = CS; B = (D-MeSer^3)CS [24]; C = [D-MeSer^3-D-Ser-(O-Gly)^8]CS hydrochloride. CS and analog solutions (1 mg/ml) were prepared in DMSO. The solutions in HBS were prepared just before the experiment (▲). Controls correspond to CYP in the absence of peptide (■) and CYP first incubated during 2 h with peptides (□). The fact that in A and C the two curves (▲ and □) do not coincide at least after 40 min is due to insufficient precision of manual pipetting.

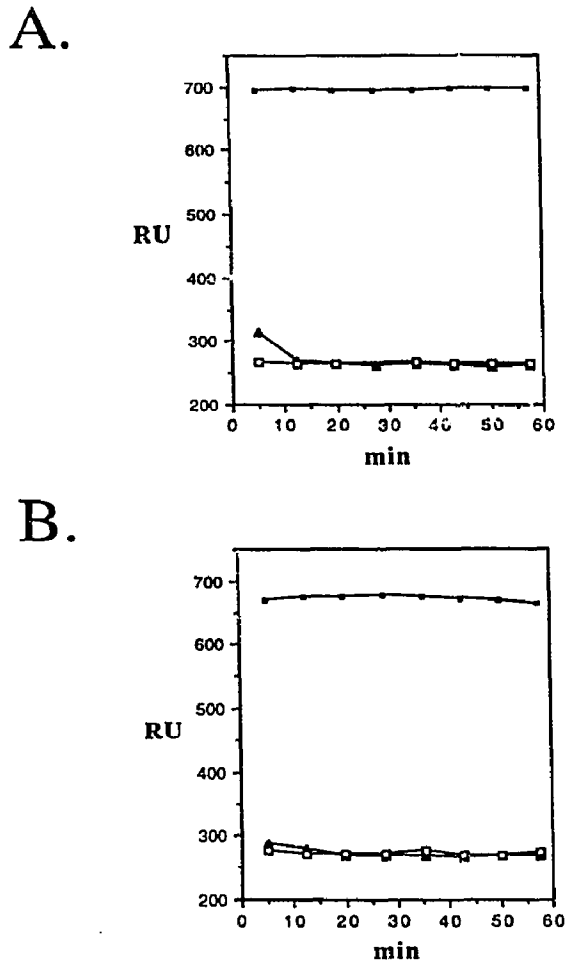

Fig. 3. Changes in the CYP–CS interaction with time, when the initial 1 mg/ml CS solutions were prepared either in THF (A) or THF/LiCl (B). The solutions in HBS were prepared just before the experiment (▲). Controls correspond to CYP in the absence of peptide (■) and CYP first incubated during 2 h with peptides (□).

ratio of different conformers may occur during the first 5 min following addition of the protein and cannot be monitored in our system.

3.2. Experimental data for equilibrium affinity measurements

The equilibrium affinity constants of CYP for the two analogs were measured three to four times on different sensor surfaces. They were $(2.0 \pm 0.15) \cdot 10^8$ and $(1.4 \pm 0.4) \cdot 10^8$ for analogs [D-MeSer^3-D-Ser-(O-Gly)^8]CS and (D-MeSer^3)-CS respectively. Typical Scatchard plots are shown in Fig. 4 for the interaction of CYP with the two analogs. The equilibrium affinity constant for CS calculated here $[(2.0 \pm 0.30) \cdot 10^7 M^{-1}]$ is similar to the previously published constant of $(2.6 \pm 0.7) \cdot 10^7 M^{-1}$ [19]. This value is in good agreement with those derived from calorimetric measurements $(2 \cdot 10^7 M^{-1})$ [24] and from fluorescence measurements $(0.5 \cdot 10^7 M^{-1})$ [1].

The affinity of CYP for the two analogs

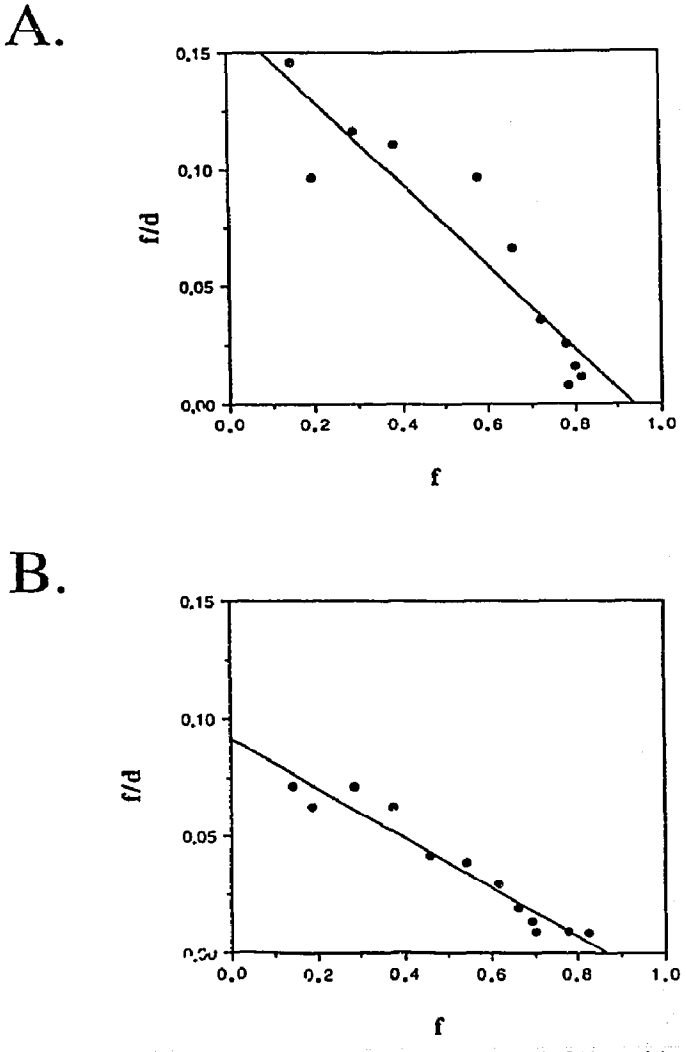

Fig. 4. Scatchard plots for the interaction of CYP with analogs (D-MeSer^3)CS (A) and [D-MeSer^3-D-Ser-(O-Gly)^8]CS (B); f is the ratio of bound CYP to total CS concentration and d the free CYP concentration. The fitted line equations are: $y = 0.164 - 0.176x$ and $y = 0.091 - 0.105x$ for (A) and (B) respectively. The coefficient of correlation is in both cases higher than 0.9.

modified at position 3 is thus ten times higher than for CS. The CS residues that are implicated in the recognition of CYP have been identified by functional [25] and structural [10,11] studies. The surface of CS, which is recognized by CYP involves residues 1, 2, 3, 9, 10 and 11. Because Sar-3 is located on the edge between CYP and calcineurin while D-Ala-8 is on the effector site (part which binds to calcineurin), it is reasonable to expect that a modification at position 3 will affect the binding affinity for CYP, while a modification at position 8 will have no effect. The gain in affinity for CYP observed in the case of the two analogs must be compensated by a loss in affinity for calcineurin because both CS-analogs have equipotent immunosuppressive activities compared to CS.

4. Conclusions

An important advantage of the BIAcore technique is that binding measurements can be performed within a few minutes after mixing of the ligands. A rapid complex formation between CYP and two CS analogs was observed, which implies that they interact with the protein in the same conformation as the "active" 9,10-*trans* conformer. Furthermore, the results reveal that the rate-limiting step of the complex formation between "native" CS and CYP is determined by the rate of CS *cis–trans* isomerisation in position 9,10.

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