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Peroxidase activity of annexin 1 from *Arabidopsis thaliana* ☆,☆☆

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Abstract

On the basis of earlier reports suggesting that annexin A1 from *Arabidopsis thaliana* (AnnAt1) participates in limiting the excessive levels of reactive oxygen species during oxidative burst in plants, we examined the sensitivity of recombinant AnnAt1 to hydrogen peroxide and its peroxidase activity. Purified recombinant protein remains mostly α-helical and binds to lipids in a calcium-dependent manner. Upon oxidation recombinant AnnAt1 exhibits a tendency to form dimers in vitro. AnnAt1 is also sensitive to the presence of reducing agents, suggesting that AnnAt1 is a redox sensor in plant cells. Moreover, using two independent methods we found that AnnAt1 displayed peroxidase activity which is probably related to the presence of a heme-binding domain within AnnAt1, as present in other peroxidases. Indeed, site-directed mutagenesis within this domain resulted in a complete abrogation of the activity of AnnAt1. Furthermore, this activity was found to be sensitive to the phosphorylation state of the protein.

Keywords: Plant annexins; Oxidative stress; Oligomerization; Peroxidase activity; *Arabidopsis thaliana*

Plants are able to cope with the effects of overproduction of partly reduced oxygen species (ROS) during oxidative stress by activating numerous protective mechanisms, including the regulation of antioxidant enzymes such as catalase, superoxide dismutase, glutathione transferases, and peroxidases, by replenishing the cellular levels of natural antioxidants such as the reduced form of glutathione, or by turning on and off the expression of multiple genes encoding various antioxidant proteins, such as thioredoxin and related molecules [1]. For many of the potentially involved proteins, including some plant annexins as AnnAt1 [2–4], experimental evidence supporting their participation in the cellular response to the oxidative stress remains scant.

AnnAt1 is a 317-amino acid residue protein with an M_r of 37 kDa (p*I* 5.0) which belongs to a multifunctional family of Ca^{2+}- and membrane-binding proteins encoded in *Arabidopsis thaliana* by seven genes denoted *AnnAt1–7*. Northern blot analysis revealed that AnnAt1 mRNA represents the predominant annexin transcript in plant green organs [5]. Intracellularly, AnnAt1 mainly exists in a soluble form but is also associated with the plasma membrane and membranes of cellular organelles. Part of the membrane-associated AnnAt1 is resistant to the treatment with EGTA and can be solubilized only with ionic detergent [6]. AnnAt1, as most annexins, does not possess sequences directing it into nucleus; however, translocation of these

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☆☆ *Abbreviations:* Ann, annexin from *A. thaliana*; Anx, vertebrate annexin; DMA, dimethyl adipimidate-2 HCl; FTIR, Fourier transform infrared; IPTG, isopropyl-β-D-thiogalactopyranoside; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride.
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proteins to the nucleus has been observed upon stress stimulation or during development [7–10], often accompanied by their posttranslational modification [11]. Limited experimental evidence suggests that AnnAt1 may be involved in the response of plant cells to osmotic stress [12].

In silico analysis of the AnnAt1 primary structure revealed several potential functionally important sequences, among others the presence of a sulfur cluster in AnnAt1, similar to that originally identified in annexin Gh1 from *Gossypium hirsutum* (cotton fiber) [13], suggesting that AnnAt1 could play a role in oxidative stress response. The presence of a histidine residue in AnnAt1 which is conserved in plant peroxidases (within a peroxidase-like domain) may imply that this residue is involved in the peroxidase activity of AnnAt1 (Fig. 1). Consistent with this type of activity, the expression of some annexins [14] is induced by factors affecting the redox state of the cell. In addition, it has been suggested that annexins may contribute to the regulation of ROS levels during the oxidative burst, as described for annexins from *Medicago sativa* [8] and mammalian AnxA1, AnxA5, and AnxA6 [10,15]. It has been also reported that in a heterologous system AnnAt1 is able to protect cells from oxidative stress [4,16]. The molecular mechanism behind such protective properties of AnnAt1 is largely unknown.

In plant cells, annexins could be regarded as one of the major calcium sensors [17]. They are relatively abundant, since they can comprise as much as 0.1% of total soluble protein [18]. They are considered an important element of calcium signaling pathways [3]. Despite their relatively high abundance and their importance as calcium sensors, annexin structure and functions in plant cells remain poorly characterized. In this report, we provide experimental evidence that recombinant AnnAt1 from *A. thaliana*, expressed in *Escherichia coli* and *Nicotiana benthamiana*, could act as a peroxidase enzyme. Recombinant AnnAt1 is mostly α-helical and can bind to lipids in a calcium-dependent manner [19]. We also examined the redox sensitivity and peroxidase activity of AnnAt1. Since numerous unrelated proteins [11,20,21], as well as plant annexins [13,22], exhibit tendency to form oligomers in response to oxidative stress, we investigated whether AnnAt1 is also able to form oligomers and whether the oligomerization state is affected by the changes in redox state. Furthermore, we characterized the enzymatic activity of recombinant AnnAt1 by measuring oxidation of luminol or Amplex Red reagent in the presence of H_2O_2. To determine the role of potential posttranslational modifications of AnnAt1, we compared the properties of AnnAt1 and its mutants expressed in prokaryotic (*E. coli*) as well as in eukaryotic (*N. benthamiana*) systems.

