

Comparative mutational analysis of peptidyl prolyl *cis/trans* isomerases: active sites of *Escherichia coli* trigger factor and human FKBP12

Thomas Tradler, Gerlind Stoller, Karl P. Rücknagel, Angelika Schierhorn, Jens-U. Rahfeld, Gunter Fischer*

Forschungsstelle 'Enzymologie der Proteinfaltung' der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V., Kurt-Mothes-Str. 3, D-06120 Halle/S., Germany

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Abstract A low degree of amino acid sequence similarity to FK506-binding proteins (FKBPs) has been obtained for the peptidyl prolyl *cis/trans* isomerase (PPIase) domain of *E. coli* trigger factor (TF) that was thought to be significant with regard to the enzymatic properties of the bacterial enzyme. We examined whether the alteration of a negatively charged side-chain at position 37 (FKBP numbering) and a phenylalanine at position 99, both highly conserved through both types of enzymes, leads to parallel effects on the catalytic activity of both FKBP12 and TF-PPIase domain in a series of tetrapeptide substrates with different P₁ subsites. For the latter enzyme, substitution of Glu¹⁷⁸ by Val or Lys, which aligns to Asp³⁷ in human FKBP12, enhanced the PPIase activity, whereas a strongly decreased enzymatic activity was determined for the Asp³⁷Leu and Asp³⁷Val variants of FKBP12. Regardless of the P₁ subsite of the substrate used for the assay, mutation of Phe²³³Tyr generated a protein variant of the TF-PPIase domain with about 1% of the wild type PPIase activity. Dependent on the substrate nature, a moderate decrease as well as a 4.8-fold increase in k_{cat}/K_M could be determined for the corresponding Phe⁹⁹Tyr FKBP12 variant. Neither of the mutations of the TF-PPIase domain was able to implant FK506 inhibition found as a major characteristic of the FKBP family of PPIases.

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Key words: Peptidyl prolyl *cis/trans* isomerase; Trigger factor (*E. coli*); FKBP; Site-directed mutagenesis

1. Introduction

The 48 kDa trigger factor (TF) of *E. coli* is an abundant cytosolic peptidyl prolyl *cis/trans* isomerase (EC 5.2.1.8) with a unique efficacy in catalyzing *trans* to *cis* prolyl bond isomerization during the refolding of denatured proteins [1]. Its extraordinary catalytic power results from the modular structure of TF. It functionally combines the central PPIase domain (TF-PPIase domain), which encompasses residues Gln¹⁴⁸ to Thr²⁴⁹, with the C-terminal and N-terminal extensions presenting chaperone-like properties toward unfolded protein substrates [2]. Interestingly, the protein was first described

as a molecular chaperone specifically involved in the secretion machinery of *E. coli* [3–5]. In the context of a possible role as a folding catalyst it is important to note that TF does not only represent a peripheral component of the 50S subunit of the *E. coli* ribosome [1,6], but was also found to associate with nascent protein chains of the translating ribosome [7,8]. Apparently involved in a different pathway TF was described to be an essential factor in the GroEL-stimulated proteolysis of misfolded proteins [9].

The *E. coli* TF-PPIase domain and human FKBP12 share only 25.3% amino acid sequence identity [1]. However, the apparent relationship between TF-PPIase domains and the FKBP family of PPIases became more visible by hydrophobic cluster analysis, which revealed a hydrophobic binding pocket quite similar to that of FKFBPs for the inhibitor FK506 [10]. Even if direct proof by three-dimensional structures of FKBP/substrate complexes is still lacking, this pocket may be identical to the catalytic site. This point of view is supported, by analogy, considering the X-ray structures of complexes of human cytosolic cyclophilin (Cyp18). For this archetype of another PPIase family structural data of complexes with the tight binding inhibitor cyclosporin A [11] and with various substrates [12,13] exist. In the Michaelis complexes of substrates the proline ring fits well into the MeVal¹¹ position of the enzyme/inhibitor complex. For the FKBP-like PPIases the proposed similarity of the active site of FKFBPs and TF-PPIase domains is at variance with the lack of inhibition of the latter enzyme up to 20 μ M concentrations of FK506 [14]. With respect to FK506 inhibition the full-length TF behaves similarly to its catalytic domain encompassing residues 145–251 of the protein [14]. Thus, a functional relationship between both types of PPIases still remains an open question.

Functional analyses of FKBP12 variants engineered by site-directed mutagenesis were reported allowing conclusions concerning the major determinants for FK506 binding. Numerous FKBP12 variants exhibit FK506 affinities in the same range as the wild type enzyme [15–18]. As exceptions, Asp³⁷ and Phe⁹⁹ have been identified as major determinants of both enzyme activity and tight binding of FK506 [18–20].

These observations indicate that mutational analysis of Asp³⁷ and Phe⁹⁹ in conjunction with the mutations at the corresponding positions Glu¹⁷⁸ and Phe²³³ in TF-PPIase domains may reflect a possible functional relationship between TF and FKFBPs. In this study we report results concerning functional properties of the active site using 6 different mutant TF-PPIase domains and the equivalent FKBP12 variants. We demonstrate differential effects of the mutations for both types of enzymes. None of the TF-PPIase domain variants could be inhibited by FK506.

*Corresponding author. Fax: (49) (345) 551 197 2.

Abbreviations: TF, trigger factor; TF-PPIase domain, trigger factor segment Arg¹⁴⁵–Glu²⁵¹; PPIase, peptidyl prolyl *cis/trans* isomerase; FKBP, FK506-binding protein; FKBP12, human cytosolic 12 kDa FK506-binding protein; PMSF, phenylmethylsulfonyl fluoride; TLCK, 1-chloro-3-tosylamido-7-amino-L-2-heptanone; MALDI-MS, matrix assisted laser desorption/ionization-mass spectrometry; ESI, electrospray ionization; TOF, time-of-flight

2. Materials and methods

2.1. Materials

Restriction endonucleases and T4-DNA ligase were purchased from Boehringer Mannheim (Germany). T7-Sequencing Kit was from Pharmacia Biotech (Freiburg, Germany). GeneCleanII Kit were obtained from Bio101 (Vista, California, USA). Sequencing grade [³⁵S]dATP and [³²P]dATP were purchased from ICN Pharmaceuticals GmbH (Göttingen, Germany). PrimeZyme polymerase was from Biometra (Göttingen, Germany). Oligonucleotides were obtained from Eurogentec (Seraing, Belgium). Ni²⁺-NTA agarose and pQE30 were from Qiagen (Hilden, Germany). DNA molecular weight markers were from Gibco (Paisley, UK). Fractogels EMD DEAE-650(M) and EMD SO₃⁻-650(M) were from Merck (Darmstadt, Germany). Protein molecular weight markers were obtained from Serva (Heidelberg, Germany). Agarose, buffers and media were purchased from Serva, Gibco, AppliChem (Darmstadt, Germany), Biomol (Hamburg, Germany) and Roth (Karlsruhe, Germany). The PPIase substrates (Suc-Ala-Xaa-Pro-Phe-NH-Np) were purchased from Bachem (Heidelberg, Germany).

