J Hum Genet (2008) 53:812–824
DOI 10.1007/s10038-008-0316-9

ORIGINAL ARTICLE

Structural characterization of mutant α-galactosidases causing Fabry disease

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Received: 2 April 2008 / Accepted: 6 June 2008 / Published online: 17 July 2008

Abstract Fabry disease is an inborn error of glycolipid catabolism resulting from lesions in the gene encoding α-galactosidase (GLA). To elucidate the basis of Fabry disease, we constructed structural models of mutant GLAs responsible for the disease and calculated indexes, i.e., the numbers of atoms affected in the main chain and side chain of each mutant GLA, the root-mean-square distance values, and the solvent-accessible surface-area values, based on 212 Fabry amino acid substitutions previously reported (196 classic and 16 variant). As two therapeutic options, enzyme replacement and enzyme enhancement, are now available for this disease, proper prediction of the natural outcome and therapeutic efficiency based on the molecular evidence for individual cases are critical for patients' quality of life. Our results revealed that structural changes in the classic Fabry group were generally large and tended to be in the core region of a protein or located in the functionally important region, including the active-site pocket. On the other hand, structural changes in the variant Fabry group were small or localized on the surface of the molecule far away from the active site. We focused on structural changes due to amino acid substitutions for which substrate analogues are effective for improving the stability or transportation of mutant GLAs, and the results of the study revealed that they are small or localized on the molecular surface, regardless of the phenotype. Coloring of affected atoms based on distances between wild type and mutant ones clearly showed the characteristic structural changes in the GLA protein geographically and subquantitatively. Structural investigation is useful for elucidation of the basis of Fabry disease and predicting disease outcome.

Keywords Fabry disease · α-Galactosidase · Amino acid substitution · Protein structure

Disclaimers: None.

Electronic supplementary material The online version of this article (doi:10.1007/s10038-008-0316-9) contains supplementary material, which is available to authorized users.

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Introduction

Lysosomal α-galactosidase (GLA, EC3.2.1.22) catalyzes hydrolysis of terminal α-D-galactosyl residues of glycoconjugates, predominantly globotriaosylceramide (GL-3), in lysosomes. The enzyme is encoded by the *GLA* gene on the long arm of the X-chromosome and is synthesized on endoplasmic reticulum (ER)-bound ribosomes as a precursor form, which consists of 429 amino acid residues. Then, the enzyme is translocated into the lumen of the ER, with subsequent cleavage of the signal peptide consisting of 31 residues. Then, the enzyme is modified in the ER by the addition of *N*-linked oligosaccharides. The oligosaccharides are then trimmed in the ER, and the enzyme is transferred to the Golgi apparatus, where further modification of sugar chains and the addition of mannose

6-phosphate residues occur. The enzyme, having mannose 6-phosphate residues at the nonreducing ends of sugar chains, is transported to endosomes via mannose 6-phosphate receptors. Subsequently, the enzyme is transported to lysosomes, where it exerts its function as a mature form consisting of 398 residues. The native GLA from humans is thought to have a homodimeric structure.

A genetic defect causes progressive accumulation of GL-3, which results in Fabry disease (MIM 301500) (Desnick et al. 2001). This disease exhibits a wide clinical spectrum. Patients with the classic form having no GLA activity develop systemic manifestations, including pain in peripheral extremities, hypohidrosis, angiokeratoma, corneal clouding, renal failure, and cardio- and cerebrovascular disorders. On the other hand, there are also variant Fabry-disease patients with residual GLA activity and milder clinical manifestations, sometimes limited to heart disorders. So far, more than 500 genetic mutations causing Fabry disease have been reported (Desnick et al. 2001). Among them, gross alterations of the *GLA* gene have been identified in patients with the classic form, but missense mutations comprising the majority of mutations have been found in both classic and variant forms.

Recombinant GLAs produced in Chinese hamster ovary cells and human fibroblasts have been developed and are clinically available for enzyme replacement therapy for Fabry disease (Eng et al. 2001a, b; Schiffmann et al. 2000). Recently, another potential approach for treating Fabry disease was developed, and a clinical trial has been performed. This enzyme enhancement therapy is based on the ability of substrate analogues including galactose and 1-deoxygalactonojirimycin to improve the stability or transportation of mutant GLAs in cells, but the therapy is only efficient in a limited group of patients having specific missense mutations (Frustaci et al. 2001; Yam et al. 2006; Fan and Ishii 2007). As a high incidence of variant Fabry disease has been revealed by newborn screening (Spada et al. 2006), prediction of the clinical outcome of the disease is becoming more and more important to determine a proper schedule for treating the disease.

Previously, we built structural models of mutant GLAs resulting from 161 missense mutations by means of homology modeling with SYBYL/BIOPOLYMER (TRIPOS, St Louis, MO, USA) and examined the correlation between structural changes in GLAs and clinical and biochemical phenotypes (Matsuzawa et al. 2005).

Recently, we developed a structural analysis system for mutant proteins involving molecular modeling software, TINKER, developed by Ponder et al. (Department of Biochemistry and Molecular Biophysics, Washington University) (Ren and Ponder 2003), which is available worldwide. We applied it to investigations on lysosomal diseases including mucopolysaccharidosis type 6 (Saito et al. 2008), mucopolysaccharidosis type 1 (Sugawara et al. 2008), and Tay-Sachs disease (Ohno et al. 2008). We believe that the standardization of a structural analysis method will enable us to compare the results for different genetic disorders, which will provide us with a deeper insight into the basis of genetic disorders. Furthermore, because TINKER is free software, other researchers can easily conduct follow-up studies.

In this study, we conducted further structural investigation of Fabry disease using the same structural analysis system. We increased the number of Fabry patients for the analysis and examined structural changes in GLAs due to 212 amino acid substitutions by determining the number of atoms affected, the root-mean-square distance (RMSD), and the solvent-accessible surface area (ASA). Then, we paid attention to mutant GLAs for which substrate analogues are effective for stabilization or transportation to lysosomes and characterized their structural changes by coloring the affected atoms.

