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The impact of Lys→Arg surface mutations on the crystallization of the globular domain of RhoGDI

Jan Czepas,[a]‡§ Yancho Devedjiev,[a]§ Daniel Krowarsch,[b] Urszula Derewenda,[a] Jacek Otlewski[b] and Zygmunt S. Derewenda[a]*

[a]Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA 22908-0736, USA, and [b]Institute of Biochemistry and Molecular Biology, University of Wroclaw, 50-137 Wroclaw, Poland

‡ Present address: Department of Molecular Biophysics, University of Lodz, 90-237 Lodz, Poland.
§ These authors contributed equally.

Correspondence e-mail: zsd4n@virginia.edu

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The potential of rational surface mutagenesis for enhanced protein crystallization is being probed in an ongoing effort. In previous work, it was hypothesized that residues with high conformational entropy such as Glu and Lys are suitable targets for surface mutagenesis, as they are rarely incorporated in crystal contacts or protein–protein interfaces. Previous experiments using Lys→Ala, Glu→Ala and Glu→Asp mutants confirmed that mutated proteins were more likely to crystallize. In the present paper, the usefulness of Lys→Arg mutations is studied. Several mutations of the globular domain of human RhoGDI were generated, including the single mutants K105R, K113R, K127R, K138R and K141R, the double mutants K(98,99)R and K(199,200)R and the triple mutants K(98,99,105)R and K(135,138,141)R. It is shown that Lys→Arg mutants are more likely to crystallize than the wild-type protein, although not as likely as Lys→Ala mutants. Out of the nine mutants tested, five produced diffracting crystals, including the K(199,200)R double mutant, which crystallized in a new space group and exceeded by ∼1.0 Å the resolution of the diffraction of the wild-type crystal. Major crystal contacts in the new lattice were created by the mutated epitope.

1. Introduction

Crystallization remains a critical bottleneck in structural characterization of proteins. Recent data from high-throughput structural genomics studies indicate that approximately 10% of proteins expressed in a soluble state form crystals suitable for X-ray investigations (Claverie *et al.*, 2002; Ding *et al.*, 2002; Sulzenbacher *et al.*, 2002). It is also well established that some proteins crystallize readily under many conditions, whereas others appear to be recalcitrant to crystallization regardless of the extent of the screening process. Thus, the protein itself should be regarded as the key variable in protein crystallization (Dale *et al.*, 2003). Recombinant methods offer unique opportunities to modify the protein to make it more susceptible to crystallization, yet to date few rational strategies have been proposed. To this end, we initiated a systematic study of specific surface mutations designed to create epitopes that mediate suitable crystal-packing interactions. Briefly, we hypothesized that replacing bulky hydrophilic residues such as Lys and Glu with smaller amino acids would lead to a reduction in the excess surface conformational entropy which impedes crystal formation (Longenecker, Garrard *et al.*, 2001; Mateja *et al.*, 2002). We chose the globular domain of human RhoGDI (residues 67–204) as the test system, as this protein is rich in both Glu and Lys and is recalcitrant to crystallization in its wild-type form. Initial results with Lys→Ala single and multiple mutants were very encouraging, as mutated RhoGDI crystallized more readily

and some crystals obtained in this way diffracted to higher resolution in comparison to the wild-type protein (Longenecker, Garrard *et al.*, 2001). Furthermore, these studies demonstrated that the proposed approach actually leads to crystal packing mediated by the mutated epitopes. A subsequent study of Glu→Ala and Glu→Asp mutants (Mateja *et al.*, 2002) was equally promising, but it was noted that the mutations reduce the stability of the protein.

