The functional consequences of mis-sense mutations affecting an intra-molecular salt bridge in arylsulphatase A

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Metachromatic leukodystrophy is a lysosomal storage disorder caused by the deficiency of arylsulphatase A. We describe the functional consequences of three mis-sense mutations in the arylsulphatase A gene (Asp-335–Val, Arg-370–Trp and Arg-370–Gln), affecting an apparent intramolecular Asp-335 to Arg-370 salt bridge, and interpret the effects and clinical consequences on the basis of the three-dimensional structure of arylsulphatase A. Asp-335–Val and Arg-370–Trp substitutions each cause a complete loss of enzyme activity and are associated with the most severe form of the human disease, whereas the Arg-370–Gln-substituted enzyme retains some residual activity, being found in a patient suffering from the milder juvenile form of the disease. Detailed analysis reveals that formation of the apparent salt bridge depends critically on the presence of aspartic acid and arginine residues at positions 335 and 370, respectively. Substitution by various other amino acids, including glutamic acid and lysine, affects enzyme function severely.

Biosynthesis and immunoprecipitation studies indicate that the Asp-335–Val substitution affects folding of arylsulphatase A more severely than either the Arg-370–Trp or Arg-370–Gln substitutions. *In vitro* mutagenesis data show that clinical severity correlates with the space occupied by residue 370. The combination with structural data suggests that the bulky tryptophan residue broadens the cleft held together by the apparent salt bridge, whereas the smaller glutamine residue still allows the cleft to close, yielding a less severely affected enzyme. The position of residue 370 in the three-dimensional structure of the enzyme provides a plausible explanation for the differing severities in loss of enzyme function caused by the mutations and thus the clinical phenotype.

Key words: lipidosis, lysosomal storage disease, metachromatic leukodystrophy, sulphatide.

INTRODUCTION

Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused by the deficiency of arylsulphatase A. Arylsulphatase A catalyses the first step in the degradation of the sulphated glycosphingolipid 3-*O*-sulphogalactosylceramide, abbreviated to ‘sulphatide’. This lipid occurs in membranes of various cell types but is found in particularly high concentrations in myelin where it constitutes 3–4% of total membrane lipids. The disease is characterized pathologically by a progressive demyelination which causes various neurological symptoms. Symptoms are progressive and patients finally die in a decerebrate state (for review see [1]).

More than 60 mutations in the arylsulphatase A gene have been described [1], but few of these mutations have been characterized biochemically with regard to their effect on the properties of the enzyme. Some mutations that have been investigated biochemically cause retention of mutant arylsulphatase A in the endoplasmic reticulum (ER) [2–4], and others allow proper biosynthesis and intracellular sorting of arylsulphatase A but cause intralysosomal instability [5,6]. A genotype–phenotype correlation for MLD has been determined based on biochemical and genetic data. Patients who are homozygous for alleles that do not allow the synthesis of a functional enzyme suffer from the most severe or late infantile form of disease. Patients who are heterozygous for null alleles and alleles associated with some residual enzyme activity experience the less serious juvenile form of the disease, whereas those who are

homozygous for alleles conferring residual enzyme activity mostly suffer from the mild adult form of the disease [7,8].

It has not been possible, so far, to explain the different effects of mis-sense mutations at a structural level. Arylsulphatase A has been crystallized recently and its three-dimensional structure determined [9]. This is a prerequisite for understanding the effect of amino acid substitution on enzyme function. Arylsulphatase A displays a central wall of 13 mostly parallel strands forming a β -pleated sheet, which is surrounded on both sides by several helices (Figure 1). Separated from the central β -sheet there is an additional β -pleated sheet of four anti-parallel strands more peripherally situated in the enzyme. An apparent salt bridge is found between residues Asp-335 and Arg-370. Asp-335 is located at the beginning of one of the helices close to the centrally placed large β -sheet, whereas Arg-370 is found at the start of the small peripheral β -pleated sheet. The regions adjacent to the apparent salt bridge do not contribute directly to the formation of the active centre of the enzyme. In addition to crystallization we have also generated monoclonal antibodies which react only with the properly folded native enzyme. Their epitopes have been localized [10,11]. The availability of structure-sensitive monoclonal antibodies and three-dimensional models of the enzyme allows one to analyse and describe the structural effects of mis-sense mutations in arylsulphatase A in detail.

