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Interaction of cyclosporin A with an Fab fragment or cyclophilin

Affinity measurements and time-dependent changes in binding

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Different conformers of the immunosuppressant cyclosporin A have been observed in structural studies of the isolated molecule and of its complex with cyclophilin or with an Fab fragment. The factors that control this conformational change are not well understood. Variations in the amount of complex formed with cyclophilin or with the antibody were measured as a function of time after adding cyclosporin to the proteins, using the Pharmacia BIAcore biosensor instrument. Up to 1 hour was needed to reach maximum complex formation in solution, which is likely to reflect the time needed for a conformational transition of cyclosporin. The equilibrium affinity constant of both proteins for cyclosporin has been measured.

Cyclosporin; Cyclophilin; Antibody fragment; Affinity; Peptide conformation; Kinetics

1. INTRODUCTION

Cyclosporin A (CS) is a cyclic peptide composed of 11 residues which is used as an immunosuppressive drug under the trade name Sandimmune. Recently the structures of two complexes of CS with a protein have been published. The complex between CS and the Fab fragment of a monoclonal antibody has been solved by crystallographic analysis to 2.65 Å resolution [1]. The structure of CS complexed to cyclophilin (CYP), its binding protein in vivo, has also been obtained both by X-ray crystallography [2] and nuclear magnetic resonance [3,4]. The conformation of CS in the two complexes is similar and totally different from its conformation in an isolated form as determined by X-ray and NMR analyses in organic solvents [5,6]. A striking difference between isomers is that the MeLeu^{9}-MeLeu^{10} peptide bond is *cis* in isolated CS and *trans* in complexed CS. Moreover, isolated CS contains three intramolecular hydrogen bonds, while in complexed CS, carbonyles and nitrogens are available for hydrogen bonding to the proteins or to water.

CS can thus adopt at least two conformations, but the factors that control its conformational change are not well understood. The observation that CS complexed to the Fab has all trans amide bonds suggested that the proteins bind the trans conformation that could preexist in aqueous solution [1]. This hypothesis was strenghtened by results of Kofron and coworkers [7] who have shown the presence of different conformers in solution, the ratio of which depends on the initial solvent used for dissolution. These authors also showed a time-dependent inhibition of the rotamase activity of CYP, which was interpreted as a slow interconversion between two conformers, the *trans* conformer being bound by CYP, and the *cis* conformer not.

Changes in the ability of CS to bind to the Fab or to CYP as a function of time have been directly measured, using a new biosensor technology (BIAcore, Pharmacia). These experiments clearly demonstrate that up to one hour is needed to reach maximum complex formation in solution, implying that reproducible binding measurements with CS require carefully controlled experimental conditions. We have also compared the affinity of CYP and of the Fab fragment of an anti CS monoclonal antibody for CS. The results thus obtained with the biosensor technology are compatible with the hypothesis of a time consuming 'hydrophobic collapse' of CS in aqueous media to generate the bioactive drug shape prior to binding to CYP [8].

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2. MATERIALS AND METHODS

2.1. *Fab preparation and purification*

Antibody preparation and papain digestion were conducted as described previously [9], except that gel filtration was performed on a Hiload 26/60 Superdex 200 column (Pharmacia) instead of a Sephacryl S-200 superfine (Pharmacia) column. Concentration of the purified Fab was determined by the Bradford [10] colorimetric micromethod assay, using bovine immunoglobulins (BioRad, ref. 500-0005) as standard [11].

