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Biochemical and Biophysical Research Communications 306 (2003) 293–297
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Oligomerization capacity of two arylsulfatase A mutants: C300F and P425T

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Received 5 May 2003

Abstract

Arylsulfatase A (ARSA) is a lysosomal enzyme implicated in most cases of metachromatic leukodystrophy (MLD). The quaternary structure of ARSA is pH-dependent: at neutral pH, ARSA is a homodimeric protein; at lysosomal (acidic) pH, ARSA is homo-octameric. This dimer–octamer transition seems to be of major importance for the stability of the enzyme in the lysosomal milieu. Sedimentation analysis was used to study the oligomerization capacity of C300F and P425T-substituted ARSA, two MLD-associated forms of the enzyme displaying reduced lysosomal half-lives. P425T-ARSA displays a modest reduction in its octamerization capacity. In contrast, the C300F mutation strongly interferes with the octamerization process of ARSA but not with its dimerization capacity. Interestingly, a major fraction of dimeric ARSA-C300F is composed of covalently linked ARSA molecules, through a thiol-cleavable bond that probably involves Cys414 residues from each monomer. Our data support the notion that the reduced lysosomal half-life of some mutated forms of ARSA is related to deficient octamerization.

Keywords: Arylsulfatase A; Metachromatic leukodystrophy; Protein stability; Lysosome

Arylsulfatase A (ARSA) is a lysosomal enzyme involved in the catabolism of various sulfate-containing substrates. The major physiologic substrate of ARSA is 3-*O*-sulfogalactosyl ceramide (sulfatide) which accounts for 3.5–4% of the total lipids of myelin.

ARSA has been crystallized and its three-dimensional structure has been solved. The core of the enzyme consists of two β-pleated sheets, linked together by several hydrogen bonds and one disulfide bridge (Cys300–Cys414). The large central β-pleated sheet is decorated on both sides by several helices such that the enzyme has high structural resemblance to bacterial alkaline phosphatase [1].

The quaternary structure of ARSA is highly pH dependent. The enzyme oscillates between two states: dimeric and octameric. At neutral pH the dimeric form predominates; at the lysosomal acidic pH the enzyme exists mainly as a homo-octamer, composed of four dimers arranged in a ring-like structure. This pH-dependent dimer–octamer equilibrium is regulated by deprotonation–protonation of Glu424, whereas the non-covalent dimerization involves other regions of the enzyme and is not pH dependent [1].

Mutations in the ARSA gene are, by far, the most frequent cause of metachromatic leukodystrophy (MLD), a lysosomal storage disorder with autosomal recessive inheritance. The intralysosomal accumulation of sulfatide occurs mainly in oligodendrocytes, Schwann cells and neurons, and to a lesser extent in visceral organs like kidney, gallbladder, and liver [2].

Presently, more than 90 different disease-causing mutations in the ARSA gene are known (HumanGeneMutationDatabase). For many of these mutations a clear relationship between the observed genetic alteration and the enzymatic deficiency can be easily established. This is the case for some non-sense mutations or for site-splicing mutations that strongly

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0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)00969-0

interfere with the production of a normal mRNA [3–5]. However, in the case of missense mutations this task requires extensive biochemical experiments, despite the fact that the three-dimensional structure of ARSA is already known [1]. A recent report on the properties of ARSA-P426L, a mutated ARSA form displaying a normal enzymatic specific activity, illustrates this point [6]. X-ray analysis of crystallized ARSA-P426L failed to reveal any major structural alteration. However, ARSA-P426L is a very unstable enzyme in lysosomes. A complete biochemical characterization of this protein provided the answer to this problem: the P426L mutation negatively interferes with the octamerization of ARSA, a process that must occur in order to yield a protease-resistant enzyme capable of surviving in the hostile lysosomal compartment. Thus, even a subtle conformational alteration in the structure of ARSA may produce a dramatic effect on its in vivo stability.

