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Three-step purification of a fragment of the large immunophilin FKBP52

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Abstract

PPIases catalyze the interconversion of *cis* and *trans* isomers of peptidyl–prolyl (Xaa–Pro) bonds in peptide and protein substrates. The PPIase family comprises three subfamilies, two of which interact with immunosuppressant drugs and are therefore termed immunophilins. One subgroup of the immunophilins are the FK506 binding proteins (FKBPs). FKBPs of a relative molecular mass higher than 40 000 also display chaperone activity and are part of the multichaperone complex that Hsp90 forms with substrate proteins. Their function in this chaperone complex is still enigmatic. To further characterize the function of FKBP52 we want to analyze constructs of FKBP52-fragments. Here we describe a fast and effective three-step purification procedure for a fragment of FKBP52 with a relative molecular mass of 48 000, termed FKBP52–123, consisting of affinity chromatography, anion-exchange column and gel-permeation chromatography. A yield of 1 mg pure protein per gram of cells was achieved.

Keywords: Immunophilins; FK506 binding proteins; Proteins

1. Introduction

Conversion from *cis* to *trans* prolines is a rate limiting step in protein folding. Enzymes that catalyze this step are called peptidyl–prolyl-*cis*-*trans* isomerases (PPIases, rotamases). To date, three subfamilies of this protein family have been identified: FK506 binding proteins (FKBPs), cyclophilins and parvulins [1]. FKBPs and cyclophilins share similarities in that both protein classes bind to and are inhibited by immunosuppressive drugs. Thus, both are termed immunophilins.

The immunophilin immunosuppressant complex, made up of cyclophilins and cyclosporin A or FKBPs and FK506, respectively, seems to be the basis for the immunosuppressive action of these drugs. When calcineurin binds to this complex it looses its ability to dephosphorylate the IL-2 specific transcription factor NF-AT. In this state NF-AT cannot migrate into the nucleus and IL-2 transcription is not initiated [1].

Immunophilins of higher relative molecular mass (>40 000) comprise a PPIase domain and additional domains which may account for an increase in function. These so-called large immunophilins (FKBP52, FKBP51, cyclophilin40 and its yeast homologs Cpr6 and Cpr7) were found in association with steroid receptors [2,3]. In their unliganded state steroid receptors are bound to a multichaperone complex containing Hsp90. The composition of this complex changes as it undergoes maturation. In the last mature step before the receptor binds the ligand and dissociates from the chaperone complex it is associated with Hsp90, a small acidic protein called p23 and one of the large immunophilins (FKBP51,

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FKBP52 or Cyp40). The binding of immunophilins to Hsp90 seems to occur via the tetratricopeptide repeats [4–6] in their sequence. All three large immunophilins apparently compete for one single binding site on Hsp90 [7].

Similar to Hsp90, large immunophilins display chaperone activity and can prevent aggregation of model proteins such as citrate synthase in vitro [8]. Additionally they were reported to bind GTP/ATP [9] and to have a C-terminal motif for Calmodulin binding [10].

Sequence and hydrophobic cluster analysis predict the division of FKBP52 in different domains [11]. Thus, for a more in depth characterization of FKBP52 functions, the analysis of fragments which lack certain domains of this protein would be of use.

In this paper the fast and effective purification of a fragment with a relative molecular mass of 48 000 that lacks the C-terminal Calmodulin binding sequence is described. Since some of the respective functional assays planned with this protein require highly purified protein we decided to use a GST fusion construct to allow for affinity purification as the first and capturing step of the purification procedure. Extensive purity of the protein was achieved by further purifying the glutathione Sepharose pool by anion-exchange and gel-permeation chromatography.

2. Experimental

2.1. Material

All chemicals used were analytical-reagent grade and were obtained from Merck (Darmstadt, Germany) or ICN (Meckenheim, Germany). All prepacked columns and column materials were from Pharmacia (Uppsala, Sweden). Human thrombin and the protease inhibitor Pefabloc were obtained from Boehringer (Mannheim, Germany).

The pGEX expression construct for FKBP52 as a fusion protein with GST was provided by Beatrice Chambraud [12].

2.2. Protein expression

For high level expression of FKBP52–123 in *E. coli* this plasmid was transformed into an *E. coli* BL21 [DE3] strain which contains the T7 polymerase structural gene under control of the lacUV5 promoter. LB medium containing 100 μg/ml ampicillin was inoculated with a stationary overnight culture (ratio 1/50 of the final culture volume). This culture was grown at 37°C to an $O.D._{600}$ of 0.4. Protein expression was induced with IPTG at a final concentration of 1 m*M*. Expression of the protein was performed at 26°C for 5 h with medium agitation (200 rpm). The cells were harvested by centrifugation at 4000 *g* for 15 min at 4°C. For storage the bacterial pellet was frozen at −20°C.

