Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, *Pichia pastoris* and its identification as zeta crystallin

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ABSTRACT

A single-stranded DNA (ssDNA)-binding protein (SSB) that binds to specific upstream sequences of alcohol oxidase (*AOX1*) promoter of the methylotrophic yeast *Pichia pastoris* has been isolated and identified as zeta crystallin (ZTA1). The cDNA encoding *P.pastoris* ZTA1 (PpZTA1) was cloned into an *Escherichia coli* expression vector, the recombinant PpZTA1 was expressed and purified from *E.coli* cell lysates. The DNA-binding properties of recombinant PpZTA1 are identical to those of the SSB present in *P.pastoris* cell lysates. PpZTA1 binds to ssDNA sequences >24 nt and its DNA-binding activity is abolished by NADPH. This is the first report on the characterization of DNA-binding properties of a yeast ZTA1.

INTRODUCTION

Single-stranded DNA (ssDNA)-binding proteins (SSBs) perform several essential functions in cells. For many species, SSBs that bind to ssDNA with little or no sequence specificity have been identified, and such proteins have been identified in almost all living cells. SSBs can be divided into several classes ranging from monomeric proteins of bacteriophages such as gene-32 protein (1) to dimeric SSBs of filamentous phages (2) to heterotrimeric nuclear SSBs in eukaryotes such as replication protein A (3) and finally to homotetrameric SSBs (4–6). Zeta Crystallin (ZTA1), an NADPH-dependent quinone oxidoreductase, is a major eye lens protein in certain vertebrates such as guinea pigs and camels (7,8) but not in others such as humans, mice or cattle. It is a soluble enzyme and is distinct from membrane-bound quinone oxidoreductase, the large complex in the respiratory chain (9). It is also distinct from the mammalian quinone oxidoreductase called DT-diaphorase, a flavin adenine dinucleotide-containing enzyme that catalyses NAD(P)H-dependent two-electron reduction of quinones (10). ZTA1 reduces naturally occurring quinones such as 1,2-naphthoquinone and phenanthraquinone

and the physiological function of ZTA1 is speculated to be detoxification or metabolism of a quinone (7,11,12). Genes encoding ZTA1 homologues are widely distributed from bacteria to higher plants and animals and the crystal structure of *Escherichia coli* quinone oxidoreductase/ZTA1 is known (13). Among the mammalian zeta crystallins, the bovine ZTA1 was shown to be capable of binding to single-stranded as well as double-stranded Z-DNA which could be competed with NADPH (14). Bovine ZTA1, also known as RF-36, was shown to play pleiotropic roles in gene expression, growth and differentiation of bovine lens (15–19). A well-characterized nucleotide-binding domain (Rossmann fold) as well as a pyridine nucleotide-binding sequence (GxxGxxG) or its variant are present in ZTA1 of all species (12,13,20,21). Genes encoding ZTA1 are reported in the genomes of *Saccharomyces cerevisiae* (http://db.yeastgenome.org/cgibin/locus.pl?locus=zta1) and *Pichia pastoris* (http://ergo.integratedgenomics.com/ERGO/CGI/prot.cgi?prot=RPPA06848) but the proteins are not biochemically characterized.

In the methylotrophic yeast *P.pastoris*, alcohol oxidase (AOX) is the first enzyme involved in the methanol metabolism. It is encoded by two methanol-inducible genes, *AOX1* and *AOX2*, of which transcription of *AOX1* gene is induced to very high levels by methanol (22,23). Therefore, the upstream region of *AOX* promoter between −735 and −1 is widely used for heterologous expression of a number of foreign proteins in *P.pastoris* (http://faculty.kgi.edu/cregg/index.htm). Recently, a protein referred to as methanol expression regulator 1 (Mxr1p) binding to the upstream region of *P.pastoris AOX1* promoter was identified (24). Mxr1p binds to the *AOX1* upstream region between −415 and −172 *in vitro* and deletion of this region results in a significant decrease in *AOX1* gene expression *in vivo*. However, deletion of sequences outside Mxr1p-binding region in the *AOX1* promoter also results in a decrease in *AOX1* gene expression indicating the involvement of multiple regulatory circuits in *AOX1* gene regulation (24). In this study, we employed a biochemical approach to identify proteins interacting with specific upstream sequences of *AOX1* promoter. Such efforts have led to the isolation of an SSB which was purified and identified as the *P.pastoris* homologue of ZTA1.

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MATERIALS AND METHODS

Growth of *P.pastoris* cells and preparation of whole cell extracts

P.pastoris (GS115 strain) cells were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C with vigorous shaking. Stationary phase cells were centrifuged once at 2000 *g*, washed with water and resuspended in buffer A (20 mM Tris–HCl, pH 8.0, 10 mM MgCl$_2$, 7 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl flouride and 1 μg/ml each of pepstatin, aprotinin and leupeptin) containing 50 mM NaCl. An equal volume of acid-washed glass beads (0.45 mm) suspended in ice-cold buffer A were added and cells were lysed at 4°C by vigorous intermittent vortexing for 30 min or using a bead beater (Biospec Products, USA). The whole cell extracts obtained after centrifugation at 10 000 *g* for 20 min at 4°C were stored at −80°C.

Heparin agarose and DEAE cellulose chromatography

The whole cell extracts were loaded onto heparin agarose column (20 mg protein/ml resin) equilibrated with buffer A, the unbound protein was collected and stored separately as the flow through fraction. The column was washed with 2 column volumes of buffer A and the bound protein was eluted sequentially with buffer A containing 200 mM (E1), 300 mM (E2) and 400 mM NaCl (E3). These eluate fractions were dialysed against buffer A at 4°C and stored at −80°C in aliquots. The E1 fraction was loaded onto a DEAE cellulose column equilibrated with buffer A, the unbound protein fraction (flow through) was collected and stored at −80°C. The column was washed with buffer A, the bound proteins were eluted in buffer A containing 200 mM NaCl and stored at −80°C in aliquots after dialysis against buffer A.

