R73A and H144Q mutants of the yeast mitochondrial cyclophilin Cpr3 exhibit a low prolyl isomerase activity in both peptide and protein-folding assays

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Abstract Previously we reported that the R73A and H144Q variants of the yeast cyclophilin Cpr3 were virtually inactive in a protease-coupled peptide assay, but retained activity as catalysts of a proline-limited protein folding reaction [Scholz, C. et al. (1997) FEBS Lett. 414, 69–73]. A reinvestigation revealed that in fact these two mutations strongly decrease the prolyl isomerase activity of Cpr3 in both the peptide and the protein-folding assay. The high folding activities found previously originated from a contamination of the recombinant Cpr3 proteins with the *Escherichia coli* protein SlyD, a prolyl isomerase that co-purifies with His-tagged proteins. SlyD is inactive in the peptide assay, but highly active in the protein-folding assay.

Key words: Cyclophilin; Immunophilin; Prolyl isomerization; Protein folding; Mitochondrion

1. Introduction

Cyclophilins catalyze the *cis*/*trans* isomerizations of prolyl peptide bonds and bind tightly to cyclosporin A (CsA), which inhibits this activity in a competitive manner [1–3]. Prolyl isomerization can be a rate-limiting reaction in protein folding [4]. Previously we reported that two variants of the yeast mitochondrial cyclophilin Cpr3 with substitutions close to the prolyl isomerase active site (R73A and H144Q) are virtually inactive towards a tetrapeptide substrate in a protease-coupled activity assay, but show high residual activities as catalysts of a proline-limited[1] protein folding reaction [5,6]. A reinvestigation of the enzymatic properties of the wild-type and mutated forms now shows that the R73A and H144Q mutations strongly decrease the activity of Cpr3 in both the peptide and the protein-folding assay. The high folding activities found in the previous work originated from a contamination of the recombinant Cpr3 proteins with the *Escherichia coli* protein SlyD. This protein co-purifies with His-tagged proteins [7] and shows an extremely high prolyl isomerase activity in the protein-folding assay, but not in the chymotrypsin-coupled peptide assay.

2. Materials and methods

(S54G,P55N)-RNase T1 was purified and carboxymethylated as described [8]. The concentrations of RCM-(S54G/P55N)-RNase T1 were determined spectrophotometrically using an absorption coefficient of $\varepsilon_{278} = 21\,060\ M^{-1}\ cm^{-1}$ [9]. The concentrations of wild-type and mutant Cpr3 and of SlyD were determined spectrophotometrically using ε_{280} values of $16\,800\ M^{-1}\ cm^{-1}$ and $5480\ M^{-1}\ s^{-1}$, respectively.

The R73A and H144Q mutations in the mitochondrial cyclophilin Cpr3 of yeast are analogous to the R55A and H126Q mutations in human cyclophilin A, respectively [10]. They were engineered and cloned in the bacterial His_6 expression plasmid pTrcHisB (Invitrogen, San Diego, CA, USA), as described [6]. The wild-type and the mutant proteins lack the mitochondrial leader sequence but carry a hexahistidine tag and a cleavage site for enterokinase at the amino-terminus.

Three hours after induction, Cpr3-producing cells were harvested by centrifugation ($10\,000 \times g$, 20 min). The pellet was resuspended in 50 mM Tris-HCl, pH 7.9, 250 mM NaCl, 10 mM β-mercaptoethanol and 8 mM imidazole (5 ml/g of cell pellet). To minimize proteolysis, Complete, a mixture of protease inhibitors (Boehringer, Mannheim, Germany), was added to the suspension. Cells were lysed by sonication, and the lysate was cleared by centrifugation ($10\,000 \times g$, 20 min, 4°C). The supernatant was removed, filtered (Qiafilter MaxiCartridge from Qiagen, Hilden, Germany) and applied to a Ni-NTA Superflow column (Qiagen, Hilden, Germany) with a flow rate of 1 ml/min. Bound proteins were washed with ≥300 ml of the lysis buffer and then eluted by 50 ml 1.0 M imidazole buffered in 50 mM Tris-HCl, pH 7.9. Protein-containing fractions were pooled, dialyzed against 20 mM HEPES pH 7.5 and applied on a TMAE anion exchange column equilibrated with the same buffer. The column was washed with at least five volumes of column buffer. Cpr3 and SlyD could be separated by applying a linear gradient from 0 to 1.0 M NaCl in 300 ml 20 mM HEPES pH 7.5. Cpr3 eluted at 0.25 M NaCl, SlyD at 0.5 M NaCl. Protein-containing fractions were analyzed by the fluorescence emission of Tyr at 305 nm and of Trp at 350 nm, after excitation at 280 nm, and assayed for prolyl isomerase activity (see below). Only Cpr3 contains Trp residues. The Cpr3- and SlyD-containing fractions were separately purified further by size exclusion chromatography on a Superdex 75 HiLoad column (Amersham-Pharmacia, Uppsala, Sweden). The eluting proteins were assayed for prolyl isomerase activity in the RCM-(S54G/P55N)-RNase T1 folding assay. Both the Cpr3 proteins and SlyD showed purities higher than 95% as judged by denaturing gel electrophoresis and Coomassie staining.

