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Research Article

Human and yeast ζ-crystallins bind AU-rich elements in RNA

M. R. Fernández†, S. Porté†, E. Crosas, N. Barberà, J. Farrés, J. A. Biosca and X. Parés*

Department of Biochemistry and Molecular Biology, Faculty of Biosciences, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona (Spain), Fax: +34 93 581 1264, e-mail: xavier.pares@uab.es

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Abstract. ζ-crystallins constitute a family of proteins with NADPH:quinone reductase activity found initially in mammalian lenses but now known to be present in many other organisms and tissues. Few proteins from this family have been characterized, and their function remains unclear. In the present work, ζ-crystallins from human and yeast (Zta1p) were expressed, purified and characterized. Both enzymes are able to reduce *ortho*-quinones in the presence of NADPH but are not active with 2-alkenals. Deletion of the *ZTA1* gene makes yeast more sensitive to menadione and hydrogen peroxide, suggesting a role in the oxidative stress response. The human and yeast enzymes specifically bind to adenine-uracil rich elements (ARE) in RNA, indicating that both enzymes are ARE-binding proteins and that this property has been conserved in ζ-crystallins throughout evolution. This supports a role for ζ-crystallins as *trans*-acting factors that could regulate the turnover of certain mRNAs.

Keywords. ARE-binding protein, medium-chain dehydrogenases/reductases, oxidative stress, quinone reductase, RNA, ζ-crystallin.

Introduction

ζ-crystallin/NADPH:quinone reductases are included in the protein superfamily of medium-chain dehydrogenases/reductases [1, 2]. ζ-crystallins have not been studied extensively, and their functional role is still unclear. ζ-crystallin was described initially as a protein found in high amounts in guinea pig and camel lenses [3], and it was reported that a mutation in guinea pig ζ-crystallin gene is associated with congenital development of cataracts [4]. The guinea pig ζ-crystallin catalyzes the reduction of *ortho*-quinones to their corresponding semiquinone radical through one-electron transfer using exclusively NADPH as a cofactor. This radical is rapidly reoxidized to the quinone form in the presence of oxygen, yielding superoxide anions. It has been proposed that the guinea pig ζ-crystallin could be implicated in the regulation of NADPH homeostasis or have a role in quinone detoxification [5]. The gene of guinea pig ζ-crystallin contains two different promoter elements, one for high expression, specific for lenses, and another for expression at low level in various tissues. The human gene does not contain the element for high expression level [6]. In *Arabidopsis thaliana*, the ζ-crystallin homolog is induced under oxidative stress [7] and is able to catalyze not only reduction of *ortho*-quinones but also hydrogenation of the α,β-unsaturated bond of 2-alkenals, such as 4-hydroxy-2-*trans*-nonenal [8].

Recently, the binding of ζ-crystallin to nucleic acids has been reported. Bovine and *Pichia pastoris* ζ-crystallins are able to bind to single-stranded DNA, and this binding is disrupted by NADPH competition [9, 10]. Moreover, ζ-crystallin from rat kidney could be implicated in the posttranscriptional regulation of

† These authors contributed equally to this work.
* Corresponding author.

mitochondrial glutaminase expression [11]. Glutaminase increases its levels under chronic acidosis by increasing the half-life of its mRNA. An 8-nucleotide direct repeat rich in adenine-uracil has been identified as the structure responsible for mRNA stabilization under acidotic conditions. Compared to normal conditions, extracts from renal cortex obtained in acidotic conditions showed higher binding of a protein that was proposed to act by mediating the pH-responsive stabilization of glutaminase mRNA [12]. A protein of 36 kDa that binds specifically to the adenine-uracil rich element (ARE) was purified from renal cortex and identified as ζ-crystallin. Rat ζ-crystallin also binds to the 3′-untranslated region (3′-UTR) of mRNA from glutamate dehydrogenase mRNA, which contains ARE sequences that are 88 % identical to one of two glutaminase AREs. Rat ζ-crystallin may mediate the pH-responsive stabilization of both mRNAs under metabolic acidosis [11].

In the present study, we have characterized human ζ-crystallin and its yeast homolog (Zta1p), a protein not previously investigated. Both enzymes were expressed, purified and kinetically characterized with quinones and 2-alkenals. The yeast ζ-crystallin gene (*ZTA1*) was deleted and the resulting phenotype studied under different growth conditions. In addition, we have demonstrated the specific binding of both enzymes to ARE elements in RNA.