Synthesis of oligonucleotides and preparation of radiolabelled DNA probes

Oligonucleotides listed in Table 1 were purchased from Sigma-Aldrich Chemicals, Bangalore, India. High-specific activity ^{32}P-radiolabeled probes were prepared by random prime labelling and lower specific activity probes were prepared by 5′ end labelling. The former (288*, 229* and 174*) were generated by first annealing complementary oligonucleotides (288 and 288c1, 229 and 229c1, 174 and 174 c1) by heating at 75°C for 15 min in presence of 10 mM Tris–HCl, pH 7.5, and 100 mM NaCl followed by slow cooling to room temperature. These partially double-stranded DNA (dsDNA) molecules were then radiolabelled in presence of dATP, dGTP, dTTP, [α-^{32}P]dCTP and klenow fragment of DNA polymerase I at 37°C for 20 min (Figure 1A). End-labelled DNA probes were generated by annealing complementary oligonucleotides (288 and 288c, 229 and 229c, 174 and 174c) and then incubating these double-stranded oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP at 37°C for 45 min (Figure 2A). After radiolabelling, unincorporated radionucleotides were removed by sephadex G-50 chromatography.

Table 1. Oligonucleotides spanning the near upstream region of *P.pastoris AOX1* promoter

(−288 to −230)	
288	5′-ACCCGCTTTTTGGATGATTATGCATTGTC-TCCACATTGTATGCTTCCAAGATTCTGGTGG-3′
288c	3′-TGGGCGAAAAACCTACTAATACGTAACAG-AGGTGTAAGATACGAAGGTTCTAAGACCACC-5′
288C1[a]	3′-ACGAAGGTTCTAAGACCACC-5′
(−229 to −175)	
229	5′-GAATACTGCTGATAGCCTAACGTTCATG-ATCAAAATTTAACTGTTCTAACCCCT-3′
229c	3′-CTTATGACGACTATCGGTAAGCAAGAT-CTAGTTTTAAATTGACAAGATTGGGGA-5′
229c1[a]	3′-TAAATTGACAAGATTGGGGA-5′
(−174 to −115)	
174	5′-ACTTGACAGCAA**TATATAAA**CAGAAGGAA-GCTGCCCTGTCTTAAACCTTTTTTTTTATCA-3′
174c	3′-TGAACTGTCGTTATATATTTGTCTTCCTTC-GACGGGACAGAATTTGGAAAAAAAAATAGT-5′
174c1[a]	3′-AATTTGGAAAAAAAAATAGT-5′

The *AOX1* promoter sequences are numbered relative to initiator codon. The putative TATA box between −162 and −155 is indicated in boldface. The major transcription start site at −115 is underlined. The upstream region covered by the oligonucleotides is indicated in parentheses.
[a]These short oligonucleotides were used for random prime labelling.

Electrophoretic mobility shift assay (EMSA)

Protein preparations (5–10 μg) obtained from heparin agarose and DEAE cellulose chromatography were incubated with radiolabelled oligonucleotides (40 000 CPM) in a 30 μl reaction containing 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM DTT, 0.05% NP-40, 6% glycerol and 100 ng of poly(dI–dC) for 30 min at 4°C. The reaction mixtures were subjected to electrophoresis at 4°C on 5% non-denaturing polyacrylamide gel in a buffer containing 7 mM Tris–HCl (pH 7.4), 3 mM boric acid and 1 mM EDTA. The gels were dried and autoradiographed. In some experiments, *P.pastoris* cell extract was replaced by recombinant ZTA1. For the calculation of dissociation constant, EMSA was carried out with 15 ng of recombinant ZTA1 and increasing amounts of end-labelled ssDNA probes. The amount of oligonucleotide bound by protein was calculated as the difference between the known total input concentration of oligonucleotide and the amount detected in the position for free oligonucleotide on the mobility shift gels. K_d values were determined by quantitative analysis using a Scatchard plot.

Production and purification of recombinant *P.pastoris* ZTA1 (PpZTA1)