2.3. Cell lysis

The lysis buffer was phosphate buffered saline (PBS: 150 m*M* NaCl, 16 m*M* Na_2HPO_4, 4 m*M* NaH_2PO_4, pH 7.3). Cell pellets were thawed and resuspended in lysis buffer, supplied with 1 mg/ml of lysozyme and incubated at 4°C for 30 min. Cell lysis was performed by ultrasonication for three shots of 30 s (sonifier B-12, Branson Sonic Power). Cell debris was removed by centrifugation at 40 000 *g* for 40 min at 4°C. The supernatant was used for the purification procedure.

2.4. Purification

For the purification of FKBP52–123 a three-step procedure was applied.

The chromatographic system used was a Hiload System by Pharmacia (Uppsala, Sweden). All purification steps were performed at 4°C. The buffers for column chromatographies were filtered and degassed before use.

The affinity chromatographic step was performed as a batch technique with glutathione Sepharose [13], equilibrated with lysis buffer. Cleavage of FKBP52–123 from the glutathione Sepharose with 15 U human thrombin was performed for 1 h at room temperature in 50 m*M* Tris, pH 8.0. The reaction was stopped with 4 m*M* Pefabloc.

The anion-exchange column Resource Q was equilibrated in 100 m*M* Tris, 2 m*M* EDTA, pH 7.5 and was run at a flow-rate of 0.5 ml/min.

The protein solution was concentrated between the Resource Q run and the gel-permeation column using

the Amicon ultrafiltration method (Amicon, Witten, Germany).

The gel-permeation column used was a Superdex-75pg equilibrated in 50 m*M* Tris, 2 m*M* EDTA, 200 m*M* NaCl, pH 7.5. The flow-rate was again 0.5 ml/min and 1.5 ml fractions were collected.

The purification steps were performed by following the clearly visible bands of the overexpressed FKBP52–123 on an SDS-polyacrylamide gel [14] and collecting the appropriate fractions. The identity of the protein was further confirmed by Western Blot analysis [15] using a specific antibody against FKBP52.

Protein concentration was determined using the theoretical extinction coefficient of 0.919 at 280 nm for a 1 mg/ml solution at a pathlength of 1 cm.

To check whether the protein finally obtained was in the native conformation circular dichroism measurements (J715 spectropolarimeter with PTC343 peltier unit, Jasco, Tokyo, Japan) were performed.

PPIase activity of the final protein pool was checked by a coupled assay with chymotrypsin using the synthetic peptide N-Suc–Ala–Ala–Pro–Phe–*p*-nitroanilide. *p*-Nitroanilide can only be cleaved off by chymotrypsin when the Ala–Pro bond is in the *trans* configuration. The release of *p*-nitroanilide results in the absorbance increase at 390 nm by which the kinetics of the isomerization reaction can be measured [16].

3. Results

3.1. Protein expression

The plasmid expressing FKBP52–123 was transformed into an *E. coli* BL21 [DE3] strain which contains the T7 polymerase structural gene under control of the lacUV5 promoter and can thus be induced with isopropyl-β-D-thiogalactopyranoside (IPTG).

For large scale expression of FKBP52–123, LB medium containing 100 μg/ml ampicillin was inoculated with a stationary overnight culture. This culture was grown at 37°C to an $O.D._{600}$ of 0.4. Protein expression was induced with IPTG.

The best conditions for expression of the protein were 26°C for 5 h with medium agitation (200 rpm). After this, the cells were harvested by centrifugation at 4000 *g* for 15 min at 4°C. For storage the bacterial pellet was frozen at −20°C.

3.2. Cell lysis

Cell pellets were thawed and resuspended in lysis buffer, supplied with lysozyme and incubated at 4°C for 30 min. Ultrasonication after this step led to the cells being opened. Cell debris was removed by centrifugation at 40 000 *g* for 40 min at 4°C. The supernatant contained the soluble proteins and was used for purification of FKBP52–123; the pellet was discarded.

3.3. Affinity chromatography

The supernatant of the centrifugation step after cell lysis was applied to the glutathione Sepharose batch material [13] which had been equilibrated in lysis buffer. The protein was allowed to bind to the column for 30 min at 4°C before the supernatant was removed by centrifugation and the batch material was washed twice with lysis buffer to remove unspecifically bound protein.