Materials and methods

Cloning of human ζ-crystallin and yeast ZTA1 DNA. The human ζ-crystallin cDNA was amplified from Mammalian Gene Collection (MGC) clones from Invitrogen (MGC:48707 IMAGE:6067125) with the following oligonucleotides, which contain the ligation-independent cloning (LIC) tags: forward, 5′-GGT ATT GAG GGT CGC ATG GCG ACT GGA CAG-3′; reverse, 5′-AGA GGA GAG TTA GAG CCT CAT AAG AGA AGA AT-3′. The amplification was performed with a hot start at 95°C for 5 min; 30 cycles at 95°C for 2 min, 20°C for 1 min and 72°C for 3 min; a final extension at 72°C for 1 min; and ending at 4°C. The yeast gene *YBR046C* (*ZTA1*) was amplified from yeast FY834 genomic DNA with the following oligonucleotides, containing the LIC tags: forward, 5′-GGT ATT GAG GGT CGC ATG AAA TGT ACT ATA CC-3′; reverse, 5′-AGA GGA GAG TTA GAG CCC TAT TGT GGT ATT TCA AGA AC-3′. The amplification was performed with a hot start at 95°C for 5 min prior to the addition of 10 U/mL Vent DNA polymerase (New England Biolabs). This was followed by 30 cycles at 95°C for 1 min, 51°C for 1 min and 72°C for 1 min, a final extension at 72°C for 1 min and ending at 4°C. The amplified products were introduced into the pET-30 Xa/LIC vector from Novagen as indicated by the supplier. After sequence verification, vectors containing the human ζ-crystallin or the *ZTA1* gene were transformed into *E. coli* BL21 (DE3) for their expression.

Protein expression and purification. For yeast protein expression, 10 mL of the transformed *E. coli* saturated preculture was inoculated into 1 L LB medium containing 30 μg/mL kanamycin. The culture was incubated at 23–24°C for 5–8 h, and then expression was induced by adding 1 mM IPTG. Cells were collected 16–17 h later and resuspended in 6 mL Tris buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 20 mM imidazole, 10 % glycerol). Cells were lysed by sonication in the presence of 1 mg/mL lysozyme, 5 μg/mL DNase and 1 % Triton X-100 and were centrifuged at 36 000 × g for 20 min. For Zta1p purification, the homogenate was incubated with Ni-Sepharose™ High Performance (GE Healthcare) for 2 h at 4°C in an orbital shaker. The suspension was centrifuged at 44 × g for 1 min, and the sedimented resin was washed five times with ten volumes Tris buffer for 10 min. After the last wash, the resin was incubated with 250 units Xa Factor in 20 mM Tris-HCl pH 8.0/2 mM $CaCl_2$/0.5 M NaCl to elute Zta1p. For human ζ-crystallin, *E. coli* BL21 strain transformed with the plasmid pET-30 Xa/LIC was grown in 2xYT medium at 24°C for 8 h. Protein expression was induced by the addition of 1 mM IPTG, and cells were collected after 16 h. After cell lysis by sonication, the homogenate was applied to a nickel-charged chelating Sepharose™ Fast Flow (GE Healthcare) column (5 mL). After washing with 60 mM imidazole in 50 mM NaH_2PO_4/0.5 M NaCl pH 8.0, the enzyme was eluted with a 0.06–1.0 M imidazole gradient in the same buffer. Protein fractions were concentrated, and imidazole was removed with an Amicon ultra device (Millipore). The protein was stored at 4°C in 50 mM NaH_2PO_4/0.5 M NaCl pH 8.0.

Molecular weight determinations. The *Mr* of the native enzymes was determined by gel-filtration chromatography on a Superdex 200 HR 10/30 (GE Healthcare) column connected to an HPLC system (Waters) and equilibrated with two volumes 50 mM sodium phosphate pH 7.0, 0.15 M NaCl and 30 % glycerol. The column was run at a flow rate of 0.2 mL/min. Calibration was performed with gel-filtration molecular weight markers (Sigma). The subunit structure of the enzyme was determined under denaturing conditions by SDS-polyacrylamide gel electrophoresis [13] and silver staining.

Kinetic characterization. Activities were determined in 50 mM Hepes pH 7.5 in the presence of 0.2 mM NADPH with freshly prepared substrate solutions. Stock quinone solutions were prepared in ethanol, resulting in final concentrations lower than 3 % v/v ethanol in the assay mixture. NADPH kinetic values were determined in the presence of 0.1 mM 9,10-phenanthrenequinone. The enzymatic activity was measured in a Cary 400 Bio (Varian) spectrophotometer by following the consumption of NADPH at 340 nm ($\varepsilon_{NAD(P)H} = 6200\ M^{-1}\,cm^{-1}$). Controls lacking either substrate or enzyme were run routinely. The reactions started with the addition of the enzyme. The initial velocities were measured in duplicate with five different substrate concentrations, and the kinetic constants were calculated using the nonlinear regression program Grafit 5.0 (Eritacus Software Ltd.). All reported values are expressed as the mean ± SE of at least three independent experiments.

Determination of coenzyme binding by fluorescence quenching. NAD(P)H binding to ζ-crystallins was followed by measuring the quenching in protein fluorescence upon binding of cofactors. Fluorescence experiments were performed using a Perkin-Elmer 650–40 spectrofluorimeter. All fluorescence intensities were determined at 25°C in 100 mM sodium phosphate buffer, pH 7.0. The assay cuvette contained 50 μg enzyme in 1 mL total volume, to which 5-μL aliquots from concentrated cofactor solution were added up to 2 μM NADPH with human ζ-crystallin and up to 20 μM for the remaining assays. Protein concentration was determined using the Bradford method, with BSA as a standard. Fluorescence spectra were obtained by exciting tryptophan (280 nm) and measuring the emission intensity (340 nm). Binding curves are reported as fluorescence quenching. The dissociation constant (K_D) for the binding of NADPH to ζ-crystallin was calculated using a previously described procedure [14, 15].