The chromogenic peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide and chymotrypsin were used in the protease-coupled assay [1,11] as described in [5]. In the protein folding assays RCM-(S54G/P55N)-RNase T1 was used as the substrate. These assays were performed as described in [5]. Fluorescence was measured in a Hitachi F4010 fluorescence spectrophotometer.

The specificity constants k_{cat}/K_M of the prolyl isomerases in the various assays were determined by measuring the rate constants of the spontaneous isomerization, k_o, and of catalyzed isomerization, k_{obs}, as a function of the enzyme concentration at substrate concen-

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Abbreviations: CsA, cyclosporin A; RNase T1, ribonuclease T1; RCM-(S54G/P55N)-RNase T1, disulfide-reduced and S-carboxymethylated form of a variant of RNase T1 with Ser^{54} and Pro^{55} replaced by Gly and Asn, respectively; Cpr3, mitochondrial cyclophilin of *Saccharomyces cerevisiae*

[1] To facilitate reading we use the expression 'proline-limited' protein folding for a folding reaction that involves the isomerization of a Xaa-Pro peptide bond as the rate-limiting step.

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trations much smaller than K_M. Under these conditions k_{cat}/K_M is derived from the dependence of k_{obs} on enzyme concentration.

To measure CsA binding 0.9 ml of 0.1 μM wild-type or mutant Cpr3 in 0.1 M Tris-HCl pH 8.0 was placed in the thermostatted fluorimeter cell and titrated with CsA at 15°C. The initial additions were made with a 5 μM, followed by a 50 μM and finally a 500 μM CsA solution in 50% ethanol. The total volume added during the titrations was less than 25 μl. Control titrations showed that equal volumes of 50% ethanol did not significantly affect the fluorescence of the Cpr3 proteins. The extent of complex formation was determined from the increase in fluorescence emission at 340 nm (10 nm bandwidth) upon excitation at 290 nm (3.0 nm bandwidth) using a Hitachi F4010 fluorescence spectrometer. After each addition of CsA the system was equilibrated for at least 2 min. The binding curves were analyzed on the assumption that binding of CsA to Cpr3 is reversible.

Circular dichroism (CD) spectra of the Cpr3 proteins were measured with a Jasco J600A spectropolarimeter. Protein concentrations were 5.0 μM in 20 mM Na phosphate buffer, pH 7.0 at 20°C. The spectra were recorded in 0.1 cm cells with a speed of 20 nm/min, a response time of 2 s and a bandwidth of 2 nm. The spectra were measured eight times and averaged.

3. Results and discussion

The Cpr3 proteins and SlyD co-eluted from the Ni-NTA column. They were separated by anion exchange chromatography over a TMAE column and further purified by gel chromatography. Residual SlyD could not be detected in the Cpr3 samples by SDS-PAGE. We did not succeed in removing the His tag from the recombinant Cpr3 proteins by incubation with enterokinase, probably because the cleavage sites were not accessible in the fusion proteins. Therefore all comparative work was performed with the Cpr3 proteins carrying an N-terminal extension of 31 amino acids.

Wild-type Cpr3 and the R73A and H144Q variants are stably folded proteins and show virtually identical CD spectra in the amide region (data not shown). Binding of CsA to cyclophilins leads to an increase of the intrinsic tryptophan fluorescence [2]. When 10 μM CsA was added to 1 μM Cpr3, the fluorescence intensities of the wild-type protein, the R73A and the H144Q variants increased by 35, 30, and 45%, respectively, which indicates that they interact with CsA. The affinity for CsA was, however, strongly reduced (by about two orders of magnitude) by the R73A and H144Q mutations. The apparent dissociation constants as derived from fluorescence titrations are ≤10 nM for wild-type Cpr3, 0.30 μM for the R73A, and 0.25 μM for the H144Q variant (Table 1). This agrees well with the finding that these variants do not bind appreciably to CsA in a drug binding assay [6].