Materials and methods

Amino acid substitutions causing classic and variant Fabry disease

In this study, we analyzed 212 missense mutations (196 classic and 16 variant) responsible for Fabry disease. Amino acid substitutions, phenotypes, and references are summarized in Table 1.

Development of a structural analysis system for mutant proteins

We developed a structural analysis system for mutant proteins to examine their structural changes responsible for genetic diseases from various viewpoints. This system comprises six stages: (1) modeling mutant proteins, (2) determining the number of atoms affected by amino acid substitutions, (3) determining the RMSD values of all atoms in the mutant proteins, (4) determining ASA values of amino acid residues in the mutant proteins, (5) statistical analysis, and (6) coloring the atoms affected in the mutant proteins based on the differences between wild-type and mutant ones. Then, we applied the system to elucidation of the basis of Fabry disease.

Structural modeling of mutant GLAs responsible for Fabry disease and determination of the number of atoms affected by amino acid substitutions

Structural modeling of mutant GLAs was performed using molecular modeling software TINKER (Kundrot et al. 1991; Dudek and Ponder 1995; Kong and Ponder 1997;

Table 1 Fabry mutations, structural changes in α-galactosidase, and phenotypes

Mutation	Number of affected atoms		RMSD	ASA	Active-site	Phenotype	Reference
	Main chain	Side chain	(Å)	(Å^2)	pocket*		
N34S	5	2	0.028	27.2		Classic	Eng et al. (1993)
N34K	165	151	0.196	27.2		Classic	Shabbeer et al. (2006)
P40S	9	8	0.025	1.3		Classic	Koide et al. (1990)
P40L	78	75	0.108	1.3		Classic	Ashton-Prolla et al. (2000)
T41I	10	38	0.031	4		Classic	Shabbeer et al. (2006)
M42V	12	13	0.028	4.9		Classic	Davies et al. (1996)
M42T	9	9	0.023	4.9		Classic	Shabbeer et al. (2002)
G43V	182	231	0.123	0		Classic	Shabbeer et al. (2002)
G43D	136	160	0.115	0		Classic	Iga et al. (2001)
G43R	414	470	0.178	0		Classic	Germain et al. (2002)
H46Y	84	119	0.088	0		Classic	Blaydon et al. (2001)
H46R	24	23	0.041	0		Classic	Eng et al. (1997)
W47G	0	9	0.025	24.3	+	Classic	Blaydon et al. (2001)
E48K	206	287	0.127	26.6	+	Classic	Rodríguez-Marí et al. (2003)
R49S	370	373	0.255	58.1		Classic	Davies et al. (1996)
R49P	227	272	0.164	58.1		Classic	Blaydon et al. (2001)
R49L	242	259	0.201	58.1		Classic	Davies et al. (1994)
R49G	179	196	0.164	58.1		Classic	Germain et al. (2002)
F50C	2	11	0.014	3.7		Classic	Shabbeer et al. (2002)
C52S	2	1	0.022	45.9	+	Classic	Eng et al. (1994)
C52R	209	272	0.14	45.9	+	Classic	Blanch et al. (1996)
C56Y	58	67	0.131	39.8		Classic	Davies et al. (1996)
C56F	67	76	0.127	39.8		Classic	Shabbeer et al. (2005)
C56G	52	61	0.134	39.8		Classic	Eng et al. (1993)
E59K	187	203	0.216	52.5		Classic	Eng et al. (1994)
L68F	39	70	0.053	0		Classic	Shabbeer et al. (2002)
M72I	34	43	0.052	0		Classic	Germain et al. (2002)
W81S	38	46	0.05	0.4		Classic	Rodríguez-Marí et al. (2003)
Y86C	9	13	0.032	0		Classic	Eng et al. (1997)
L89P	6	12	0.022	0		Classic	Eng et al. (1997)
L89R	287	337	0.164	0		Classic	Eng et al. (1994)
D92Y	401	572	0.181	0.2	+	Classic	Eng et al. (1997)
D92H	321	477	0.139	0.2	+	Classic	Davies et al. (1996)
D93G	239	287	0.146	0.3	+	Classic	Davies et al. (1996)
D93N	64	132	0.073	0.3	+	Classic	Dobrovolny et al. (2005)
D93V	206	289	0.131	0.3	+	Classic	Shabbeer et al. (2006)
C94Y	195	236	0.179	0.2	+	Classic	Eng et al. (1997)
C94S	31	40	0.044	0.2	+	Classic	Blaydon et al. (2001)
W95S	5	12	0.021	0		Classic	Ashton-Prolla et al. (2000)
A97P	33	30	0.054	11.6		Classic	Kimura et al. (2002)
A97V	10	12	0.023	11.6		Classic	Eng et al. (1997)
R100K	55	40	0.051	29		Classic	Eng et al. (1994)
R100T	194	218	0.131	29		Classic	Eng et al. (1997)