2.2. Molecular biology techniques

The DNA sequence of the TF-PPIase domain was amplified using chromosomal DNA of the *E. coli* K12 strain DH5 α and two primers corresponding to the 5' and the 3' regions of the fragment (5'-CAGTCAGGATCCGATGACGATGACAAACGTAACAGCAG-GCGACCTGG-3' and 5'-TGAAGTGAAGCTTTATTCTGCAGTC-AGTTCCGGCAG-3'). The resulting PCR product (corresponding to residues Arg¹⁴⁵-Glu²⁵¹ of the trigger factor) was ligated into the vector pUC18 and transformed into *E. coli* DH5 α . Recombinants were screened by restriction analysis and controlled by sequencing according to the procedure of the manufacturer. A positive clone designated as TTtig3 was used for mutagenesis. To express the wild type plasmid DNA of TTtig3 was digested with *Bam*HI and *Hind*III. The resulting fragment was extracted from agarose gel and ligated into pQE30. Resulting plasmids were transformed into the *E. coli* K12 strain M15(pREP4) and recombinants were screened by restriction analysis. After controlling sequences, a clone designated as TTtig4 was used for overexpression of the wild type TF-PPIase domain.

The construction of mutant TF-PPIase domains was carried out using the overlap extension method according to published instructions [21] with three primers for all mutations (I: 5'-CGGCT-CGTATGTTGTGTGGA-3'; II: 5'-TATACGCCTGTGAAATACCGCACAG-3'; III: 5'-CCGTCAGGGCGCGTCAGCGG-3'). The following primers were used for site-directed mutagenesis of the TF-PPIase-active domain (Glu¹⁷⁸Asp: 5'-GACGGCGAAGAGTTC-GATGGCGGTAAAGCGTCTG-3', Glu¹⁷⁸Val: 5'-GACGGCGAA-GAGTTCGTAGGGCGGTAAAGCGTCTG-3', Glu¹⁷⁸Lys: 5'-GACGGCGAAGAGTTCAAAGCGGTAAAGCGTCTG-3', Phe¹⁹⁸His: 5'-CGTATGATCCCGGGCCATGAAGACGGTATCAAAG-3', Phe¹⁹⁸Trp: 5'-CGTATGATCCCGGGCTGGGAAGACGGTATCAAAG-3', Phe²³³Tyr: 5'-GGTAAAGCAGCGAAATACGCTATCA-ACCTGAAG-3'). Primary PCR products were purified by preparative electrophoresis using the GeneCleanII Kit according to instructions of the manufacturer and 30 ng of each was used for the second PCR reaction. The secondary PCR products were purified by preparative electrophoresis, digested with *Bam*HI and *Hind*III and ligated into pUC18 after purification by phenol/chloroform extraction. After transformation recombinants were screened by restriction analysis and controlled by DNA sequencing. Insert DNA of positive clones was ligated into pQE30, transformed into *E. coli* M15/pREP4 and controlled by sequencing.

For expression of wild type FKBP12 the pUC derivative pFKBP6 was used (kindly provided by Boehringer Mannheim, Germany). Mutagenesis was carried out as described above for the TF-PPIase variants. For construction of the Asp³⁷Leu FKBP12 variant the primer 5'-GAAGATGGAAAGAAATTTCTTCTCCCGGACAGA-3' was used. The plasmid expressing the Phe⁹⁹Tyr FKBP12 variant was a kind gift from G. Wiederrecht.

2.3. Overexpression and purification of the proteins

All expressed variants of the TF-PPIase domain contained an N-terminal extension with the amino acid sequence: MRGS(H)₆GS(D)₄K- followed by the TF-PPIase domain sequence Arg¹⁴⁵-Glu²⁵¹ which included the respective point mutation. The pu-

rification of the mutated variants followed the same procedure as described below for the wild type.

For overexpression of the wild type TF-PPIase domain cells of the strain *E. coli* M15(pREP4/pTTtig4) were grown in 1 l of 2 \times YT medium at 37°C until an OD₆₀₀ of 0.5 was reached. Then 2.5 mM IPTG was added and the cells were grown for 5 h. The cells were harvested by centrifugation (6000 \times g, 4°C, 15 min), resuspended in 50 mM phosphate buffer (pH 8.0), 0.3 M NaCl, 1 mM PMSF and passed through a French pressure cell from SLM Aminco (Buettelborn, Germany). All the following procedures were carried out at 4°C. Cell debris were removed by ultracentrifugation at 95000 \times g for 40 min in a 45Ti rotor from Beckman (Palo Alto, CA, USA). The supernatant was applied to a Ni²⁺-NTA column (1 \times 2 cm) equilibrated in the same buffer. Material unspecifically bound to the matrix was removed with 50 mM phosphate buffer (pH 6.0), 0.3 M NaCl, 10% glycerol (v/v). Bound proteins were eluted with a gradient of 0–0.5 M imidazole (40 ml total). The imidazole was removed by dialysis against 30 mM Tris buffer (pH 8.0). Further purification of the enriched protein was achieved by gel filtration. Samples of 1 ml were applied to a Superdex75 16/60 HiLoad FPLC column from Pharmacia (Uppsala, Sweden) equilibrated with 0.01 M HEPES (pH 7.5), 0.15 M KCl, 0.015 M MgCl₂. During all runs the flow rate was maintained at 0.8 ml/min. The eluted TF-PPIase domain was homogeneous in the SDS-PAGE as was verified by Coomassie staining.