Materials and methods

Cloning of AnnAt1 from A. thaliana, its expression in E. coli and in N. benthamiana, and preparation of polyclonal anti-AnnAt1 antibodies. To obtain a full-length cDNA encoding AnnAt1 from *A. thaliana*, total RNA was isolated from fully expanded leaves of 8-week *A. thaliana* plants. Specific primers were designed on the basis of *AnnAt1* sequence available in the NCBI database (AF083913). Cloned and purified PCR product was sequenced for identity verification, revealing 100% identity with *AnnAt1*. For expression in *E. coli*, the *AnnAt1* coding sequence was cloned into the pET 28 a(+) vector to provide an N-terminally fused His6-Tag. Recombinant AnnAt1 and the H40A AnnAt1 mutant were expressed in *E. coli* strain BL21 after induction with isopropyl-β-D-thiogaloctopyranoside (IPTG) for 4 h at 30 °C. From this step, the entire purification procedure was performed at 4 °C. The bacterial culture was centrifuged at 6000*g* for 15 min. The pellet was resuspended in a buffer containing 20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM aprotinin, 0.1 mg/ml DNase, 0.1 mg/ml RNase, and lysozyme (650 U/100 ml bacterial culture), incubated for 30 min, and sonicated three times for 1 min. The homogenate was centrifuged at 10,000*g* for 15 min. The supernatant was supplemented

A

AnxGh1	(101)	TKRWTSSNQVL**M**EIA**C**TRSA...	(235) ALLRSTVK**C**LVYPEKY**F**E
AnnAt1	(96)	TKRWTSSNQVL**M**EVA**C**TRTS...	(231) ALLRSTIQ**C**LTRPELY**F**V
AnnAt2	(96)	TKMFTKNNWVLVEIA**C**TRPA...	(231) KLLRAVIT**C**LTYPEKH**F**E
AnnAt4	(99)	LKKGEEAYNLIVEVS**C**TRSA...	(230) SLLNEALI**C**LLKPALY**F**S
AnnMs	(89)	TKMLTSNNSIIVEIASTRSP...	(223) KLLRAAIKGLTYPEKY**F**E
AnxA1	(127)	MKGLGTDEDTLIEILASRTN...	(262) KCLTAIVK**C**ATSKPAF**F**A
AnxA2	(136)	MKGLGTDEDSLIEII**C**SRTN...	(272) NAFLNLVQ**C**IQNKPLY**F**A
AnxA5	(99)	LKGAGTNEKVLTEIIASRTP...	(228) ETSGNLEQLLLAVVKSIR

B

AnnAt1	(19)	**QL**RTA**F**EGW**G**TNE--**DL**IISILA**H**
AnnAt2	(19)	**QL**HKA**F**SGW**G**TNE--K**L**IISILA**H**
AnnAt4	(19)	GMGVDENALISTL--GKSQKEHRK
peroxidase At	(166)	**QL**RQN**F**GQR**G**LSMH-**DL**VALSGG**H**
HRP type C	(147)	**QL**KDS**F**RNV**G**LNRSS**DL**VALSGG**H**

Fig. 1. Amino acid sequence alignment. (A) Evidence of the characteristic sulfur cluster comprising M107, C111, and C239 in AnnAt1. The partial amino acid sequences of the following proteins are compared: AnnGh1 from *G. hirsutum* (321 amino acid residues, NCBI 1N00_A) AnnAt1 (317 amino acid residues, NCBI NP_174810), AnnAt2 (317 amino acid residues, NCBI NP_201307), AnnAt4 (319 amino acid residues, NCBI NP_181409), and AnnMs1 from *Medicago sativa* (alfalfa) (308 amino acid residues, NCBI CAA52903), human AnxA1 (346 amino acid residues, NCBI CAI16496), human AnxA2 isoform 1 (357 amino acid residues, NCBI NP_001002858), and human AnxA5 (320 amino acid residues, NCBI NP_001145). (B) A potential peroxidase domain with active His40 residue in AnnAt1. The following sequences are aligned: AnnAt1, AnnAt2, and AnnAt4 from *A. thaliana*, a peroxidase of *A. thaliana* (317 amino acid residues, NCBI CAA67550), and a peroxidase from *Armoracia rusticana* (horseradish) (308 amino acid residues, NCBI 6ATJA).