Table 1 continued

Mutation	Number of affected atoms		RMSD	ASA	Active-site	Phenotype	Reference
	Main chain	Side chain	(Å)	(Å^2)	pocket*		
R112C	84	86	0.075	25.7		Classic	Ashton-Prolla et al. (2000)
R112S	28	40	0.038	25.7		Classic	Shabbeer et al. (2005)
A121T	32	40	0.061	1		Classic	Matsuzawa et al. (2005)
A121P	88	86	0.119	1		Classic	Kotanko et al. (2004)
G128E	37	44	0.051	39.5		Classic	Blanch et al. (1996)
L131P	57	51	0.052	0.4		Classic	Eng et al. (1994)
G132R	449	543	0.188	0.2		Classic	Shabbeer et al. (2002)
Y134S	166	231	0.128	0	+	Classic	Eng et al. (1997)
A135V	7	16	0.028	0		Classic	Dobrovolny et al. (2005)
D136H	362	463	0.248	0	+	Classic	Ashley et al. (2001)
G138R	209	258	0.279	0		Classic	Eng et al. (1997)
G138E	168	207	0.122	0		Classic	Germain et al. (2002)
T141I	59	58	0.097	0	+	Classic	Shabbeer et al. (2002)
C142Y	7	20	0.037	39.9	+	Classic	Okumiya et al. (1995a)
C142R	50	90	0.065	39.9	+	Classic	Topaloglu et al. (1999)
C142W	0	10	0.01	39.9	+	Classic	Schäfer et al. (2005)
A143P	4	5	0.014	47.1	+	Classic	Eng et al. (1994)
G144V	1	0	0.005	31.8	+	Classic	Eng et al. (1994)
S148R	137	180	0.106	0		Classic	Eng et al. (1997)
S148N	21	26	0.04	0		Classic	Ashton-Prolla et al. (2000)
D155H	404	455	0.353	0		Classic	Dobrovolny et al. (2005)
A156V	70	74	0.069	0.5		Classic	Okumiya et al. (1995a)
A156T	14	13	0.031	0.5		Classic	Schäfer et al. (2005)
W162C	35	32	0.045	24.3		Classic	Germain et al. (1996)
W162R	31	51	0.046	24.3		Classic	Eng et al. (1993)
G163V	2	7	0.017	15.2		Classic	Eng et al. (1997)
D165V	294	319	0.156	3.1		Classic	Davies et al. (1994)
L166V	14	14	0.031	0.3		Classic	Okumiya et al. (1995a)
L166G	72	85	0.072	0.3		Classic	Shabber et al. (2006)
L167P	110	120	0.094	0		Classic	Morrone et al. (2003)
L168R	0	22	0.014	2.5	+	Classic	Shabbeer et al. (2002)
D170V	89	134	0.072	0	+	Classic	Eng et al. (1997)
D170H	224	286	0.117	0	+	Classic	Rodríguez-Marí et al. (2003)
G171D	81	94	0.091	3.3	+	Classic	Shabbeer et al. (2005)
C172Y	12	26	0.041	34	+	Classic	Eng et al. (1994)
C172F	12	24	0.039	34	+	Classic	Schäfer et al. (2005)
C172R	18	41	0.048	34	+	Classic	Ashton-Prolla et al. (2000)
C172G	10	7	0.028	34	+	Classic	Yasuda et al. (2003)
G183D	262	297	0.205	3.8		Classic	Topaloglu et al. (1999)
G183S	64	108	0.09	3.8		Classic	Shabbeer et al. (2002)
M187V	7	13	0.029	0		Classic	Ashton-Prolla et al. (2000)
M187T	0	0	0.007	0		Classic	Shabber et al. (2006)
V199M	63	88	0.056	0		Classic	Shabbeer et al. (2002)
S201F	0	2	0.007	8.2		Classic	Shabbeer et al. (2005)
S201Y	0	0	0.01	8.2		Classic	Shabber et al. (2006)
C202Y	333	387	0.195	0.4		Classic	Eng et al. (1997)
C202W	275	312	0.174	0.4		Classic	Ploos van Amstel et al. (1994)
P205T	6	16	. 0 021	0		Classic	Davies et al. (1996)

Table 1 continued

Mutation	Number of affected atoms		RMSD	ASA	Active-site	Phenotype	Reference
	Main chain	Side chain	(Å)	(Å^2)	pocket*		
P205R	465	584	0.26	0		Classic	Shabbeer et al. (2002)
Y207S	3	4	0.018	47.5	+	Classic	Shabbeer et al. (2002)
Y216D	190	251	0.148	7.6		Classic	Eng et al. (1997)
I219N	2	9	0.014	0		Classic	Eng et al. (1994)
C223Y	455	548	0.221	0		Classic	Shabbeer et al. (2002)
C223R	516	589	0.256	0		Classic	Shabbeer et al. (2002)
N224S	40	42	0.052	0		Classic	Ashton-Prolla et al. (2000)
N224D	68	72	0.061	0		Classic	Guffon et al. (1998)
W226R	30	35	0.035	0.4		Classic	Ashton-Prolla et al. (2000)
R227Q	86	119	0.082	12.9	+	Classic	Enget et al. (1993)
A230T	0	0	0.003	57.7	+	Classic	Ashton-Prolla et al. (2000)
D234Y	362	472	0.274	36.6		Classic	Shabbeer et al. (2002)
D234E	20	25	0.043	36.6		Classic	Shabbeer et al. (2005)
S235C	0	0	0.004	51.5		Classic	Topaloglu et al. (1999)
W236L	0	3	0.006	40.8		Classic	Topaloglu et al. (1999)
W236C	2	7	0.012	40.8		Classic	Davies et al. (1996)
W236R	6	23	0.025	40.8		Classic	Shabber et al. (2006)
I239T	90	107	0.1	0.1		Classic	Kotanko et al. (2004)
I242N	13	14	0.032	0.6		Classic	Takata et al. (1997)
L243F	1	7	0.012	0.1		Classic	Germain et al. (2002)
D244N	16	44	0.037	68.8		Classic	Eng et al. (1994)
D244H	211	276	0.118	68.8		Classic	Topaloglu et al. (1999)
G260A	3	1	0.009	9.3		Classic	Okumiya et al. (1995b)
G261D	338	327	0.22	0		Classic	Takata et al. (1997)
N263S	20	36	0.04	3.4		Classic	Eng et al. (1997)
D264V	256	348	0.135	10.5		Classic	Eng et al. (1993)
D264Y	101	128	0.086	10.5		Classic	Shabbeer et al. (2005)
D266V	23	40	0.036	4.9	+	Classic	Eng et al. (1993)
D266H	435	585	0.196	4.9	+	Classic	Ashton-Prolla et al. (2000)
D266E	42	70	0.062	4.9	+	Classic	Germain et al. (2002)
D266N	44	68	0.056	4.9	+	Classic	Lee et al. (2000)
M267I	72	100	0.093	5.3	+	Classic	Topaloglu et al. (1999)
M267R	123	181	0.095	5.3	+	Classic	Shabber et al. (2006)
V269A	9	17	0.029	0		Classic	Davies et al. (1993)
V269M	118	132	0.107	0		Classic	Shabber et al. (2006)
G271C	51	54	0.063	0		Classic	Shabbeer et al. (2002)
G271S	59	57	0.068	0		Classic	Shabber et al. (2006)
G271V	197	239	0.149	0		Classic	Shabber et al. (2006)
N272K	62	102	0.067	3.9		Classic	Eng et al. (1994)
N272S	2	3	0.013	3.9		Classic	Verovnik et al. (2004)
S276G	0	7	0.009	46.7		Classic	Shabbeer et al. (2005)
Q279H	103	123	0.094	17.4		Classic	Blaydon et al. (2001)
Q279R	51	54	0.057	17.4		Classic	Rodríguez-Marí et al. (2003)
Q280K	29	41	0.041	0		Classic	Dobrovolny et al. (2005)
T282N	6	9	0.019	0.1		Classic	Ashley et al. (2001)
Q283P	302	320	0.165	0		Classic	Shabber et al. (2006)
M284T	0	3	0.011	0		Classic	Blanch et al. (1996)
A285D	30	29	0 041	0		Classic	Shabber et al. (2006)