In a previous work we described two MLD-associated mutations in the ARSA gene [7]. The first mutation, ARSA-C300F, was found in a homozygotic state in a child displaying a severe phenotype of MLD. The second mutation, ARSA-P425T, was found in a juvenile patient, in heterozygosity with one of the most frequent mutations in MLD, the P426L mutation [4]. More recently, we demonstrated that ARSA-C300F displays an almost null specific enzymatic activity whereas ARSA-P425T was found to have 12% of the activity of the normal enzyme. Data suggesting that both mutated forms are correctly targeted to the lysosomal compartment were also provided. However, pulse-chase analysis revealed that both mutated enzymes are rapidly degraded in the lysosome [8].

As an attempt to understand the reduced half-lives of these two ARSA mutant forms, we have analyzed their homopolymerization capacity. Our results indicate that ARSA-P425T displays a reduction in its octamerization capacity. The octamerization capacity of ARSA-C300F is highly reduced. Interestingly, ARSA-C300F is able to form dimers implying that formation of the Cys300–Cys414 disulfide bond is not a prerequisite for the dimerization process of the enzyme. Furthermore, a major fraction of dimeric ARSA-C300F is composed of covalently linked ARSA molecules, through a thiol-cleavable covalent bond that probably involves opposing Cys414 residues from each monomer. It is hypothesized that the structural alterations occurring at the monomer–monomer interface in the ARSA-C300F enzyme change the association angle of the two monomers impairing the octamerization process.

These data lend support to the notion that a deficiency in the octamerization process of ARSA may be the reason behind the reduced lysosomal half-lives of some mutated forms of this enzyme.

Materials and methods

Materials. Cell culture media, antibiotics, and fetal calf serum were from Invitrogen Life Technologies. Mammalian protease inhibitor cocktail was from Sigma. Centricon-10 concentrators were from Amicon. The High Molecular Weigh Calibration Kit was from Amersham–Pharmacia Biotech. Other reagents were from Sigma.

Arylsulfatase A activity determination. ARSA activity determination was done using the artificial ARSA substrate *p*-nitrocatechol sulfate [9].

Sample preparation. Stably transfected Ltk$^-$ cell lines expressing C300F-ARSA, P425T-ARSA, and WT-ARSA have been previously generated [8]. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 5% fetal calf serum. Sixteen confluent 75 cm^2 flasks of Ltk$^-$ cells expressing C300F-ARSA, P425T-ARSA, or WT-ARSA were harvested. The cells were sonicated in 0.02% (w/v) Triton X-100 with 520 μM AEBSF, 0.4 μM aprotinin, 10.5 μM leupeptin, 18 μM bestatin, 7.5 μM pepstatin A, 7 μM E-64, 50 μM iodoacetamide, 10 μM PMSF, and 1 mM EDTA. The suspensions were first clarified by a 20 min centrifugation at 15,000*g* and the supernatants were then subjected to a 20 min ultracentrifugation at 145,000*g* using a T-1270 rotor (Sorvall). The cleared supernatants were concentrated fourfold using Centricon-10 concentrators from Amicon.

Sedimentation analysis. Cell extracts containing 240 mU WT-ARSA activity or, in the case of the mutants, an equivalent amount of ARSA protein as quantified by Western blotting analysis (see below), were diluted with appropriated buffer (10 mM Tris–HCl, 150 mM NaCl (pH 7.0) or 100 mM NaAc/HAc, and 150 mM NaCl (pH 4.8 or pH 5.2)) to a final volume of 500 μl. The samples were then applied onto the top of discontinuous sucrose gradients (1.8 ml of 7.5%, 1.8 ml of 10%, 1.7 ml of 15%, 1.6 ml of 21%, 1.5 ml of 25%, and 1.4 ml of 30% (w/v) sucrose in the buffer used to prepare the corresponding sample). After centrifugation at 165,000*g* for 16 h, at 4 °C, in a TST 41.14 swing-out rotor (Sorvall), 12 fractions of 0.86 ml were collected from the bottom of the tube.

The High Molecular Weigh Calibration Kit from Amersham–Pharmacia Biotech was used to calibrate the gradients.