We have described recently two mutations (Asp-335–Val and Arg-370–Trp) in patients with the severe, late infantile form of MLD [2,12]. In this paper we describe a new mutation, Arg-370–Gln, found in a late-onset patient. Whereas it is known that

Abbreviations used: BHK, baby hamster kidney; DMEM, Dulbecco's modified essential medium; ER, endoplasmic reticulum; MLD, metachromatic leukodystrophy.

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Figure 1 Three-dimensional model of arylsulphatase A

The picture shows a ribbon-string model of the three-dimensional structure of arylsulphatase A (NCBI database accession number 1AUK). Amino acids Asp-335 and Arg-370 forming the apparent salt bridge are depicted as space-filling symbols. C β S and P β S depict the central and peripheral β -pleated sheets, respectively.

the Asp-335–Val-substituted enzyme is retained and degraded in the ER, to date neither the Arg-370–Trp- nor the Arg-370–Gln-substituted enzymes have been investigated biochemically. When the Asp-335–Val and Arg-370–Trp mutations were first described the three-dimensional structure of arylsulphatase A, and thus the potential salt-bridge formation between these residues, was still unknown. Here we examine the Asp-335–Val, Arg-370–Trp and Arg-370–Gln substitutions, comparing the possible structural effects and clinical consequences of these three salt-bridge mutations.

EXPERIMENTAL

Materials

Enzymes used for DNA modification or synthesis were obtained from New England Biolabs (Frankfurt am Main, Germany) or Invitrogen (Karlsruhe, Germany). [35 S]Methionine (specific activity > 39 TBq/mmol) and [32 P] P_i (specific activity > 111 TBq/mmol) were purchased from Amersham Biosciences (Uppsala, Sweden). Suspensions of *Staphylococcus aureus* used in immunoprecipitations were bought from Calbiochem (Darmstadt, Germany). Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany) or Eurogentec (Seraing, Belgium). The preparation and characterization of the monoclonal antibodies have been described previously [10,11].

Cell culture and transfection

Ltk $^-$ cells and baby hamster kidney (BHK) cells were maintained in Dulbecco's modified essential medium (DMEM) in the presence of 10% fetal calf serum, penicillin and streptomycin. For

transient transfections BHK cells were transfected by lipofection. For transfection 2×10^5 cells were plated on to a 3.5 cm cell-culture dish. Next day medium was removed and cells were washed with DMEM devoid of supplements. Plasmid DNA (2 μ g) was mixed with 750 μ l of DMEM containing 5 μ l of LIPOFECTAMINE $^{\text{TM}}$ reagent (Life Sciences). After 30 min of incubation the DNA–LIPOFECTAMINE complexes were added to the cells in a total volume of 1.5 ml. After 5 h of incubation LIPOFECTAMINE-containing medium was removed and replaced by DMEM/fetal calf serum. Cells were harvested and analysed for enzyme activity and protein 48 h after transfection. In the case of stable transfection, Ltk $^-$ cells were transfected by calcium phosphate precipitation as described previously. Also in the case of stable transfection one tenth of transfected plasmids was pSV $_2$ neo, carrying a neomycin-resistance gene. Cells were selected in 800 μ g/ml G418 (Invitrogen) and single colonies were screened for expression of arylsulphatase A mRNA by Northern blot analysis.

Arylsulphatase A activity determination

Arylsulphatase A activity was measured with 10 mM *p*-nitrocatecholsulphate in 170 mM NaCl, 0.5 M sodium acetate pH 5.0, 0.3% Triton X-100 and 1 mg/ml BSA. This substrate solution (200 μ l) was incubated with 5–50 μ l of cell homogenates. Reaction was performed at 37 $^{\circ}$ C for various time periods and stopped with 1 M NaOH. Absorption was read at 515 nm. To obtain measurements in the linear range, only samples with an extinction of below 0.7 were included; otherwise the determination was repeated with shorter incubation times.