The approach suggested by our studies has been successfully applied to several new proteins that are otherwise difficult to crystallize. The RGSL (also known as LH) domain of the PDZRhoGEF exchange factor has been crystallized as a triple mutant (K463A, E465A and E466A) and the well diffracting crystals have been shown to be largely held together by a crystal contact formed by the mutated epitope (Longenecker, Lewis *et al.*, 2001). The major antigen of *Yersinia pestis*, LcrV, was crystallized by the same strategy (Derewenda *et al.*, 2004) and a double mutant of the N-terminal DCX domain of doublecortin was also crystallized, thus circumventing problems with the wild-type protein (M. H. Kim *et al.*, unpublished data). There are also successful examples from other laboratories. The 1.7 Å resolution structure of the CUE–ubiquitin complex was solved based on a crystal of a complex incorporating a ubiquitin mutant K435A/K436A (Prag *et al.*, 2003). Very recently, investigators from the Merck Research Laboratories succeeded in the preparation of crystals of the apo unactivated insulin-like growth-factor receptor kinase, with diffraction to 1.5 Å, using a double mutant E1067A/E1069A (Munshi *et al.*, 2003). Prior studies of this protein were limited to 2.7 Å resolution based on the wild-type crystals (Munshi *et al.*, 2002).

The present paper reports efforts intended to probe the impact of Lys→Arg mutations. In contrast to Lys→Ala and Glu→Ala mutations, which reduce the conformational entropy of the protein by ∼8 kJ mol^{-1}, there is no comparable advantage to Lys→Arg mutations, as arginine has a similar conformational entropy to lysine. However, because of the planarity of the guanidinium group, the associated hydrogen-bonding network is more favored at protein–protein interfaces (Conte *et al.*, 1999). Also, Lys→Arg mutations are expected to affect the stability and solubility of the protein to a lesser extent than elimination of hydrophilic Glu and Lys side chains. This strategy was originally suggested by Dasgupta *et al.* (1997) based on a statistical analysis of crystal contacts. We report that Lys→Arg mutations appear to have a positive impact on the crystallization properties of RhoGDI, although not as dramatic as those previously reported for Lys→Ala mutants. As expected, the Lys→Arg mutants show small stability differences and some are more stable than the wild-type protein.

2. Materials and methods

2.1. Recombinant methods and mutagenesis

The methods used in this study have been described in detail elsewhere (Longenecker, Garrard *et al.*, 2001; Mateja *et al.*, 2002). Briefly, the pGST-Parallel1 expression vector encoding the Δ66 RhoGDI in fusion with GST and an rTEV cleavage site was used (Sheffield *et al.*, 1999). Single and multiple mutants were made using single primers and the QuikChange mutagenesis kit (Stratagene). Primers were purchased from Life Technologies. DNA manipulations were carried out in the following *Escherichia coli* strains: Epicurian Coli XL-10 Gold Ultracompetent Cells (Stratagene) and Library Efficiency DH5α Competent Cells (Life Technologies). Positive clones were verified by DNA sequencing.

2.2. Protein expression and purification

Protein expression was carried out in XL-10 Gold and Epicurian Coli BL21 (DE3) Competent Cells (Stratagene) in Luria–Bertani medium (LB). Proteins were produced after induction with 1 m*M* IPTG (at an OD_{600} of 0.4–0.6). Cells harvested by centrifugation were lysed by sonication and the soluble protein fraction was isolated by centrifugation. GST-fusion protein was separated by GST-affinity chromatography on Glutathione Sepharose 4B columns (Pharmacia Biotech). Subsequently, protein was eluted with buffer containing 10 m*M* glutathione and dialysed against TEV buffer pH 8.0. GST was removed by GST-affinity chromatography and gel filtration on a Superdex 75 column (Pharmacia Biotech) after cutting with rTEV.

2.3. Crystallization and data collection

Mutant proteins were crystallized by vapor diffusion in 96-well Compact Clover plates (Emerald Biostructures) using protein concentrations typically in the range 14–20 mg ml^{-1}. To screen for crystallization conditions, we used Crystal Screen I and Crystal Screen II (Hampton Research) as well as Emerald Wizard I and Emerald Wizard II screens (Emerald Biostructures). Crystals suitable for X-ray diffraction experiments were flash-frozen with the use of 20% glycerol as a cryoprotectant and their diffraction was assessed at the SER-CAT beamline at APS. X-ray data were only collected for mutant crystals that diffracted better than the wild-type RhoGDI (2.5 Å resolution). The diffraction limits of poorly diffracting crystals were determined by analysis of the spots on a single image and space-group determination was not attempted. Crystals of the K(199,200)R mutant were obtained from 30% PEG 4000 in 0.1 *M* Tris pH 8.5 with the addition of 0.2 *M* lithium sulfate (Hampton Screen, No. 17) and were harvested and flash-frozen directly from the screen droplet. In all other cases, a standard optimization procedure was applied including only the major variables pH and precipitant concentration, as well as the addition of 0.1%(*w*/*v*) β-octyl glucoside in some cases. The quality of the crystals was assessed at the home X-ray source: the best crystal diffracted to 2.1 Å resolution (data not shown). The same crystal was used to collect data at higher resolution (1.6 Å) at APS beamline 19ID. Processing and merging of the data was carried out with *HKL*2000 (Otwinowski & Minor, 1997).