PpZTA1 cDNA was isolated by RT–PCR from *P.pastoris* cellular RNA using the primer pair 5′<u>ggatcc</u>atgactacaattccc 3′ and 5′<u>aagcttt</u>cattgagggatctc 3′ and cloned into BamHI and HindIII sites of pRSETA vector (Invitrogen, USA) using standard molecular biology protocols (25). The restriction sites in the primers are underlined. The recombinant plasmid was transformed into *E.coli* BL21(DE3)pLysS cells and expression of the recombinant protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside. Three hours after induction, cells were harvested and resuspended in a buffer B (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 10%
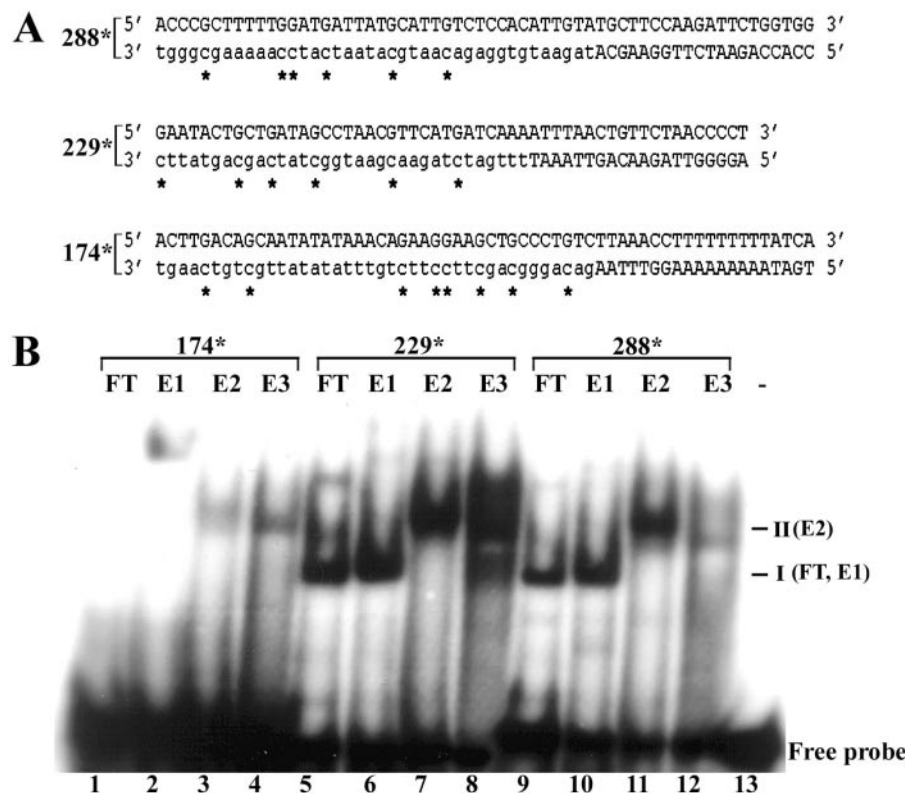
Figure 1. Identification of proteins binding to radiolabelled upstream sequences of *P.pastoris AOX1* promoter by EMSA. (**A**) Strategy for generating ^{32}P-labelled probes (288*, 229*, 174*) using random prime labelling by incorporation of radioactive nucleotide (asterix) into the newly synthesized DNA (lower case) by Klenow polymerase using annealed partially dsDNA molecules (upper case) as templates. (**B**) EMSA of flow through (FT) and eluate (E1, E2, E3) fractions (5 μg) obtained by heparin agarose chromatography. Protein–DNA complexes I and II generated by FT, E1 and E2 fractions are indicated.

glycerol and 10 mM β-mercaptoethanol) containing 10 mM imidazole. Cells were lysed by sonication and the lysate was centrifuged at 10 000 *g* at 4°C for 30 min. The clear supernatant was loaded onto a Ni²⁺-NTA agarose column (Qiagen, USA). The column was washed with buffer B containing 50 mM imidazole and the Histidine-tagged PpZTA1 protein was eluted with buffer B containing 150 mM imidazole. The protein was dialysed against buffer A and stored in aliquots at −80°C after ascertaining its purity by SDS–PAGE.

RESULTS

Identification of an SSB in *P.pastoris* cell extracts

The upstream region of *AOX1* promoter between −735 and −1 has been extensively used for methanol-inducible expression of a number of proteins. This region was shown to harbour multiple regulatory elements including the binding site for the recently identified transcription factor Mxr1p (24). In this study, we first focused our attention on the sequences in the near upstream region of AOX1 promoter between −288 and the major transcription start site (0–115) of *AOX1* promoter. Oligonucleotides spanning this region were designed (Table 1) and their ability to interact with proteins present in whole cell extracts of *P.pastoris* cells grown in YPD was examined by EMSA. Since EMSA with whole cell extracts did not yield significant results, we enriched DNA-binding proteins by heparin agarose chromatography

and also generated high-specific activity ^{32}P-labelled DNA probes (Materials and Methods and Figure 1A). The results presented in Figure 1B indicate that 229* and 288* DNA probes formed protein–DNA complexes (E1, E2 and E3) while no significant super shift was observed with probe 174*. In this study, we focussed our attention on complex I generated by the E1 fraction (Figure 1B).

In the next set of experiments, we generated 5′ end-labelled DNA probes using polynucleotide kinase and [γ-32P]ATP (Figure 2A) and examined their ability to form complex I by EMSA. Surprisingly, complex I was generated by ssDNA probes (Figure 2B, lanes 1, 2, 4, 5 and 8) except 174c (Figure 2B, lane 7) but not by dsDNA probes (Figure 2B, lanes 3, 6 and 9). These results indicate that complex I is formed by an SSB protein present in the E1 fraction.