2.2. *Equipment and reagents*

The BIAcore system, sensor chips CM5, surfactant P20, *N*-hydrox-

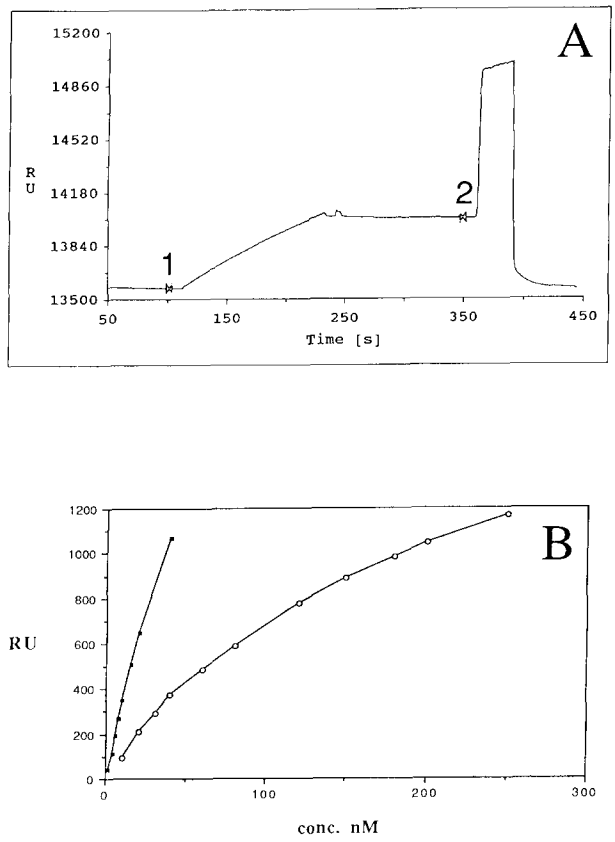

Fig. 1. (A) Sensorgram corresponding to the injection of a 15 nM Fab solution on a BSA/CS surface. The difference in response between report points 2 and 1 corresponds to the RU value reported in the calibration curve. (B) Example of calibration curves constructed with known concentrations of Fab (black squares) or CYP (open circles). These curves are used for the evaluation of free protein in protein–CS mixtures.

ysuccinimide (NHS), *N*-ethyl-*N'*-(3-dimethyl-aminopropyl)carbodiimide hydrochloride (EDC) and 1M ethanolamine-hydrochloride pH 8.5 were obtained from Pharmacia Biosensor AB, Uppsala Sweden.

CS, CYP A, monoclonal antibody V45-271-11 and a D-Lys^{8}-CS/BSA conjugate [12] (BSA/CS) were kindly provided by Drs. H.-P. Kocher, V.F.J. Quesniaux, M. Schreier and M. Zurini (Sandoz, Basel). The antibody to CS used in the present study is antibody V45-271-11 [12]. This antibody differs from antibody R45-45-11 used for crystallographic analysis of an Fab–CS complex [1] by a single amino acid change of Phe to Tyr at position 58 (Kabat numbering, [13]) located in the H2 loop [14]. Crystallographic analysis shows that this amino acid does not contact CS [15].

2.3. *Immobilization of BSA–CS conjugate*

The standard immobilization technique was used to couple the BSA/CS conjugate through primary amino groups to the carboxylated dextran matrix of a CM5 sensor chip [16,17]. The flow rate was 5 μl/min, using as running buffer HBS, pH 7.4 (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.05% surfactant P20). In order to immobilize large amounts of the conjugate, activation of the carboxylated dextran matrix of CM5 sensor chips by the EDC/NHS mixture was increased to 8 min, and BSA/CS was injected in three successive steps of 8, 3 and 3 min at a concentration of 7.5 μg/ml in 10 mM formate buffer, pH 3.0. Remaining NHS-ester groups were blocked by injection of 35 μl of ethanolamine. Non-covalently attached protein was removed through washing the surface with 15 μl of 10% acetonitrile in 0.1 M NaOH. In these conditions 3000–5000 resonance units (RUs) of BSA–CS conjugate were immobilized.

2.4. *Monitoring of changes in protein–CS interaction with time*

CS was dissolved from lyophilized powder at a concentration of 1 mg/ml in either dimethyl sulfoxide (DMSO), or trifluoroethanol (TFE), or TFE containing 0.47 M of lithium chloride (LiCl). 120 to 250 nM solutions of CS in HBS buffer were prepared from the 1 mg/ml stock solution. The amount of free protein (antibody V45-271-11 or CYP) present in a CS–protein mixture was followed over time by injecting repeatedly the CS–protein mixture on a BSA/CS immobilized surface. In order to shorten the time between two successive injections, only 20 μl were injected at a flow rate of 10 μl/min, followed by 4 μl of the regeneration solution, which was 0.1 M HCl in the case of CYP and 10% acetonitrile in 0.1 M NaOH in the case of the antibody. The protein response was read 10 s before regeneration. Readings were thus obtained every 7 min, starting 5 min after adding CS to the protein. The antibody and CYP concentrations were 50 and 100 nM, respectively. Concentrations of CS were chosen so that approximately 50% of the protein remained free for binding to BSA/CS. These concentrations were 75 and 250 nM for the antibody and CYP respectively.

