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Structure comparison of native and mutant human recombinant FKBP12 complexes with the immunosuppressant drug FK506 (tacrolimus)

SUSUMU ITOH[1] AND MANUEL A. NAVIA

Vertex Pharmaceuticals Incorporated, Cambridge, Massachusetts 02139-4211

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Abstract

The consequences of site-directed mutagenesis experiments are often anticipated by empirical rules regarding the expected effects of a given amino acid substitution. Here, we examine the effects of "conservative" and "nonconservative" substitutions on the X-ray crystal structures of human recombinant FKBP12 mutants in complex with the immunosuppressant drug FK506 (tacrolimus). R42K and R42I mutant complexes show 110-fold and 180-fold decreased calcineurin (CN) inhibition, respectively, versus the native complex, yet retain full peptidyl prolyl isomerase (PPIase) activity, FK506 binding, and FK506-mediated PPIase inhibition. Interestingly, the structure of the R42I mutant complex is better conserved than that of the R42K mutant complex when compared to the native complex structure, within both the FKBP12 protein and FK506 ligand regions of the complexes, and with respect to temperature factors and RMS coordinate differences. This is due to compensatory interactions mediated by two newly ordered water molecules in the R42I complex structure, molecules that act as surrogates for the missing arginine guanidino nitrogens of R42. The absence of such surrogate solvent interactions in the R42K complex leads to some disorder in the so-called "40s loop" that encompasses the substituent. One rationalization proposed for the observed loss in CN inhibition in these R42 mutant complexes invokes indirect effects leading to a misorientation of FKBP12 and FK506 structural elements that normally interact with calcineurin. Our results with the structure of the R42I complex in particular suggest that the observed loss of CN inhibition might also be explained by the loss of a specific R42-mediated interaction with CN that cannot be mimicked effectively by the solvent molecules that otherwise stabilize the conformation of the 40s loop in that structure.

Keywords: calcineurin; immunophilins; site-directed mutagenesis; structure-based drug design; X-ray crystallography

FK506 (United States Adopted Names Council of the American Medical Association, Chicago, Illinois [USAN], *tacrolimus*) is a natural product screening lead (Kino et al., 1987) now approved for therapeutic use as an immunosuppressant in Japan, the USA, Germany, and other countries. FK506, in complex with its 12-kDa M_r binding protein FKBP12, exerts its immunosuppressive effects through the inhibition of calcineurin (CN), an intracellular Ca^{+2}-calmodulin-dependent phosphatase (Klee & Cohen, 1988). CN inhibition, in turn, interrupts the induction of IL-2 and other T-cell activation events (Friedman & Weissman, 1991; Liu et al., 1991). A homologous natural product, rapamycin (AY-22,989; USAN, *sirolimus*), which was initially discovered as an antifungal agent (Sehgal et al., 1975), can antagonize the CN inhibitory activity of FK506 (Bierer et al., 1990a; Dumont et al., 1990b), even though it is itself an immunosuppressant by a different mechanism (Bierer et al., 1990a; Dumont et al., 1990a). These differences in CN inhibitory activity between the agonist FK506 and the antagonist rapamycin in their complexes with FKBP12 (Table 1), were first explained within the framework of an elegant model (Schreiber, 1991) that focused attention on the corresponding differences in chemical structure between the two ligands in their so-called "effector domain" region (see, e.g., Fig. 1). X-ray crystallographic studies have provided support for this model (van Duyne et al., 1991a, 1991b, 1993; Becker et al., 1993; Rotonda et al., 1993; Connelly et al., 1994; Wilson et al., 1995), by showing that the effector

Reprint requests to: Manuel A. Navia, Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, Massachusetts 02139-4211; e-mail: navia@vpharm.com.

[1] Visiting from: Chugai Pharmaceuticals Co. Ltd., 1-135 Komakado, Gotemba Shizuoka 412, Japan.

Abbreviations: CN, calcineurin; PPIase, peptidyl prolyl isomerase; FK506, USAN tacrolimus; FKBP12, 12-kDa M_r FK506 binding protein; FKBP13, 13-kDa M_r FK506 binding protein; USAN, United States Adopted Names Council of the American Medical Association, Chicago, Illinois.