SDS–PAGE and Western blot. Proteins present in each gradient fraction were precipitated with 10% (w/v) trichloroacetic acid. After 30 min on ice, the precipitated protein was pelleted (15 min at 15,000*g*), washed with acetone, solubilized in Laemmli sample buffer [10], and analyzed by SDS–PAGE and Western blotting using an ARSA antiserum, raised against recombinant ARSA in rabbits. SDS–PAGE was performed in 1.0 mm thick, 10% polyacrylamide gels using the Laemmli discontinuous buffer system [10].

Results and discussion

Sedimentation analysis was used to compare the oligomerization capacity of C300F and P425T-ARSA mutants with WT-ARSA. As shown in Fig. 1, at pH 4.8 about 75% of WT-ARSA is found in fractions 4–6 corresponding to an apparent molecular mass of c.a. 500 kDa. This value is in good agreement with a molecular mass of about 496 kDa, the theoretical value expected for the ARSA octamer. About 20% of ARSA is found in fractions 8–10 (estimated molecular mass of 120 kDa c.a.), indicating that dimeric WT-ARSA (calculated molecular mass of 124 kDa) is also present under these experimental conditions. At pH 5.2, the amount of wild type ARSA in the octameric form decreases to about 60% of total (Fig. 1A, fractions 4–6)