Table 1 continued

Mutation	Number of affected atoms		RMSD	ASA	Active-site	Phenotype	Reference
	Main chain	Side chain	(Å)	(Å^2)	pocket*		
A285P	23	30	0.044	0		Classic	Shabbeer et al. (2005)
W287G	6	12	0.022	0.2		Classic	Davies et al. (1996)
W287C	15	14	0.027	0.2		Classic	Eng et al. (1997)
A288P	54	66	0.071	0		Classic	Shabbeer et al. (2002)
A288D	21	11	0.045	0		Classic	Eng et al. (1994)
I289F	319	347	0.182	0		Classic	Topaloglu et al. (1999)
M290I	14	22	0.029	3.2		Classic	Shabber et al. (2006)
P293A	73	52	0.068	2.5		Classic	Shabbeer et al. (2002)
P293T	14	19	0.032	0		Classic	Shabber et al. (2006)
S297F	208	264	0.144	0		Classic	Eng et al. (1993)
S297C	0	1	0.012	0		Classic	Germain et al. (2002)
N298S	18	19	0.031	0		Classic	Eng et al. (1997)
N298K	106	182	0.091	0		Classic	Blanch et al. (1996)
R301P	184	246	0.158	41.8		Classic	Ashley et al. (2001)
R301G	142	194	0.151	41.8		Classic	Lai et al. (2001)
I303N	3	3	0.016	25.9		Classic	Shabbeer et al. (2002)
L310F	71	88	0.085	0.1		Classic	Calado et al. (2004)
Q312H	61	69	0.069	23.5		Classic	Shabber et al. (2006)
D313Y	80	116	0.096	38.3		Classic	Eng et al. (1993)
N320Y	237	277	0.182	0		Classic	Ashton-Prolla et al. (2000)
N320K	165	167	0.109	0		Classic	Okumiya et al. (1995b)
Q321E	32	32	0.042	37.5		Classic	Topaloglu et al. (1999)
Q321R	22	24	0.035	37.5		Classic	Shabber et al. (2006)
Q327K	129	109	0.087	2.7		Classic	Davies et al. (1993)
G328A	158	151	0.104	0		Classic	Eng et al. (1993)
G328R	559	582	0.282	0		Classic	Ishii et al. (1992)
G328V	218	239	0.158	0		Classic	Shabbeer et al. (2005)
E338K	267	287	0.148	0		Classic	Shabbeer et al. (2005)
E341D	283	285	0.166	0.1		Classic	Shabbeer et al. (2002)
R342Q	51	31	0.059	0		Classic	Ploos van Amstel et al. (1994)
A348P	11	1	0.035	11.2		Classic	Shabber et al. (2006)
A352D	69	67	0.062	0		Classic	Morrone et al. (2003)
N355K	163	188	0.134	0		Classic	Germain et al. (2002)
R356W	98	114	0.076	13.5		Classic	Bernstein et al. (1989)
E358K	575	662	0.294	84.8		Classic	Miyazaki et al. (1998)
E358G	119	176	0.101	84.8		Classic	Germain et al. (2002)
E358A	107	177	0.093	84.8		Classic	Shabbeer et al. (2005)
G360S	2	2	0.026	54.2		Classic	Dobrovolny et al. (2005)
G361R	3	2	0.019	25.9		Classic	Davies et al. (1993)
P362L	4	8	0.021	99.2		Classic	Shabbeer et al. (2002)
R363H	63	59	0.079	33.7		Classic	Cooper et al. (2000)
R363C	167	196	0.254	33.7		Classic	Shabbeer et al. (2002)
G373S	1	0	0.004	0.3		Classic	Okumiya et al. (1995b)
G373D	105	118	0.118	0.3		Classic	Germain et al. (2001)
A377D	79	89	0.101	0.6		Classic	Blaydon et al. (2001)
C378Y	187	193	0.217	0		Classic	Topaloglu et al. (1999)
C382Y	242	245	0.203	0		Classic	Rodríguez-Marí et al. (2003)
I384N	5	4	0.016	0		Classic	Shabbeer et al. (2002)