DNA preparation and sequence analysis

Genomic DNA was prepared from cultivated skin fibroblasts of the patient as described in [13]. The arylsulphatase A gene was amplified in two overlapping fragments and sequenced entirely from the PCR fragments. The sequencing strategy and the sequence of primers has been described in detail [12]. DNA was sequenced with the dideoxy dye-terminator kit from ABI (Weiterstadt, Germany) and analysed on an ABI 310 automatic DNA sequence analyser.

Site-directed mutagenesis

The entire transcription unit of the vector pBEH [14] containing the arylsulphatase A cDNA was cloned into the polylinker site of the pALTER plasmid (Promega, Mannheim, Germany). *In vitro* mutagenesis was performed according to the protocol supplied by the manufacturer. In all cases introduction of the mutation was verified by DNA sequencing.

Metabolic labelling and immunoprecipitation

Metabolic labelling, immunoprecipitation and endoglucosaminidase H digestion procedures have been described in detail elsewhere [15]. In experiments in which secretion of newly synthesized enzymes was enhanced by the addition of NH_4Cl , the drug was added at a final concentration of 0.01 M and was present during labelling and chase periods. When immunoprecipitation was performed under non-denaturing conditions, SDS was omitted from all solutions and the immunoprecipitation procedure was modified accordingly. In this case, cells were harvested in 0.05 M Tris/HCl, pH 7.0/0.2% Triton-X-100 containing 25 μ g/ml leupeptin, 1 mM PMSF, 5 mM iodoacetamide and 5 mM EDTA. After removing lysate debris by centrifugation at 10000 *g* for 10 min the supernatants were adjusted to contain 5% BSA, 0.2% Triton X-100, 0.1% sodium deoxycholate and

0.15 M NaCl (buffer A). The adjusted supernatants were absorbed twice for 30 min with 100 μ l of a 10% *S. aureus* suspension, which was removed by centrifugation at 10000 *g* for 10 min. Monoclonal antibodies and antisera were added to the cleared supernatants and incubation was continued for 16 h at 4 °C. Thereafter, 5 μ g of an anti-mouse IgG antiserum, raised in rabbits, was added to the samples containing the monoclonal antibodies and incubation proceeded for another 2 h. Arylsulphatase A-antibody complexes were collected with 25 μ l of a 10% *S. aureus* suspension for 30 min. *S. aureus* pellets were washed twice in ice-cold buffer A and once with PBS.

Patients' history

Patients 1 and 2 are children of Moroccan Jewish non-consanguineous parents. Patient 1 was apparently normal until the age of 5.5 years when he presented the first neurological symptoms. With time he developed spasticity in all four limbs and by the age of 13 he became profoundly demented, quadriplegic and bedridden. Computer tomography showed, 5 years later, marked central and peripheral atrophy of the grey matter accentuated on the right hemisphere, including basal ganglia. Patient 2 began to show signs of illness at the age of 4 years with a rapid deterioration. At the age of 5 he was bedridden followed by minimal spontaneous movements. In both cases the diagnosis was confirmed by arylsulphatase A activity determinations. Patients homozygous for the Asp-335-Val and Arg-370-Trp mutations have been described recently [2,12]. Both developed symptoms before the age of 18 months and suffered from the severe late infantile form of the disease.

RESULTS

DNA sequence analysis

The arylsulphatase A gene of patient 1 was amplified in two overlapping fragments and sequenced completely. The patient was found to be homozygous for a G-to-A transition at gene nucleotide position 2094 in exon 7, causing the substitution of Arg-370 by Gln (Arg-370-Gln; results not shown).

Effect of the salt-bridge mutation on arylsulphatase A activity

The three-dimensional structure of arylsulphatase A shows that the Arg-370-Gln substitution, as well as the two previously described mutations Arg-370-Trp and Asp-335-Val, affect an apparent intramolecular salt bridge [9]. To assess the effect of these amino acid substitutions on arylsulphatase A function, BHK cells were transiently transfected with a vector expressing either wild-type arylsulphatase A cDNA, or with cDNAs encoding the substituted enzymes. Results of the transfection experiments are shown in Figure 2. All amino acid substitutions cause a severe decrease in the expression of enzyme activity in transient transfections. No enzyme activity could be expressed from plasmids coding for Asp-335-Val- and Arg-370-Trp-substituted arylsulphatase As, whereas residual enzyme activity can be expressed in the vector containing the Arg-370-Gln-substituted arylsulphatase A cDNA. An aliquot of the cell homogenate was analysed by Western blot. Arylsulphatase A cross-reacting material was present in homogenates of BHK cells expressing wild-type and mutant arylsulphatase As but not in mock-transfected controls.