Purification and characterization of SSB from *P.pastoris* cell extracts

To purify the SSB present in *P.pastoris* cell extract, the E1 fraction was fractionated on a DEAE-Sepharose column, the flow through and 200 mM NaCl eluate fractions were analysed by SDS–PAGE (Figure 3A) and EMSA (Figure 3B). The SSB activity was detected in the flow through fraction (Figure 3B, lane 1) but not in the eluate (Figure 3B, lane 2). UV cross-linking of DEAE flow through fraction and ^{32}P-labelled 229 DNA followed by SDS–PAGE and autoradiography lead to the identification of a radioactive band of ∼35–40 kDa molecular weight (data not shown).
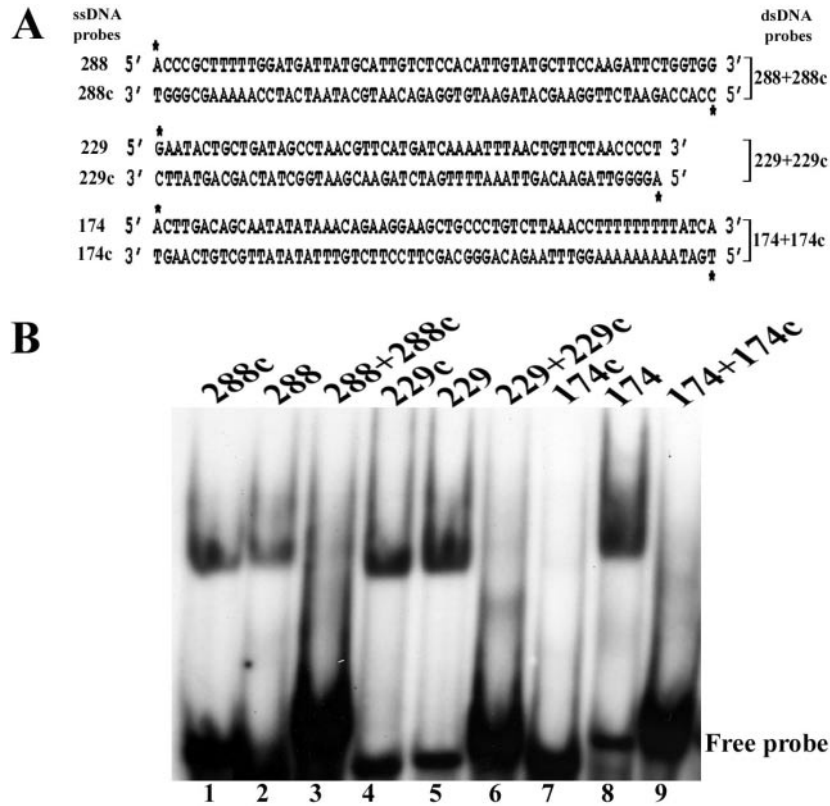
Figure 2. Detection of SSB activity in *P.pastoris* cell extract. (**A**) Generation of 5′ end-labelled ss and dsDNA probes by 5′ end labelling. (**B**) EMSA of Heparin agarose-E1 fraction using 5′ end-labelled ssDNA (lanes 1, 2, 4, 5, 7 and 8) and dsDNA (lanes 3, 6 and 9) probes.
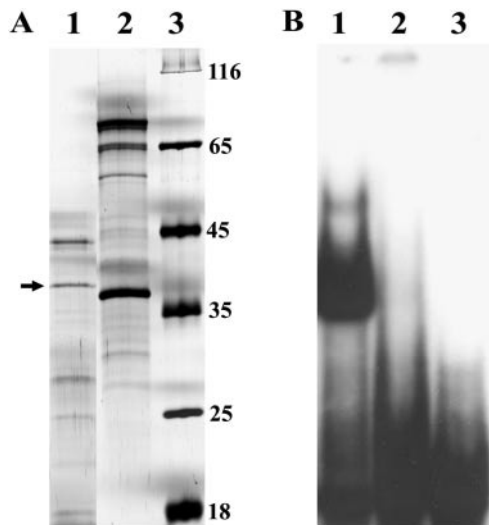

Figure 3. Partial purification of *P.pastoris* SSB. (**A**) Analysis of DEAE flow through (lane 1) and eluate (lane 2) fractions by SDS–PAGE followed by Coomassie blue staining of the gel. Lane 3, protein molecular weight markers (kDa). A protein band of ∼37 kDa which was excised and sequenced is indicated by an arrow. (**B**) EMSA of DEAE flow though (lane 1) and eluate (lane 2) fractions using 5′ end-labelled 229 probe. Lane 3, free probe.

Since a protein of similar molecular weight was enriched in the DEAE flow through fraction (indicated by an arrow in Figure 3A), this protein band was excised from the gel, subjected to trypsin digestion and the amino acid sequence of one of its tryptic peptides was determined. BLASTP search of this amino acid sequence (AYLSPSTFAQYTK) against the *S.cerevisiae* genome database revealed it to be homologous to a 37 kDa protein annotated as YBR046c/zeta crystallin (ZTA1) (http://db.yeastgenome.org/cgibin/locus. pl?locus=zta1). BLASTP search of ScZTA1 amino acid sequence with the *P.pastoris* genome database (http://ergo. integratedgenomics.com) led to the identification of a protein (ID # RPPA06848) annotated as quinone oxidoreductase. Multiple sequence alignment of amino acid sequences of ZTA1 from different species indicated that the NADPH-binding motif (AXXGXXG) present in the ZTA1 isolated from *E.coli* and mammalian cells is conserved in the yeast ZTA1 as well (Figure 4A). Yeast ZTA1 had ∼55% and 46–49% homology to *E.coli* and mammalian ZTA1, respectively (Figure 4B). Since the molecular weight as well as the amino acid sequence of the protein sequenced in this study was similar to that encoded by *S.cerevisiae* YBR046c/ZTA1 and identical to that encoded by *P.pastoris* RPPA06848 genes, we conclude that the protein sequenced in this study is the *P.pastoris* homologue of *E.coli* and mammalian ZTA1 (PpZTA1).

Characterization of SSB activity of recombinant PpZTA1

To examine whether PpZTA1 is an SSB, oligonucleotide primers were designed based on the Pp*ZTA1* nucleotide sequence and the full-length Pp*ZTA1* cDNA was isolated