Table 1. *Biochemical properties of the native and mutant complexes*[a]

Mutant	Calcineurin K_i (nM)	Reduction vs. native complex	FK506 K_i (nM)	Specific PPIase activity ($s^{-1}\ \mu M^{-1}$)
Native[b]	5.5 (1.8)	–	0.6 (0.2)	4.3 (0.4)
R42K[b]	590 (200)	107.3	0.6 (0.2)	3.8 (0.3)
R42I[b]	970 (150)	176.4	0.1 (0.1)	2.5 (0.3)
R42Q[c]	325 (150)	59.1	4.3 (2.0)	3.0 (0.3)
Native[d]	7.9 (3.0)	–	0.4 (0.2)	2.2 (0.2)
R42Q[d]	850 (250)	107.6	1.7 (0.6)	1.3 (0.3)
R42A[d]	280 (80)	35.4	0.2 (0.1)	1.1 (0.2)
Chimera[d,e]	19 (2)	2.4	0.4 (0.2)	0.57 (0.05)

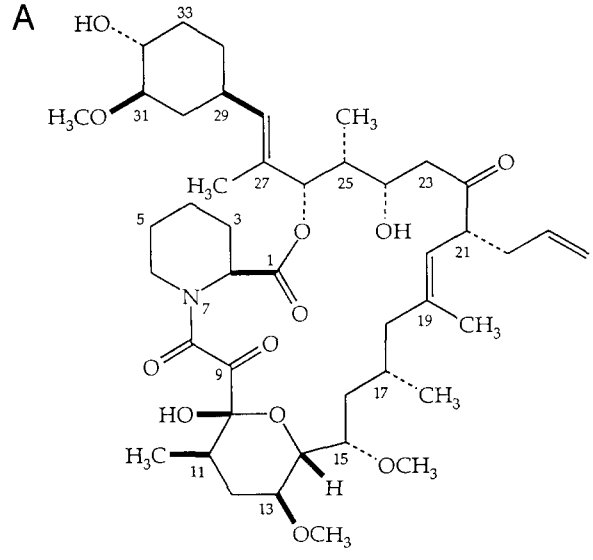
[a] Summary of published biochemical data for native and mutant FKBP12 proteins and their complexes with FK506 and calcineurin. The inhibition constant for calcineurin by native and mutant FKBP12 complexes with FK506 is given, along with the FKBP12 inhibition constants versus FK506 and the PPIase specific activity of the various FKBP12s versus a synthetic substrate.

[b] Aldape et al. (1992).

[c] Futer et al. (1995).

[d] Yang et al. (1993).

[e] Substitute FKBP12 residues 40–44 (-RDRNK-) with the corresponding residues (-LPQNQ-) from FKBP13.



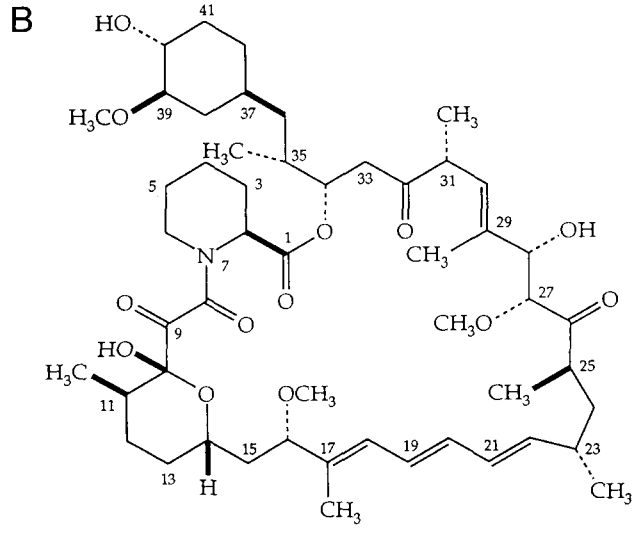


Fig. 1. A: Chemical structure of FK506 (USAN, tacrolimus). The effector domain of FK506 (Schreiber, 1991) corresponds to those portions of the ligand (C18–C23 and substituents in the macrocycle, and C26–C34 in the cyclohexyl ring) that have been shown crystallographically to protrude from the surface of its complex with FKBP12; structural elements in common between rapamycin and FK506 have been shown crystallographically to bind the PPIase active site of FKBP12 in the same manner (van Duyne et al., 1991a, 1991b, 1993; Becker et al., 1993; Rotonda et al., 1993; Armistead et al., 1995; Itoh et al, 1995; Wilson et al., 1995). **B:** Chemical structure of rapamycin (AY-22989, USAN, sirolimus). The rapamycin effector domain corresponds to atoms C15–C29 in the macrocycle and C36–C42 in the cyclohexyl ring.

domains of FK506 and rapamycin do indeed protrude from the surface of their respective protein–ligand complexes (Fig. 2A) with distinct conformations that might be compatible with CN binding and inhibition for the one ligand, but not for the other. In turn, those chemical structure elements shared by the two ligands (Fig. 1) were shown in those studies to constitute FKBP12 "binding domains," allowing a rationalization of the reciprocal antagonism between the two ligands in terms of their competition for a common FKBP12 binding site.