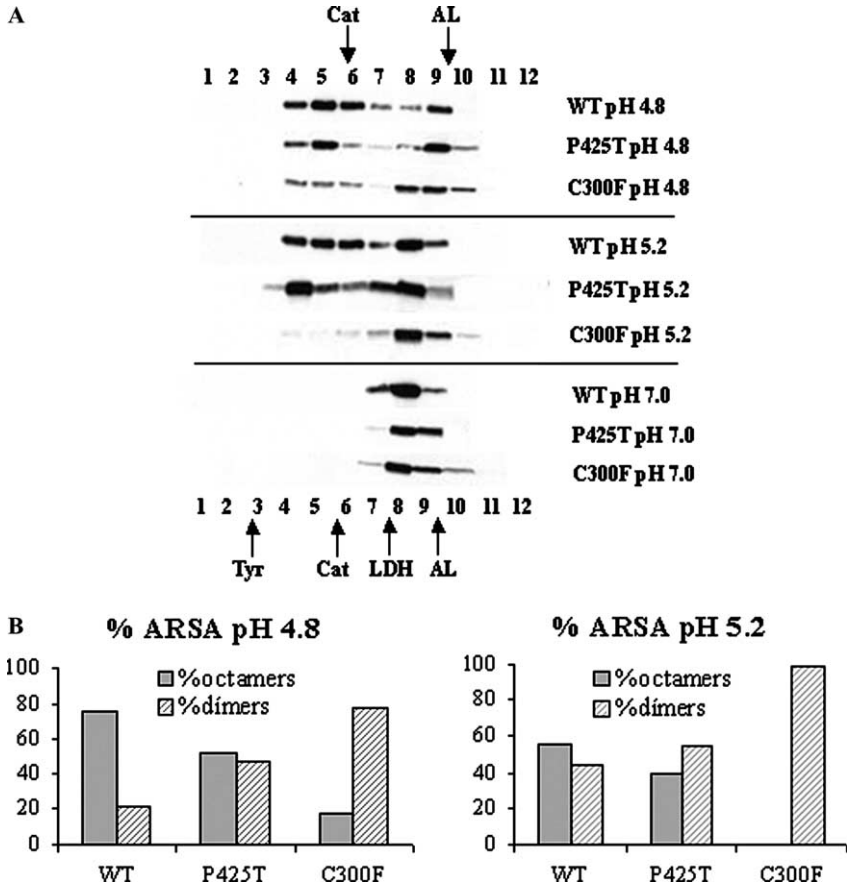

Fig. 1. Oligomerization of WT-ARSA, C300F-ARSA, and P425T-ARSA at different pH. WT, C300F, and P425T-ARSA were subjected to sucrose gradient centrifugation at pH 4.8, pH 5.2, and pH 7.0. After fractionation of the gradients (1–12 fractions from bottom to top) proteins were analyzed by SDS–PAGE and Western blot. Native molecular mass markers used to calibrate the gradient (pH 7.0): thyroglobulin (Tyr, fraction 3, 669 kDa), catalase (Cat, fraction 6, 232 kDa), lactate dehydrogenase (LDH, fraction 8, 140 kDa), and bovine serum albumin (AL, fractions 9 and 10, 67 kDa). At pH 4.8, the thyroglobulin and the lactate dehydrogenase precipitated, but catalase and bovine serum albumin maintained their localization. The results presented in the graphics result from the quantification of Western blot signals by laser scan densitometry of X-ray films. At pH 4.8, we considered dimers at fractions 8–10 and octamers at fractions 4–6. At pH 5.2, we considered dimers at fractions 7–9 and octamers at fractions 4–6, with the exception of P425T-ARSA octamers that we considered at fractions 3–5.

while the amount in the dimeric form increases to about 40% (Fig. 1A, fractions 7–9). At pH 7.0, the vast majority of the wild type enzyme is detected as a dimer (fractions 7–9, Fig. 1) as expected [1].

When the sedimentation properties of ARSA-P425T were analyzed, a different behavior was observed. At pH 4.8, the amount of ARSA-P425T in the octameric state is clearly lower than the one observed for the normal enzyme (see Fig. 1B). At pH 5.2, this difference is still detected, although not so markedly, suggesting that the P425T substitution not only shifts the pH at which half of ARSA exists as a octamer towards lower values (c.a. pH 4.8), but also the profile of the dimer–octamer equilibrium as a function of pH. Although no attempts were made to quantify these two variables, the data obtained for ARSA-P425T are very similar to the results described recently for the P426L mutation [6]. No effects on the dimerization capacity of ARSA-P425T were noted in these experiments (see Fig. 1, panel "pH 7.0").

In contrast with the results described above, the C300F mutation was found to have a dramatic effect on the octamerization process of the enzyme. Indeed, as shown in Fig. 1, only a small fraction of ARSA-C300F behaves as an octameric protein at pH 4.8. At pH 5.2, octameric ARSA-C300F is no longer detected (Figs. 1A and B). These data indicate that the C300F mutation induces conformational alterations in ARSA such that the octamerization process of the enzyme is highly affected.

As mentioned above, Cys300 forms an intramolecular disulfide bridge with Cys414. These two cysteines localize at the monomer–monomer interface (and not at the dimer–dimer interface). Thus, the observation that C300F mutation interferes with the octamerization process of ARSA would be easy to understand if dimerization of ARSA-C300F was impaired. Strikingly, this is clearly not the case. As shown in Fig. 1, the majority of ARSA-C300F behaves as a dimeric protein upon sedimentation analysis at all the pHs tested. How can this result be explained? Perhaps the easiest explanation would be to

assume that the C300F substitution results in a distortion of the monomer–monomer interface changing the association angle of the two molecules. Such dimers could still interact with other dimers but a stable ring of four dimers would be difficult to obtain. Although this hypothesis remains speculative, data showing that indeed some structural alterations at the monomer–monomer interface are introduced by the C300F mutation were obtained when the behavior of ARSA was analyzed by non-reducing SDS–PAGE. As shown in Fig. 2, when WT-ARSA is subjected to SDS–PAGE only a single band (apparent molecular mass of 62 kDa) corresponding to the monomeric protein is observed, as expected. In contrast, ARSA-C300F displays a dual behavior: a fraction of the enzyme co-migrates with the WT-ARSA; the other fraction of ARSA-C300F migrates as a 124 kDa protein. Under reducing conditions this 124 kDa protein band disappears with the concomitant increase of the 62 kDa protein band (monomeric ARSA-C300F; see lane 4). These data, together with the observation that only one protein (corresponding to ARSA) is immunoprecipitated from ^{35}S-labeled Ltk$^-$ cells expressing ARSA-C300F by an anti-ARSA antibody [8], indicate that the 124 kDa species represents a dimer of ARSA in which the two monomers are linked together by at least one disulfide bridge.