A

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Sc ZTA1        MKCTIPEQQKVILIDEIG-GYDVIKYE-DYPVPSISEEELLIKNKYTGVNYIESYFRKGI 58
Pp ZTA1        -MTTIPKTQKVILFEETSQDLSVLQYK-DFPVPEISADEILIKNKYAGVNFIEAYFRSGL 58
E.coli ZTA1    -------MATRIEFHKHG-GPEVLQAV-EFTPADPAENEIQVENKAIGINFIDTYIRSGL 51
Bovine ZTA1    -MATGQKLMRAIRVFEFG-GPEVLKLQSDVAVPIPKDHQVLIKVQACGVNPVDTYIRSGT 58
Guinea pig ZTA1 -MATGQKLMRAIRVFEFG-GPEVLKVQSDVAVPIPKDHQVLIKVHACGINPVETYIRSGT 58
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Sc ZTA1        YP--CEKPYVLGREASGTVVAKGKGVTNFEVGDQVAYISNSTFAQYSKISSQG--PVMKL 114
Pp ZTA1        YP--SKTPYVLGREASGVVVAKGDNVTKFKVGDKVAYLSPSTFAQYTKVDGTTGIRILKL 116
E.coli ZTA1    YPP-PSLPSGLGTEAAGIVSKVGSGVKHIKAGDRVVYAQ-SALGAYSSVHNIIADKAAIL 109
Bovine ZTA1    HNIKPLLPYTPGFDVAGIIEAVGESVSAFKKGDRVFTTR-TISGGYAEYALAADHTVYTL 117
Guinea pig ZTA1 YTRIPLLPYTPGTDVAGVVESIGNDVSAFKKGDRVFTTS-TISGGYAEYALASDHTVYRL 117
                :      * . *   :.:*  :    *..*. :: **:*    *::.           *

Sc ZTA1        PKGTSDEELKLYAAGLLQVLTALSFTNEAYHVKKGDYVLLFAAAGGVGLILNQLLKMKGA 174
Pp ZTA1        KDDASEEDLKLFGSVLVQALTALTFVYEPYNVKSGDFILIHAAAGGVGQLLTQLAASKGA 176
E.coli ZTA1    PAAIS---FEQAAASFLKGLTVYYLLRKTYEIKPDEQFLFHAAAGGVGLIACQWAKALGA 166
Bovine ZTA1    PEKLD---FKQGAAIGIPYFTAYRALLYSAPVKPGESVLVHGASGGVGIAACQIARAYGL 174
Guinea pig ZTA1 PEKLD---FRQGAAIGIPYFTACRALFHSARAKAGESVLVHGASGGVGLAACQIARAYGL 174
                    .    :: .:  :   :*.      *  .: .*...*:****   *        *

Sc ZTA1        HTIAVASTDEKLKIAKEYGAEYLINASKEDILRQVLKFTNGKGVDASFDSVGKDTFEISL 234
Pp ZTA1        KVIATASTDAKLQIAKELGASFLINSSTEDIVEKVKEFTNGVGVNAVYDGIGKATFEASF 236
E.coli ZTA1    KLIGTVGTAQKAQSALKAGAWQVINYREEDLVERLKEITGGKKVRVVYDSVGRDTWERSL 226
Bovine ZTA1    KVLGTASTEEGQKIVLENGAHKVFNHKEANYIDKIKKSVGEKGVDVIIEMLANVNLSNDL 234
Guinea pig ZTA1 KVLGTAGTEEGQKVVLQNGAHEVFNHRDAHYIDEIKKSIGEKGVDVIIEMLANVNLSNDL 234
                :  :....*    :. : ** ::*        . *  .: .*...*:  *. .   *:

Sc ZTA1        AALKRKGVFVSFGNASGLIPPFSITRLSPK-NITLVRPQLYGYIADPEEWKYYSDEFFGL 293
Pp ZTA1        ESLAPKGTFVSFGNASGAVPPFSISRLAPK-NIAILRPQLFGYTNTPETWEKYSKELLGL 295
E.coli ZTA1    DCLQRRGLMVSFGNSSGAVTGVNLGILNQKGSLYVTRPSLQGYITTREELTEASNELFSL 286
Bovine ZTA1    NLLSHGGRVIVVG-SRGTIEINPRDTMTKE----SSIKGVTLFSSTKEEFQQFAAALQAG 289
Guinea pig ZTA1 KLLSCGGRVIIVG-CRGSIEINPRDTMAKE----STISGVSLFSSTKEEFQQFASTIQAG 289
                   *    *   .:: .*  . * *:      ::          : :     :  : :. 

Sc ZTA1        VNSKKLNIKIY--KTYPLRDYRTAAADIESRK-TVGKLVLEIPQ 334
Pp ZTA1        LSDGKLKFSIT--KVYSLKDYPKAAQELESRA-TTGKLTLEIPQ 336
E.coli ZTA1    IASGVIKVDVAEQQKYPLKDAQRAHEILESRA-TQGS-SLLIP- 327
Bovine ZTA1    MEIGWLRPVIG--PQYLLEKATQAHENIIHSSGATGKMILLLD- 330
Guinea pig ZTA1 MELGWVKPVIG--SQYPLEKASQAHENIIHSSGTVGKTVLLM-- 329
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B

	S. cerevisiae	*P. pastoris*	*E. coli*	*Guinea pig*	*Bovine*	*Mouse*	*Human*
S. cerevisiae	100	58 (74)	36 (56)	26 (46)	27 (49)	26 (48)	26 (47)
P. pastoris	58 (74)	100	36 (54)	23 (48)	25 (48)	26 (47)	27 (49)

Figure 4. Comparison of amino acid sequences of ZTA1 from different species. (**A**) Multiple sequence alignment of yeast, *E.coli* and mammalian ZTA1. The amino acid sequence of the *P.pastoris* trypic peptide is underlined. The conserved nucleotide-binding motif is boxed. (**B**) Percent identity and homology (parantheses) among various ZTA1.