2.5. *Equilibrium affinity measurements*

Constant CS concentrations were mixed with increasing amounts of protein and incubated for 1 h. The protein-CS mixture was 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 protein concentrations on the same surface. Data were analysed using Scatchard plots.

The procedure used for calibration of the surface with known protein concentrations and titration of free protein in protein–CS mixtures was as described above. A sensorgram corresponding to the injection of a 10 nM Fab solution is shown in Fig. 1A. The Fab fragment was used instead of the whole antibody to avoid problems linked with antibody bivalency. Typical calibration curves, obtained by injecting the Fab at concentrations comprised between 2 and 40 nM, or CYP at concentrations ranging from 5 to 250 nM, are shown in Fig. 1B.

3. RESULTS AND DISCUSSION

3.1. *Monitoring changes in protein–CS interaction with time*

CS solutions in HEPES buffer were prepared from 1 mg/ml solutions in DMSO, TFE or TFE/LiCl. The level of free protein (CYP or antibody) was measured every 7 min following addition of CS in HEPES buffer to the proteins, by repeated injection of the mixture on a BSA/CS immobilized sensor surface. Fig. 2 shows results obtained with the antibody at room temperature. The level of free antibody decreases in the first half-hour following the addition of CS to the antibody, indicating that the amount of complex formed in solution increases with time. The time needed to reach a stable level of CS–antibody complex is likely to reflect a conformational transition of CS. Kofron et al. [7] observed different behaviours for CS initially dissolved in TFE and TFE/LiCl. In our case, readings obtained with CS dissolved in TFE/LiCl (Fig. 2B) were only slightly more stable than those obtained with CS dissolved in TFE (Fig. 2A).