with imidazole and NaCl to final concentrations of 10 and 300 mM, respectively. The eukaryotic expression system (*N. benthamiana*) was based on a deconstructed virus strategy [23,24] and was used due to the presence of numerous potential posttranslational modification sites in the primary structure of AnnAt1. Recombinant AnnAt1 expressed in *N. benthamiana* was obtained by homogenization of leafs in liquid nitrogen followed by resuspending the obtained powder in 20 mM Tris–HCl, pH 8.0, 300 mM NaCl, and 10 mM imidazole, and centrifugation at 12,500*g* for 10 min (this step has been repeated). AnnAt1 expressed in *E. coli* or in *N. benthamiana* was finally purified to homogeneity on a Ni–agarose (Qiagen) column eluted with a linear gradient of imidazole (10–200 mM). The purity of the obtained proteins was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining. Anti-AnnAt1 antibodies were raised in rabbits against the full-length recombinant protein expressed in bacteria at the Medical Research Center in Warsaw. The antigen (total 400 μg of protein) was injected into animals with Freund's complete adjuvant (first injection) and Freund's incomplete adjuvant (second and third injections). The antibody was purified by Protein A (Bio-Rad) affinity chromatography.

Fourier transform infrared (FTIR) spectroscopy. Recombinant AnnAt1 purified from *E. coli* (20 mg protein/ml) was dissolved in ^2H_2O buffer containing 80 mM Tris–HCl, p^{2}H 7.5, 15 μM EGTA and kept in this solution for 1 h to assure full ^1H:^2H exchange. The p^{2}H of the solution was determined with a glass electrode and was corrected by a value of 0.4 [25]. Infrared spectra (128 interferograms with 2 cm^{-1} resolution) were acquired with a Nicolet 510M FTIR spectrometer equipped with a DTGS detector. The cell (model TFC-M25, Harrick Scientific Corp.) with CaF_2 windows was kept constantly at 25 °C. The path length was 50 μm. Nine individual spectra (from three distinct batches of samples) were measured under the same conditions and were co-added to obtain the final averaged spectrum. The final spectrum of AnnAt1 was not smoothed but was corrected for H_2O vapor absorption as previously described for mammalian AnxA6 [26].

Binding of AnnAt1 to asolectin liposomes. Large unilamellar liposomes were prepared from asolectin in the presence of 0.25 M sucrose, as described previously [27]. To determine the binding of AnnAt1 to liposomes, 5 μg of the protein was incubated at 4 °C for 45 min with 10 μg lipids, in a total volume of 25 μl. The assay medium contained 10 mM Tris–HCl, pH 7.5, 1 mM EGTA, 50 mM NaCl, 0.25 M sucrose, and $CaCl_2$ in a concentration range from 0 to 5 mM. Liposomes were centrifuged at 12,000*g* for 10 min. The protein composition of supernatants and pellets was then analyzed by SDS–PAGE.

Oligomerization of AnnAt1. The oligomerization of AnnAt1 in solution was determined by three methods. First, 200 μg of AnnAt1 and 600 μg of molecular weight markers (ovalbumin of M_r 45 kDa, bovine serum albumin of M_r 67 kDa, and aldolase of M_r 160 kDa, 200 μg each) were loaded on the top of a 15 ml sucrose concentration step gradient (7 layers from 5% to 35% sucrose) in the absence and in the presence of 1 mM $CaCl_2$ and centrifuged at 104,000*g* for 24 h. The gradients were then fractionated (0.5 ml fractions beginning from the top) and their protein pattern was analyzed by means of SDS–PAGE on 14% gels. The protein amount in each fraction was quantified using the Ingenius system (Syngene). As a second method, fast performance liquid chromatography (FPLC) has been employed. AnnAt1 (1 mg) in 50 mM NaCl, 20 mM Tris–HCl, pH 7.5 (total volume 300 μl) was loaded on a Superdex 200 column (Amersham) and eluted with the same buffer at a flow rate of 0.5 ml/min. As a third method, AnnAt1 (5 μg per sample) was incubated in 50 mM NaCl, 10 mM Tri-s-HCl, pH 7.5, 1 mM EGTA, either in the absence or presence of various concentrations of H_2O_2 (as indicated in the abscissa to Fig. 4B) in combination with reducing agents dithiothreitol (DTT, up to 30 mM) or β-mercaptoethanol (up to 20 mM) and 2 mM (2-carboxyethyl)phosphine hydrochloride (TCEP) at 4 °C. Samples were collected after 15 min and analyzed by non-reducing SDS–PAGE, followed by Western blot identification of AnnAt1.

Peroxidase activity. Peroxidase activity of recombinant proteins expressed in eukaryotic and prokaryotic systems was tested using two independent methods. The first method is based on the chemiluminescence of oxidized luminol, as follows:

$$\text{luminol} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{oxidized product of luminol} + \text{light}$$