2.4. Structure solution and refinement of the K(199,200)R mutant crystal structure

The structure of the K(199,200)R mutant was solved by molecular replacement with *AMoRe* (Navaza, 1994). As a search model, the structure of the quadruple mutant of the RhoGDI at 2.0 Å resolution (Longenecker, Garrard *et al.*, 2001) was used (PDB code 1fso). Refinement was carried out using *REFMAC*5 within the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

2.5. Stability measurements

The stability of the mutants was assessed by thermal denaturation using an FP-750 spectrofluorimeter (Jasco), measuring the fluorescence at 348 nm on excitation at 295 nm. 5 nm emission and excitation band widths with a response time of 10 s were utilized. Protein samples in the concentration range 3–5 μg ml^{-1} in 25 m*M* Tris, 0.5 *M* guanidinium chloride pH 8.3 were used and the heating rate was 0.25 K min^{-1}. A probe immersed in the cuvette and controlled using an ETC-272 Peltier attachment (Jasco) was used to directly monitor the temperature of the protein samples. The data were analysed assuming a two-state reversible equilibrium transition. *PeakFit* software (Jandel Scientific Software) was used for the analysis.

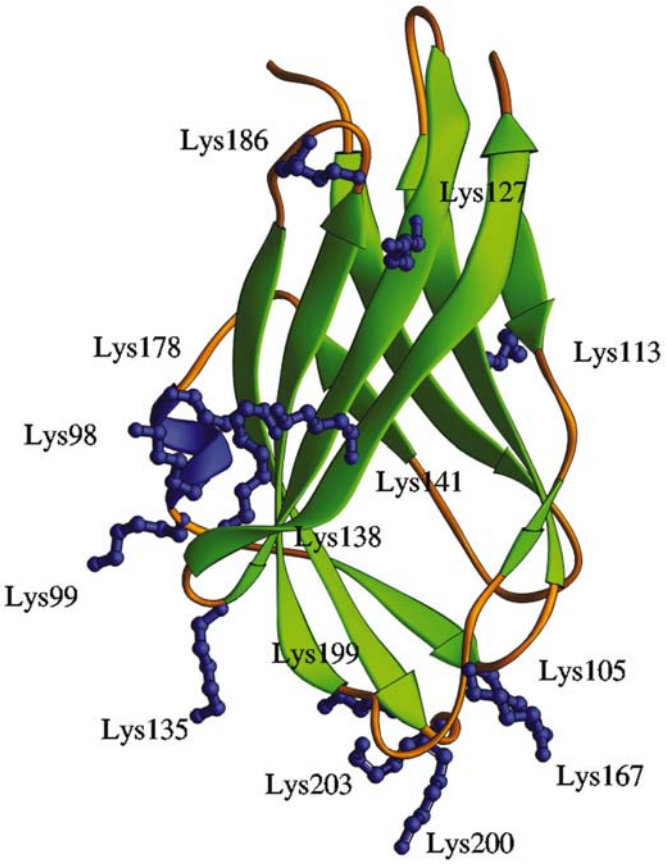

Figure 1
Ribbon presentation of RhoGDI Δ66 fragment with mutated lysine side chains colored blue. The numbering of the amino-acid residues is according to the sequence of the full-chain protein. The figure was prepared using *RIBBONS*.

Table 1
Thermal denaturation of RhoGDI and Lys→Arg mutants.