from *P.pastoris* cellular RNA by RT–PCR. The cDNA was cloned into an *E.coli* expression vector (Figure 5A) and the recombinant PpZTA1 was purified as a histidine-tagged protein using Ni^{2+}-affinity chromatography (Figure 5B). The ability of recombinant PpZTA1 to bind ssDNA was examined by EMSA. The results indicate that the SSB properties of recombinant PpZTA1 are indistinguishable from those of the SSB purified from *P.pastoris* cell extracts (compare Figures 5C and 2). To gain further insights into the DNA-binding properties of PpZTA1, we chose 5′ end-labelled 288c and 229c ssDNA probes and annealed them to a number of short complementary oligonucleotides to generate partial duplex DNA molecules for use in EMSA as depicted in Figure 6A. The results indicate that the DNA-binding activity of ZTA1 is not abolished when the 3′ end (Figure 6B, lanes 2 and 2a) or a region near the 3′ end of ssDNA molecule (Figure 6B, lanes 5 and 5a, and Figure 6C, v) is double-stranded. However, PpZTA1 failed

to bind partial duplex DNA with double-stranded regions in the middle (Figure 6B, lanes 3 and 3a), 5′ end (Figure 6B, lanes 4 and 4a) or near the 5′ end (Figure 6B, lanes 6 and 6a). The results of EMSA are summarized in Figure 6C. Dissociation constants (K_d) for the interaction between recombinant PpZTA1 and three different oligonucleotides were calculated using scatchard plots and the results indicate that PpZTA1 binds 174 ($K_d = 1 \times 10^{-10}$M) and 229 ($K_d = 2 \times 10^{-10}$M) ssDNA probes with higher affinity than 288 ssDNA ($K_d = 9 \times 10^{-10}$M). To identify the minimal ssDNA region required PpZTA1 binding, short oligonucleotides of 20–32 nt in length were designed (Figure 7A) and used in EMSA. The results presented in Figure 7B indicate that PpZTA1 binds to ssDNA of 28 and 32 nt in length but not to shorter oligonucleotides. Since *E.coli* and mammalian ZTA1 are NADPH quinone oxidoreductases capable of binding to NADPH through the AXXGXXG nucleotide-binding motif, we examined the effect of NADPH on the
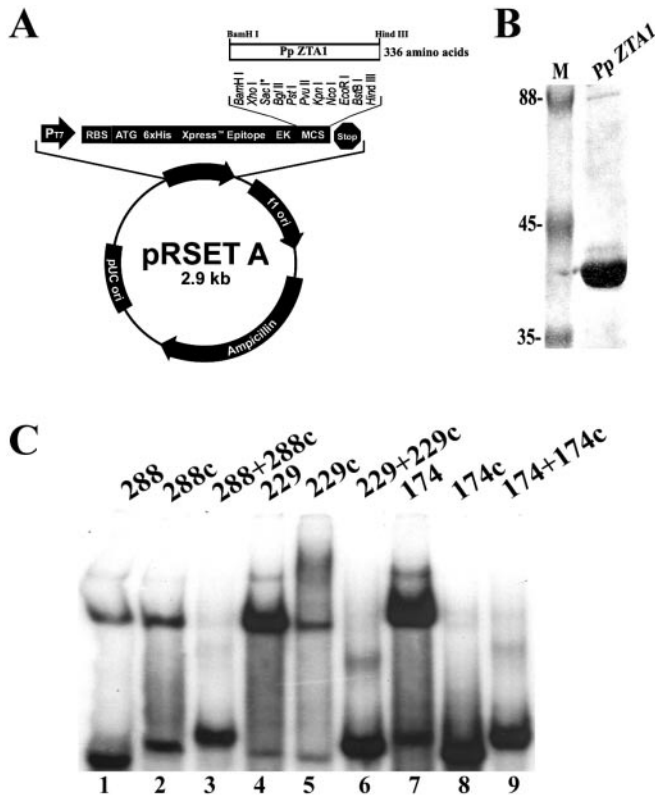
Figure 5. Production and purification of recombinant PpZTA1. (**A**) Strategy for cloning cDNA encoding PpZTA1 into *E.coli* expression vector. (**B**) Visualization of purified PpZTA1 (lane 2) in a Coomassie blue-stained SDS–polyacrylamide gel. Lane M, protein molecular weight markers (kDa). (**C**) EMSA of recombinant PpZTA1 using radiolabelled ssDNA (lanes 1, 2, 4, 5, 7 and 8) and dsDNA (lanes 3, 6 and 9) DNA probes.

SSB activity of PpZTA1. The results presented in Figure 8 indicate that SSB activity of recombinant PpZTA1 (Figure 8) is abolished in presence of NADPH but not NADH. Finally, gel filtration chromatography indicates that PpZTA1 exists as a dimer in solution since it co-elutes with albumin (Figure 9).

DISCUSSION

Our efforts to identify proteins interacting with the upstream sequences of *P.pastoris AOX1* promoter have led to the isolation of a SSB. This SSB could be identified in *P.pastoris* cell extracts primarily because of our strategy of radiolabelling oligonucleotides by method A as well as enrichment of DNA-binding proteins by heparin agarose chromatography. Radiolabelling of oligonucleotides by method A (Figure 1A) resulted in the generation of full-length as well as partially double-stranded radiolabelled DNA molecules and the latter are responsible for binding to the SSB enriched in the E1 and flow through fractions (Figure 1B). The results presented in Figure 2 clearly demonstrate that complex I is indeed generated by an SSB present in the E1 fraction. Partial purification and sequencing of a ~37 kDa protein enriched in the DEAE flow through fraction led to the identification