Wild type FKBP12 and the Asp³⁷Leu variant contained the N-terminal extension MTMITNSM(H)₄(D)₄K- flanked by the 108 amino acid segment of FKBP12. Numbering of the mutational sites referred to mature FKBP12 consisting of 107 amino acids. The FKBP12 variants were expressed in *E. coli* strain K12 DH5 α . All His-tagged variants were purified as described for the TF-PPIase domain. The expressed Phe⁹⁹Tyr variant of FKBP12 lacked an engineered N-terminal extension. Therefore, the first step of purification was anion exchange chromatography on a Fractogel EMD DEAE-650(M) column (1.6 \times 9 cm) equilibrated with 20 mM HEPES (pH 7.3). The supernatant of the ultracentrifugation step, which had been adjusted to the same buffer conditions, was loaded onto the column with a flow rate of 1.5 ml/min. The FKBP variant was found in the flow through fractions which were loaded onto a Fractogel EMD SO₃⁻-650(M) column (1 \times 8 cm) equilibrated in 20 mM MES (pH 6.3). The protein was eluted with 100 ml of a 0–1 M KCl gradient. Final purification was achieved by gel filtration as described above. The homogeneity of the proteins was verified by Coomassie staining following SDS-PAGE.

2.4. Protein analysis

The purified proteins were desalted on the LC-10A reversed phase HPLC system from Shimadzu (Kyoto, Japan) using a Nucleosil 500–5 C₃-PPN column (125 \times 4 mm, guard column 11 \times 4 mm) from Macherey-Nagel (Düren, Germany). The column was pre-equilibrated with 0.09% aqueous trifluoroacetic acid in 1% acetonitrile (v/v). The proteins were eluted with a gradient of 25–60% acetonitrile in 0.08% trifluoroacetic acid within 25 min at 40°C at a flow rate of 1 ml/min. Runs were monitored by UV/VIS absorbance at 215 nm and 280 nm.

Electrospray mass spectra were obtained on a VG Bio-Q triple-quadrupole mass spectrometer from Fisons Instruments (Manchester, UK) equipped with an electrospray ion source. 4 μ l of the sample solution was injected directly into the electrospray source via a loop injector at a solvent (acetonitrile:water 1:1 v/v, 1% formic acid) flow rate of 4 μ l/min. Scanning was performed from *m/z* 500 to *m/z* 1400 in 10 s and this mass scale was calibrated using horse heart myoglobin.

MALDI-MS was performed on a REFLEX reflection-type time-of-flight spectrometer from Bruker-Franzen Analytik (Bremen, Germany). Ions formed by laser desorption at 337 nm (N₂ laser) were recorded at an acceleration voltage of 28.5 kV in the linear mode. A saturated solution of sinapinic acid in acetonitrile:methanol:water 3:2:5 (v/v) was used as a matrix. Spectra were calibrated with angiotensin II, insulin and cytochrome *c* as external standards.

The molecular masses of the TF-PPIase variants were determined using ESI-MS. The theoretical and experimental values, respectively, are 13833.7 and 13834.3 \pm 7.08 Da for the wild type, 13819.7 and 13820.6 \pm 4.4 Da for the Glu¹⁷⁸Asp variant, 13832.8 and 13833.0 \pm 3.3 Da for the Glu¹⁷⁸Lys variant, 13804.7 and 13812.2 \pm 11.0 Da for the Glu¹⁷⁸Val variant, 13872.7 and 13873.3 \pm 3.3 Da for the Phe¹⁹⁸Trp variant (11661.5 and 11668.3 \pm 10.0 Da for the truncated fragment), 13823.7 and

13 834.8 ± 7.4 Da for the Phe¹⁹⁸His variant (11 612.5 and 11 615.5 ± 9.3 Da for the shorter fragment), 13 849.7 and 13 856.6 ± 4.9 Da for the Phe²³³Tyr variant.

In consideration of the lack of the N-terminal Met for the mature proteins, the molecular masses for the His-tagged wild type FKBP12 and its Phe⁹⁹Tyr variant were calculated as 13 865.9 Da and 11 835.1 Da, respectively. These values are in accordance with the experimental data from MALDI-TOF-MS that revealed respective molecular masses of 13 867.6 Da and 11 834 Da. In addition, the integrity of the Phe⁹⁹Tyr FKBP12 variant was confirmed by ESI-MS revealing a value of 11 834.1 ± 3.8 Da. Using ESI-MS the molecular mass of the His-tagged Asp³⁷Leu variant was determined to be 13 864.7 ± 5.7 Da, which matched the sequence-derived value of 13 863.9 Da. Approximately 20% of the purified recombinant FKBP12 and its Asp³⁷Leu variant were N-terminally acetylated.

Besides mass spectroscopic analyses of the purified protein a tryptic digestion of the Phe⁹⁹Tyr FKBP12 variant was performed. The protein sample (200 pmol) was pyridylethylated, desalted by HPLC and digested with trypsin in 0.1 M ammonium bicarbonate at an enzyme substrate ratio of 1:20 at room temperature overnight. Tryptic peptides were separated by HPLC and analyzed by MALDI-MS using a saturated solution of α-cyano-4-hydroxycinnamic acid in acetone as matrix. The determined molecular masses of the generated peptides matched perfectly with calculated values.

Circular dichroism was measured using the J-710 spectropolarimeter from JASCO (Tokyo, Japan). Far-UV CD measurements were carried out in a thermostatted 0.1 cm quartz cell using 0.01 M sodium phosphate (pH 7.0) at 25°C. The spectra were recorded 20 times and averaged. The protein concentrations for the different variants of the TF-PPIase domain were: wild type 10.2 μM, Glu¹⁷⁸Asp 11.9 μM, Glu¹⁷⁸Val 10 μM, Glu¹⁷⁸Lys 10.2 μM, Phe²³³Tyr 10.3 μM. The concentrations for the FKBP variants were: wild type 10.8 μM, Asp³⁷Leu 9.5 μM and Phe⁹⁹Tyr 10.0 μM. Data were analyzed using the software provided by the instrument manufacturer JASCO.