Mutant	T_{den} (K)	ΔH_{den} (kJ mol^{-1})
K(98,99,105)R	322.41	355
K(199,200)R	324.30	386
K105R	324.64	413
K(98,99)R	325.54	498
Wild type	327.04	440
K(135,138,141)R	327.84	486
K141R	327.88	465
K138R	328.61	423
K113R	329.01	360
K127R	329.33	380

3. Results and discussion

A total of nine Lys→Arg mutants were generated using the N-terminally truncated (Δ66) variant of human RhoGDI (residues 67–204; Fig. 1). Five were single-site mutants: K105R, K113R, K127R, K138R and K141R. There were two double mutants, K(98,99)R and K(199,200)R, and two triple mutants, K(98,99,105)R and K(135,138,141)R. Four mutants, specifically K113R, K141R, K(98,99,105)R and K(135,138,141)R, were analogous to the corresponding Lys→Ala mutants used in our earlier study (Longenecker, Garrard *et al.*, 2001).

The thermal denaturation studies revealed that the stability of the Lys→Arg mutants, as indicated by the denaturation temperature T_{den}, differs marginally from the stability of the wild-type protein: five mutants have increased stability by up to 2.3 K and the other four have lower stability in comparison with the wild type (Table 1). Most of the single mutants have higher stability, with the exception of the K105R mutant. Both the double mutants, K(98,99)R and K(199,200)R, are less stable. The triple mutant K(98,99,105)R has the lowest stability, with a denaturation temperature 4.6 K lower than the wild type. In general, single mutants were more stable than wild type and multiple mutants. The relative differences when compared with the wild type are significantly smaller than for the Glu→Ala mutants (Mateja *et al.*, 2002). These results reaffirm our expectation that Lys→Arg mutations are benign from the point of protein stability owing to the preservation of the salt bridges and long-range electrostatic interactions on the surface of the protein.

For the initial search, we chose four of the most popular commercially available crystallization screens. This combination provides an optimal number of non-redundant crystallization condition within commercially available screens and offers a uniform measure for the crystallizability of the mutant crystals compared with the wild-type RhoGDI, which was crystallized under the same conditions and with similar protein concentration. A summary of successful crystallization conditions ('hits') and some characteristics of the obtained crystals are presented in Table 2. In total, seven of the mutants (78%) gave 'hits' in the initial stage of searching for crystals. Polyethylene glycols as well as ammonium sulfate were suitable precipitants for crystallization of most of the mutants,

Table 2
Summary of the crystallization results of the RhoGDI Δ66 Lys→Arg mutants.

RhoGDI Δ66	Screen†	Crystallization conditions	Crystal morphology
K105R	WZ2 No. 4	2.0 *M* $(NH_4)_2SO_4$ pH 6.3, 200 m*M* NaCl	Large prisms, unstable
K113R	None	—	
K127R	HR2 No. 31	20% Jeffamine M-600 pH 7.5	Thin plates
	WZ1 No. 2	10% 2-propanol pH 7.3, 200 m*M* NaCl	
	WZ1 No. 3	15% ethanol pH 9.4	
	WZ1 No. 8	2.0 *M* $(NH_4)_2SO_4$ pH 5.3	
K138R	HR2 No. 14	2.0 M $(NH_4)_2SO_4$ pH 5.6, 200 m*M* K Na tartrate	
	WZ1 No. 1	20% PEG 8000 pH 9.6	
K141R	WZ1 No. 8	2.0 *M* $(NH_4)_2SO_4$ pH 5.3	Plates, twinned
K(98,99)R	HR1 No. 17	30% PEG 4000 pH 8.5, 200 m*M* Li_2SO_4	Plates
	HR1 No. 29	0.8 *M* K Na tartrate pH 7.5	
	HR2 No. 26	30% PEG 5000 MME pH 6.5, 200 m*M* $(NH_4)_2SO_4$	
K(199,200)R	HR1 No. 9	30% PEG 4000 pH 5.6, 200 m*M* NH_4OAc	Rods
	HR1 No. 15	30% PEG 8000 pH 6.5, 200 m*M* $(NH_4)_2SO_4$	
	HR1 No. 17	30% PEG 4000 pH 8.5, 200 m*M* Li_2SO_4	
	HR2 No. 26	30% PEG MME 5000 pH 6.5, 200 m*M* $(NH_4)_2SO_4$	
K(98,99,105)R	HR1 No. 42	20% PEG 8000, 50 m*M* KH_2PO_4	Plates
	WZ1 No. 10	20% PEG 2000 MME pH 7.0	
	WZ1 No. 21	20% PEG 8000 pH 7.5	
	WZ1 No. 28	20% PEG 3000 pH 7.4, 200 m*M* NaCl	
K(135,138,141)R	None	—	

† HR1, Hampton Research Crystal Screen 1; HR2, Hampton Research Crystal Screen 2; WZ1, Emerald Biostructures Wizard 1; WZ2, Emerald Biostructures Wizard 2.