Deletion of the YBR046C gene. The coding region from the gene *ZTA1* (*YBR046C*) was substituted with the *kanMX*4 cassette [16] using the one-step gene replacement method [17]. Yeast colonies that had integrated the *kanMX4* cassette were selected in rich medium YPD (1 % yeast extract, 2 % peptone, 2 % agar, 2 % glucose) supplemented with 200 μg/mL G418. Positive colonies were verified by PCR analysis.

Drop-test assay. Serial dilutions of yeast cultures grown to exponential phase (at the same cell density) were performed for

Table 1. NADPH-dependent reductase activity of human ζ-crystallin and Ztalp with different compounds.

Substrate	Concentration (mM)	% Activity	
		Human ζ-crystallin	Zta1p
1,2-naphthoquinone	0.1	100*	100*
9,10-phenanthrenequinone	0.1	77	100
1,4-naphthoquinone	0.1	3	ND
menadione (2-methyl-1,4-naphthoquinone)	0.1	<1	<1
nonanal	0.5	ND	4
2-*trans*-pentenal	0.5	ND	<1
2-*trans*-nonenal	0.5	NA	1
2-*trans*-nonene	0.5	ND	<1
4-hydroxy-2-*trans*-nonenal	0.5	ND	2
all-*trans*-retinaldehyde	0.05	NA	NA

*Activities are expressed as the percentage of the 1,2-naphthoquinone reductase activity.
NA, no activity detected; ND, not determined.

the deleted Δ*zta1* and the wild-type strains. From each dilution, 4-μL aliquots were plated on rich medium YP (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v agar) containing different carbon sources: 2 % w/v glucose, 2 % w/v maltose, 2 % w/v galactose, 3 % w/v potassium acetate, 2 % w/v lactate, 3 % v/v glycerol or 3 % v/v ethanol. The growth on oleic acid as a carbon source was also tested in YNO plates (0.67 % w/v yeast nitrogen base without amino acids and without ammonium sulfate, 0.5 % w/v ammonium sulfate, 0.1 % w/v yeast extract, 0.1 % v/v oleic acid, 0.05 % v/v Tween-40, 2 % w/v agar, supplemented with 40 μg/mL adenine, 40 μg/mL histidine, 60 μg/mL leucine, 40 μg/mL lysine and 25 μg/mL uracil. In addition, yeasts were plated on YPD medium containing different drugs. To induce oxidative stress, 50 μM or 100 μM menadione or 1 mM diamide was added to the solid medium. To induce DNA damage, 0.035 % ethyl methanesulfonate was added to the solid medium. Osmotic stress was tested in YPD plates with either 1 M sorbitol, 0.1 M LiCl or 0.6 M NaCl. In each experiment, a control plate without any treatment was prepared in order to check that the same cell quantity was inoculated from both yeast strains and that differences in cell density were due to the treatment. To test hydrogen peroxide sensitivity, cells were grown to the same cell density and treated with 10 mM hydrogen peroxide in the liquid media for 1 h prior to being plated on YPD agar.

***In vitro* transcription.** A 100-bp fragment within the 3′-UTR from the *GAL3* gene, from 1546 nt to 1646 nt downstream of the start codon, was amplified from yeast genomic DNA with *Kpn*I and *Xho*I sites at the 5′-end and 3′-end, respectively. The resulting product was introduced into the pBluescript II SK(+) vector after digestion with *Kpn*I and *Xho*I. Prior to transcription, the vector was digested with *Eco*RI. The DNA template for the $A(UUUA)_5$ probe was obtained from two overlapping oligonucleotides (forward: 5′-AAG CTT CAT ATG ATT TAT TTA TTT ATT TAT TTAG-3′ and reverse: 5′-AAT TCC TAA ATA AAT AAA TAA ATA AAT CAT ATG A-3′) that were annealed and ligated to the *Eco*RI and *Hind*III sites of pBluescript II SK(+). Prior to transcription, the vector was digested with *Not*I. The template for the transcription of the $A(UUUA)_6$ RNA probe was obtained by partially overlapping oligonucleotides containing the T7 promoter (underlined) in the forward primer (forward: 5′-<u>TAA TAC GAC TCA CTA ATA</u> GGG AGG AAA AAAA AAA AAA AAU UUA UUU AUU UAU UUA UUU AUU UAA AAA AAA AAA AAA AA-3′ and reverse: 5′-TTT TTT TTT TTT TTT TAA ATA AAT AAA TAA ATA AAT TTT TTT TTT TTT TTC CTC CC-3′) that had been made dsDNA by annealing and treatment with Klenow DNA polymerase in the presence of dNTPs. In all cases the DNA templates were transcribed from the T7 promoter. The transcription reaction was performed in 20 μL containing 0.5 ng template DNA; 20 μCi [α-^{32}P]-UTP; 0.5 mM each ATP, CTP and GTP; 50 μM unlabeled UTP; 20 units RNAsin; 10 mM dithiothreitol; and 20 units T7 DNA polymerase. After incubating at 37°C for 1 h, 1 unit RNase-free DNase was added, and the reaction mixture was further incubated at 37°C for 15 min. The RNA was then separated from the oligonucleotides by Micro Bio-spin column chromatography (BioRad), and its concentration determined by scintillation counting. For the unlabeled probe, similar reactions were performed in the absence of [α-^{32}P]-UTP but with 0.5 mM of each NTP and 1 μg template. The RNA quantification was performed by measuring the absorbance at 260 nm.