Protein concentration was determined spectrophotometrically using the molar extinction coefficients at 280 nm (6970 M⁻¹ cm⁻¹ for the wild type and the Glu¹⁷⁸ variants of the TF-PPIase domain, 8250 M⁻¹ cm⁻¹ for the Phe²³³Tyr variant, 9530 M⁻¹ cm⁻¹ for the wild type FKBP12 and the Asp³⁷Leu variant and 10 810 M⁻¹ cm⁻¹ for the Phe⁹⁹Tyr FKBP12 variant). All data were calculated from the amino acid composition according to the method of Gill and von Hippel [22].

2.5. PPIase assay and FK506 inhibition

PPIase activity measurements and inhibitory studies were carried out using a slightly modified protease-coupled assay as described pre-

viously [1]. Inhibition by FK506 was assayed utilizing Suc-Ala-Phe-Pro-Phe-NH-Np as substrate. Stock solutions of 6.2 mM FK506 were prepared in ethanol. Inhibition of the TF-PPIase domain variants was tested up to a final concentration of 24 μM of FK506 in the assay. This concentration resulted from 252-fold dilution of the inhibitor stock solution into the quartz cell. Reference values of enzyme activity were determined in the presence of an equivalent volume of ethanol.

For determining the K_i values of the Asp³⁷Leu and the Phe⁹⁹Tyr variants of FKBP12 the FK506 concentrations ranged from 0 to 1.4 μM. The data were fitted non-linearly to competitive tight binding equation [23].

2.6. Computer methods

Pairwise sequence alignments were performed using the BestFit and Gap programs from the UWGCG (University of Wisconsin Genetics Computer Group) software package, version 8.1. The SWGAP-PEP.CMP symbol comparison table was used. Gap creation penalty was set at 3, gap extension penalty was 0.1. Quality values were obtained as described in the manual of the BestFit program. Significance of the alignment was evaluated by determining the average quality of 10 alignments to randomized sequences of the same length and composition [24–27].

3. Results

3.1. Molecular cloning and isolation of FKBP12 and TF-PPIase domain variants

Our mutagenesis experiments with the TF-PPIase domain were guided by results of site-directed mutagenesis on FKBP12, providing information about residues being important for the formation of a functional active site in FKBP12. Among the three positions selected for point mutations, only Phe⁹⁹ is conserved in the TF-PPIase domains, but Asp³⁷ and Trp⁵⁹ are conservatively replaced by Glu and Phe, respectively (Fig. 1). In order to allow reliable comparison of the kinetic constants measured here, mutated TF-PPIase domains as well as corresponding FKBP12 variants, Asp³⁷Leu and Phe⁹⁹Tyr, have been produced.

The DNA sequence of the TF-PPIase domain was cloned into vectors pUC18 and pQE30 corresponding to the clones TTtig3 and TTtig4, respectively. The strain TTtig4 was used for overexpression of the wild type TF-PPIase domain that

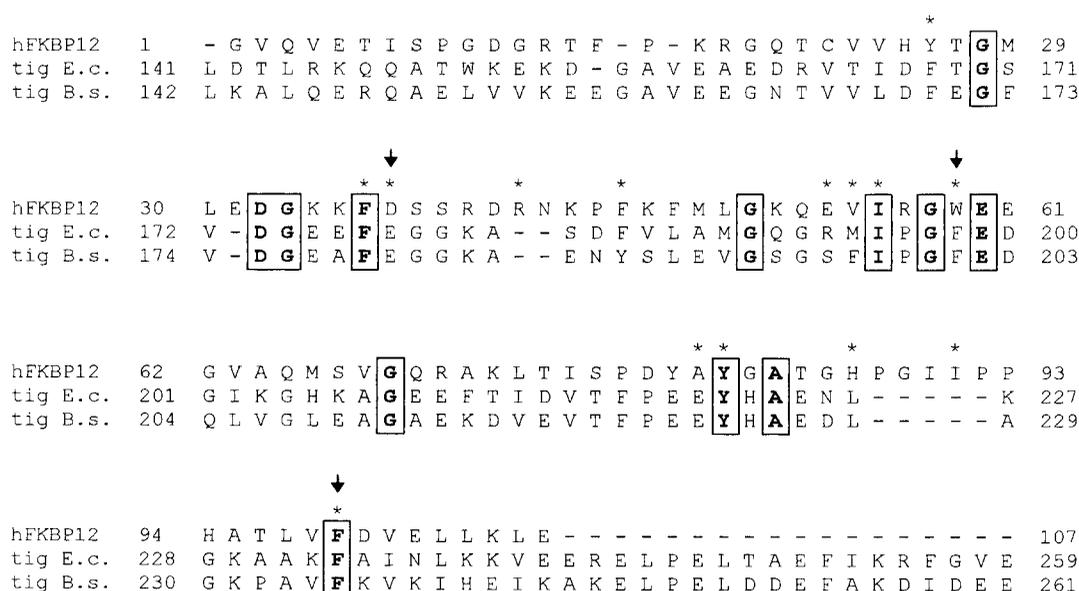


Fig. 1. Partial sequence alignment of the PPIase domains of the enzymatically active TFs from *E. coli* [28] and *B. subtilis* (EMBL Z75208) with FKBP12 [29]. Asterisks mark residues of FKBP12 involved in the FK506 binding [18,30–32]. Boxed residues are conserved between the *E. coli* TF, *B. subtilis* TF and FKBP12. The positions of residues mutated in the *E. coli* TF-PPIase domain are marked by arrows.