Table 3
Summary of some characteristics of the crystals of RhoGDI Δ66 Lys→Arg mutants.

Protein	Unit-cell parameters (Å, °)	Space group	V_M (Å^3 Da^{-1})	Mols. per AU	Resolution (Å)
Wild type†	$a = b = 156.6$, $c = 132.5$, $\gamma = 120$	*R*32	3.26	3	2.5
K138R	Not determined	—			3.8
K141R	Not determined	—			4.0
K(98,99)R	$a = 79.5$, $b = 111.8$, $c = 37.9$	$P2_12_12$	2.63	2	2.3
K(199,200)R	$a = 148.7$, $b = 58.2$, $c = 75.1$, $\beta = 92.5$	*C*2	2.56	4	1.6
K(98,99,105)R	$a = 79.1$, $b = 112.2$, $c = 37.6$	$P2_12_12$	2.61	2	2.9

† Keep *et al.* (1997).

which crystallized in a wide range of pH values between 5.3 and 9.6. Only the K113R and K(135,138,141)R mutants did not produce any crystals. Crystals typically grew over a period of time between a few days and one week. Some of the crystals, however, were obtained in two or three weeks and one mutant (K141R) produced crystals after three months. Four out of five single mutants crystallized (Table 2). While the K113A and K141A mutants produced crystals diffracting to 2.6 and 2.8 Å, respectively, the K113R mutant did not crystallize at all, while the K141R mutant produced crystals diffracting to 4.0 Å resolution. Most of the multiple mutants also yielded crystals in initial screens. The K(199,200)R and K(98,99,105)R mutants produced crystals in four different screen conditions. The K(98,99,105)A mutant produced crystals from nine screen conditions, but all of them showed large cells. The K(98,99,105)R mutant crystals diffracted to 2.9 Å and the cell was identical to K(98,99)R mutant crystals. The K(98,99)R double mutant crystallized from three conditions and the single mutant K138R from two different conditions. The K105R mutant gave crystals from one set of conditions, in contrast to K105A, which crystallized from six different conditions (Longenecker, Garrard *et al.*, 2001). However, neither mutant gave crystals that were useful for diffraction studies. K105R mutant crystals, in particular, were not stable and had a tendency to liquify.

Table 4
Data-collection and refinement statistics for the K(199,200)R mutant.

Values in parentheses are the relevant value for the last resolution shell (1.66–1.60 Å).

Source	APS, 19ID
Space group	*C*2
Unit-cell parameters (Å, °)	$a = 148.7$, $b = 58.2$, $c = 75.1$, $\beta = 92.5$
Resolution (Å)	1.6
Total No. reflections	311750
No. unique reflections	84662
Redundancy	3.7 (3.4)
Completeness (%)	99.6 (99.4)
R_{sym}† (%)	5.3 (38.2)
$I/\sigma(I)$	15.2 (2.7)
Resolution (Å)	27.1–1.6
No. reflections	82685
R_{cryst}/R_{free} (%)	16.5/21.9
R.m.s.d. bonds (Å)	0.016
R.m.s.d. angles (°)	1.6
R.m.s.d. chiral	0.12

† $R_{sym} = \sum |I_i - \langle I \rangle| / \sum I$, where I_i is the intensity of the Ith observation and $\langle I \rangle$ is the mean intensity of the reflections.