Stability and sorting of mutant enzymes

In order to evaluate the effects of the amino acid substitutions in more detail, we created Ltk⁻ cells, which express stably the

Figure 2 Transient transfection of BHK cells with wild-type and mutant arylsulphatase A cDNAs

BHK cells were transfected with pBEH expression plasmids containing either the wild-type (wt) or Asp-335-Val-, Arg-370-Trp- and Arg-370-Gln-substituted enzymes. Columns show arylsulphatase A (ASA) activities per mg of cell protein; bars show maximal deviations ($n = 3$). Aliquots of the cell homogenates were subjected to Western blot analysis using an arylsulphatase A polyclonal antiserum to demonstrate the presence of cross-reacting material (bottom). Differences between the Arg-370-Trp and Arg-370-Gln ($P < 0.01$), the Asp-335-Val and Arg-370-Gln ($P < 0.022$), Arg-370-Gln and control (Co; $P < 0.03$) are statistically significant.

mutant cDNAs encoding Arg-370-Gln-, Arg-370-Trp- or Asp-335-Val-substituted arylsulphatase A.

Pulse-chase experiments were performed to investigate the stability of the mutant enzyme. Stably transfected cells expressing the normal, Arg-370-Gln- or Arg-370-Trp-substituted arylsulphatase A cDNAs were labelled for 3 h with [³⁵S]methionine and chased for 16 h (Figure 3A). In contrast to the wild-type enzyme the majority of the mutant enzymes were degraded within 16 h.

In the Golgi apparatus lysosomal enzymes are specifically recognized by a phosphotransferase, which initiates the transfer of phosphate to mannose residues of their oligosaccharide side chains. Lysosomal enzymes bind via these phosphorylated mannose residues to mannose 6-phosphate receptors, which mediate their further transport to the lysosome [16].

Figure 3(A) shows an experiment in which lysosomal targeting of newly synthesized arylsulphatase A was blocked by the addition of NH₄Cl. NH₄Cl interferes with the intracellular sorting of lysosomal enzymes in a post-Golgi compartment and leads to the secretion of newly synthesized enzymes [17]. Cells expressing the normal and mutant Arg-370-Gln- or Arg-370-Trp-substituted arylsulphatase A cDNAs were pulse-labelled with

Figure 3 Stability and sorting of Arg-370-Trp- and Arg-370-Gln-substituted arylsulphatase A

(A) Stability and secretion of Arg-370-Trp- and Arg-370-Gln-substituted arylsulphatase A. Ltk⁻ cells stably expressing arylsulphatase A cDNAs coding for the wild-type (WT; control), or the Arg-370-Trp- and Arg-370-Gln-substituted enzymes were pulse-labelled for 3 h with [³⁵S]methionine, then chased for 16 h in the presence or absence of NH₄Cl. Arylsulphatase A was immunoprecipitated from cells and media. Exposure time of Arg-370-Trp immunoprecipitates was five times longer than for control and Arg-370-Gln. (B) The wild-type (control) and the Arg-370-Gln-substituted enzyme are phosphorylated to a similar extent. Ltk⁻ cells stably expressing the wild-type arylsulphatase A, Asp-335-Val- or Arg-370-Gln-substituted arylsulphatase A were simultaneously pulse-labelled with [³⁵S]methionine and [³²P]P_i for 5 h. Arylsulphatase A was immunoprecipitated from the cells and the immunoprecipitates were split into three aliquots: two aliquots were incubated in the absence or presence of endoglucosaminidase H (EndoH) and one aliquot was frozen immediately. The Asp-335-Val-substituted enzyme was used here as a negative control; this result has been published before [2]. Top: exposure of the SDS/PAGE gel directly to X-ray film. Bottom: the weak β -emissions of ³⁵S were blocked with a piece of paper placed between the gel and the film. This part shows ³²P signals only.