Samples containing the proteins to be analyzed were separated by non-denaturating electrophoresis, were transferred onto a nitrocellulose membrane, covered with the developing solution (ECL kit, Amersham) containing luminol, and exposed to X-ray medical film for 1 h according to the manufacturer's protocol. Alternatively, peroxidase activity of recombinant proteins including AnnAt1 H40A mutant was determined using a fluorometric method with Amplex Red reagent (Molecular Probes) on a Fluorolog 3 spectrofluorimeter (Jobin Yvon Spex, Edison, NJ) with 1-nm slits for both excitation and emission. The assay medium (total volume of 100 μl) contained 50 M potassium phosphate buffer, pH 7.4, 2 mM H_2O_2, Amplex Red reagent at a final concentration of 100 μM, and proteins and other additions as described in the figure legend. Measurements were made in quartz cuvettes of optical path length of 10 mm (0.1 ml volume). Fluorescence emission of the product of Amplex Red reagent oxidation, resorufin, was recorded at λ_{em} 590 nm (λ_{exc} 560 nm). The reaction was as follows:

$$\text{Amplex Red} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{resorufin} + H_2O$$

For the determination of the effect of protein phosphorylation on peroxidase activity, prior to measurements, AnnAt1 was incubated with alkaline phosphatase (Sigma, 15 U/ml) at 36 °C for 10 min in a potassium phosphate buffer, pH 7.4. A sample without AnnAt1 was used as a control.

Other procedures. Protein concentration was determined according to the Bradford method [28] with bovine serum albumin as a standard. SDS–PAGE (reducing and non-reducing conditions) was performed on 6% stacking and 12% or 14% resolving gels. Gels were stained with Coomassie brilliant blue [29] or silver nitrate [30] and scanned using Ingenius software to perform semi-quantitative analysis. For Western blots, nitrocellulose was incubated first in 5% bovine serum albumin for 1 h at room temperature and then with the anti-AnnAt1 antibody in concentration of 1:10,000 (in 3% bovine serum albumin) at 4 °C overnight, followed by incubation with anti-rabbit antibody (Amersham) at the same concentration (in 3% bovine serum albumin) for 1 h at room temperature. Western blots were visualized with the ECL kit (Amersham).

Results and discussion

Biochemical properties of recombinant AnnAt1

Fig. 2A shows a part of the infrared spectrum of recombinant AnnAt1 expressed in *E. coli*. This spectrum is similar to that of mammalian AnxA6 [30], indicating that AnAt1 contains mostly α-helical structures as evidenced by the presence of the major peak centered at 1652 cm^{-1} (amide 1 region). This is consistent with the X-ray structure of AnnAt1 (PDB Acc. No. 1YCN), annexin Gh1 [13] and annexin 24 from *Capsicum annuum* [31]. However, the electrophoretic mobilities (under non-denaturating conditions) of AnnAt1 expressed in *E. coli* and that expressed in *N. benthamiana* differ significantly (Fig. 2B, lanes 1c and 2c). No such difference is observed in the protein mobility on the gels run under denaturating conditions (Fig. 2B, lanes 1b and 2b). This finding may suggest that AnnAt1 undergoes posttranslational modifications that may affect biochemical properties of AnnAt1 and resulting different activities. As a typical member of the annexin family of proteins, AnnAt1 should bind lipids in a calcium-dependent manner. As shown in Fig. 3, the binding of protein to asolectin liposomes is largely prevented in the presence of a chelating agent, while it is promoted in the presence

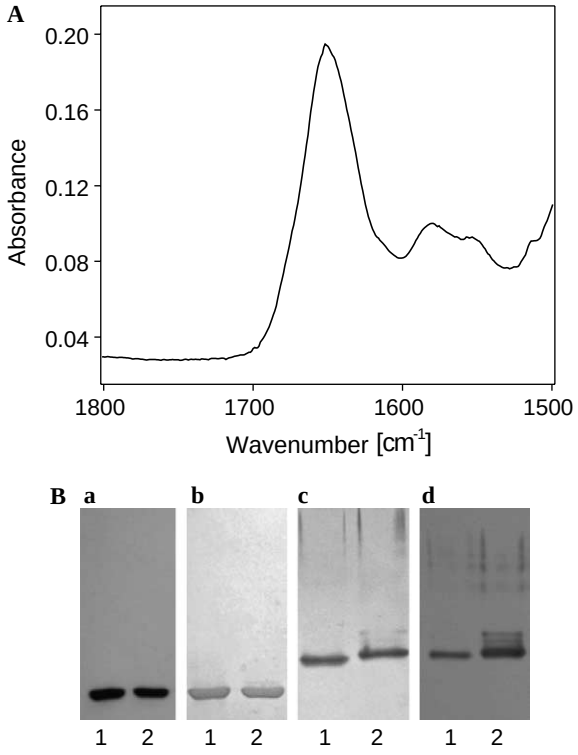

Fig. 2. Structural properties of recombinant AnnAt1. (A) Non-smoothed averaged infrared spectrum of AnnAt1 (expressed in *E. coli*) recorded in the absence of Ca^{2+}. The infrared spectrum of AnnAt1 (20 mg/ml) was recorded in a buffer containing 80 mM Tris–HCl, p^2H 7.5, 15 μM EGTA. A total of nine spectra were collected from three independent protein batches. They were co-added, averaged, and corrected for water vapor. (B) Western blot analysis (a,d) and electrophoretic pattern (b,c) of AnnAt1 expressed in *N. benthamiana* (lanes 1, 5 μg protein) or in *E. coli* (lanes 2, 5 μg protein). The gels (12%) were run either under denaturating (b) or non-denaturating conditions (c). For immunochemical identification of proteins, the polyclonal anti-AnnAt1 antibodies raised in rabbit were used (see Materials and methods).