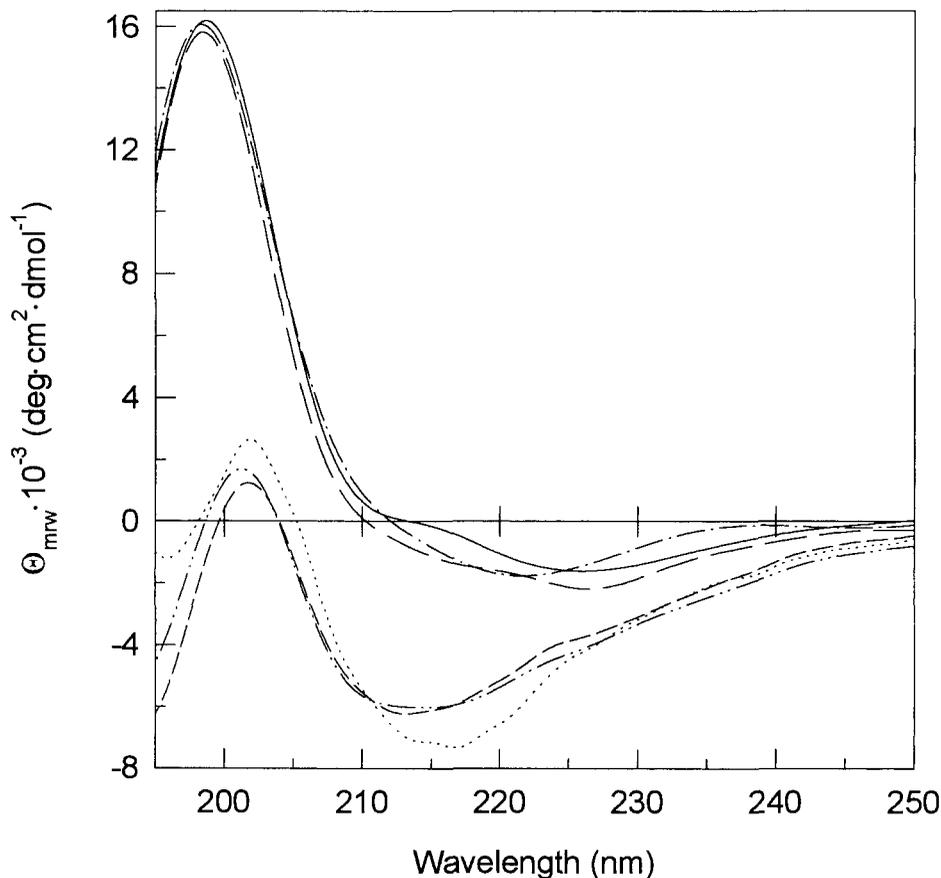


Fig. 2. Far-UV CD spectra of TF-PPIase domain variants and FKBP12 variants are shown as mean residue molecular ellipticity versus wavelength. Spectra were recorded at 25°C in 10 mM sodium phosphate (pH 7.0) (for further details see Section 2.4). The individual mutant proteins are designated as follows: (—) wild type TF-PPIase domain, (---) Glu¹⁷⁸Lys variant, (- · -) Phe²³³Tyr variant, (- - -) wild type FKBP12, (- - -) Asp³⁷Leu variant and (· · ·) Phe⁹⁹Tyr variant of FKBP12. The CD spectra of the Glu¹⁷⁸Asp and Glu¹⁷⁸Val variants of the TF-PPIase domain were identical to the Glu¹⁷⁸Lys spectrum.

finally carried an N-terminal His-tag. About 50 mg of pure recombinant protein was produced from 1 liter of overexpression culture. A similar expression level and final yield of purified protein was achieved for all of the TF-PPIase domain variants.

In contrast to the stability of the other mutated TF-PPIase domains the Phe¹⁹⁸Trp and the Phe¹⁹⁸His variants were found to be unstable. Thus, the protein fractions after chromatographic purification on Ni²⁺-NTA agarose of the Phe¹⁹⁸Trp and Phe¹⁹⁸His variants were contaminated with truncated polypeptide chains to about 5% and 20%, respectively, as was estimated by evaluation of Coomassie stained SDS-polyacrylamide gels or the peak areas of the HPLC profile. Despite addition of protease inhibitors like PMSF and TLCK, the proteins were cleaved at the Lys²³²-Phe²³³ site as determined by Edman degradation and MALDI mass spectrometry. In the course of the protein purification by gel filtration separation of the intact TF-PPIase domain variants from these enzyme fragments could not be achieved.

The expressed wild type FKBP12 and its Asp³⁷Leu variant bore a His-tag adjacent to the respective N-termini of the FKBP12 sequence, whereas the Phe⁹⁹Tyr variant was devoid of such N-terminal extension. In its mature form the first methionine was found to be cleaved off as was proven by MALDI and ESI mass spectrometry (see Section 2.4). The amino acid sequences of all proteins used in this study were

verified by combinations of mass spectrometry, tryptic digestion and Edman degradation (see Section 2.4 for details). To clarify the extent to which the occurrence of the engineered N-terminal segments would affect the enzymatic properties of the proteins, the enzymatic activity of both the His-tagged form and the mature wild type FKBP12, where the His-tag was removed by enterokinase cleavage, was determined. In the protease coupled PPIase assay using Suc-Ala-Leu-Pro-Phe-NH-Np as substrate, there was no difference in the values of k_{cat}/K_M detectable (T. Zarnt, unpublished results).

The similarity of the far-UV CD spectra of all FKBP12 variants and mutated TF-PPIase domains with those of the respective wild type proteins revealed a lack of structural disturbances relevant for the mutations (Fig. 2). Based on this information it becomes quite obvious that functional differences determined for the protein variants were not due to structural instabilities of the proteins.

3.2. Enzymatic properties

In contrast to an assay using a polypeptide as a substrate, the tetrapeptide-based PPIase assay revealed an enzymatic efficiency for the wild type TF-PPIase domain similar to the full-length TF [14]. Thus, basic features of the catalytic mechanism of TF, which is thought to be indicative of the construction of the active site, are retained in the TF-PPIase domain. We utilized the second-order rate constants k_{cat}/K_M

of a series of tetrapeptide substrates modified in P₁ position as well as inhibition of enzyme activity by FK506 to collect information about these catalytic events.

All mutant proteins characterized here were enzymatically active in the tetrapeptide assay even if the Phe²³³Tyr TF-PPIase domain displayed only a very weak catalytic action toward Suc-Ala-Glu-Pro-Phe-NH-Np (Table 1). For FKBP12 the mutations generally decreased k_{cat}/K_M with the exception of the Phe⁹⁹Tyr variant when assayed with Suc-Ala-Glu-Pro-Phe-NH-Np. In this case a 4.8-fold increase was noted when compared with the wild type FKBP12, however, on a low level of absolute activity (Table 1).