The three-dimensional structure of WT-ARSA reveals that from the 14 cysteines present in the molecule only Cys38 and Cys294 are in a reduced state. In ARSA-C300F, an additional reduced cysteine would be expected: Cys414. Although all these three cysteines are located at the monomer–monomer interface, it is plausible to assume that the structure of Cys414 will be the most affected by the C300F substitution. The presence of the hydrophobic and bulky side chain of a phenylalanine at this position could not only induce a reorientation of the sulfide group of Cys414 towards the exterior of the monomer but also bring in close proximity, at the monomer–monomer interface, the sulfide groups of Cys414 from each monomer.

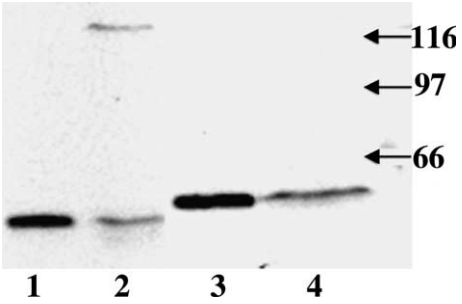

Fig. 2. Non-reducing SDS–PAGE analysis of the C300F-ARSA mutant. Proteins in homogenates of Ltk$^-$ cells, stably expressing WT (lanes 1 and 3; 80 μg protein/lane) and C300F-ARSA (lanes 2 and 4; 200 μg protein/lane), were resolved by SDS–PAGE, under reducing or non-reducing conditions, and analyzed by Western blot. Lanes 1 and 2—the homogenates were incubated with 20 mM IAA, for 10 min, at room temperature and with Laemmli sample buffer, without DTT, for 5 min at 90 °C, before being applied on the SDS–PAGE gel. Lanes 3 and 4—the homogenates were incubated with 20 mM IAA for 10 min, at room temperature, and with 100 mM DTT and Laemmli sample buffer for 5 min at 90 °C, before being applied on the SDS–PAGE gel. The numbers in the right indicate the molecular masses of the applied standards in kDa.

The aim of the work presented here was to address the relationship between the reduced in vivo half-life of some MLD-associated ARSA mutant forms and their oligomerization capacities. As suggested recently [6], mutations in the ARSA protein impairing its pH-dependent octamerization result in unstable enzymes rapidly degraded by lysosomal cathepsins. We selected for this study two mutated forms of the enzyme: ARSA-P425T and ARSA-C300F, both displaying a reduced half-life [8]. The first mutation involves an amino acid residue adjacent to Glu424, the proposed molecular switch controlling the pH-dependent dimer–octamer transition of ARSA [1]. Thus, considering the short half-life of ARSA-P425T, a deficient octamerization capacity for this mutated ARSA form would be expected, as described recently for ARSA-P426L [6]. Indeed, such phenomenon was observed—although ARSA-P425T is able to octamerize, the efficiency of this process is clearly lower than the one displayed by the normal enzyme.

The second mutation characterized in this work involves the substitution of cysteine 300 by a phenylalanine. In the normal enzyme, Cys300 lies at the monomer–monomer interface establishing an intramolecular disulfide bridge with Cys414. Thus, a priori, some effect (if any) on the dimerization process of this mutated ARSA enzyme would be expected. Strikingly, the octamerization (and not the dimerization) process of ARSA-C300F is highly impaired, a property that probably reflects the existence of an altered association angle of the two ARSA monomers. As described for the ARSA-P426L mutated form [6], it is possible that this incapacity of ARSA-C300F in forming an octameric structure results in an increased accessibility to the lysosomal proteases.

In summary, the data presented here suggest that an impairment in the octamerization process of ARSA may be the reason behind the lysosomal instability of some mutated ARSA forms.

Acknowledgments

The authors would like to thank A.M. Damas and P. Pereira for valuable discussions regarding the effect of the mutations on the three-dimensional structure of ARSA. A.M. was supported by Grant PRAXIS XXI/BD/16058/98 from FCT (Portugal).

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