The effector domain model has retained broad acceptance as a first approximation to the complicated problem of immunosuppressive drug design, in part because of its consistency with the observed loss of CN inhibitory activity that follows even minor variation in the chemical structure of FK506 (Goulet et al., 1994). By limiting the role of the FKBP12 protein to that of a presenter of ligand functionality to CN (Schreiber, 1991; Rosen & Schreiber, 1992; Schreiber et al., 1993), the model reduces the scope of the drug design problem to one of simple mimicry of the conformation of the FK506 effector domain that protrudes from the surface of the native FKBP12–FK506 complex. Unfortunately, these efforts have yet to produce a linear or macrocyclic drug lead—let alone a clinical candidate—that exceeds the potency of FK506 (Itoh et al., 1995); ligands predicted on the basis of this model have all turned out to be antagonists of FK506 (see, e.g., Bierer et al., 1990b; Somers et al., 1991; Armistead et al., 1995).

Experimental evidence for a more complicated interaction between CN and the FKBP12–FK506 complex first emerged from a systematic examination of the biochemical properties of site-directed mutants of charged residues on the surface of FKBP12 (Aldape et al., 1992). The critical involvement of the "40s loop" and "80s loop" regions of the protein (as defined in Fig. 2B) was established for this interaction by these and subsequent studies (Yang et al., 1993; Futer et al., 1995), leading to a generalization of the effector domain model in the direction of a composite "effector surface" of both protein and ligand structural elements. Elsewhere, we have explored the structural consequences that follow substitutions in the 80s loop region of FKBP12 (Itoh et al., 1995) and have identified composite features on the FKBP12-