RNA-electrophoretic mobility shift assay (EMSA). Purified protein (10–500 ng) was incubated at room temperature for 10 min in a 10-μL reaction mixture containing 10 mM Hepes pH 7.4, 25 mM potassium acetate, 2.5 mM magnesium acetate, 0.5 μg yeast tRNA, 0.5 % Igepal CA 630, 5 % glycerol, 1 mM dithiothreitol and 10 units RNAsin. Subsequently, 25 fmol labeled RNA were added. For competition experiments, specific or nonspecific competitors (10- to 100-fold excess) were also added. Samples were incubated for 20 min at room temperature and then subjected to electrophoresis in a 5 % polyacrylamide gel at 170 V for 90 min using 90 mM Tris, 110 mM boric acid and 2 mM EDTA as a running buffer. After electrophoresis, gels were incubated for 20 min in an aqueous solution of 20 % ethanol and 10 % acetic acid, introduced into plastic bags and imaged using a PhosphorImager screen (BioRad).

Results

Expression and purification of human and yeast ζ-crystallins. The human and yeast ζ-crystallins were expressed in *E. coli* and purified to homogeneity (as judged from SDS-PAGE) by affinity chromatography, with an approximate yield of 12 and 16 mg, respectively, from 1 L *E. coli* culture.

Molecular weight determination. Human ζ-crystallin and Zta1p showed a subunit molecular weight of 36 kDa by denaturing polyacrylamide gel electrophoresis. Analysis by molecular exclusion chromatography indicated a molecular weight of 140 kDa for the

Table 2. Catalytic properties of human, yeast and guinea pig ζ-crystallins.

Substrate	Human ζ-crystallin			Zta1p			Guinea pig ζ-crystallin*		
	Km (μM)	*kcat* (min^{-1})	*kcat*/*Km* (min^{-1} mM^{-1})	*Km* (μM)	*kcat* (min^{-1})	*kcat*/*Km* (min^{-1} mM^{-1})	*Km* (μM)	*kcat* (min^{-1})	*kcat*/*Km* (min^{-1} mM^{-1})
1,2-naphthoquinone	29.0±1.0	170±3	5900±320	9.0±1.0	250±10	27 800±3280	10	600	30 000
9,10-phenanthrenequinone	1.5±0.2	130±4	87 000±12 000	4.0±0.5	250±8	62 500±8060	13	300	11 500
NADPH	5.0±0.7			70±10			5		

For *kcat* calculation, the subunit molecular mass (36 kDa) was used.
* Results taken or estimated from Ref. [5].

human enzyme and 70 kDa for the yeast enzyme. This suggests that human ζ-crystallin is a homotetramer, like guinea pig ζ-crystallin [18], while yeast Zta1p is a homodimeric protein, like the homolog proteins from *E. coli* and *Thermus thermophilus* [19, 20]. The purified proteins were quite unstable and became completely inactive after 1 wk at 4°C. They showed a high tendency to aggregate and precipitate once purified.

Enzymatic characterization of human ζ-crystallin and Zta1p. Activity was assayed with quinones, nonanal, 2-alkenals and retinaldehyde in the presence of NADPH as a coenzyme (Table 1). Human ζ-crystallin and Zta1p showed maximum activity with the *ortho*-quinones 1,2-naphthoquinone and 9,10-phenanthrenequinone, while they exhibited very low activity against the *para*-quinone 1,4-naphthoquinone and near undetectable activity with menadione. With nonanal and 2-alkenals, no activity and very faint activity were measured for human ζ-crystallin and Zta1p, respectively. No activity was detected with retinaldehyde. The *Km* values for *ortho*-quinone substrates were in the 1.5 to 30 μM range, with the best catalytic efficiency (*kcat/Km*) for 9,10-phenanthrenequinone. The *Km* values for *ortho*-quinones were in the range of those previously reported for guinea pig ζ-crystallin [5], while *kcat* values were significantly lower (Table 2). Human and yeast ζ-crystallins were completely specific for NADPH as coenzyme. The *Km* value of the human enzyme for NADPH was about 10-fold lower than that of Zta1p. No activity was detected with NADH. As judged from analysis of fluorescence quenching, human ζ-crystallin bound NADPH with higher affinity (K_D =160 nM) than the yeast enzyme (Fig. 1). In this latter case, a K_D value could not be calculated, since only a 40% decrease in fluorescence intensity was observed with a cofactor concentration as high as 20 μM. The human protein did not bind NADH, as the fluorescence curve could not be distinguished from that obtained with BSA.