The goal of the present study was to find out whether Lys→Arg mutations would result in better crystallization properties of the protein and whether the resulting crystals were of high quality. Crystals of five mutants were assessed by X-ray diffraction: K138R, K141R, K(98,99)R, K(199,200)R and K(98,99,105)R (Table 3). The K(199,200)R double mutant yielded the best results, with diffraction to beyond 1.6 Å at a synchrotron source. The molecular model was refined to an *R* factor of 16.5% and an R_{free} of 21.9% (Table 4). The crystal exhibited *C*2 symmetry and revealed a new type of crystal packing for RhoGDI, with four molecules within the asymmetric unit arranged as two non-crystallographic dimers, molecules *A*/*B* and *C*/*D*, related by translational non-crystallographic symmetry (Fig. 2). It has been noted by us previously that RhoGDI has a tendency to dimerize across diverse crystal forms, so that the hydrophobic entrances to the central prenyl-binding cavity face each other (Longenecker, Garrard *et al.*, 2001). The dimers observed in this study again follow this paradigm, suggesting that this association mode

might have some physiological consequence. Compared with other RhoGDI crystal structures there are no significant differences other than selected surface side chains that have conformations altered by solvent structure or by proximity to crystal contacts. The major lattice contact is mediated by the mutated epitope and specifically two symmetry-related side chains of Arg199 brought together by an intricate charge constellation involving Asp204, its counterpart from a symmetry-related molecule and two sulfate ions. These four negatively charged ions compensate for repulsion between the arginines, so that the latter stack closely with intimate van der Waals contacts (Fig. 3). This contact is observed between molecules *A* and *B* in the adjacent asymmetric unit and is repeated between molecules *C* and *D* in another asymmetric unit. Thus, the engineered crystal contact is the principal contact in the lattice, the other contact being the typical interface in the non-crystallographic RhoGDI dimer. This novel crystal packing contributes to a significant enhancement of diffraction quality in comparison to the wild-type crystals.

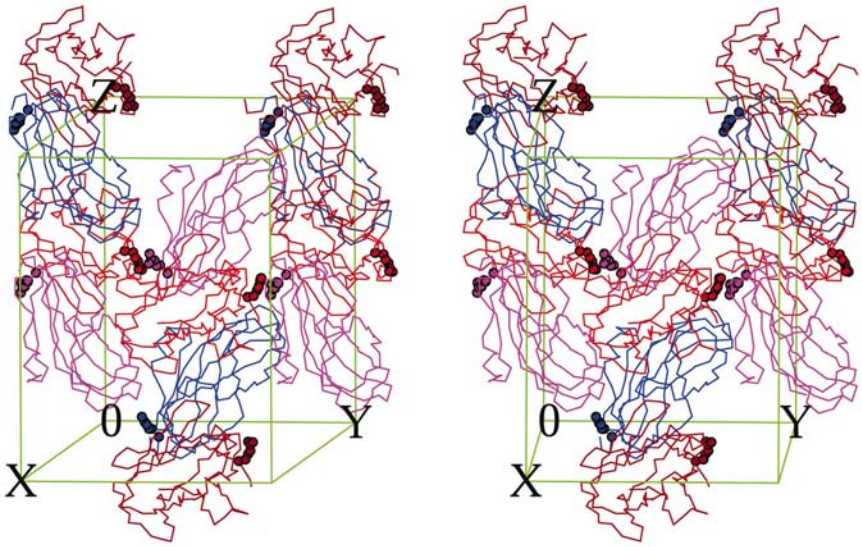

Figure 2
A view of the packing of RhoGDI molecules in the *C*2 crystal form, which shows the essential contribution of mutated arginines in the formation of the major lattice contacts. Four molecules in the asymmetric unit are color coded: *A*, brown; *B*, magenta; *C*, blue; *D*, red. See text for details. The figure was produced with *MOLSCRIPT* (Esnouf, 1997).