Elution of FKBP52–123 from the glutathione Sepharose material was achieved by cleavage with 15 U of human thrombin at room temperature for 1 h. The reaction was stopped by addition of 4 m*M* Pefabloc. In the following centrifugation step the FKBP52–123 without the GST-fusion was found in the supernatant (Fig. 1, lane 3) whereas the GST was still bound to the glutathione Sepharose beads. The Sepharose material was regenerated for further use by eluting the bound GST by incubation in 50 m*M* Tris, 10 m*M* GSH, pH 8.0.

3.4. Anion-exchange chromatography

A Resource Q column (6 ml) was equilibrated with a buffer containing 0.1 *M* Tris, 2 m*M* EDTA, pH 7.5 prior to use. The eluate from the glutathione Sepharose was applied onto this column with a flow-rate of 0.5 ml/min. Here, the FKBP52–123 was found in the flowthrough (Fig. 1, lane 4), whereas most of the contaminating proteins bound to the Resource Q and were eluted with an increasing gradient of NaCl.

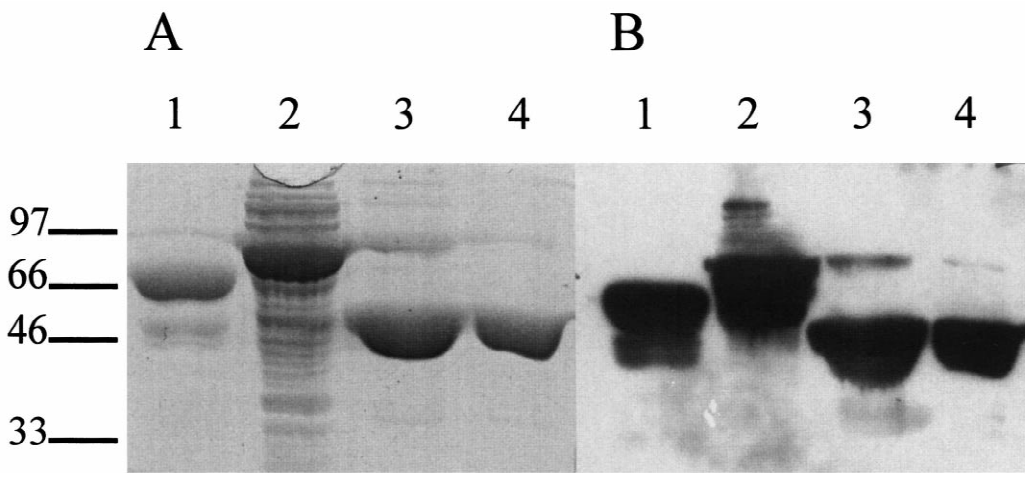

Fig. 1. Affinity chromatography and anion-exchange column. (A) SDS-polyacrylamide gel showing the efficiency of the glutathione Sepharose and the Resource Q steps. The gel shows the protein pool on the glutathione Sepharose material before (lane 2) and after eluting by cleavage with 15 U of human thrombin (lane 3). Lane 4 shows the flowthrough of the Resource Q with the contaminating fusion protein band. The protein in lane 1 is a FKBP52-standard. Western Blot of the SDS-polyacrylamide gel shown in (A) to confirm the identity of the purified protein. The small band at a relative molecular mass of about 80 000 represents the uncleaved form of FKBP52–123-GST which partly coelutes from the glutathione Sepharose.

3.5. Gel-permeation column

The flowthrough of the Resource Q column was concentrated by ultrafiltration and applied with a flow-rate of 0.5 ml/min onto a gel-permeation column (Superdex 75 pg), which had been equilibrated with a buffer containing 50 m*M* Tris, 2 m*M* EDTA, 200 m*M* NaCl, pH 7.5. Fractions of 1.5 ml were collected. The FKBP52–123 eluted in a distinct peak between 61 and 66 ml (Fig. 2A) elution volume and was pooled. The purity of the pooled fractions was verified on an SDS-polyacrylamide gel [14] (Fig. 2B). No contaminating proteins were found in the pooled fractions.

The protein pool was dialysed against 50 m*M* Tris, 2 m*M* EDTA, pH 7.5, concentrated up to 1.9 mg/ml, frozen in liquid nitrogen and stored at −80°C.

To confirm that the obtained FKBP52–123 was in native conformation circular dichroism measurements were performed (data not shown).

To check whether the purified protein pool was native the PPIase activity of FKBP52–123 was monitored by a protease coupled assay [16]. The k_{cat}/K_m value which was found in this assay for the purified protein corresponds well with the value for the entire protein.