Effect of the disruption of yeast *ZTA1*. The coding region of the yeast *ZTA*1 gene was deleted by

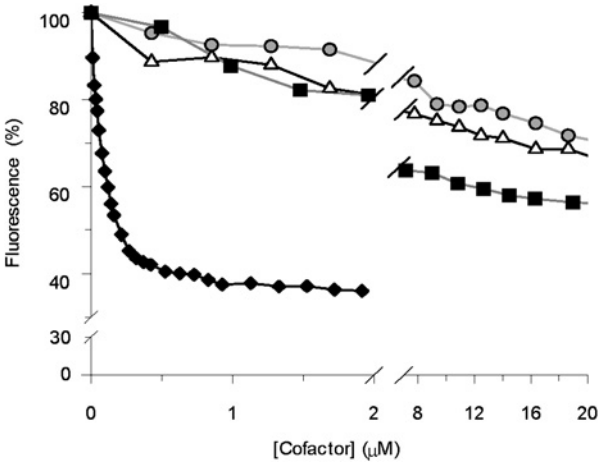

Figure 1. Fluorescence quenching upon NADPH or NADH binding to human and yeast ζ-crystallins. Change in the protein fluorescence intensity (in percentage) upon addition of cofactor. Human and yeast ζ-crystallins and bovine serum albumin (BSA) were used at a concentration of 50 μg/mL in 100 mM sodium phosphate pH 7.5 at 25°C (◆ human ζ-crystallin + NADPH; ● human ζ-crystallin + NADH; ■ Zta1p + NADPH; △ BSA + NADH).

substitution with a *kanMX4* cassette. To test the effect of the deletion, we assayed the growth capacity of wild-type and deleted Δ*zta*1 strains under different conditions. Compared to the wild-type strain, no effect was detected when growing the yeast cells on rich medium containing either fermentative (glucose, maltose or galactose) or non-fermentative (potassium acetate, lactate, glycerol or ethanol) carbon sources. No effect was found when using oleic acid as a carbon source for peroxisome induction. No effect was detected either in rich medium with ethyl methanesulfonate, which has been reported to induce DNA damage. Long-term growth of yeast cells at 37°C under acidic pH conditions also had no effect. In contrast, growth in the presence of menadione revealed that viability was higher for the wild-type than for the Δ*zta*1 strain. No effect on viability was observed in the presence of diamide. Treatment with 10 mM hydrogen peroxide for 1 h prior to plating showed that Δ*zta*1 was more sensitive to this agent than the wild-type yeast (Fig. 2).

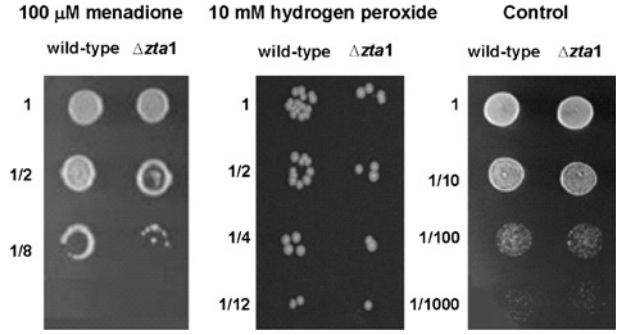

Figure 2. Tolerance of wild-type and Δ*zta*1 strains to menadione and hydrogen peroxide. Culture cells were grown to logarithmic phase at the same cell density. Cell suspensions were serially diluted (fold dilutions are indicated on the left side) and spotted as 4 μL on plates with menadione (left), after 1 h of incubation with H_2O_2 (center) or without treatment (control, right). Each assay was performed at least three times.

Interaction of human ζ-crystallin and yeast Zta1p with AU-rich sequences. We investigated whether human ζ-crystallin and Zta1p specifically bind to AREs such as those recognized by the ζ-crystallin rat homolog. Thus, we screened the 3′-UTR sequences in the yeast genome for the presence of AREs similar to those of the rat glutaminase. From the different positives, the 3′-UTR from the *GAL3* gene, coding for a transcriptional regulator involved in the activation of *GAL* genes, was selected for the binding assay. The *GAL3* 3′-UTR mRNA contains two AREs in tandem. One of them exhibits 100 % identity to one ARE from rat glutaminase mRNA, while the other is 75 % identical to the second ARE from rat glutaminase mRNA. The *GAL3* 3′-UTR containing those AREs was *in vitro* transcribed in the presence of labeled UTP, and its binding to human ζ-crystallin and Zta1p was assayed using RNA-EMSA. Human ζ-crystallin and Zta1p were able to specifically bind to the labeled *GAL3* RNA probe (Fig. 3a). The specificity for Zta1p was tested using competition assays with an excess of unlabeled *GAL3* RNA or 18S rRNA. The interaction was disrupted by competition with *GAL3* RNA but not with 18S rRNA, indicating a specific interaction with the *GAL3* RNA probe (Fig. 3b). The binding with a five tandem repeat (or six in the case of Zta1p) of the ARE consensus sequence (AUUUA) that is specifically recognized by all ARE-binding proteins showed that both human and yeast ζ-crystallins interact specifically with this element, indicating that they are ARE-binding proteins (Fig. 4). Human ζ-crystallin specificity was tested by performing RNA-EMSA with a labeled $A(UUUA)_5$ probe or a fragment of the pBluescript vector transcript. The human enzyme bound to the $A(UUUA)_5$ probe but not to the RNA derived from the vector multicloning site, indicating a specific interaction with the adenine-uracil-rich sequence (Fig. 4a).