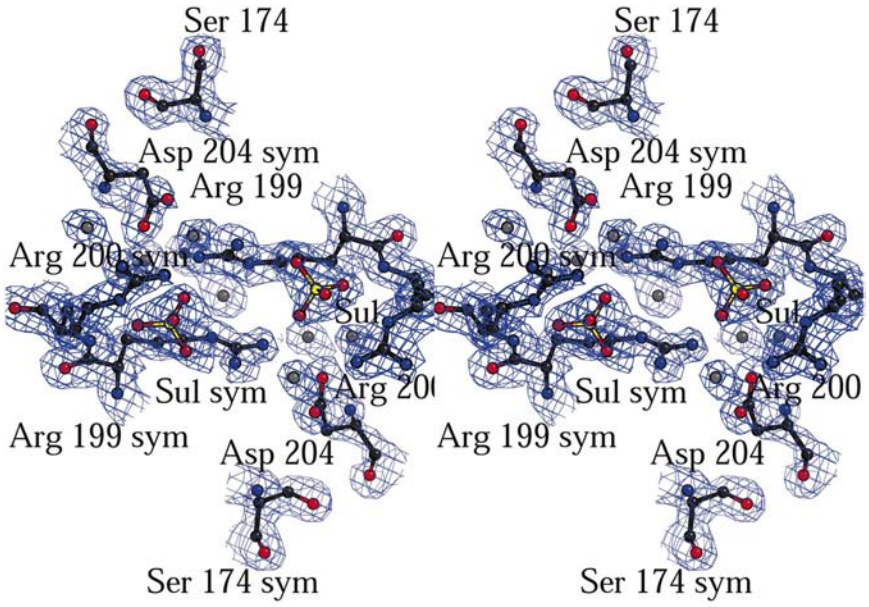

Figure 3
Electron-density map in the region of the lattice contact contoured at the 1σ level, involving mutated Arg199, its symmetry-related pair Arg199 sym as well as the charge constellation including the negatively charged residues Asp204, Asp204 sym and two solvent sulfates. Additional hydrogen bonds involving Arg200, Ser174 and their symmetry-related partners contribute to stability of the lattice contact. The figure was prepared with *BOBSCRIPT* (Esnouf, 1997).

Our previous studies of Lys→Ala, Glu→Ala and Glu→Asp surface mutants of the globular domain of RhoGDI demonstrated that multiple mutations at the surface of the protein lead to formation of epitopes that readily mediate novel crystal contacts (Longenecker, Garrard *et al.*, 2001; Mateja *et al.*, 2002). In contrast, single mutations seem to impact on the kinetics of crystallization and thus on the crystal growth, but do not readily change crystal contacts and thus lead to the same space group. As the diffraction quality is dependent on the lattice, not on the protein, some mutants yield crystals that diffract to much higher resolution than the wild type. For example, a dramatic enhancement of resolution to 1.2 Å was observed for the E(154,155)A double mutant (Mateja *et al.*, 2002). We do note, however, that replacement of surface Lys and Glu with alanines inevitably compromises solubility, which in some cases may make it difficult to apply this strategy for crystallization.

In the present study, we investigated whether mutation of lysines to arginines would also provide X-ray quality crystals. As expected, the purified proteins behaved very similarly to the wild-type with respect to solubility. The single Lys→Arg mutants show only limited potential. Four out of five single mutants produced crystals, but none of the crystals were suitable for high-resolution X-ray studies. Small crystals of the K127R mutant were obtained from four different solutions, but their morphology (thin plates) was such that no diffraction experiments were possible. In the case of the K138R mutant, the crystals were well shaped but showed poor diffraction to only 3.8 Å resolution. Crystals of K141R mutant appeared after almost four months as well shaped plates, but diffracted poorly (to 4.0 Å resolution). Crystals of the K(98,99)R

double mutant exhibited the symmetry of space group $P2_12_12$ (Table 3) and diffracted to higher resolution (2.3 Å), but did not yield a structure owing to a twinning problem (data not shown). However, the crystals belong to a novel orthorhombic space group and the packing probably involves a dimer in the asymmetric unit. The quality of crystals of the triple mutant K(98,99,105)R was not as high (2.9 Å resolution) and data collection was not attempted.

4. Conclusions

The results of this work provide further evidence that rational surface mutagenesis may help significantly in the preparation of X-ray quality crystals. While Lys→Ala and Glu→Ala mutations have already proven their potential, Lys→Arg mutants have been systematically tested here for the first time. Single mutations appear to have a limited impact and in the case of RhoGDI did not generate a useful novel form. However, of the nine tested mutants several showed potential which we did not pursue, while the double K(199,200)R mutant was readily solved and refined at high resolution. As was the case with Lys→Ala and Glu→Ala mutations, the crystal contacts in the mutant structure are mediated by the mutated epitope. We conclude that in the case of proteins showing limited solubility or with few Glu/Lys clusters on the surface, Lys→Arg mutations offer a potentially useful alternative strategy.

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