4. Discussion

FKBP52–123 was expressed well in *E. coli* BL21 [DE3]. The glutathione Sepharose proved to be a very reliable method for capturing the GST-fused FKBP52–123 from the cell lysate, even though a certain fraction of the protein remained in the lysate and did not bind to the Sepharose material even after repeated incubation steps. Elution of the FKBP52–123 from the batch material by incubation with 15 U of human thrombin provided an additional level of purity and removed the GST tag from the N-terminus of the protein leaving only seven additional amino acids (SPEFREE). Further digest of FKBP52–123 into smaller domains by thrombin was successfully prevented by addition of 4 m*M* Pefabloc.

Since this first purification step removed the majority of the *E. coli* proteins the risk of degradation by resident proteases was minimized.

The physicochemical properties of FKBP52–123 dictated the next purification steps. At pH 7.5 most

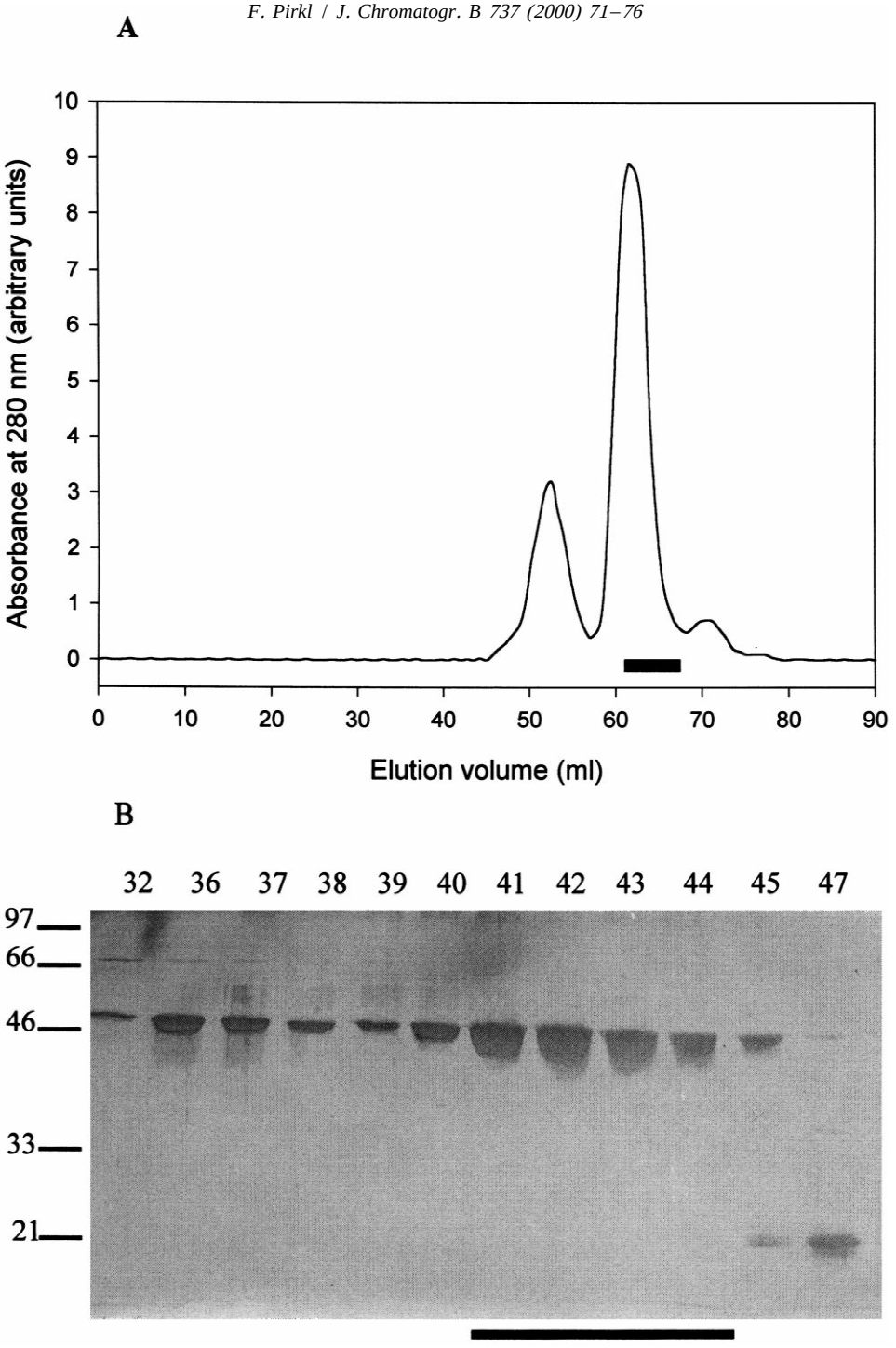

Fig. 2. Gel-permeation column Superdex 75 pG. (A) Elution profile of the Superdex 75 pG run. The flow-rate was 0.5 ml/min. Fractions of 1.5 ml were collected. The elution of the protein was monitored by measuring the absorbance at 280 nm. The pure FKBP52–123 fractions which were finally pooled are indicated by a vertical bar below. (B) SDS-polyacrylamide gel showing single fractions from the gel-permeation run. The bars at the left-hand side depict molecular mass markers. The lanes are numbered according to the fractions numbers collected. The large bar below the gel indicates the fractions pooled as pure.