To test whether the coenzyme-binding site was involved in RNA binding, competition with increasing amounts of NADPH or NADH was performed in RNA-EMSA (Fig. 5). NADPH efficiently competed with the $A(UUUA)_5$ probe for the binding to human ζ-crystallin. NADH, which does not bind to ζ-crystallins, did not show any competition. This suggests that the coenzyme-binding site is involved in ζ-crystallin binding to RNA. For Zta1p, weak competition was observed only at high NADPH concentrations (above 70 μM, results not shown), consistent with its lower affinity for NADPH.

Discussion

In the present study, we have demonstrated that two evolutionarily distantly related proteins, human ζ-crystallin and the yeast homolog Zta1p, catalyze the reduction of *ortho*-quinones (1,2-naphthoquinone and 9,10-phenanthrenequinone) in the presence of NADPH as a coenzyme. In contrast, they were inactive with menadione (2-methyl-1,4-naphthoquinone or vitamin K_3). Similar substrate specificity has been reported for guinea pig ζ-crystallin [5] and the homologous quinone reductase QorA from *Staphylococcus aureus* [21]. In contrast to *Arabidopsis* P1-ζ-crystallin [8], the human and yeast enzymes were not active in the reduction of 2-alkenals. Thus, human ζ-crystallin and Zta1p have similar substrate specificity, only being active for the reduction of quinones in the *ortho* configuration.

Human ζ-crystallin and Zta1p were completely specific for NADPH as a coenzyme. Indeed, the measurement of coenzyme binding by a fluorescence-quenching assay demonstrated binding of the human enzyme to NADPH but not NADH. A similar high NADPH specificity had been reported for the guinea pig ζ-crystallin [5]. The yeast enzyme bound NADPH with lower affinity than the human enzyme, consistent with its higher *Km* value.

Yeast mutant Δ*zta1* grew like the wild-type strain under standard conditions, but it was more sensitive to oxidative stress induced by menadione and hydrogen peroxide. These results suggest that yeast Zta1p is involved in protection against oxidative stress. Consistently, the *ZTA1* homolog from *Staphylococcus aureus* (*qorA* gene) was induced under the oxidative stress produced by treatment with 9,10-phenanthrenequinone [21]. Moreover, P1- and P2-ζ-crystallins accumulated rapidly in *Arabidopsis* plants under various oxidative stress conditions [7]. Finally, in *S. cerevisiae,* the level of *ZTA1* mRNA was increased