Table 1 continued

Mutation	Number of affected atoms		RMSD	ASA	Active-site	Phenotype	Reference
	Main chain	Side chain	(Å)	(Å^2)	pocket*		
T385P	34	32	0.046	18.2		Classic	Shabbeer et al. (2002)
Q386P	208	236	0.149	7.9		Classic	Shabber et al. (2006)
E398K	144	172	0.183	72.4		Classic	Shabbeer et al. (2002)
I407K	169	195	0.173	4.8		Classic	Rodríguez-Marí et al. (2003)
P409S	7	4	0.021	42.1		Classic	Germain et al. (2002)
P409A	13	10	0.049	42.1		Classic	Blaydon et al. (2001)
G411D	274	294	0.2	0.7		Classic	Matsuzawa et al. (2005)
L414S	0	0	0.003	0		Classic	Rodríguez-Marí et al. (2003)
G35R	1	0	0.008	52.2		Variant	Davies et al. (1994)
E66Q	23	29	0.047	21.4		Variant	Ishii et al. (1992)
M72V	6	6	0.025	0		Variant	Okumiya et al. (1998)
I91T	0	3	0.011	0.1		Variant	Eng et al. (1997)
R112H	70	71	0.082	25.7		Variant	Eng et al. (1994)
F113L	4	2	0.017	3.6		Variant	Eng et al. (1997)
P146S	13	21	0.026	6.9		Variant	Ploos van Amstel et al. (1994)
N215S	0	0	0.004	77.2		Variant	Eng et al. (1993)
S247C	0	2	0.004	34.5		Variant	Germain et al. (2002)
P259L	10	8	0.03	56.8		Variant	Topaloglu et al. (1999)
Q279E	33	25	0.034	17.4		Variant	Ishii et al. (1992)
M296V	7	12	0.022	0		Variant	von Scheidt et al. (1991)
M296I	11	17	0.023	0		Variant	Nakao et al. (1995)
R301Q	100	146	0.119	41.8		Variant	Sakuraba et al. (1990)
I317T	2	0	0.01	16		Variant	Sachdev et al. (2002)
T410A	0	0	0 006	18 7		Variant	Yang et al. (2003)

Amino acid substitutions for which substrate analogues are effective are indicated in *red*

RMSD root-mean-square distance, *ASA* solvent-accessible surface area

Pappu et al. 1998; Ren and Ponder 2003). The crystal structure of human GLA (Garman and Garboczi 2004) (PDB: 1R46) was used as a template, and energy minimization was performed. The root-mean-square gradient value was set at 0.05 kcal/mol Å. Each mutant model was then superimposed on the wild-type GLA structure based on Cα atoms by the least-square-mean fitting method (Kabsch 1976, 1978; Sakuraba et al. 2000, 2004). In this study, we defined that the structure was affected by an amino acid substitution when the position of an atom in a mutant differed from that in the wild type by more than the cutoff distance (0.15 Å) based on total RMSD, as described previously (Matsuzawa et al. 2005). Then, we determined the numbers of atoms affected in GLA main chain and side chain.

Determination of RMSD values of all atoms in mutant GLAs

RMSD values of all atoms in mutant GLAs were determined according to Weiner's method (Weiner et al. 1984) to predict the degrees of GLA structural changes, and the average RMSD values for the classic and variant Fabry groups were determined and compared with each other, as described previously (Sugawara et al. 2008).

Determination of ASA values of amino acid residues in mutant GLAs

To predict the position of a substituted amino acid residue in the GLA molecule, the ASA value of each residue in the wild-type GLA was calculated using ACCESS (McDonald and Thornton 1994). The average ASA values of the residues for which a substitution had been found in the classic and variant Fabry groups were determined and compared with each other, as described previously (Saito et al. 2008; Sugawara et al. 2008).

Statistical analysis

Statistical analysis to determine differences in the numbers of atoms affected, RMSD values, and ASA values between

classic and variant Fabry groups was performed using the *F* test and then Welch's *t* test, it being taken that there was a significant difference if $P < 0.05$.

Coloring the atoms affected in mutant GLAs for which substrate analogues are effective

To determine the influence of amino acid substitutions geographically and semiquantitatively, coloring the affected atoms in the three-dimensional structure of GLA based on the distances between the wild-type and mutant ones was performed, followed by determination of the numbers of affected atoms, RMSD values, and ASA values. We analyzed amino acid substitutions including E59K, E66Q, M72V, I91T, A97V, R112H, F113L, A156V, L166V, N215S, G260A, Q279E, M296I, M296V, R301Q, R356W, and G373S, for which substrate analogues are effective for stabilization or transportation of mutant enzymes to lysosomes (Okumiya et al. 1995b; Yam et al. 2006; Ishii et al. 2007).

Results

Localization of amino acid substitutions responsible for Fabry disease

According to the crystallographic structure of human GLA (Garman and Garboczi 2004), the enzyme unit comprises two domains: an *N*-terminal $(\beta/\alpha)_8$-barrel domain and a *C*-terminal antiparallel β-sheet domain. The active-site pocket is localized in the *C*-terminal of the β-sheet of the *N*-terminal domain. We determined the locations of residues of which amino acid substitutions have been identified in Fabry disease patients in the homodimeric enzyme structure (Fig. 1a). Then, we localized the residues of which amino acid substitutions are responsible for classic (Fig. 1b, c) and variant (Fig. 1d, e) Fabry groups in the GLA subunit to compare them with each other. In the classic Fabry group, amino acid substitutions were distributed all over the enzyme protein molecule, including the active-site pocket. On the other hand, in the variant Fabry group, they were located far from the active-site pocket, and most of them were localized on the molecular surface of the protein.

Numbers of atoms affected by amino acid substitutions responsible for Fabry disease

We built structural models of the mutant GLAs and calculated the number of atoms affected by the amino acid substitution for each mutant model (Table 1), the results being summarized in Fig. 2.