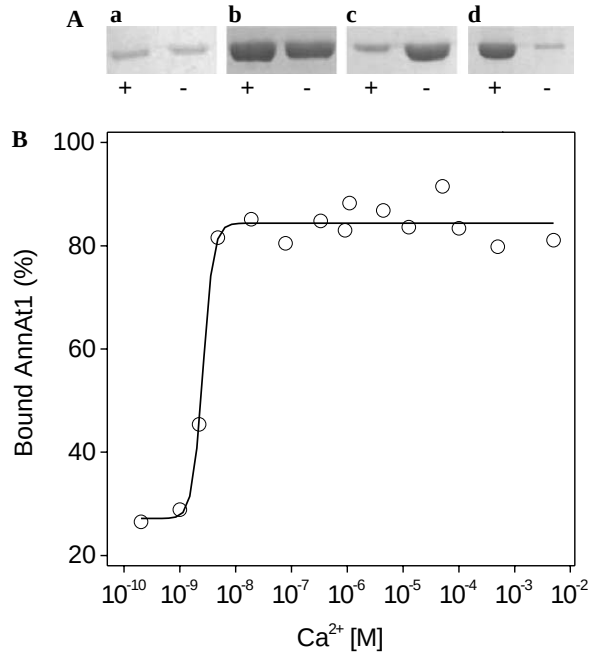

Fig. 3. Functional properties of recombinant His-tagged AnnAt1 expressed in *E. coli*. Ca^{2+}-dependent AnnAt1 binding to asolectin liposomes. (A) AnnAt1 was incubated in the absence (−) or presence (+) of asolectin liposomes in a buffer supplemented either with 20 mM EGTA (a,b) or with 5 mM $CaCl_2$ (c,d). After 30 min of incubation, samples were centrifuged at 12,000*g* for 10 min. Supernatants (b,c) and pellets (a,d) were analyzed by SDS–PAGE in 14% gels. (B) Calcium concentration dependence of AnnAt1 binding to liposomes. For further details, see Materials and methods. The mean values of three experiments varying by 5–7% are shown.

of Ca^{2+} at millimolar concentrations. A detailed analysis of calcium dependence revealed that AnnAt1 binds to liposomes with K_D for Ca^{2+} equal to $2.6 \pm 0.18 \times 10^{-9}$ M (Fig. 3). Such low calcium concentration requirement for lipid binding is not frequently found under in vitro conditions for mammalian annexins which are characterized by a calcium concentration requirement that is three orders of magnitude higher (for review see [32]). In addition, there is a significant population of the AnnAt1 molecules which bind to liposomes in the absence of calcium (Fig. 3). This is in agreement with the observations on annexins behaving as integral membrane proteins and exhibiting ion channel activity (for review, see [33]). In summary, the data presented above confirmed the suitability of recombinant protein to be used for functional investigations.

Formation of oligomers by AnnAt1 is induced by H_2O_2 and prevented by reducing agents

One of the characteristic features of plant annexins is the formation of oligomers in the presence of calcium [22]. Using recombinant AnnAt1 expressed in *E. coli* and ultracentrifugation in a sucrose concentration gradient, we confirmed that AnnAt1 remains in an equilibrium between monomer and dimer (with an apparent molecular weight of 75 kDa) when compared to marker proteins, ovalbumin, bovine serum albumin, and aldolase (Fig. 4A). Some higher molecular weight oligomers can be also detected, as observed using cross-linking agents: glutaraldehyde or DMA (not shown). By FPLC we detected protein dimers in a buffer containing no calcium (not shown), as was also observed for the calcium-binding protein calmyrin [34]. The existence of the sulfur cluster in plant annexins [13] encouraged us to hypothesize that, instead of or in addition to calcium, there may be other agents affecting oligomerization of the protein. Fig. 4B shows that also H_2O_2 in a concentration range from 50 to 500 μM is a potent inducer of oligomer formation. The formation of AnnAt1 oligomers can be fully prevented by DTT (30 mM; Fig. 4B), β-mercaptoethanol (20 mM), and TCEP (2 mM). At lower concentrations DTT (2 mM) and β-mercaptoethanol (2 mM) only partially prevented oligomerization in the presence of H_2O_2 what implies the sensitivity of AnnAt1 to the redox state. By performing computer modeling of AnnAt1 [35] we found that the S_3 cluster (M107–C111–C239) originally identified in annexin Gh1 [13] is also present in AnnAt1 (Fig. 1A). However, the possibility that the S_3