[³⁵S]methionine for 3 h and chased in the presence of NH₄Cl for 16 h. In cells expressing the wild-type arylsulphatase A most of the newly synthesized arylsulphatase A is secreted into the medium upon addition of NH₄Cl. The same applies for the Arg-370-Gln- and Arg-370-Trp-substituted enzymes (Figure 3A).

The extent of mannose residue phosphorylation in the Golgi apparatus was determined in experiments in which cells expressing the Arg-370-Gln-substituted enzyme were labelled simultaneously with [³⁵S]methionine and [³²P]P_i. The arylsulphatase A immunoprecipitates obtained from such cells were digested with endoglucosaminidase H, which removes high-mannose-type oligosaccharide side chains and, thus, the phosphate label. This signal represents the [³⁵S]methionine incorporation into arylsulphatase A. A piece of paper placed in between the film and the

Figure 4 Immunoprecipitation of mutant arylsulphatase As with various monoclonal antibodies

BHK cells were transiently transfected with expression plasmids coding for the wild-type, Arg-370-Gln-, Arg-370-Trp- and Asp-335-Val-substituted arylsulphatase As. 48 h after transfection cells were labelled for 3 h with [³⁵S]methionine and arylsulphatase A (ASA) was immunoprecipitated with various monoclonal antibodies; A2, A5, B1, C, D and F. For each immunoprecipitation a control with polyclonal antiserum (P) was done in parallel. Co designates the pre-immune serum control. Immunoprecipitation was done without SDS. Numbers below the lanes give the percentage of cross-reacting material precipitated with the respective antibodies (% prec. ASA). The amount precipitated with the polyclonal antiserum was taken as 100%. Values were determined with a Fuji Bio-Imager. n.d., not detectable.

gel absorbs the weak ³⁵S β -emissions, allowing the selective detection of ³²P incorporation. Figure 3(B) demonstrates that Arg-370-Gln-substituted arylsulphatase A is phosphorylated to the same extent as wild-type arylsulphatase A.

Reactivity of mutant enzymes with monoclonal antibodies

As a means of investigating the structural integrity of the salt-bridge-deficient enzymes, mutant arylsulphatase As were immunoprecipitated using various monoclonal antibodies. These antibodies have been shown to recognize epitopes that depend on the native state of the enzyme and are lost upon denaturation [10,11]. BHK cells were transiently transfected with plasmids expressing wild-type, Asp-335-Val-, Arg-370-Trp- or Arg-370-Gln-substituted arylsulphatase A. The enzymes were separated by immunoprecipitation in the presence of 0.2% SDS, from cells metabolically labelled with [³⁵S]methionine. Whereas all monoclonal antibodies precipitated the wild-type enzyme, the mutant enzymes were only recognized by the polyclonal antiserum and not by any of the monoclonal antibodies (results not shown). The Arg-370-Gln-substituted enzyme, however, reacted weakly with antibodies A2, A5 and B1. For this reason the immunoprecipitation, which is performed routinely in the presence of 0.2% SDS followed by washing with SDS-containing high-salt buffers, was done in the absence of both SDS and high-salt (Figure 4). Under these conditions the Arg-370-Trp- and the Arg-370-Gln-substituted arylsulphatase A were partially precipitable with mAbs A2, A5, B1 and C, but not with D or F. The Asp-335-Val-substituted enzyme was less well precipitated by monoclonal antibodies A2, A5 and B1 and could not be immunoprecipitated by C, D or F.

Figure 5 Effects of various amino acid substitutions at residues 335 and 370

Various amino acid substitutions were introduced by *in vitro* mutagenesis into the wild-type arylsulphatase A cDNA and the mutated cDNAs were transiently expressed in BHK cells. Arylsulphatase A (ASA) activity and protein were determined in four independent experiments. Means $\pm$ S.D. of the activity determined are shown. The background activity measured in mock-transfected cells is not shown but was subtracted from each value. Western blot analysis was performed to demonstrate the presence of arylsulphatase A cross-reacting material. The lower band of the doublet represents arylsulphatase A, whereas the top band is frequently also found in non-transfected cells (not shown). The left-hand three lanes are from a different experiment than the other lanes.