The classic Fabry group showed a wide distribution. Averages for the affected atoms in the main chain and side chain were 108 and 130, respectively. In particular, regarding the former, 108 of the 196 classic cases (56%) had 50 atoms or more affected. There were 36 amino acid substitutions located in the active-site pocket, all of which cause the classic Fabry phenotype. These cases are colored *red* for their differentiation from other cases, which are colored *black*.

In contrast, the number of affected atoms in the variant Fabry group was low, and the distribution was narrower. These cases are colored *black*. Averages of the affected atoms in the main chain and the side chain were 18 and 21, respectively. In particular, regarding the main-chain atoms, 14 of the 16 variant Fabry cases (88%) had 49 atoms or less affected.

The *F* test showed that the distribution exhibited unequal variance ($P < 0.05$) between the classic and variant Fabry groups, and thus, Welch's *t* test was performed. Results revealed that there were significant differences in the numbers of affected atoms in both the main chain and side chain between the two groups ($P < 0.05$).

RMSD values for amino acid substitutions responsible for Fabry disease

The RMSD values for the classic and variant Fabry groups were determined. Results are shown in Fig. 3. The average RMSD values in the classic and variant Fabry groups were 0.089 and 0.029 Å, respectively. In 115 of the 196 classic Fabry cases (59%), the RMSD value was ≥0.05 Å. On the other hand, it was <0.05 Å in 14 of the 16 variant Fabry cases (88%). Results of the *F* test followed by Welch's *t* test showed that there was a significant difference in the RMSD values between the two groups.

ASA values of amino acid substitutions responsible for Fabry disease

To determine and compare locations of amino acid residues in the GLA molecule associated with the classic (126 residues) and variant (15 residues) Fabry cases, the ASA values of the residues in the wild-type GLA structure were calculated, the results being shown in Fig. 4 (the result for each residue is presented in "Supplementary data No. l"). In the classic Fabry group, the average ASA value for the 126 residues analyzed was 13.3 $Å^2$, 93 of them (74%) being <20 $Å^2$. In the variant Fabry group, the average ASA value for the 15 residues analyzed was 25.1 $Å^2$, eight of them being ≥20 $Å^2$ (53%).

The *F* test followed by Welch's *t* test revealed that *P* was 0.09. Results suggest that the residues associated with

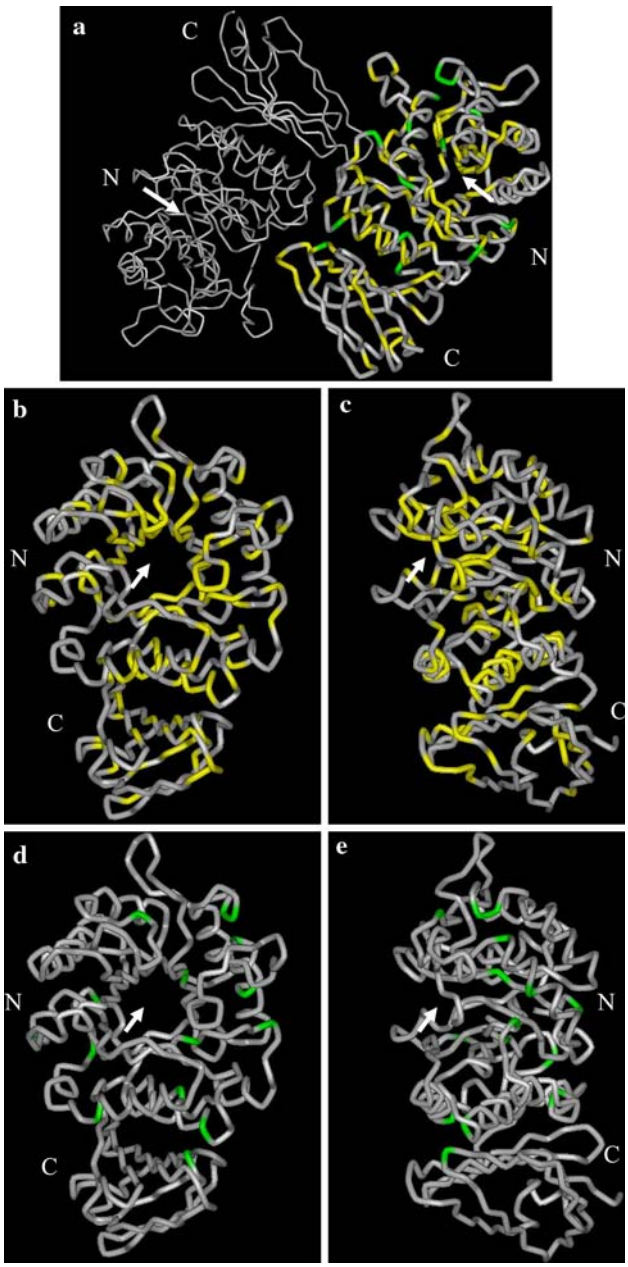

Fig. 1 Localization of amino acid substitutions responsible for Fabry disease in the α-galactosidase (GLA) structure. Secondary structures in GLA are shown as a tube drawing. Locations of amino acid substitutions identified in the classic and variant Fabry groups are shown in *yellow* and *green*, respectively. *N* The *N*-terminal $(\beta/\alpha)_8$-barrel domain. *C* The *C*-terminal antiparallel *β*-sheet domain. An *arrow* indicates the active-site pocket. **a** Homodimeric GLA structure, classic and variant Fabry groups. **b** Front view of the GLA subunit, classic Fabry group. **c** Side view of the GLA subunit, classic Fabry group. **d** Front view of the GLA subunit, variant Fabry group. **e** Side view of the GLA subunit, variant Fabry group