The catalytic activities of the three proteins as measured by the chymotrypsin-coupled peptide assay are shown in Table 1. This assay is based on the conformational specificity of chymotrypsin, which cleaves the peptide Suc-Ala-Ala-Pro-Phe-4-nitroanilide only when the Xaa-Pro bond is *trans*. Purified wild-type Cpr3 shows a k_{cat}/K_M value of 5.8×10^6 M^{-1} s^{-1}, which resembles the k_{cat}/K_M values that were found previously for other cyclophilins [3,12]. The activities of both variants are strongly reduced to levels below 1% of the activity of the wild-type protein (Table 1). These residual activities are fully inhibited by 4.5 μM CsA, which shows that they do not originate from *E. coli* prolyl isomerases of the FKBP or parvulin type. Neither are they likely to originate from the *E. coli* cyclophilins, because those are only weakly inhibited by 5 μM CsA [13,14]. CsA binds to wild-type Cpr3 with a very high affinity (Table 1) and accordingly inhibits its prolyl isomerase activity with an inhibition constant of 3.8×10^{-9} M (data not shown). The activities of the wild-type form and the two variants of Cpr3 decrease only slightly when they are incubated with 30 μM chymotrypsin for 10 min (Table 1). SlyD, however, is hydrolyzed very rapidly by this protease and is thus inactive in the chymotrypsin-coupled assay (Table 1 and [15,16]).

The activities of the Cpr3 proteins and of SlyD as catalysts of a proline-limited protein folding reaction were measured as before by using the reduced and carboxymethylated form of S54G/P55N-RNase T1 as a substrate protein. Its refolding is limited in rate by the *trans*→*cis* isomerization of the Tyr^{38}-Pro^{39} bond. In this folding assay wild-type Cpr3 showed a k_{cat}/K_M value of about 70 000 M^{-1} s^{-1} (Table 2), which is similar to the activities of other cyclophilins in this assay. This activity is completely inhibited by 4.5 μM CsA.

In the protein-folding assay the catalytic activities of the two Cpr3 variants are strongly reduced as well. The R73A variant shows a k_{cat}/K_M value of about 1000 M^{-1} s^{-1}, which, in the presence of 4.5 μM CsA, is reduced to about 700 M^{-1} s^{-1}. The k_{cat}/K_M value for the H144Q variant is about 550 M^{-1} s^{-1} in the absence of CsA. In the presence of 4.5 μM CsA H144Q-Cpr3 is inactive. This shows that the variants show about 0.5% of the folding activity of the wild-type protein. The H144Q variant seems to be extremely pure, the R73A variant still contains a very small amount of SlyD. SlyD is a member of the FKBP family but is not inhibited by FK 506. It shows an enormous activity in the folding assay (see below).

Together, the results in Tables 1 and 2 indicate that both mutations are strongly inactivating, and that, contrary to our previous results [5,6], the inactivation is reflected in both the peptide and protein-folding assays. The extent of inactivation is similar to the inactivations of human cyclophilin A by the analogous mutations [10].

SlyD is rapidly hydrolyzed by chymotrypsin and is there-

Table 1
Activities of wild-type Cpr3, its R73A and H144Q variants and of SlyD in the protease-coupled peptide assay[a]

Species	k_{cat}/K_M (M^{-1} s^{-1})	Activity relative to wild-type Cpr3 (%)	k_{cat}/K_M after 10 min incubation with 30 μM chymotrypsin (M^{-1} s^{-1})	Inhibition by 4.5 μM CsA	K_D of complex with CsA[b]
Wild-type Cpr3	5.8×10^6	100	5.6×10^6	yes	≤10 nM
R73A-Cpr3	6 700	0.11	5 100	yes	0.30 μM
H144Q-Cpr3	16 700	0.28	13 500	yes	0.25 μM
SlyD	≤500	≤0.008	≤500	–	–

[a]The activities were measured using the peptide succinyl-Ala-Ala-Pro-Phe-4-nitroanilide and the chymotrypsin-coupled assay in 0.1 M Tris-HCl, pH 8.0 at 15°C. The protein concentrations were 10 nM (wild-type Cpr3), 200 nM (R73A- and H144Q-Cpr3), and 200 nM (SlyD). The uncertainties in the activity measurements are $\pm0.2\times10^6$ M^{-1} s^{-1} for wild-type Cpr3 and ±2000 M^{-1} s^{-1} for the Cpr3 mutants.
[b]The dissociation constants K_D of the complexes with CsA were determined by fluorescence titrations in 0.1 M Tris-HCl, pH 8.0 at 15°C as described in Section 2.