of PpZTA1. Studies carried out with recombinant PpZTA1 clearly establish its identity as an SSB. Although gene sequences encoding ZTA1 have been reported for a number of organisms (http://www.ncbi.nlm.nih.gov/entrez/query. fcgi?db=protein&cmd=search&term=zeta?crystallin), thus far only the bovine ZTA1 was shown to have SSB activity (14,18,19). Our studies indicate that PpZTA1 is similar to bovine ZTA1 since it also binds ssDNA. In this study, we focused our attention on the DNA-binding activity of PpZTA1 using oligonucletodies of defined length and sequence since such studies have not been carried out thus far. We have also calculated the K_d values which are comparable with those for other well-characterized DNA-binding proteins (26). Of the six different single-stranded oligonucleotides examined in this study, ZTA1 binds to five of them (Figure 5, lanes 1, 2, 4, 5 and 7) but does not bind to 174c oligonucleotide (Figure 5, lane 8), which is complementary to 174. This is a very interesting observation since it demonstrates for the first time that the binding of ZTA1 to ssDNA may be sequence-specific. However, the results presented in Figure 6 does not completely support this notion. To address these issues, we are now carrying out DNA-binding studies using chimeric oligonucleotides in which specific regions in 174 ssDNA are replaced by corresponding regions in 174c ssDNA. We are also examining the ability of ZTA1 to bind to homopolymers such as poly(dA), poly(dT), poly(dC) and poly(dG). Such studies aimed at understanding the molecular mechanism of ZTA1 DNA binding will be published in a separate study.

Earlier studies on analysis of SSB of bovine ZTA1 were carried out using denatured or high molecular weight bovine lens DNA and Z-DNA (14,18,19). For the first time, we report the purification of ZTA1 based on its ability to bind to ssDNA sequences of defined length. Further, the SSB activity of PpZTA1 is abolished in presence of NADPH as observed in case of bovine ZTA1 (14). Among the mammalian ZTA1 proteins studied thus far, bovine but not guinea pig ZTA1 binds ssDNA and the theoretical pI of bovine and guinea pig ZTA1 calculated based on their amino acid sequences is 8.61 and 7.81, respectively (14). It was suggested that the increased positive charge may contribute to the high affinity of bovine ZTA1 for ssDNA (14). The theoretical pI of PpZTA1 calculated based on the amino acid sequence is 8.93 which is similar to that of bovine ZTA1 rather than guinea pig ZTA1. Thus, there appears to be a good correlation between the net positive charge of ZTA1 of different species and the SSB activity.

The redox potential of cells vary during the metabolism of different carbon sources and proteins which act as redox sensors as well as regulators of gene expression may play an important role in cellular homeostasis. A number of pyridine nucleotide-binding proteins possessing nucleotide-binding domains also bind nucleic acids indicating a dual role for these metabolic enzymes in enzyme catalysis as well as gene regulation (27). It is tempting to speculate that ZTA1 may have such dual functions wherein, it not only performs catalytic function in the cytoplasm but also under conditions of reduced cytosolic NADPH levels, translocates to nucleus, bind to single-stranded regions in the promoters of genes such as *AOX1* and functions as a regulator of gene expression. ZTA1 is localized in cytoplasm as well as nucleus
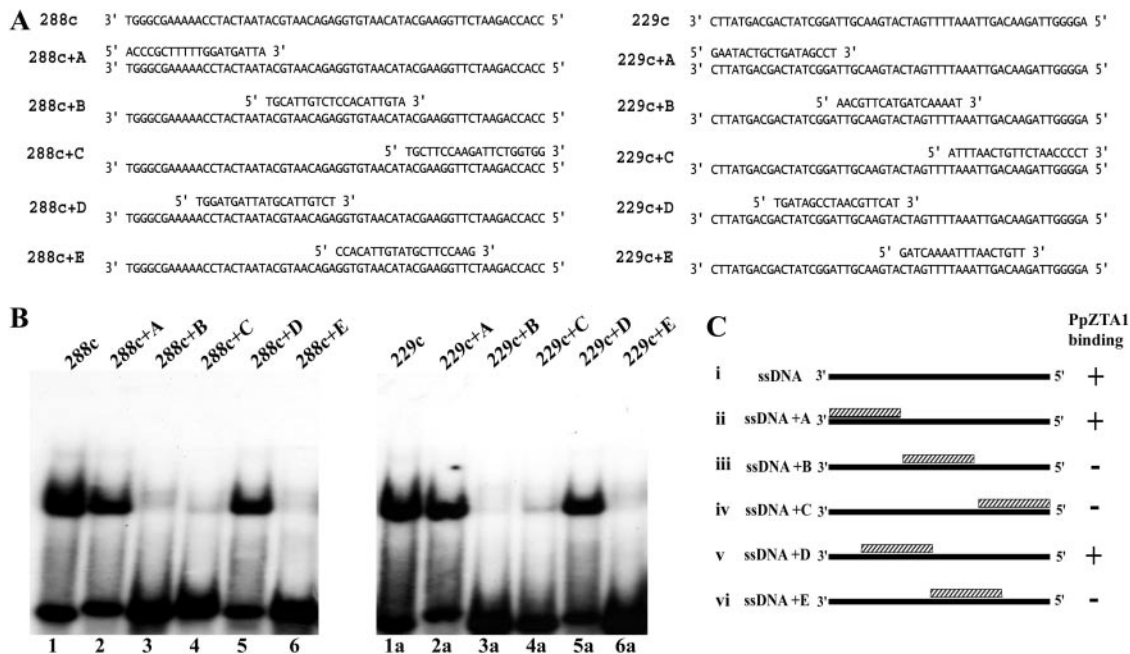
Figure 6. Analysis of SSB properties of PpZTA1. (**A**) Nucleotide sequence of ssDNA and partial duplex DNA probes used in EMSA. (**B**) EMSA of PpZTA1 using various radiolabelled probes. (**C**) Schematic representation of results of EMSA. Double-stranded regions are indicated by shaded boxes.
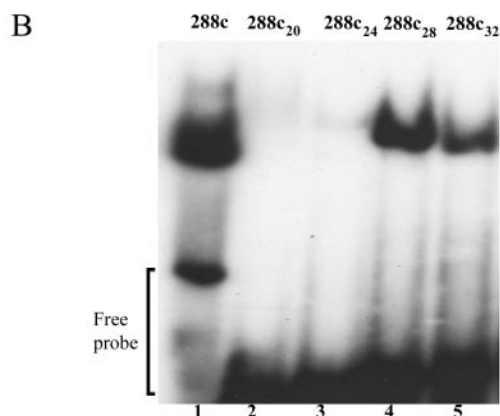