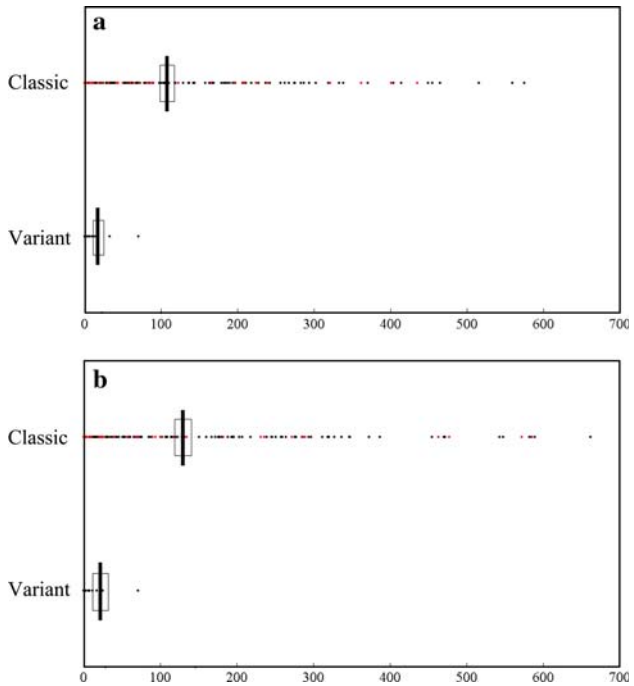

Fig. 2 Numbers of atoms in the **a** main chain and the **b** side chain of the α-galactosidase (GLA) protein affected by amino acid substitutions. *Classic* classic Fabry group. *Variant* variant Fabry group. Mutations located in the active-site pocket are colored *red*, others being colored *black*. *Boxes* indicate mean ± standard errors of mean

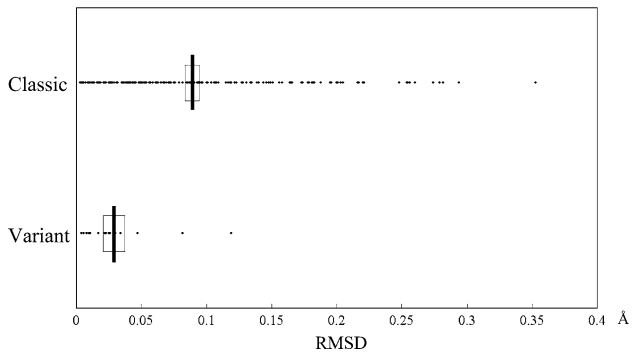

Fig. 3 Root-mean-square distance (*RMSD*) values for classic and variant Fabry mutations (*Å*). *Boxes* indicate mean ± standard errors of mean

classic Fabry mutations tend to be less solvent-accessible than those associated with variant ones, although this could not be confirmed statistically.

Coloring the affected atoms due to amino acid substitutions for which substrate analogues are effective

Regarding amino acid substitutions responsible for Fabry disease, we paid attention to 17 mutations for which substrate analogues improved the stability or transportation of mutant GLAs in cells, i.e., E59K (classic), E66Q (variant), M72V (variant), I91T (variant), A97V (classic), R112H (variant), F113L (variant), A156V (classic), L166V (classic), N215S (variant), G260A (classic), Q279E (variant),

M296I (variant), M296V (variant), R301Q (variant), R356W (classic), and G373S (classic). Coloring the affected atoms in the three-dimensional GLA structure was performed for these mutations, the results being shown in Fig. 5.

Coloring the affected atoms clearly allowed visualization of structural changes. Determining the numbers of atoms affected and RMSD and ASA values confirmed the results. In most mutant GLAs, the predicted structural changes were small (numbers of affected atoms in both the main chain and side chain <50, and RMSD < 0.05: E66Q, M72V, I91T, A97V, F113L, L166V, L215S, G260A, Q279E, M296I, M296V, and G373S), or localized on the molecular surface, although the structural changes were not small (ASA ≥ 20: E59K, R112H, and R301Q), regardless of phenotype. There were only two exceptions, A156V and R356W, and their clinical phenotype was classic. None of the amino acid substitutions for which substrate analogues are effective caused any structural changes in the active site.

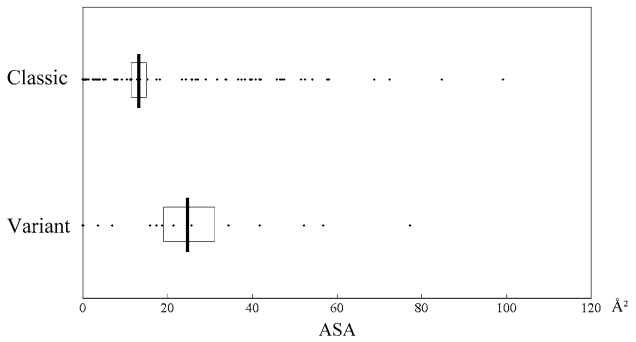

Fig. 4 Solvent-accessible surface area (*ASA*) values of amino acid residues associated with classic and variant Fabry disease (Å^2). *Boxes* indicate mean ± standard errors of mean

Discussion

Considering the results of newborn screening, the incidence of Fabry disease is unexpectedly high (1 in 3,000–4,000 male newborns), especially the variant form (Spada et al. 2006). It is very important to examine the structural changes in the enzyme protein responsible for the different phenotypes to elucidate the basis of Fabry disease and to predict disease outcome. Garman and Garboczi calculated the side-chain-accessible surface area and revealed that the residues involved in Fabry mutations tend to be less solvent-accessible than the typical residues and that most of them lead to disruption of the hydrophobic core of the protein (Garman and Garboczi 2004; Garman 2007). However, there is little structural information on defective GLA proteins, although a large number of gene mutations responsible for Fabry disease have been reported so far.