The residue Glu¹⁷⁸ of TF corresponds to Asp³⁷ of FKBP12, which was shown by computer simulations to be involved in the binding of an oligopeptide substrate by hydrogen bonding [33]. In order to create an Asp at this position and to explore the importance of the negative charge for the catalytic site, Glu¹⁷⁸ of the TF-PPIase domain was mutated to either Asp, Val or Lys. The replacement of Glu¹⁷⁸ by Asp showed only little effect on PPIase activity. Moreover, it did not implant inhibition by FK506 to the TF-PPIase domain, which was tested up to an inhibitor concentration of 24 μM . Both Glu¹⁷⁸Val and Glu¹⁷⁸Lys mutations mostly led to an increase in catalytic efficiency for the corresponding enzymes, up to 2.4-fold for the Glu¹⁷⁸Lys mutant (Table 1). The pattern of catalytic specificity regarding to the P₁ subsite exhibited minor alterations even if the charge of the side chain was reversed in the Glu¹⁷⁸Lys mutant.

The difference of the Glu¹⁷⁸Val and Glu¹⁷⁸Lys variants of TF-PPIase domain to the Asp³⁷Leu FKBP12 variant was manifested by a decreased level of PPIase activity ranging from 3 to 6% of the wild type enzyme (Table 1). As an exception, Glu in the P₁ position [34] of the substrate caused an elevated k_{cat}/K_M , but on a very low level of absolute activity. This mutation also resulted in a 700-fold lower affinity to FK506 with a K_i value of 349 ± 26 nM when compared with the wild type ($K_i = 0.5$ nM [35]).

A gain of FK506 affinity should be anticipated for the replacement of the corresponding residue Phe¹⁹⁸ of TF by Trp in the TF-PPIase domain, because the hydrophobicity of the binding pocket will be enhanced by this substitution. This hypothesis is in line with published data on FKBP12 that the more hydrophilic His residue in this position greatly deteriorates FK506 binding and PPIase activity in parallel [20]. We found that mutation of the Phe¹⁹⁸ to either a Trp or a His residue rendered the TF-PPIase domain unstable, leading to partial degradation by proteases during the purification procedure. Therefore, the enzymatic activities of these protein variants were estimated using an enzyme fraction contaminated with partially degraded protein. Considering the pro-

teolytic sensitivity of the mutant proteins, the protease coupled PPIase assay was substituted by an alternative, non-proteolytic method utilizing solvent jumps with Suc-Ala-Phe-Pro-Phe-NH-Np as substrate [36]. The estimates gave 56% and < 0.2% of the wild type enzyme activity for the Phe¹⁹⁸Trp and Phe¹⁹⁸His mutant, respectively. The detection of the enzyme activity of the Phe¹⁹⁸His variant required an enzyme concentration of 8 μM in the assay. Thus, it cannot be ruled out that the very low enzymatic activity of the Phe¹⁹⁸His variant has been caused by a minor PPIase contamination of about 0.3% in the Phe¹⁹⁸His fraction.

A close proximity of Phe⁹⁹ to the pipercolinyl ring and the C9 keto oxygen of FK506 in FKBP12 along with unique conservation among the FKBP12 and TFs has to be noted. Subtle alterations introduced by the Phe⁹⁹Tyr (Phe²³³Tyr) mutation produced stable protein variants, but influenced both kinds of enzymes differently. With the exception of the inert Suc-Ala-Glu-Pro-Phe-NH-Np, the TF-PPIase domain variant yielded $1.1 \pm 0.4\%$ of the wild type activity when assayed with the series of substrates modified in the P₁ position. Contrary to this finding, the FKBP12 variant affected the substrate specificity profile in a range between 480% and 5% of the specificity constants for the wild type enzyme. Its binding affinity for FK506 (K_i value 118 ± 9 nM) was lowered 240-fold as compared to the wild type protein. However, none of the TF-PPIase domain variants could be competitively inhibited by 24 μM FK506.

4. Discussion

In this paper we have shown by examining enzymatic properties of point mutated protein variants of the TF-PPIase domain and FKBP12 that considerable differences exist for both types of PPIases. Inhibition by the peptidomacrolides FK506 and rapamycin are major characteristics of the FKBP12. The low degree of amino acid sequence conservation throughout this family of PPIases indicates a less stringent requirement for structural retention of the active site. Consequently, studies by site-directed mutagenesis, which included many residues of the FK506-binding pocket, revealed that both FK506 affinity and PPIase activity are rather resistant to point mutations. In fact, an enzymatically inactive FKBP12 variant with a proven structural integrity of the protein has not been reported so far. Strikingly, the FKBP-like TF cannot be inhibited by FK506 despite maintaining most of the critical hydrophobic residues forming the putative active site [10].

In consideration of the close proximity (< 3.9 Å) of the indole ring of Trp⁵⁹ to the C ^{β} , C ^{γ} , C ^{δ} carbon edge of the pipercolinyl ring of the peptidomacrolides in the FK506/FKBP12 complex, the replacement of Trp by Phe in TF

Table 1

Subsite specificity (k_{cat}/K_M , mM⁻¹ s⁻¹) of the *E. coli* TF-PPIase domain variants and FKBP12 variants towards Suc-Ala-Xaa-Pro-Phe-NH-Np, measured in the protease coupled assay at 10°C in 35 mM HEPES (pH 7.5)

| Xaa | TF-PPIase domain | | | | Phe ²³³ Tyr | FKBP12 | | |
|-----|------------------|------------------------|------------------------|------------------------|------------------------|-----------|-----------------------|-----------------------|
| | wild type | Glu ¹⁷⁸ Asp | Glu ¹⁷⁸ Val | Glu ¹⁷⁸ Lys | | wild type | Asp ³⁷ Leu | Phe ⁹⁹ Tyr |
| Phe | 1060 | 950 | 1720 | 2520 | 14 | 780 | 48 | 64 |
| Leu | 650 | 620 | 970 | 1500 | 10 | 1240 | 66 | 64 |
| Ala | 240 | 230 | 350 | 470 | 1.9 | 100 | 32 | 37 |
| Lys | 250 | 220 | 130 | 89 | 1.6 | 58 | 1.8 | 6.9 |
| Glu | 6.9 | 9.5 | 16 | 42 | n.d. | 1.5 | 1.2 | 7.2 |

n.d.: PPIase activity was not detectable up to a final enzyme concentration of 5 μM in the cuvette.

may be unfavorable for the formation of the FK506 binding pocket. For Cyp18, which represents the prototype of the cyclophilin family of PPIases, Trp¹²¹ was found to be very important for binding of cyclosporin A [37]. Consequently, the Phe/Trp substitution at position 121 has been shown to strengthen the CsA/Cyp18 greatly. However, we could not implant competitive inhibition by FK506 by a Phe¹⁹⁸Trp replacement in the TF-PPIase domain. The Phe side chain at position 59 in FKBP52 seems to be sufficient to provide strong hydrophobic contacts to FK506. For FKBP12.6, which already has this substitution in a wild type FKBP, FK506 binding was found to be affected to a limited extent only [38].