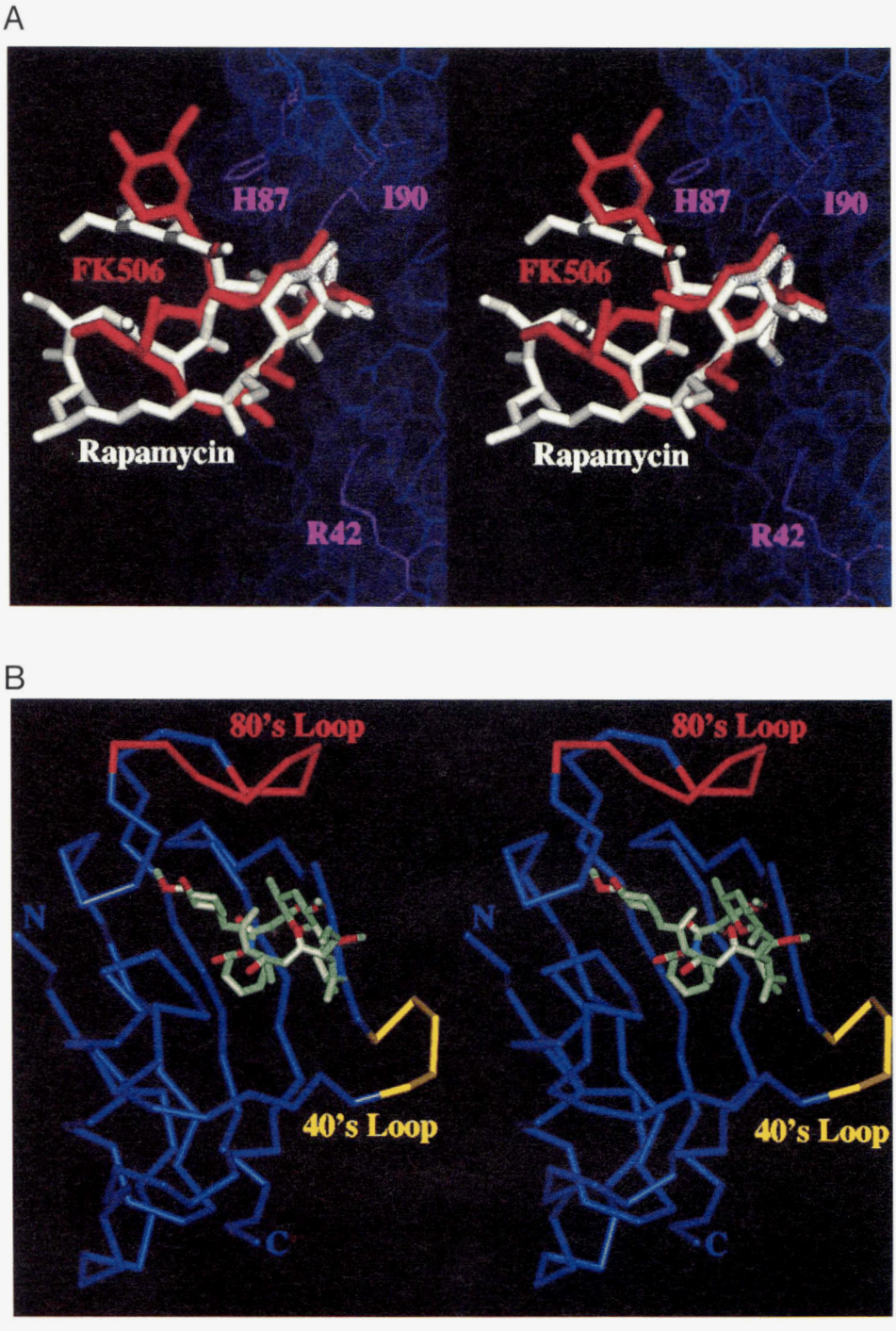


Fig. 2. A: Comparison of the structures of the FKBP12 complexes with FK506 (in red) and with rapamycin (in white). A dot representation of the surface of native FKBP12 (Wilson et al., 1995) is shown in blue and is representative of the surface of the FKBP12 complex structures with FK506 and rapamycin. **B:** Conformation of the backbone Cα of FKBP12 in its complex with FK506 is shown in blue. The 40s loop and 80s loop regions of the FKBP12 protein are specifically identified in yellow and red, respectively. The 40s loop is made up of residues 40–44 in FKBP12, which form a bulge on the third β-strand of the protein, as defined by van Duyne et al. (1991a). The 80s loop includes residues 84–91 on the edge of the fifth β-strand of the structure.

FK506 effector surface that might be candidate CN recognition and binding elements (Wilson et al., 1995). In this paper we focus on structural elements in the 40s loop region of FKBP12 and, in particular, on substitutions at residue 42 of the protein, which demonstrate profound (Table 1) but complicated effects on the CN inhibitory potency of the corresponding mutant complexes with FK506. These mutant data have led to proposed mechanisms of action, which are distinctly different in their character and consequences, that need to be resolved (Clardy, 1995).

Results

Crystallization, data collection, and refinement statistics for the native and mutant complex structures reported here are given in Table 2, along with RMS differences in conformation and mobility versus the native complex structure; biochemical data are summarized from the existing literature (Aldape et al., 1992; Yang et al., 1993; Futer et al., 1995) in Table 1. In all the complexes studied, the FKBP12 fold (Fig. 2B) that was seen in the

Table 2. *Summary of the wild-type and mutant complex structure analyses*[a]

	Wild type	R42K	R42I
Area detector used	Siemens	Rigaku	Rigaku
$P4_22_12$ unit cell; a, c (Å)	58.39, 55.76	58.31, 55.93	58.25, 55.98
Resolution (Å)	6.0~1.5	6.0~1.5	6.0~1.6
No. observations	76,344	43,803	44,188
% Reflection ($I > 2\sigma$)	81.4	88.1	89.7
R-merge (%)	3.99	5.88	3.15
R-factor (%)	16.6	18.7	16.8
No. water molecules	85	83	87
RMS bond length error (Å)	0.016	0.016	0.018
RMS bond angle error (deg)	2.74	2.85	2.93
Avg. FK506 B-factors (Å^2)	12.3	15.3	11.0
FK506 RMS diff vs. wt (Å)	–	0.139	0.123
FKBP12 RMS diff vs. wt (Å)	–	0.146	0.147

[a] Native and mutant FKBP12 complexes all share the native crystal form first reported by van Duyne et al. (1991a).

native complex structure (van Duyne et al., 1991a) is strongly conserved, consistent with the observed retention of PPIase and FK506 binding activity (Table 1). In this study, a considerable effort was made to crystallize all the complexes reported in a common crystal form (Table 2), in order to facilitate a direct comparison between structures. This crystal form turned out to be that of the native FKBP12–FK506 complex (van Duyne et al., 1991a), as a consequence of seeding mutant complex crystallization experiments with microcrystals of the native complex and subsequently using crystals from those solutions as macroseeds leading to data quality mutant complex crystals.