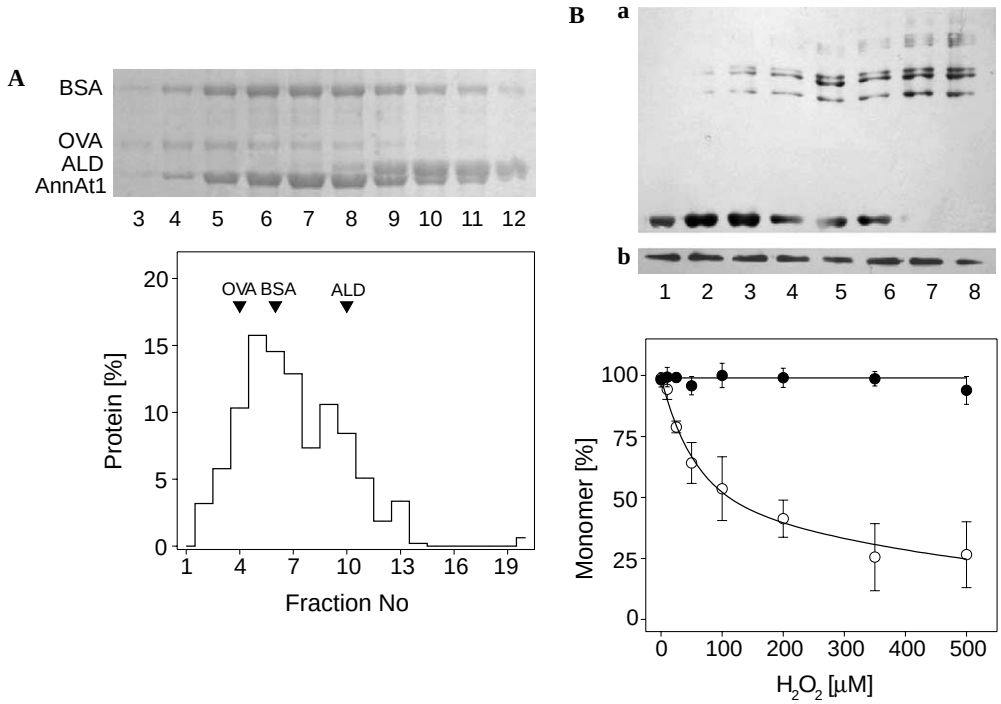

Fig. 4. Oligomer formation induced by Ca^{2+} or H_2O_2. (A) Oligomerization of AnnAt1 in the presence of 1 mM $CaCl_2$ was examined by sucrose density gradient centrifugation in the presence of bovine serum albumin (BSA—67 kDa), ovalbumin (OVA—45 kDa), and aldolase (ALD—160 kDa) as molecular weight markers. The upper panel represents the protein pattern of fractions #3 to #12 (numbered from the top of the gradient) obtained in the course of a typical experiment. The SDS–PAGE was performed on 14% gel stained with Coomassie brilliant blue. The lower panel represents a semi-quantitative analysis of AnnAt1 distribution in sucrose gradient fractions. The positions of the peaks for bovine serum albumin, ovalbumin, and aldolase are indicated. The mean values of at least three experiments varying by 4–8% are shown. (B) Inhibition of AnnAt1 oligomerization induced by H_2O_2 by reducing agents. Western blot of AnnAt1 separated by gel electrophoresis under non-reducing conditions (12% gel), preincubated in the presence of H_2O_2 at following concentrations 0 (lane 1), 10 (lane 2), 25 (lane 3), 50 (lane 4), 100 (lane 5), 200 (lane 6), 350 (lane 7), and 500 (lane 8) μM without DTT (a) or with 30 mM DTT (b) for 15 min. The lower panel represents a semi-quantitative analysis of monomers of AnnAt1 (5 μg) distribution at different H_2O_2 concentrations. Symbols: filled circles, protein after treatment with 30 mM DTT; open circles, protein without treatment with reducing agent.

cluster is involved in the oligomerization remains hypothetical. The cysteine side chains could form a disulfide bridge as evidenced by the mammalian AnxA2 crystal structure [36]. We prefer a model in which the M107–C111–C239 residues donate electron to ROS [13], and may thus constitute a redox sensor of the molecule inducing specific conformational changes of AnnAt1 or interactions with other proteins by reducing intra- or intermolecular disulfide bonds, necessary for certain complex formation as suggested by Hofmann et al. [13].

Peroxidase activity of AnnAt1

There is strong evidence that AnnAt1 in heterologous systems can protect cells against oxidative stress [16,37]. This annexin is also able to restore the ability of ΔOxyR mutants of *E. coli* to survive and to grow in the presence of H_2O_2 [4]. This could be related to a peroxidase activity of AnnAt1 or could result from the AnnAt1 ability to modulate endogenous antioxidant systems [16]. In this report, we provide experimental evidence that recombinant AnnAt1 exhibits peroxidase activity (Fig. 5). By using the H_2O_2–luminol system, widely employed for chemiluminescence detection of protein–antibody complexes, we demonstrated that AnnAt1 expressed in *N. benthamiana* is able to catalyze a peroxidase reaction similar to that of horseradish peroxidase (Fig. 5). AnnAt1 is active at both pH 7.0 and 9.0, irrespective of the presence of liposomes (Figs. 5C and D) but not at pH 5.5 (not shown). Under these experimental conditions (Fig. 5A), AnnAt1 expressed in *E. coli* has no, or a very low, activity. This may suggest that the peroxidase activity is dependent on posttranslational modifications of the protein as implied from the results of gel electrophoresis performed under non-denaturating conditions (Fig. 2B).