The highly conserved Asp³⁷ of the FKBP52 was identified experimentally as a major determinant for FK506 binding. Thus, the conservative replacement by Glu in TFs was thought to be another potential candidate for the explanation of the abolished FK506 affinity. There are two additional examples for a replacement of Asp by Glu at this position. They comprise the third FKBP-like domain of FKBP52 of the steroid receptor and an *E. coli* ORF (FKBP16) [30]. Like the TF-PPIase domain both sequences have an exceptionally low overall similarity to other FKBP52s.

Principally, a Glu residue at position 178 does not impair important steps in catalysis, because the k_{cat}/K_M values for the TF-PPIase domain and for FKBP12 were of the same order of magnitude (Table 1). Based on the assumption of a FKBP-like structure of TF, the Glu¹⁷⁸Asp TF-PPIase domain variant was designed with the idea to restore FK506 binding, and to add a functional link between FKBP52s and TFs. However, the failure of the TF variant to be sensitive toward FK506 provides evidence for a distinct active site of TF and FKBP52s. This point of view is supported by the different effects on the enzymatic activity produced by mutations of the Glu¹⁷⁸ residue. For the Asp³⁷Leu FKBP12 variant, a reduction of k_{cat}/K_M to 3–6% of the wild type value was determined for most tetrapeptide substrates used here. This observation agrees well with the reported data of 9% for the Asp³⁷Val replacement [17]. In contrast, the considerable increase in catalytic power of the Glu¹⁷⁸Val TF-PPIase domain, which can be seen throughout the series of tetrapeptide substrates, does not indicate any significant contribution of the carboxylate group to the catalytic mechanism as has been suggested for FKBP52s [33]. Even a positively charged side chain like in the Glu¹⁷⁸Lys variant cannot deteriorate the active site of TF.

Moreover, the specificity constants determined for the Phe⁹⁹Tyr FKBP12 variant (Phe²³³Tyr in TF) do not support a conserved active site structure in both types of PPIases. A previous study of this FKBP12 mutation reported complete loss of both PPIase activity and binding of a FK506 derivative [20]. In contrast, we determined a K_i value of 118 ± 9 nM for the Phe⁹⁹Tyr FKBP12 variant, which is reduced only 200-fold as compared to the wild type FK506 affinity. Within the series of tetrapeptide substrates at least 5% of wild type activity was found for this protein variant, but also an enhanced activity could be obtained. Obviously, the P₁ subsite specificity of the enzyme is altered by this replacement. Only a few other FKBP12 mutations like Tyr⁸²Phe, His⁸⁷Leu and the Tyr²⁶-Phe/Tyr⁸²Phe double mutant FKBP12 resulted in altered P₁ subsite specificity [18]. In the Michaelis complex of FKBP12, Phe⁹⁹ is thought to make a hydrogen bond to the imide carbonyl group. The electronic state of this bond may provide an important contribution to the energetic state of the rotational

barrier. Mutation of Phe to Tyr will maintain this interaction, but will allow for subtle alterations of the hydrogen bond energy and the spatial alignment of the substrate within the active site. Various catalytic factors, like transition state stabilization by desolvation, enzyme-assisted substrate autocatalysis and ground-state destabilization are believed to contribute to the catalytic machinery of FKBP52s [33,39]. For example, rate enhancement for hydrophobic substrates will be especially susceptible to the desolvation component of catalysis. The substrate dependent variability of the Phe⁹⁹Tyr enzyme variants activity may reflect the distinct influence of the point mutation on the individual components of the catalytic machinery.

In this respect, the Phe²³³Tyr TF-PPIase domain behaves unlike the corresponding FKBP12 mutation. There is an invariantly strong reduction in catalytic activity for the Phe²³³Tyr TF-PPIase domain, even if charged side chains are present in position P₁ of the substrates (Table 1). This indicates that the catalytic component, which is retained on a low level of activity in this protein variant, is not related to a mechanism of transition state stabilization sensitive to substrate hydrophobicity. Besides of mechanistic aspects, the uniformly low activity of this protein variant makes it a suitable candidate for complementation experiments in order to assess the role of TF catalytic activity in cell function.

Taken together the functional differences of the enzyme variants described above argue against the presence of a potential FK506-binding pocket within the TF active site.

Recently we were able to show that two *E. coli* proteins, SlyD and FKBP16 (SlpA), are indeed PPIases that cannot be inhibited by FK506. Originally, they were assigned by sequence comparison to belong to the FKBP52s. As for the TF-PPIase domain, only a small degree of sequence conservation relative to other FKBP52s has been obtained (Hottenrott, Schumann, Plückthun, Fischer and Rahfeld, accepted). Unfortunately, any detailed functional characterization, such as was provided for the TF-PPIase domain by the data presented above, does not yet exist for these enzymes. Do trigger factors along with other non-FK506-binding FKBP52s make up a new family of PPIases?

The alignment of the TF-PPIase domain with the PPIase domain of the FK506-inhibitable, Mip-like FKBP22 from *E. coli* revealed similarity and identity values of 55.4% and 28.7%, respectively. Quality values for this alignment of 45 and 49.4 exceed only by 10 and 14 the average quality values of 10 alignments to randomized sequences. Alignments of more closely related FKBP52s, either SlyD with SlpA or FKBP12 with FKBP22, gave quality values of 89.7 and 80.4. These numbers are higher by 40 and 46 than the average values of the randomized alignments. On the other hand, alignments of TF-PPIase domain with the PPIase domains of the non-FK506-binding SlyD and SlpA produced even lower similarities and identities, 48.9% (21.3% identity) and 53.6% (17.4% identity), respectively. Quality values of 49.5 and 44.6, higher by only 10 and 7 than the average values from randomizations, were obtained for both alignments. Due to the very low level of sequence similarity, these data cannot assist in establishing a new PPIase family made up by non-FK506-binding FKBP52s and trigger factors.