Effect of various amino acid substitutions at residues 335 and 370

To elucidate the effect of amino acid substitutions on the apparent salt bridge in more detail, residues Asp-335 and Arg-370 were replaced by various amino acids using *in vitro* mutagenesis. The mutated cDNAs were transiently expressed in BHK cells and arylsulphatase A activity was measured in cell homogenates (Figure 5). In parallel, Western blot analysis was performed to demonstrate the presence of cross-reacting material. All amino acid substitutions at position 335 (Arg, Glu, Ile, Leu, Ala) cause a severe reduction in the expression of enzyme activity to an average of about 4% of normal. Surprisingly, even the conservative Asp-335-Glu substitution also leads to the expression of only very low residual enzyme activity. In general, residue 370 is less sensitive to amino acid substitution. Arg-370-Lys-, Asp-, Ala- or Ile-substituted arylsulphatase A cDNAs display slightly higher residual enzyme activities in the range 6–12% of normal. In order to investigate the contribution of the interaction between residues 335 and 370 to enzyme function, amino acid substitutions were performed simultaneously at both positions. Neither the introduction of a 'reversed' salt bridge (Asp-335-Arg and Arg-370-Asp; see Figure 5), nor the introduction of various pairs of apolar amino acids or two cysteines (results not shown), leads to the expression of a fully functional enzyme.

DISCUSSION

The recent elucidation of the three-dimensional structure of arylsulphatase A is a requirement for understanding the effect

of mutations leading to amino acid substitutions at a structural level. Residues Asp-335 and Arg-370 form an apparent salt bridge in arylsulphatase A [9]. Amino acid sequence comparisons among various sulphatases [18] show that, except for iduronate sulphatase, Asp-335 in arylsulphatase A is conserved among various lysosomal and non-lysosomal sulphatases, suggesting an important structural role for this residue throughout the entire sulphatase family. In a position equivalent to Arg-370 either arginine or histidine residues are conserved, again with iduronate sulphatase being the only exception. Structural data are also available for arylsulphatase B, which is involved in the degradation of sulphated glycosaminoglycans [19], confirming the presence of an apparent salt bridge in this enzyme formed between the equivalent residues Asp-352 and Arg-388. Thus with the exception of iduronate sulphatase, an equivalent salt bridge seems to be conserved within the sulphatase family.

In arylsulphatase A the apparent salt bridge is located in the interior of the enzyme. The residues involved are only partially accessible to solvent [9]. This supports recent data that provide evidence that conserved, structurally important salt bridges are, in general, less accessible from the surface and are buried within the protein [20].

We have investigated three MLD patients with mis-sense mutations affecting this apparent salt bridge (Asp-335-Val, Arg-370-Trp and Arg-370-Gln). The effects of these mutations on the enzyme are different. Asp-335-Val- or Arg-370-Trp-substitution abolish the expression of enzyme activity completely, whereas low residual enzyme activity can still be expressed from the plasmid coding for the Arg-370-Gln-substituted arylsulphatase A. This is consistent with the genotype-phenotype correlation found for MLD, in which patients with low amounts of residual enzyme activity suffer from the late-onset type of the disease [7,8]. In line with these findings the Arg-370-Gln mutation was found in a juvenile patient, whereas the other mutations are found in more severely affected, late infantile patients. Western blot analysis demonstrates the presence of arylsulphatase A cross-reacting material in transiently transfected cells expressing the mutant enzymes and also the wild-type enzyme (Figure 2). Thus we conclude that the amino acid substitutions not only affect the stability but also the specific enzymic activity of the mutant arylsulphatase A. Moreover, the presence of a salt bridge appears to be essential for the proper configuration of the active centre of arylsulphatase A, although the sequences involved do not contribute directly to the active site.