In the native complex structure (red coordinates in Fig. 3A, Kinemage 1), the two guanidino nitrogens of R42 are seen to stabilize the "40s loop" of FKBP12 through their participation in a bridging network of noncovalent interactions between residues D37 and K44. In the R42I mutant complex structure (yellow coordinates in Fig. 3A, Kinemage 1), two tightly bound water molecules (yellow in Fig. 3A, Kinemage 1) substitute for the missing

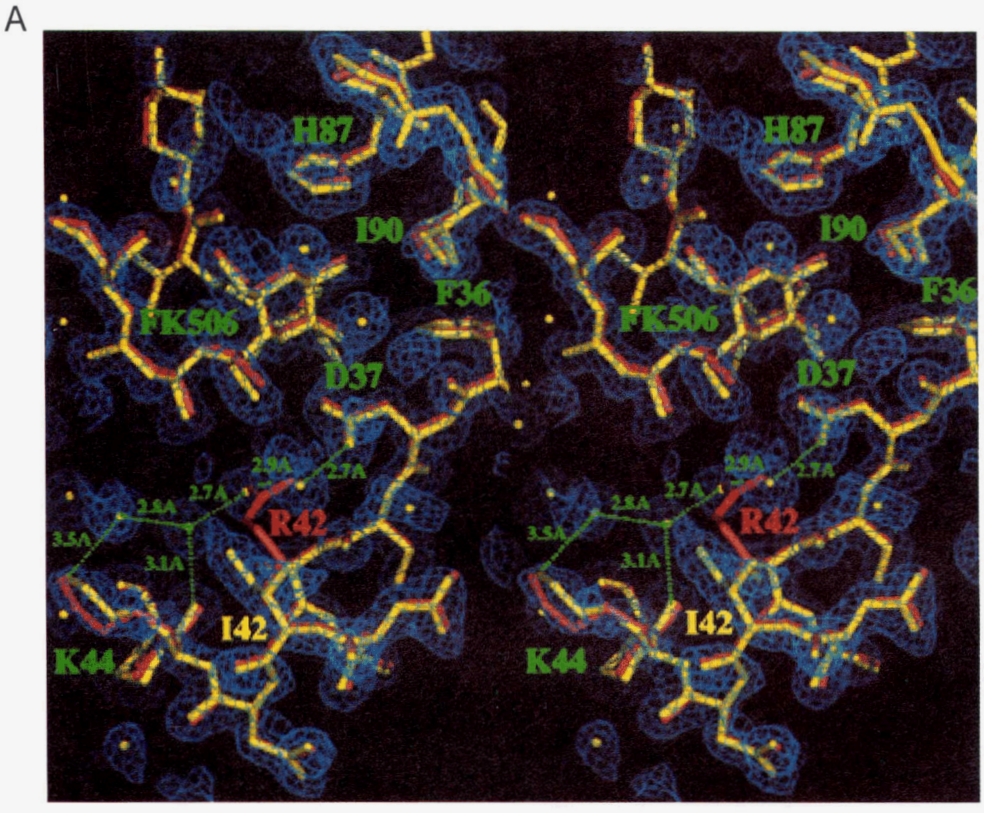


Fig. 3. A: Comparison of the structures of native (red) and R42I mutant (yellow) FKBP12 complexes with FK506. Refined coordinates (Table 2) of both complex structures are superimposed in the figure, together with the $2|F_o| - |F_c|$ electron density (in blue) of the R42I mutant complex structure, contoured at 1σ above background. Part of the FK506 binding domain is shown, as well as residues surrounding R42 and H87 in the protein. Water molecules bridging residues D37 and K44 are shown in green and yellow, corresponding to the R42I mutant complex structure. Dashed lines indicate bond distances between the water molecules. Our observations are inconsistent with the suggestion of Yang et al. (1993) that R42 mutants exert their effects indirectly, by reorienting nearby regions of the complex structure. Nor does the conformation of the 40s loop change as drastically as that of FKBP13 (Schultz et al., 1994) as a consequence of these substitutions. **B:** Dot-surface representation of the R42I mutant complex structure in the vicinity of FK506. Two water molecules (shown in green) in the R42I mutant complex structure fit readily into the gap created by the R42I substitution and act as surrogates for the missing guanidino nitrogens of R42 in bridging residues D37–K44. This interaction helps preserve the native conformation in the mutant complex and maintains PPIase and FK506 binding activity. Nonetheless, CN inhibition is drastically reduced (Table 1), suggesting a specific protein–protein interaction in the native complex that the water surrogates would be unable to mimic. **C:** Structure of the R42K mutant complex (in green) compared to that of the native complex (in red). As above, the $2|F_o| - |F_c|$ electron density corresponding to the R42K mutant complex, contoured at 1σ above background, is presented in blue. (*Continues on facing page.*)