A control corresponding to antibody in the absence

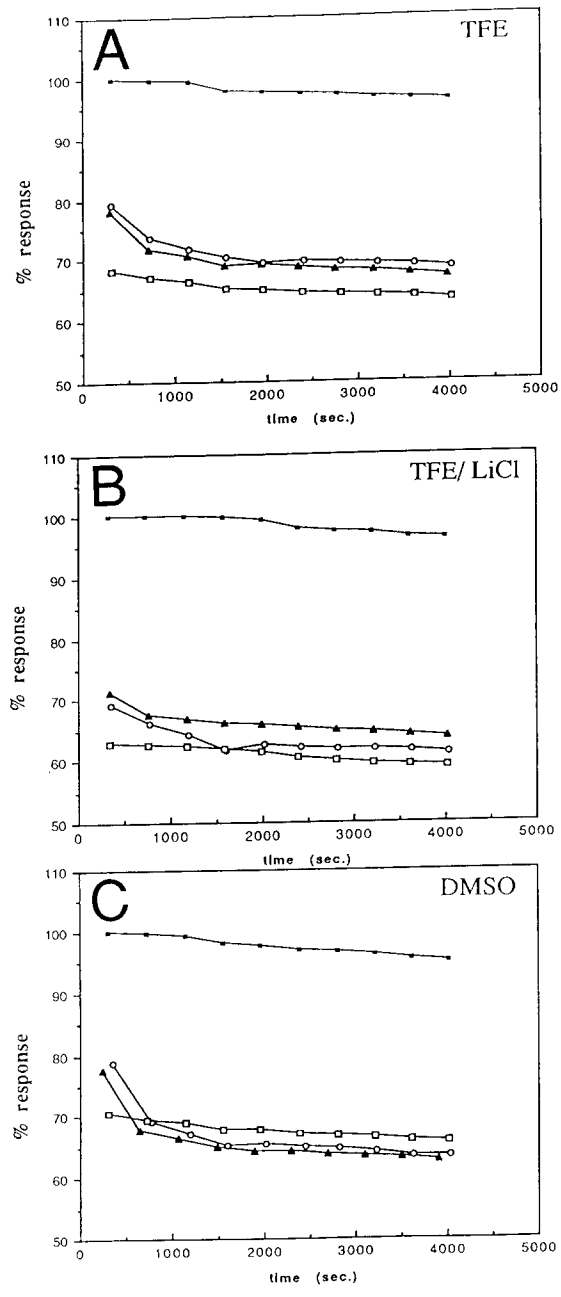

Fig. 2. Changes in the antibody-CS interaction with time, when the initial 1 mg/ml CS solutions were prepared either in TFE (A), TFE/ LiCl (B) or DMSO (C), 2 h (black triangles) or several days before the experiment (open circles). CS solution in HEPES buffer was prepared just before the experiment. Controls correspond to antibody in the absence of CS (black squares) and antibody first incubated during 2 h with CS (open squares).

of solution CS is represented by black squares (Fig. 2). The control shows a slight decrease in binding at each injection cycle. This small decrease (less than 1% per cycle) corresponds to BSA/CS leaking from the surface at each washing step. A drastic surface regeneration with 10% acetonitrile in 0.1 M NaOH was necessary to dissociate the bound antibody from the BSA/CS surface.

Open squares represent an additional control where CS and the antibody were incubated 2 h before starting to monitor the free antibody level. Readings were then constant over time, indicating that differences in binding observed immediately after mixing the proteins were real, and not an experimental artifact.

Similar results were obtained with CYP and are shown in Fig. 3 for CS initially dissolved in DMSO. The decrease in free protein level was slower at 4°C (Fig. 3A) than at room temperature (Fig. 3B). Controls corresponding to CYP in the absence of CS (black squares) or after 2 h incubation (open squares) are more stable than for the antibody because surface regeneration was less drastic (0.1 M HCl).

The CS powder was typically dissolved in apolar solvents at least 2 h before the experiment, while the CS solution in HEPES buffer was prepared just before the experiment. Fig. 3 shows that the binding capacity of CS was weak immediately after dissolving the powder (open triangles), presumably because some time is needed for complete dissolution. Superimposable results were obtained whether the CS solution in HEPES buffer was prepared immediately or several hours before the experiment (data not shown), indicating that the conformational change occurs in the presence of the proteins and not spontaneously when the drug leaves the apolar solution and enters the aqueous buffer. The most likely explanation is that the proteins shift the equilibrium between different conformers by preferential binding to one of them.

However, when the drug was initially dissolved in DMSO, the decrease in free protein level was slightly larger when the HEPES solution of CS had just been prepared, compared to a solution prepared several hours beforehand (results not shown).

3.2. *Theoretical considerations for equilibrium affinity measurements*

The range of K_a values that can be evaluated is limited by the measurable range of free ligand concentrations, as indicated by the upper and lower limits of the calibration curve (Fig. 1B). Maximum precision is reached in the steepest part of the curve. Fig. 1B shows that the lowest free protein concentrations that can be measured are roughly 2 nM for the Fab and 5 nM for CYP. This difference is due to the fact that the response in RUs of bound ligand is directly proportional to the ligand molecular weight (Fab: MW 50,000; CYP: MW 17900).

The protein to CS ratios that must be used in order to have free protein concentrations lying in this measurable range can be calculated from the equation of the law of mass action. When using the symbols of Hardie and Van Regenmortel [18], the equation reads:

$$K_a = \frac{ny}{(As - ny)\,(Bn - ny)}$$

where A = total CS concentration; s = CS valence; As

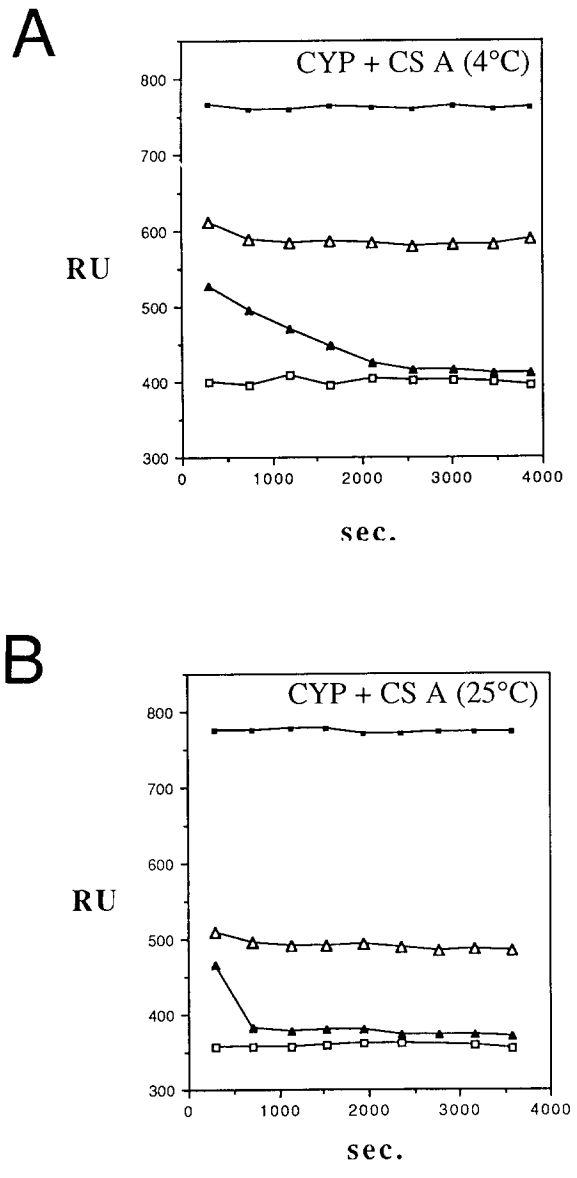

Fig. 3. Changes in the CYP–CS interaction with time, measured at 4°C (A) or at room temperature (B). CS solution (1 mg/ml) in DMSO was prepared 2 h (black triangles) or just before the experiment (open triangles). Controls correspond to CYP in the absence of CS (black squares) and CYP first incubated during 2 h with CS (open squares).

= total CS sites; B = total protein concentration; n = ligand valence; Bn = total protein sites; y = bound protein concentration; ny = bound sites.

To construct Scatchard plots, this equation is rearranged in the form:

$$\frac{f}{d} = K_a\,(s - nf)$$

where $f = y/A$ = ratio of bound protein to total CS; d = free protein concentration.

When f/d is plotted versus f, a straight line is obtained, the slope of which is K_a s. The extrapolation on the x-axis gives s/n.

In Fig. 4, the d value expected for f ranging from 0.1 to 0.9 has been calculated for K_a values of 10^7, 10^8 and 10^9 M^{-1}. The range of d values (nM) that can be most accurately measured (steepest part of the calibration curve) is shaded. From Fig. 4, it is clear that K_a values around 10^8 M^{-1} will lead to easily measurable free protein concentrations (d). K_a values that are larger than 10^9 M^{-1} cannot be determined because the free protein concentrations are too low to be measured. K_a values that are much smaller than 10^7 M^{-1} cannot be determined because free protein concentrations are too large.

3.3. *Experimental data for equilibrium affinity measurements*

Since a stable equilibrium is reached only after one hour, equilibrium affinity measurements were performed after one hour incubation time. Typical Scatchard plots for the interaction between CS and CYP or the Fab are shown in Figs. 5A and 5B, respectively. The affinity of CYP for CS is in the 100 nM range, while that of the Fab is in the nM range. As indicated in Fig. 4, f values below 0.7 could not be obtained for the CS–Fab interaction because the free Fab concentration was too low to be measured. The x-axis extrapolation (s/n) should be equal to 1 since both CS and the proteins are expected to be monovalent. For the Fab–CS interaction, s/n was close to 0.82, which could mean that either the antigen valency is larger than one, or the antibody valency is smaller than one. However, in our case, it seems likely that this value results from an error in the evaluation of active Fab and/or CS concentrations.