Another independent and more sensitive method of determination of peroxidase activity of AnnAt1 by using Amplex Red permitted us to observe this activity in both AnnAt1 expressed in *N. benthamiana* and in *E. coli*. The activity of AnnAt1 expressed in the eukaryotic system was three times higher than that of the *E. coli*-expressed protein (Fig. 6). The AnnAt1 activity was protein concentration-dependent. As negative controls, heat-inactivated proteins from *N. benthamiana* and from *E. coli,* or bovine serum albumin were used, exhibiting no activity (Table 1). The positive control, e.g., horseradish peroxidase, had an activity six orders of magnitude higher than that of AnnAt1. In summary, these findings strongly suggest that

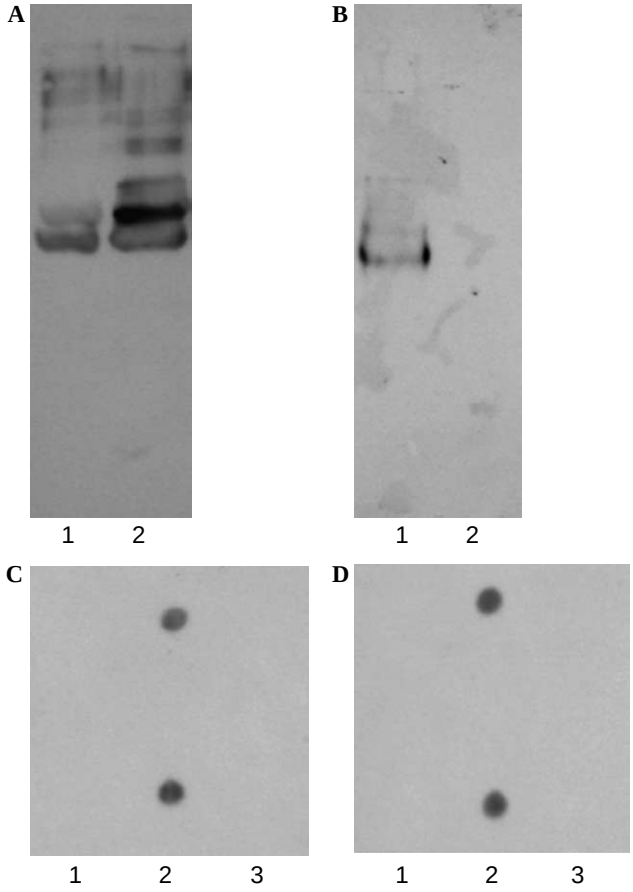

Fig. 5. Peroxidase activity of recombinant AnnAt1. AnnAt1 (5 μg) was expressed in *N. benthamiana* (A,B—lanes 1) or in *E. coli* (A,B—lanes 2), separated by gel electrophoresis under non-denaturating conditions (12% gel), electrotransferred onto nitrocellulose, and then visualized either directly with the ECL kit (A) or after incubation with anti-AnnAt1 Ab (B). Five microgram of bovine serum albumin (dot 1), 5 μg AnnAt1 expressed in *N. benthamiana* (dot 2) or 10 μg AnnAt1 expressed in *E. coli* (dot 3) were dot-blotted on nitrocellulose and developed in the ECL kit without specific antibodies at pH 7.0 (C) or pH 9.0 (D) in the absence (upper row, panels C and D) or presence of asolectin liposomes (lower row, panels C and D).

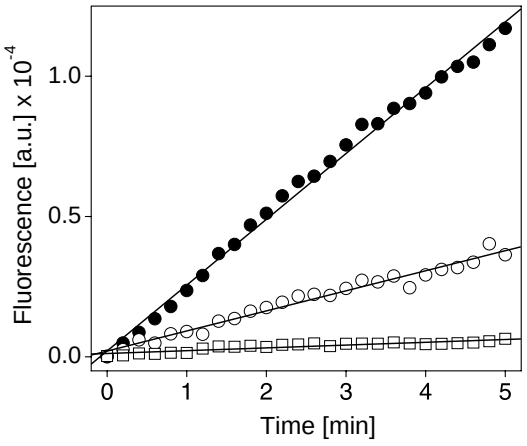

Fig. 6. Peroxidase activity of AnnAt1 measured using Amplex Red reagent—time dependence. Comparison of activities of recombinant proteins (50 μg per assay) expressed in *N. benthamiana* (filled circles), *E. coli* (open circles), and *E. coli* mutant H40A (squares).