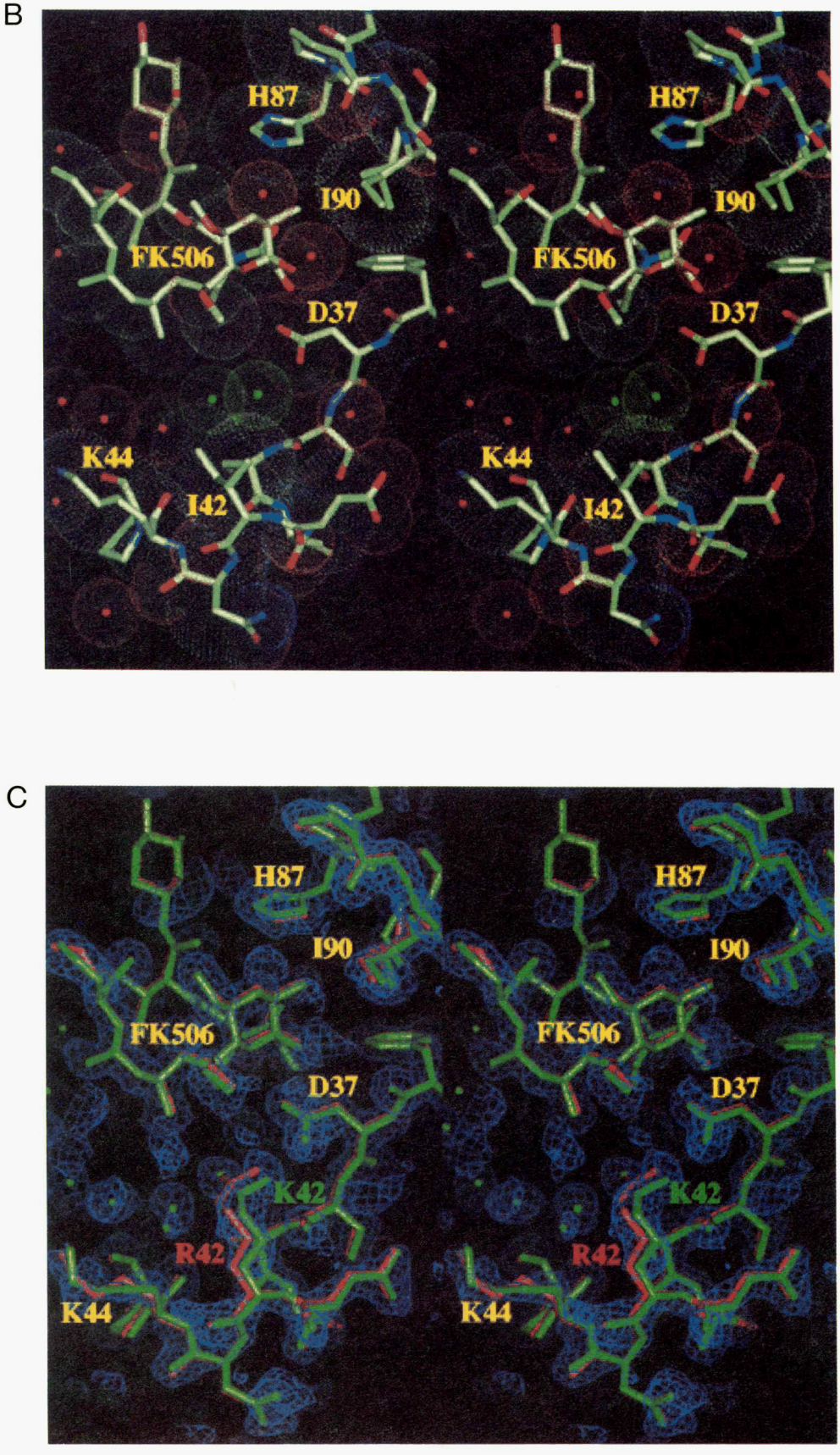


Fig. 3. *Continued.*

arginine guanidino nitrogens of R42, and are well accommodated (Fig. 3B, Kinemage 1) in the gap created by the smaller isoleucine substituent; a third water molecule (green in Fig. 3A, Kinemage 1) is apparently common to all the FKBP12 complex structures. As guanidino nitrogen surrogates, the two water molecules help to preserve the native complex 40s loop conformation in the R42I mutant complex, with an RMS deviation of 0.147 Å between the structures (Table 2). This interaction resembles one seen in the structure of a T157G mutant of T4 lysozyme (Alber et al., 1987; Matthews, 1993), where an ordered water molecule, acting as a surrogate for the missing T157 hydroxyl, preserves the pattern of stabilizing hydrogen bonding interactions seen in the native protein. It is interesting that, in spite of the high degree of conservation seen in the structure of the R42I mutant complex, CN inhibition is nonetheless reduced by ~180-fold (Table 1).