In conclusion, our functional data are indicative of an only limited relationship of the active sites of TF-PPIase domains and FK506-inhibitable FKBP52s. To include these important

functional characteristics into the classification of PPIases, we suggest that TF-PPIase domains be defined as a subfamily of FKBP's.

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References

- [1] G. Stoller, K.P. Rücknagel, K.H. Nierhaus, F.X. Schmid, G. Fischer, J.-U. Rahfeld, *EMBO J* 14 (1995) 4939–4948.
- [2] C. Scholz, G. Stoller, T. Zarnt, G. Fischer, F.X. Schmid, *EMBO J* 16 (1997) 54–58.
- [3] E. Crooke, W. Wickner, *Proc Natl Acad Sci USA* 84 (1987) 5216–5220.
- [4] E. Crooke, L. Brundage, M. Rice, W. Wickner, *EMBO J* 7 (1988) 1831–1835.
- [5] E. Crooke, B. Guthrie, S. Lecker, R. Lill, W. Wickner, *Cell* 54 (1988) 1003–1011.
- [6] R. Lill, E. Crooke, B. Guthrie, W. Wickner, *Cell* 54 (1988) 1013–1018.
- [7] Q.A. Valent, D.A. Kendall, S. High, R. Kusters, B. Oudega, J. Luirink, *EMBO J* 14 (1995) 5494–5505.
- [8] T. Hesterkamp, S. Hauser, H. Lütcke, B. Bukau, *Proc Natl Acad Sci USA* 93 (1996) 4437–4441.
- [9] O. Kandror, M. Sherman, M. Rhode, A.L. Goldberg, *EMBO J* 14 (1995) 6021–6027.
- [10] I. Callebaut, J.P. Morion, *FEBS Lett* 374 (1995) 211–215.
- [11] H. Ke, D. Mayrose, P.J. Belshaw, D.G. Alberg, S.L. Schreiber, Z.Y. Chang, F.A. Etzkorn, S. Ho, C.T. Walsh, *Structure* 2 (1994) 33–44.
- [12] Y. Zhao, H. Ke, *Biochemistry* 35 (1996) 7356–7361.
- [13] Y. Zhao, Y. Chen, M. Schutkowski, G. Fischer, H. Ke, *Structure* 5 (1997) 139–146.
- [14] G. Stoller, T. Tradler, K.P. Rücknagel, J.-U. Rahfeld, G. Fischer, *FEBS Lett* 384 (1996) 117–122.
- [15] D. Yang, M.K. Rosen, S.L. Schreiber, *J Am Chem Soc* 115 (1993) 819–820.
- [16] S. Itoh, M.T. Decenzo, D.J. Livingston, D.A. Pearlman, M.A. Navia, *Bioorg Med Chem Lett* 5 (1995) 1983–1988.
- [17] O. Futer, M.T. Decenzo, R.A. Aldape, D.J. Livingston, *J Biol Chem* 270 (1995) 18935–18940.
- [18] M.T. Decenzo, S.T. Park, B.P. Jarrett, R.A. Aldape, O. Futer, M.A. Murcko, D.J. Livingston, *Protein Eng* 9 (1996) 173–180.
- [19] B. Ludwig, J. Rahfeld, B. Schmidt, K. Mann, E. Wintermeyer, G. Fischer, J. Hacker, *FEMS Microbiol Lett* 118 (1994) 23–30.
- [20] A.P. Timerman, G. Wiederrecht, A. Marcy, S. Fleischer, *J Biol Chem* 270 (1995) 2451–2459.
- [21] I. Mikaelian, A. Sergeant, *Nucleic Acids Res* 20 (2) (1992) 376.
- [22] S.C. Gill, P.H. von Hippel, *Anal Biochem* 182 (1989) 319–326.
- [23] J.F. Morrison, *Biochim Biophys Acta* 185 (1969) 269–286.
- [24] S.B. Needleman, C.D. Wunsch, *J Mol Biol* 48 (1970) 443–453.
- [25] T.F. Smith, M.S. Waterman, *Adv Appl Math* 2 (1981) 482–489.
- [26] M. Gribskov, R.R. Burgess, *Nucleic Acids Res* 14 (1986) 6745–6763.
- [27] R. Rechid, M. Vingron, P. Argos, *CABIOS* 5 (1989) 107–113.
- [28] B. Guthrie, W. Wickner, *J Bacteriol* 172 (1990) 5555–5562.
- [29] R.F. Staendaert, A. Galat, G.L. Verdine, S.L. Schreiber, *Nature* 346 (1990) 671–674.
- [30] J.E. Kay, *Biochem J* 314 (1996) 361–385.
- [31] G.D. Van Duyne, R.F. Staendert, P.A. Karplus, S.L. Schreiber, J. Clardy, *J Mol Biol* 229 (1993) 105–124.
- [32] K.P. Wilson, M.M. Yamashita, M.D. Sintchak, S.H. Rotstein, M.A. Murcko, J. Boger, J.A. Thomson, M.J. Fitzgibbon, J.R. Black, M.A. Navia, *Acta Crystallogr D51* (1995) 511–521.
- [33] S. Fischer, S. Michnick, M. Karplus, *Biochemistry* 32 (1993) 13830–13837.
- [34] G. Fischer, *Angew Chem Int Ed Engl* 33 (1994) 1415–1436.
- [35] T. Zarnt, K. Lang, H. Burtscher, G. Fischer, *Biochem J* 305 (1995) 159–164.
- [36] M. Schutkowski, M. Drewello, S. Wöllner, M. Jakob, U. Reimer, G. Scherer, A. Schierhorn, G. Fischer, *FEBS Lett* 394 (1996) 289–294.
- [37] J. Liu, C.M. Chen, C.T. Walsh, *Biochemistry* 30 (1991) 2306–2310.
- [38] T.J. Sewell, E. Lam, M.M. Martin, J. Leszyk, J. Weidner, J. Calaycay, P. Griffin, H. Williams, S. Hung, J. Cryan, N.H. Sigal, G.J. Wiederrecht, *J Biol Chem* 269 (1994) 21094–21102.
- [39] Fischer G, Kramer M. *Biopolymers* 1997 (in press).