AnnAt1 is a peroxidase. On the basis of the analysis of the primary structure of AnnAt1, a ~40-amino acid sequence with a conserved H40 residue in the N-terminus of the molecule has been identified, exhibiting similarity to a heme-binding motif of plant peroxidases (Fig. 1B [4]). Our results indicate that AnnAt1 expressed in *E. coli* and carrying the H40A mutation lacks peroxidase activity similarly to the heat-inactivated protein, confirming the functional relevance of the conserved H40 residue (Table 1). The activity of AnnAt1 expressed in *N. benthamiana* after treatment with alkaline phosphatase was lower by 53% than the activity of non-treated AnnAt1 (Table 1). Moreover, AnnAt1 expressed in *E. coli* was less sensitive to this treatment (decrease by 24% in comparison to the not treated protein, Table 1). Both observations suggest that peroxidase activity of AnnAt1 may depend on posttranslational modifications of protein, most likely on phosphorylation.

The peroxidase activity of annexins seems to be physiologically relevant, especially in terms of plant resistance to stress. Under normal growth conditions the production of ROS is low. However, various stressful conditions (drought, desiccation, chilling, heat shock, mechanical wounding, nutrient deprivation, pathogen attack or exposure of heavy metals, UV, radiation or air pollutants) can lead to a disruption of cell homeostasis, to an enhanced production of ROS, and to the feed-back between calcium signaling and ROS accumulation [38].

Conclusions

In this report, we provide experimental evidence that AnnAt1 from *A. thaliana* expressed in heterologous systems is mostly an α-helical protein that is able to bind asolectin liposomes as model membranes at submicromolar calcium concentrations. In addition, recombinant AnnAt1 binds to a limited extent to F-actin in a Ca^{2+}-independent manner. These features are characteristic for most members of the annexin family of eukaryotic proteins examined to date, sharing highly similar secondary and tertiary structures [19]. These properties are related to the presence of functional domains in the AnnAt1 molecule such as multiple endonexin folds responsible for binding of annexins to membranous anionic lipids in a calcium-dependent manner and for the F-actin binding permitting an interaction of annexins with the cytoskeleton. By examining the primary structure of AnnAt1, other potentially functional domains present exclusively in some but not all annexins may be identified, such as a sulfur cluster formed by M107, C111, and C239 residues [13], and a heme-binding domain similar to that present in plant peroxidases [4]. These domains suggest that AnnAt1 may play a role in the cellular response to oxidative stress. In the course of our work, we found that these domains might indeed be functional in AnnAt1, at least under in vitro conditions. We observed formation of dimers and larger oligomers by AnnAt1 induced by H_2O_2. Formation of such oligomers was largely prevented by the addition of reducing agents, DTT,

Table 1
Peroxidase activity of recombinant AnnAt1 measured by using Amplex reagent

Protein expressed in biological systems and further treatment as indicated	Enzyme activity $\times 10^{-4}$ (ΔF/min/mg protein)
AnnAt1 (*N. benthamiana*)	4.88 ± 1.10 ($n = 8$)
AnnAt1 (*N. benthamiana*) treated with alkaline phosphatase	2.25 ± 0.39 ($n = 2$)
AnnAt1 (*N. benthamiana*) temperature denaturated	0.37 ± 0.12 ($n = 3$)
AnnAt1 (*E. coli*)	1.69 ± 0.25 ($n = 8$)
AnnAt1 (*E. coli*) treated with alkaline phosphatase	1.28 ± 0.12 ($n = 3$)
AnnAt1 (*E. coli*) mutant H40A	0.25 ± 0.04 ($n = 4$)
AnnAt1 (*E. coli*) temperature denaturated	0.25 ± 0.05 ($n = 3$)
Horseradish peroxidase	$3.33 \pm 0.48 \times 10^6$ ($n = 5$)
Bovine serum albumin	0 ($n = 3$)

The activity is expressed as change of fluorescence (ΔF, in arbitrary units) per minute per milligram of protein determined over a time interval within which the activity was proportional to time and to protein concentration (0–50 μg per assay) ± SD. The number of determinations (n) is given in parentheses.

β-mercaptoethanol, and TCEP. Although AnnAt1 is able to sense the redox state, the protein could also act as an antioxidant enzyme by eliminating H_2O_2, and protecting the cells from oxidative stress and apoptosis. The peroxidase activity of annexin-like protein poxy5 from *A. thaliana*, both as a recombinant protein expressed in *E. coli* and as a purified protein from *A. thaliana*, was for the first time described by Gidrol et al. [4] who identified a characteristic peroxidase motif within the poxy5 N-terminal sequence. AnnAt1 from *A. thaliana* contains the same motif, however the recombinant AnnAt1 expressed in *E. coli* is characterized by a much lower peroxidase activity under our experimental conditions than that of the protein expressed in *N. benthamiana*. AnnAt1 expressed in *N. benthamiana* revealed peroxidase activity, but also differed in some overall characteristics from *E. coli*-expressed annexin suggestive of posttranslational modifications which may be of particular importance for the biological activity of AnnAt1. Further studies are required to characterize the redox-sensing domain and the peroxidase activity of AnnAt1, as well as to identify its physiological substrates and to determine its participation in the organism's defense system against oxidative stress.

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