In the structure of the FK506 complex with the more conservative R42K mutant protein, the single ϵ-amino nitrogen of the lysine side chain is unable to substitute for both of the guanidino nitrogens of arginine (red in Fig. 3C, Kinemage 1); nor does the substitution leave enough space for an additional water molecule to insert itself as a surrogate, as shown in Figure 3B (Kinemage 1) for the R42I mutant complex. The resulting destabilization of the 40s loop is reflected in the higher temperature factors observed in that region of the protein (Fig. 4), even though the conformation of mutant protein complex still closely resembles that of the native (Fig. 3C, Kinemage 1), and FK506 binding and PPIase activity are preserved (Table 1).

Discussion

Two working models have emerged to rationalize the profound though complicated effects on CN inhibition that accompany substitutions in and around residue 42 of FKBP12. The simpler of these is made evident in the R42 single-site mutant complexes that were first characterized biochemically by Aldape et al. (1992), whose crystal structures are described here. In that model, the observed loss of CN-inhibitory activity can be immediately explained in terms of a direct and localized perturbation, by the substituted residue, of the effector surface presented to CN by the corresponding FKBP12–FK506 complex. The Merck group (Becker et al., 1993; Rotonda et al., 1993) arrives at a similar conclusion in their analysis and comparison of the structures of human and yeast FKBP12–FK506 complexes and of the human FKBP12 complex with L-685,818, an 18-hydroxy,21-ethyl analog of FK506.

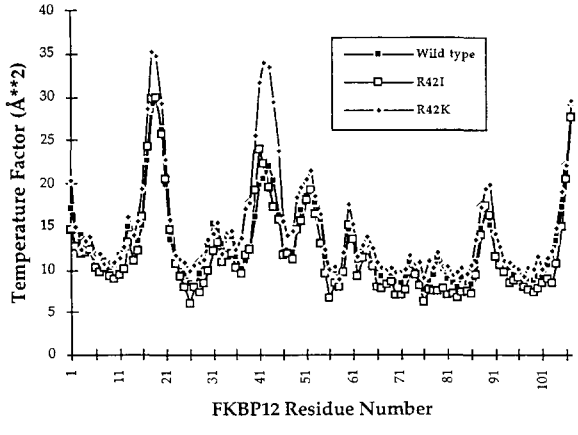


Fig. 4. Average X-ray temperature factors for the main-chain heavy atoms of FKBP12 for the native and the two mutant complex structures. Residues showing high temperature factors correspond to loop regions in the structure of FKBP12. These have been shown previously to be regions of local flexibility within the protein that were identified through a crystal structural analysis of 19 FKBP12–ligand complexes, each in a different crystal-packing arrangement (Wilson et al., 1995).

Yang et al. (1993), however, have suggested that substitutions at R42 exert their influence on CN inhibition indirectly, through a generalized conformational misorientation of nearby elements of the FKBP12–FK506 effector surface. This model is inferred from the curious pattern of CN-inhibitory activity evidenced in the FKBP12/13 chimeras studied by these workers. Substitution in FKBP12 of the corresponding 40s loop sequence from FKBP13 (i.e., replacing the sequence RDRNK with LPQNQ) leads to only a modest loss of CN-inhibitory potency (by ~2-fold to 19 nM). In turn, the single site R42Q mutant complex is severely compromised (by ~100-fold, to 850 nM), even though the R42Q substitution is incorporated in the FKBP12/13 chimera. From these observation, Yang et al. (1993) concluded that the effects of an R42 substitution would have to be strongly contextual. In other words, R42 and Q42 would each be appropriate to the 40s loop of FKBP12 and FKBP13 respectively, with only a modest loss of activity for the latter in the chimeric FKBP12/13 complex. An incompatible substitution, such as that of R42Q into FKBP12, would then lead to a significant and generalized disruption of the effector surface, an event that would be reflected in the much lower CN-inhibitory activity seen in the single-mutant complexes. Clardy (1995) has noted that the 40s loop in the structure of the native FKBP13–FK506 complex is displaced by about 2 Å RMS relative to the FKBP12–FK506 complex when these are overlapped, a point in support of the Yang et al. (1993) thesis.