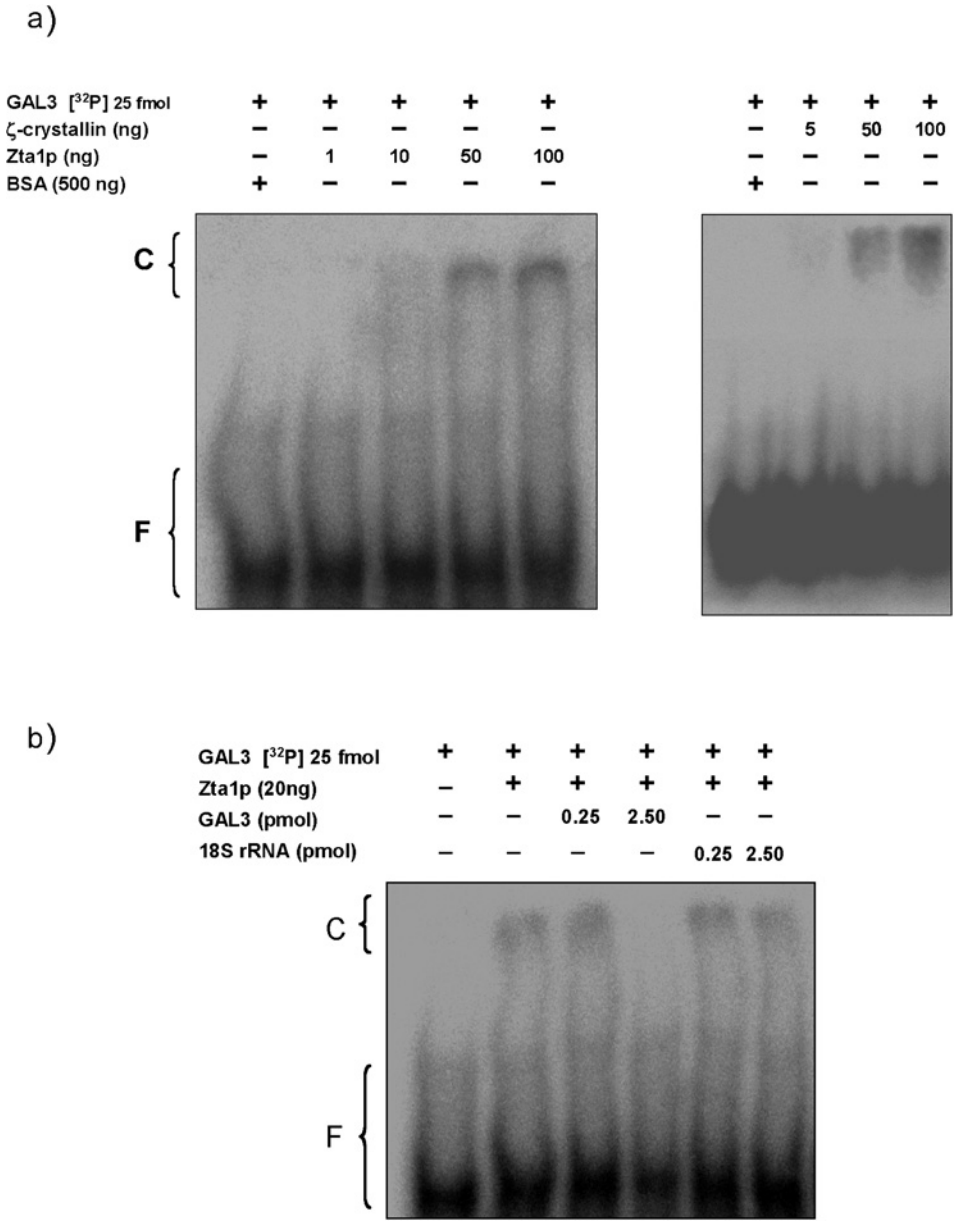

Figure 3. Analysis of Zta1p and human ζ-crystallin binding to *GAL3* ARE with RNA-EMSA. (a) A sequence of RNA containing the ARE of yeast *GAL3* RNA was labeled with ^{32}P-UTP and incubated with increasing amounts of purified Zta1p or human ζ-crystallin in the presence of 0.5 μg tRNA as a competitor for non-specific binding. (b) Specificity was also investigated by binding competition assays in the presence of 10-fold and 100-fold excess of the unlabeled *GAL3* RNA or 18S rRNA. Negative binding control assays were performed with 500 ng BSA. The C bracket indicates the main RNA-protein complex, and the F bracket indicates the free RNA.

following exposure to hydrogen peroxide, menadione and diamide (Saccharomyces Genome Database http://www.yeastgenome.org/). Taken together, these results support the involvement of the ζ-crystallin family in the oxidative stress response.

It is widely accepted that guinea pig ζ-crystallin and the *S. aureus* homolog, QorA, catalyze the NADPH-dependent one-electron reduction of quinones to produce their semiquinone radical. This radical is oxidized back to the quinone form in the presence of oxygen, resulting in the generation of superoxide anions [5]. As a consequence, it would be expected that the quinone reductase activity of ζ-crystallins should, by itself, induce oxidative stress but not protection. Therefore, the protective effect observed in our drop-test experiments has to be provided by a property of yeast ζ-crystallin other than its quinone reductase activity.

In fact, our results suggest that another ζ-crystallin feature has been conserved throughout evolution: the ability to specifically bind to adenine-uracil-rich sequences in mRNA. This property was first described for rat ζ-

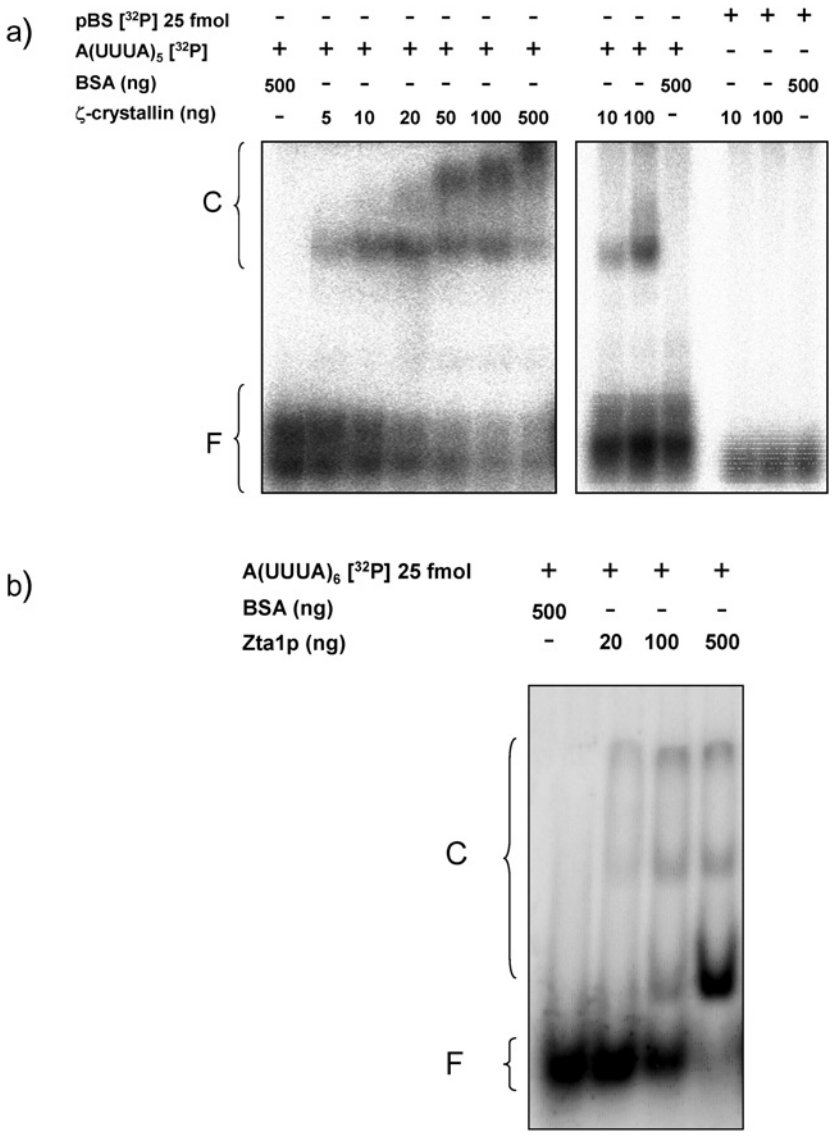

Figure 4. Binding analysis of $A(UUUA)_5$ with ζ-crystallin and $A(UUUA)_6$ with Zta1p. (a) Shift analyses of ζ-crystallin/RNA. (b) Binding of Zta1p to the $A(UUUA)_6$ probe. BSA was used to check for non-specific interactions. Position of free probe (F) and shifted complex (C) are indicated (pBS, pBlueScript SK).