In order to understand better the influence of the apparent salt bridge on enzyme activity, we performed a detailed analysis of the amino acids involved. This yielded some unexpected results. Surprisingly, both the conservative replacement of Asp-335 by glutamic acid and the replacement of Arg-370 by lysine caused a severe reduction of enzyme activity expression. Reversal of the salt-bridge dipole (Asp-335-Arg in combination with Arg-370-Asp) also did not result in the synthesis of active enzyme. In contrast to arylsulphatase A, there are examples of various other proteins in which reversal of the salt-bridge charges does not affect protein function. Examples include the cystic fibrosis transmembrane regulator [21], the α_{1b} adrenergic receptor [22] and yeast plasma membrane H^+ -ATPase [23]. This demonstrates that the detrimental effects of the amino acid substitution on arylsulphatase A activity cannot be explained simply by the improper interaction between residues 335 and 370 due to the loss of charge. Obviously, residues Asp-335 and Arg-370 contribute to the correct structure of the enzyme in addition to their role in the formation of the apparent salt bridge.

The secretion of the Arg-370-Trp- and Arg-370-Gln-substituted enzymes upon addition of NH_4Cl and mannose

phosphorylation (Figure 3) demonstrate that at least a fraction of these enzymes can pass the Golgi apparatus and thus satisfy the ER quality-control mechanisms. Our experiments do not allow us to conclude how large this fraction is and whether a portion of the mutant enzymes will be retained in the ER. However, we demonstrate that the absence of an intramolecular salt bridge does not always lead to complete retention in the ER. By contrast, the Asp-335–Val substitution causes complete arrest of the newly synthesized enzyme in the ER [2]. The retention of the Asp-335–Val-substituted enzyme shows that this residue affects folding of arylsulphatase A in a way that residue Arg-370 does not. This conclusion is supported by the reactivity of the mutant enzymes with various structurally sensitive antibodies. Compared with either Arg-370–Trp or Arg-370–Gln, the Asp-335–Val-substituted arylsulphatase A reacts less well with mAbs A2, A5 and B1 and does not react at all with mAb C (Figure 4).

Obviously the Asp-335-substituted protein does not fold properly, in a comparable way to the Arg-370-substituted enzyme. This suggests that, during arylsulphatase A maturation, residue 335 acts at an earlier stage than residue 370.

In general, residue 370 seems to be less sensitive to substitution, displaying residual enzyme activity of 6–12% of the control value (Figure 5). The only substitution at position 370, however, which leads to a complete loss of enzyme activity, is the Arg-370–Trp-substitution found in the patient. This cannot be explained by the Arg-370–Trp substitution causing gross structural alteration to the enzyme, with the milder Arg-370–Gln substitution affecting enzyme structure far less severely. Our data demonstrate that the structural differences between the Arg-370–Gln- and Arg-370–Trp-substituted arylsulphatase A cannot be that large, since reactivity with various structurally sensitive monoclonal antibodies is comparable and both enzymes are accepted by the ER folding control mechanisms. Thus, the different effects of Arg-370 substitution on enzyme function may be explained by their local effects on the three-dimensional conformation of arylsulphatase A rather than as a result of gross structural differences.

When the apparent salt bridge is lost, weaker forces such as hydrophobic interactions may compensate partially for the salt bridge, in particular because the salt bridge is located in a region that is not exposed to solvent. The replacement of residues forming a salt bridge in the Arc repressor of bacteriophage P22 with hydrophobic residues, for example, even resulted in protein of enhanced stability compared with the wild-type repressor [24]. A small amino acid residue at position 370 is likely to permit close contact between interacting regions and may even allow weak hydrophobic interactions. This may explain the considerable 12% residual enzyme activity of the Arg-370–Ala substituted enzyme (Figure 5). Residues of intermediate size (Arg, Lys, Asp, Ile, Leu and Gln) sterically weaken these contacts, resulting in a further reduction of enzyme activity. A bulky amino acid like tryptophan, however, enlarges the cleft bridged by residues 335 and 370, interrupting the contact of adjacent regions completely to yield an entirely non-functional arylsulphatase A. Thus, the three-dimensional structure of arylsulphatase A, taken together with the *in vitro* mutagenesis experiments done in this study, provide a basis to explain the different residual enzyme activities and clinical phenotypes associated with the Arg-370–Gln and Arg-370–Trp alleles.

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