Table 2
Activities of wild-type Cpr3, its R73A and H144Q variants and of SlyD in the protein-folding assay[a]

Species	k_{cat}/K_M (M^{-1} s^{-1})	k_{cat}/K_M in the presence of 4.5 μM CsA (M^{-1} s^{-1})	Activity relative to wild-type Cpr3 (%)	k_{cat}/K_M in the presence of 3.5 μM FK 506 (M^{-1} s^{-1})
Wild-type Cpr3	70 000	≤ 1 000	100	70 000
R73A-Cpr3	1 000	700	0.4	950
H144Q-Cpr3	550	≤ 100	0.7	550
SlyD	450 000	450 000	640	420 000

[a]The activities were measured by using RCM-(S54G/P55N)-RNase T1 as the refolding protein substrate. Folding was measured in 0.1 M Tris-HCl, pH 8.0 at 15°C. The protein concentrations were 200 nM (wild-type Cpr3), 1 μM (R73A- and H144Q-Cpr3), and 10 nM (SlyD). The uncertainties in the activity measurements are ±1000 M^{-1} s^{-1} for wild-type Cpr3 and SlyD and ±100 M^{-1} s^{-1} for the Cpr3 mutants.

fore inactive in the peptide assay. In the protein-folding assay, however, it shows a remarkably high activity, and, under the conditions used, it reaches a k_{cat}/K_M value of about 450 000 M^{-1} s^{-1}. This is 6–7-fold higher than the corresponding activity of wild-type Cpr3 (Table 2). SlyD is a prolyl isomerase of the FKBP type, but its high activity in the folding assay is hardly affected by 3.0 μM FK 506. Using a trypsin-coupled peptide-based assay Hottenrott et al. also found that SlyD was insensitive to inhibition by FK 506 [15].

The very high activity of SlyD in the protein-folding assay, the lack of activity in the chymotrypsin-coupled peptide assay, the fact that Cpr3 and SlyD have similar molecular weights, and the insensitivity of SlyD to FK 506 inhibition explain in large part our previous erroneous results [5,6]. In those experiments the contaminating SlyD did not contribute to the activities measured by the chymotrypsin-coupled peptide assay, because it was inactivated by the protease. It did, however, contribute strongly to the activity measured in the protein-folding assay, because in this assay SlyD is much more active than Cpr3. The insensitivity to inhibition by FK 506 was previously taken as evidence that the measured high folding activities of the Cpr3 proteins originated from a cyclophilin and not from an *E. coli* FKBP. However, SlyD binds FK 506 so weakly that it is not significantly inhibited by FK 506. In addition, SlyD contains no Trp residues and thus in the original samples contributed very little to the absorbance, which was used to determine the prolyl isomerase concentrations.

Together, our results indicate that Cpr3 resembles human cyclophilin A [10] in its sensitivity to mutations of a His and an Arg residue in the active site region. In contrast to our previous conclusions [5,6], these two mutations reduce the activities towards both peptide and protein substrates in a similar manner to less than 1% of the activity of the wild-type protein. These results are now in accord with the conclusion that Arg^{55} is a catalytic residue, as drawn from the crystal structure of human cyclophilin A complexed with a peptide [17]. The H144Q mutation in Cpr3 does not affect the folding of an imported protein in mitochondria derived from a mutant yeast strain, and the viability of this strain on lactate at 37°C is also not affected [6]. Thus, either the residual in vitro activity of the purified H144Q variant of Cpr3 is sufficient to explain the absence of effects in vivo and in mitochondria, or prolyl isomerase activity is dispensable for the in vivo activity of Cpr3. Finally, these findings for the Cpr3 active site mutants may also be relevant to our related studies of FKBP12 active site mutants [6], which will also be re-investigated.

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