E. coli proteins bind to anion-exchange columns and are usually separated from each other by elution over a gradient of 0 to 1 *M* salt. The finding that FKBP52–123 does not bind to the anion-exchange column Resource Q at this pH but is found in the flowthrough provided me with a means for further purification of this protein. After this column step most contaminating proteins were eliminated from the FKBP52–123 pool. Only a slight band in the relative molecular mass region of the original fusion protein was seen on a Coomassie stained SDS-polyacrylamide gel. The fact that this band is also stained by an antibody specific for FKBP52 further corroborates the hypothesis that this is in fact the original fusion protein which was unspecifically eluted from the glutathione Sepharose beads (possibly due to overloading) and which has due to its physicochemical properties copurified with FKBP52–123 on the Resource Q column.

Because of the considerable difference in mass between the FKBP52–123 and this contaminating protein a gel-permeation column was the method of choice to remove this contamination and obtain final purity of the FKBP52–123 pool. FKBP52–123 eluted in a distinct peak at an elution volume of 61–66 ml. The smaller peaks at 45–56 ml (resulting from the FKBP52–123-GST) and 68–75 ml represent small amount of contaminating proteins which could be removed by gel-permeation chromatography. Size-exclusion chromatography was not only used as the last polishing step in the purification but is also indispensable in that it ensures that the purified protein pool is free of aggregated forms of the wanted protein.

The circular dichroism measurements which were used to additionally confirm that FKBP52–123 has native conformation gave spectra which were typical for an FKBP.

Furthermore, it could be proven that the purified protein pool is native since it has retained its natural PPIase activity which is supposed to reside in the first domain (amino acids 1–150) of FKBP52 [12]. A yield of 1 mg pure protein per gram of cells (wet weight) was achieved using this purification protocol.

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References

[1] G. Fischer, Angew. Chem. Int. Ed. Engl. 33 (1994) 1415.
[2] D.F. Smith, L.E. Faber, D.O. Toft, J. Biol. Chem. 265 (1990) 3996.
[3] W.B. Pratt, D.O. Toft, Endocrine Rev. 18 (1997) 306.
[4] J.R. Lamb, S. Tugendreich, P. Hieter, Trends Biochem. Sci. 20 (1995) 257.
[5] C. Radanyi, B. Chambraud, E.E. Baulieu, Proc. Natl. Acad. Sci. USA 91 (1994) 11197.
[6] K. Hoffman, R.E. Handschumacher, Biochem. J. 307 (1995) 5.
[7] T. Ratajczak, A. Carrello, J. Biol. Chem. 271 (1996) 2961.
[8] S. Bose, T. Weikl, H. Bügl, J. Buchner, Science 274 (1996) 1715.
[9] S. Le Bihan, J.M. Renoir, C. Radanyi, B. Chambraud, V. Joulin, M.G. Catelli, E.E. Baulieu, Biochem. Biophys. Res. Commun. 195 (1993) 600.
[10] N. Massol, M.C. Lebeau, J.M. Renoir, L.E. Faber, E.E. Baulieu, Biochem. Biophys. Res. Commun. 187 (1992) 1330.
[11] M.C. Lebeau, N. Binart, F. Cadepond, M.G. Catelli, B. Chambraud, N. Massol, C. Radanyi, G. Redeuilh, J.M. Renoir, M. Sabbah, G. Schweizer-Groyer, E.E. Baulieu, in: V.K. Milhoud (Ed.), Steroid Hormone Receptors, Birkhäuser, Boston, 1993.
[12] B. Chambraud, N. Rouviere-Fourmy, C. Radanyi, K. Hsiao, D.A. Peattie, D.J. Livingston, E.E. Baulieu, Biochem. Biophys. Res. Commun. 196 (1993) 160.
[13] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31.
[14] U.K. Laemmli, Nature (London) 227 (1970) 680.
[15] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350.
[16] G. Fischer, H. Bang, C. Mech, Biomed. Biochim. Acta 43 (1984) 1101.