In this study, we constructed structural models of Fabry mutant GLAs and examined their structural changes from various aspects by determining the number of atoms affected, as well as RMSD and ASA values, using our structural analysis system. The results revealed that structural changes in the classic Fabry group are generally large

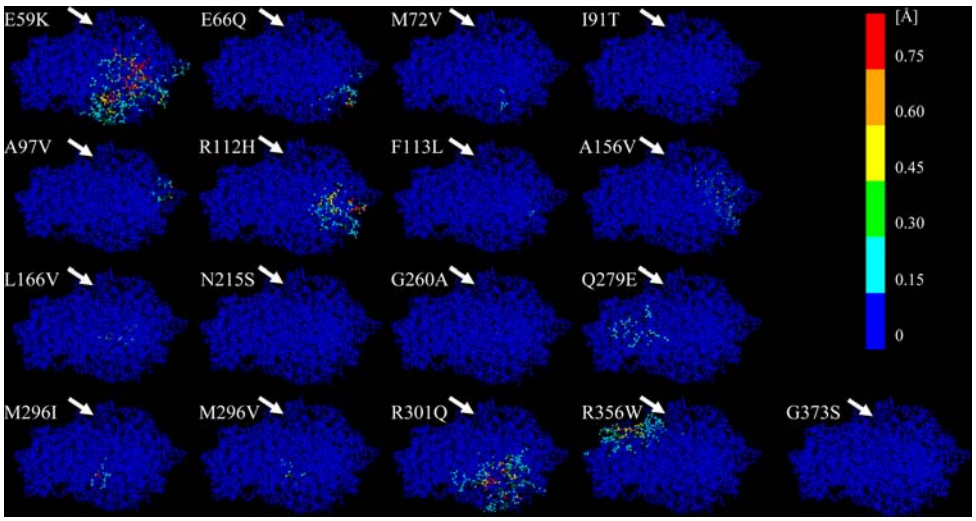

Fig. 5 Coloring the atoms in the three-dimensional structure affected by amino acid substitutions for which substrate analogues are effective. The degrees and distributions for E59K, E66Q, M72V, I91T, A97V, R112H, F113L, A156V, L166V, N215S, G260A, Q279E, M296I, M296V, R301Q, R356W, and G373S, for which substrate analogues are effective, are shown. Each atom is colored according to the distance between the atom in the mutant and the corresponding atom in the wild-type structure. The colors of the atoms show the distances as follows: *blue* <0.15 Å, 0.15 Å ≤ *cyan* < 0.30 Å, 0.30 Å ≤ *green* < 0.45 Å, 0.45 Å ≤ *yellow* < 0.60 Å, 0.60 Å ≤ *orange* < 0.75 Å, and *red* ≥ 0.75 Å. *Arrows* indicate the active-site pocket

and tend to be in the core region of the protein. About 85% (116/196) of the amino acid substitutions leading to classic Fabry disease satisfied one of the conditions given below; number of affected atoms in the main chain ≥50, number in the side chain ≥50, RMSD ≥ 0.05 Å, or ASA < 20 Å^2. They seriously affected protein folding or intracellular transport, leading to a deficiency of enzyme activity. All amino acid substitutions causing structural changes of the active-site pocket resulted in the classic phenotype. In such cases, structural changes would seriously affect expression of GLA activity, even if the number of influenced atoms is relatively small.

On the other hand, the predicted structural changes in GLA are generally small or localized on the surface of the molecule far away from the active site in the variant Fabry group. In such cases, a small amount of enzyme having GLA activity would be protected from the ER's quality control system and transported to lysosomes, resulting in residual enzyme activity.

The number of affected atoms calculated using TINKER differs from that calculated using SYBYL/BIOPOLYMER. This is not surprising, because the minimized structure depends on the minimization algorithm, force field, and computational implementation. As shown in supplementary data No. 2 and No. 3, the number of affected atoms calculated using TINKER was generally larger than that using SYBYL/BIOPOLYMER. The mutant model obtained with TINKER was well optimized compared with that with SYBYL/BIOPOLYMER, which indicates that the mutant model constructed in this study is improved compared with the previous one (Matsuzawa et al. 2005). However, the supplementary data suggest that the number of affected atoms calculated using SYBYL/BIOPOLYMER was correlated with that using TINKER. Therefore, the discussion in the previous study is thought to remain correct.

Furthermore, we focused on the structural changes due to amino acid substitutions for which substrate analogues are effective and examined them by determining the affected atoms, RMSD values, and ASA values, followed by coloring the affected atoms. Results revealed that they cause small structural changes in GLA or are localized on the molecular surface, except for a couple of exceptions. None of them affected the active site. These results suggest that binding of a substrate analogue to a mutant enzyme protein in which a small structural change has occurred on the surface of the molecule reduces its folding defect and increases its stability in cells. Previously, we expressed mutant GLAs including M72V, L156V, L166V, Q279E, and R301Q in COS-1 cells and Sf9 cells and examined their biochemical characteristics (Ishii et al. 1993; Okumiya et al. 1995a, 1998; Kase et al. 2000). The expressed products had GLA activity, but they were unstable and easily lost their activity in vitro, suggesting that their structural changes are located far from the active site and that the degree of the changes is not so large. The results are well correlated with those of the structural analysis performed this time.

In conclusion, we investigated the structural changes in GLA responsible for Fabry disease. Results showed a correlation between the three-dimensional structural changes and clinical phenotypes, and they also revealed the characteristics of the structural changes in mutant enzyme proteins for which substrate analogues are effective. Structural investigation is useful for elucidation of the basis of Fabry disease, and it will increase our ability to determine a proper therapeutic schedule for this disease.

Acknowledgments We thank Dr. J. Ponder (Department of Biochemistry and Molecular Biophysics, Washington University) for providing us with the TINKER software. We also thank I.K. McDonald, D. Naylor, D. Jones, J.M. Thornton, S. Hubbard, D.K. Smith, R. Laskowski, and G. Hutchinson for providing us with the HBPLUS. This work was partly supported by grants from the Japan Society for the Promotion of Science; the Ministry of Education, Science, Sports and Culture of Japan; the Ministry of Health and Welfare of Japan; the Japan Science and Technology Agency; and CREST.

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