Figure 7. Identification of minimal DNA-binding site of PpZTA1. Oligonucleotides of varying length were designed based on the nucleotide sequence of 288c (**A**) and their ability to supershift recombinant PpZTA1 was examined by EMSA (**B**). The nucleotide sequence of $288c_{20}$ within the 288c oligonucleotide is underlined.
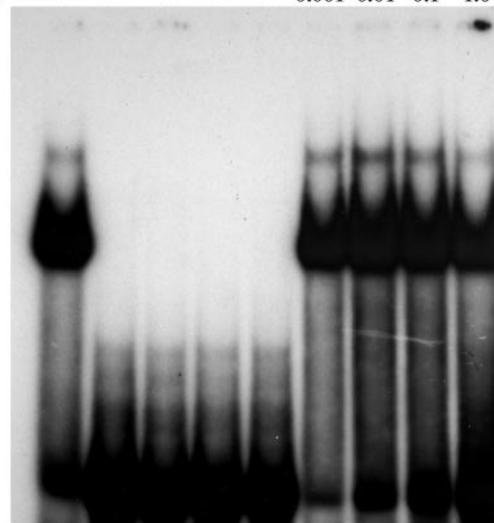

Figure 8. Effect of NADPH and NADH on the SSB activity of recombinant PpZTA1. 5′ End-labelled 229 probe was used.

in *S.cerevisiae* cells (http://yeastgfp.ucsf.edu/getOrf.php?orf =YBR046C) and preliminary studies from our laboratory indicate that PpZTA1 is also localized in both these compartments (B. V. Kranthi and P. N. Rangarajan, unpublished data). It should be noted that deletion of ZTA1 results in a

viable phenotype (http://db.yeastgenome.org/cgibin/pheno type/phenotype.pl?dbid=S000000250) in *S.cerevisiae* indicating that the function of ZTA1 may be evident only under specific growth conditions. Identification of these growth conditions as well as characterization of the DNA sequences to which ZTA1 binds *in vivo* may pave way for the understanding of the physiological functions of ZTA1.

Bovine ZTA1 was originally identified as a DNA-binding protein called regulatory factor 36 (RF-36) involved in the homeotic switch concerned with adult lens gene expression
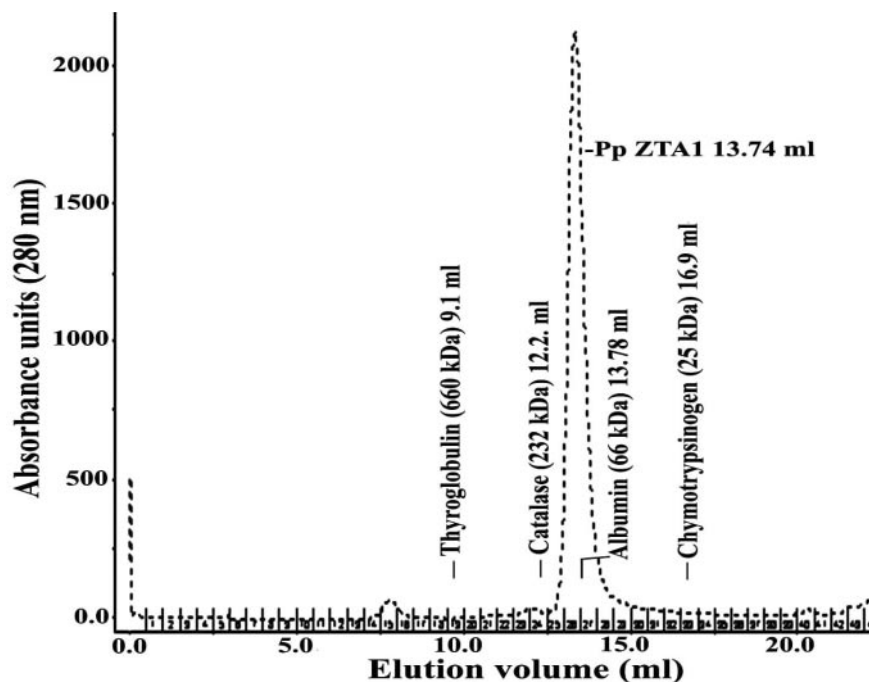
Figure 9. Gel filtration chromatography of recombinant PpZTA1. The 37 KDa PpZTA1 co-elutes with albumin indicating that it exists as a dimer in solution.

(28). RF-36 was shown to bind to alpha-crystallin A-2 gene promoter region between the TATA box and transcription initiation site which possesses a Z-DNA conformation (16,17). Addition of bovine ZTA1 (RF-36) to *in vitro* transcription reactions was shown to result in 6- to 10-fold increase in transcription of alpha-crystallin A-2 gene or purified bovine lens chromatin (15,16). It is speculated that mammalian ZTA1 may act as a transcriptional enhancer in the outer lens cortex by binding to Z-DNA sequences in the lens crystallin gene promoters (19). In the light of these observations, a role for PpZTA1 in *AOX* gene regulation cannot be ruled out. Finally, ZTA1 is induced by various oxidative stress treatments in *Arabidopsis thaliana* and it confers tolerance toward oxidative stress to yeast when introduced into the yeast, suggesting that the enzyme is involved in an antioxidative mechanism in plants (29). Whether PpZTA1 has a similar role during the oxidative stress induced by methanol and oleate metabolism in *P.pastoris* remains to be examined.

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