The structural data presented here for the R42K and R42I mutant complexes show no such significant rearrangement of the 40s loop, the 80s loop, or any other part of the FKBP12 protein (Fig. 3). Nor do we observe a change in the conformation of FK506, even though we have demonstrated elsewhere (Itoh et al., 1995) that just such a conformational transformation is present in the FKBP12 R42K-H87V double mutant complex (Kinemage 2). All of our mutant complex structures (including the Itoh et al. [1995] double-mutant complex) have been solved in the same native FKBP12–FK506 complex crystal form described by van Duyne et al. (1991a), a crystal form that includes a significant number of ligand–ligand interactions (van Duyne et al., 1993; Wilson et al., 1995) that might otherwise have compromised the comparative interpretation we've presented for these structures. Even in the R42K mutant complex, where a significant increase in the temperature factors of the 40s loop of the mutant complex structure is observed (see Fig. 4), the weakened electron density in this region (Fig. 3C) is still consistent with the conformation of the 40s loop found in the native complex structure. In the less conservative R42I mutant complex structure, however, the fortuitous and unexpected ordering of two water molecules (Fig. 3A,B) acting as surrogates of the missing guanidino nitrogens of R42, leads to a more highly conserved structure, even though the loss of CN-inhibitory activity is also greater (~180-fold at 970 nM for the R42I mutant complex versus ~110-fold at 590 nM for the R42K mutant complex; Table 1).

Elsewhere, we describe the structures of FKBP12 (Itoh et al., 1995) and FKBP13 (Griffith JP, Wilson KP, Futer O, Living-

ston DJ, Navia MA. Structure of a mutant FKBP13-FK506 complex that is a high affinity inhibitor of calcineurin [manuscript in preparation]) mutant complexes that are inconsistent with the hypothesis that significant structural rearrangements in the 80s loop region of FKBP12 are responsible (Yang et al., 1993; Clardy, 1993, 1995) for the loss of CN-inhibitory activity seen in the corresponding FKBP12 mutant complexes. Nor do the results presented here support a similar hypothesis for the 40s loop region. Given our structural observations, one might speculate that R42 participates in some direct interaction with CN that cannot be effectively mimicked by the guanidino nitrogen surrogate water molecules that stabilize the 40s loop in the R42I mutant complex structure. With the R42K mutant complex, one might further consider an intermediate level of interaction with CN, through the single ϵ-amino nitrogen of the lysine substituent, and in spite of the greater disorder in the 40s loop. Teague (1995) has recently restated the importance of a conformationally well-defined recognition surface in promoting a strong interaction between exposed hydrophobic elements, such as are found in the FKBP12–FK506 effector surface and are presumed to exist on the complementary surface of CN. Our results show that relatively minor, localized perturbations of the CN complementarity of the FKBP12–FK506 effector surface can lead to quite significant effects on the CN-inhibitory potential of the resulting mutant complexes.

Methods

Mutant FKBP12 protein was prepared as reported (Aldape et al., 1992; Park et al., 1992; Wilson et al., 1995). Native and mutant FKBP12–FK506 complexes were prepared and crystals were grown essentially as described (van Duyne et al., 1991a; Wilson et al., 1995). Native crystals were used to seed the mutant complex crystallization experiments, and all the species reported here crystallized isomorphously in the native crystal form, as shown in Table 2. Diffraction data were collected on an X1000 multiwire area detector (Siemens Analytical Instruments, Madison, Wisconsin) or on an R-Axis II image plate detector (Rigaku/MSC, Woodlands, Texas), as indicated in Table 2. Data collection and processing used software provided by the manufacturers. All data were collected at room temperature. The reported structure of native FKBP12 in complex with FK506 (van Duyne et al., 1991a; Brookhaven Protein Data Bank [Bernstein et al., 1977] entry 1FKF) was used directly as an initial model for the crystallographic refinement of the mutant and native protein complexes. Refinement was by simulated annealing using the X-PLOR program package (Brünger, 1992). Mutated amino acids were initially refined as alanine, and the actual mutant side chains were introduced as refinement progressed. Water molecules were positioned in the model with the aid of a peak search program (SERC Daresbury Laboratory, 1979). The program QUANTA (Molecular Simulations; Burlington, Massachusetts) was used to examine electron density maps and protein models and for the superposition of structures and the calculation of the RMS differences reported (Table 2). Coordinates for the structures are being deposited in the Protein Data Bank.

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Note added in proof

A structure determination of the native FKBP12-FK506 complex bound to calcineurin has now been reported (Griffith JP, Kim JL, Kim EE, Sintchak MD, Thomson JA, Fitzgibbon MJ, Fleming MA, Caron PR, Hsiao K, Navia MA. 1995. X-ray structure of calcineurin inhibited by the immunophilin-immunosuppressant FKBP12-FK506 complex. *Cell 82*:507–522). A preliminary fit of mutant FKBP12 complex structures to the native FKBP12 in the calcineurin complex is entirely consistent with the results presented in this paper.