crystallin, which was demonstrated by RNA-EMSA to be able to specifically bind to the 3′-UTR AREs from renal glutaminase and glutamate dehydrogenase mRNA [11, 22, 23]. We here show that human ζ-crystallin and Zta1p are able to bind *in vitro* to a direct repeat ARE of the *GAL3* RNA probe similar to those of rat glutaminase. This property, therefore, may be general for ζ-crystallins and conserved from yeast to mammals. This is particularly interesting because it suggests that ζ-crystallins have a role in the posttranscriptional regulation of the expression of some groups of genes under particular conditions, *i.e.* during acidosis in the case of rat kidney cells and, probably, under oxidative stress in the case of yeast cells.

The strength and the specificity of the binding of human ζ-crystallin and Zta1p to the ARE consensus sequence $A(UUUA)_{5-6}$ further demonstrate that both proteins can be classified as ARE-binding proteins [24]. This means that ζ-crystallins may act as *trans*-acting factors that could regulate the turnover of groups of mRNAs from some pathways under special conditions.

Although the binding to the consensus sequence was strong, the binding to the physiological *GAL3* RNA probe was comparatively weak, as judged from the high amount of free probe in the RNA-EMSA experiments (Fig. 3). This seems to indicate that AREs from yeast *GAL3* RNA are not the preferred targets for human ζ-crystallin and Zta1p. Further research needs to be performed to identify the specific mRNA targets.

Interestingly, two other dehydrogenases containing the Rossmann fold were recently identified as ARE-binding proteins. Glyceraldehyde-3-phosphate dehydrogenase from human ovarian cells was found to bind to the ARE within the 3′-UTR of colony-stimulating

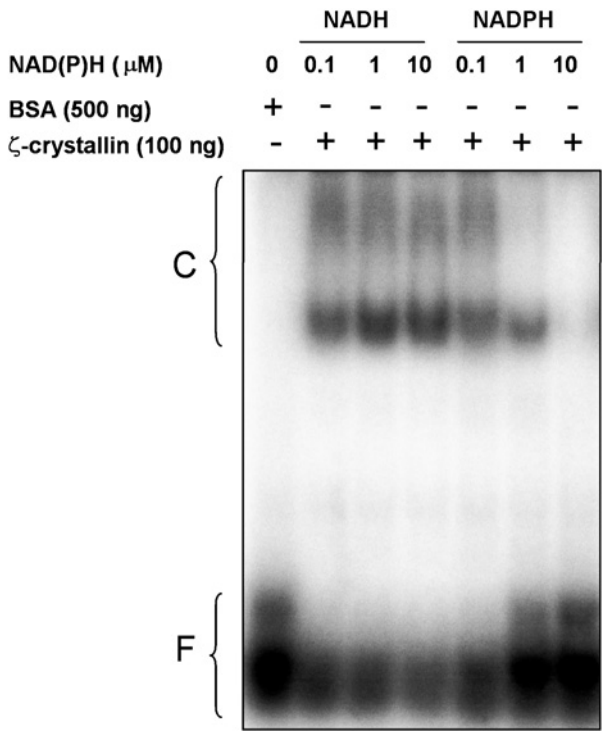

Figure 5. Binding analysis of $A(UUUA)_5$ with ζ-crystallin in the presence of NADH or NADPH. Gel shift analysis of ζ-crystallin/RNA interactions in the presence of increasing concentration of NADPH or NADH. Free probe (F) and shifted complex (C) are indicated. BSA was used to check for non-specific interactions.

factor-1 mRNA [25]. Moreover, lactate dehydrogenase (LDH) isozyme M from mouse erythroleukemia cells binds to the ARE within the 3′-UTR of granulocyte-macrophage colony stimulating factor mRNA and also interacts directly with AUF1/heterogeneous nuclear ribonucleoprotein D [26]. NAD disrupted the LDH binding to RNA, similar to the effect of NADPH on human ζ-crystallin, indicating that the coenzyme-binding site is probably involved in the interaction of both proteins with RNA. However, the binding to specific RNA sequences does not seem to be a general property of dehydrogenases. In this regard, human PIG3 (Swiss-Prot entry Q53FA7), a quinone reductase structurally related to ζ-crystallin, does not bind the ARE consensus sequence (unpublished results). We can conclude that human ζ-crystallin and Zta1p are multifunctional proteins with NADPH:quinone reductase activity and ARE-binding properties, and both capacities are conserved throughout evolution. Our results and previous reports support a role of ζ-crystallins in the oxidative stress response.

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