Experiments were performed at least twice. The equilibrium affinity constant for the Fab–CS interaction has been measured for CS initially dissolved in TFE, DMSO and TFE/LiCl. The calculated values were 3.7 ± 0.6, 3.6 ± 1.0 and $3.7 \pm 0.2 \times 10^9$ M^{-1}, respec-

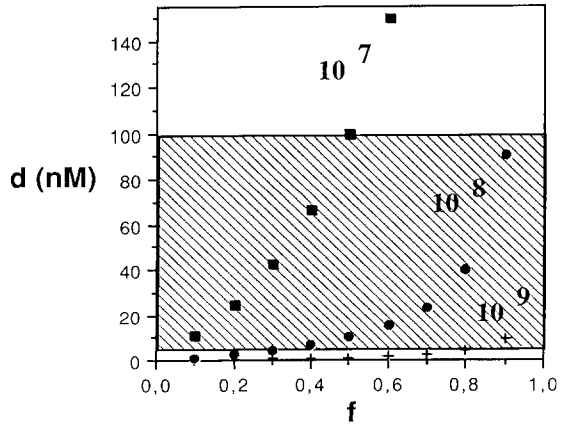

Fig. 4. Theoretical curves showing free antibody concentrations (d) expected for affinity values of 10^7, 10^8 and 10^9 M^{-1}.

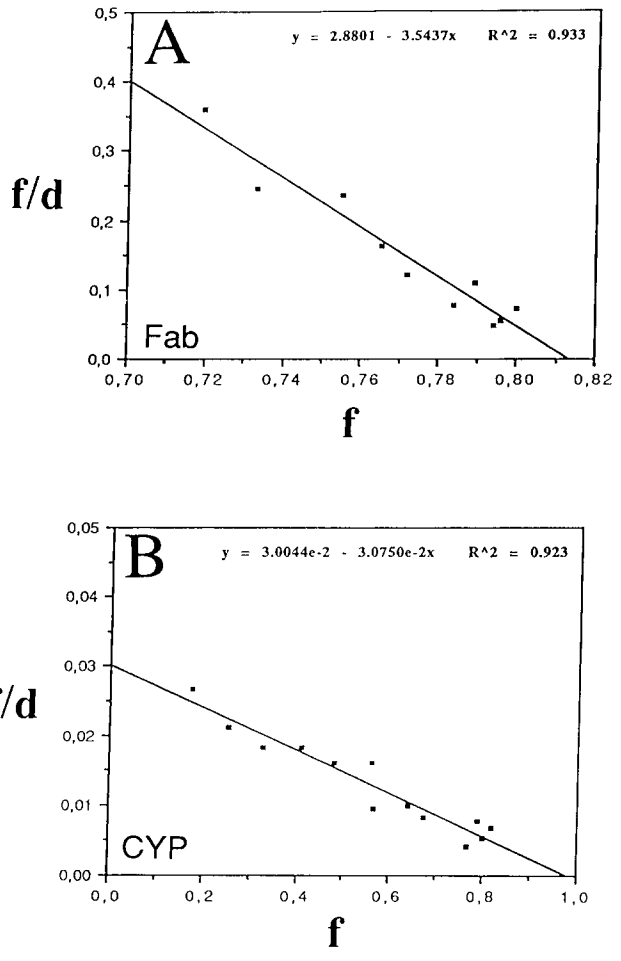

Fig. 5. Typical Scatchard plots for the interaction of CS with the Fab (A) or with CYP (B).

tively. The affinity is thus the same in all three cases indicating that the CS conformers found after one hour incubation time are similar whatever solvent was used for dissolving the CS. The equilibrium affinity constant for the CS-CYP interaction has been measured for CS initially dissolved in ethanol and is $2.6 \pm 0.7 \times 10^7$ M^{-1}, which is reasonably close to the value of 0.5×10^7 M^{-1} obtained from fluorescence measurements [19].

4. CONCLUSION

Classical methods used to measure the interaction between two reactants usually require a long time between the initial mixing of reactants and the monitoring of the interaction. For example in ELISA or RIA, readings are typically obtained 6–8 h after mixing the ligands. One advantage of the BIAcore which made the present analysis possible, is that readings can be performed within a few minutes of mixing the ligands. A complete cycle including surface regeneration lasts only 7 min. We were thus able to follow slow conformational changes of CS by directly following its binding to the antibody or to CYP.

Knowledge of the experimental conditions required to reach stable complex formation is crucial for reproducible monitoring of the drug and for the analysis of structure–activity correlates. Factors such as temperature, procedure used to dissolve CS, and time of incubation of CS with proteins must be controlled. The method presented here for analysing conformational states of CS should also be useful for the rapid screening of derivatives showing the desired CYP binding properties.

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