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Pitfalls in the interpretation of structural changes in mutant proteins from crystal structures

P. R. Pokkuluri, X. Yang, Y. Y. Londer, and M. Schiffer

Biosciences Division, Argonne National Laboratory, Lemont, IL 60439, USA

M. Schiffer: mschiffer@anl.gov

Abstract

PpcA is a small protein with 71 residues that contains three covalently bound hemes. The structures of single mutants at residue 58 have shown larger deviations in another part of the protein molecule than at the site of the mutation. Closer examination of the crystal packing has revealed the origin of this unexpected structural change. The site of mutation is within Van der Waals distance from another protein molecule related by a crystallographic twofold axis within the crystal. The structural changes occurred at or near the mutation site have led to a slight adjustment of the surface residues in contact. The observed deviations between the native and the mutant molecular structures are derived from the new crystal packing even though the two crystals are essentially isomorphous. Without careful consideration of the crystal lattice a non-expert looking at only the coordinates deposited in the Protein Data Bank could draw erroneous conclusion that mutation in one part of the molecule affected the structure of the protein in a distant part of the molecule.

Keywords

Crystal contacts; Crystal packing effects; Cytochrome *c*₇; *Geobacter sulfurreducens*; Single mutant structures; Structural changes

Introduction

X-ray diffraction data collected from crystals provide detailed structures of molecules that are valid in the context of that particular crystal packing environment. The molecule in a crystal adopts a conformation in which the inter-atomic interactions between self and neighboring molecules are optimized. The coordinates deposited in the Protein Data Bank (PDB) represent the protein that makes up the asymmetric unit (the smallest component that can be used to build up the crystal by mathematical operations). Understanding the effects of a mutation on the native protein structure requires careful consideration of the influence of the crystal lattice, rather than simple examination of only one molecule (i.e., the coordinates deposited in the PDB). The user needs to generate the neighboring molecules by using the

symmetry operators provided in the PDB and a crystallographic program. This consideration is especially important for nonspecialist users of coordinates deposited in the PDB.

The importance of considering the crystal lattice in understanding structural changes in mutants is demonstrated by a series of crystal structures of single mutants at Met58 in a small tri-heme cytochrome *c*₇ from *Geobacter sulfurreducens* (*Gs*). PpcA (periplasmic cytochrome A) is one of the most abundant proteins in the periplasm of *Gs* [1]. PpcA is part of a family of five tri-heme cytochromes *c*₇ encoded by the genome of *Gs*. We previously determined the structures of all five proteins by X-ray crystallography [2, 3]. In addition, the solution structure of fully reduced PpcA was also reported [4].

PpcA is a protein with 71 residues that contains three covalently bound hemes. It is one of the smallest *c*-type cytochromes with the highest ratio of hemes to amino acids, approximately 1 heme per 24 amino acids. The purified PpcA can reduce Fe(III), U(VI), Cr(VI) and other metal ions [1]. *Gs* can respire using various electron donors and electron acceptors [5]. PpcA is thought to be directly responsible for the reduction of soluble metal species, such as U(VI), which can enter the periplasm [1]. Furthermore, PpcA is believed to serve as an intermediate carrier of electrons obtained from oxidation of the electron donor acetate to the terminal reductase(s) localized in the outer membrane of the organism for extracellular reduction of insoluble Fe(III) oxide [1, 5, 6].

Desulfuromonas acetoxidans (*Da*) is another delta proteobacteria closely related to *Gs*. *Da* has a homologous cytochrome *c*₇ (*Dac*₇), the structure of which has been determined first in solution by NMR [7, 8] and later by X-ray crystallography [9]. The sequence identity between PpcA and *Dac*₇ is 46 % [2]. The sequence identity is largely confined to the heme binding motifs, CXXCH, in which the two cysteines are covalently attached to the heme groups through a thioether bond, and the histidine serves as the proximal ligand to the heme Fe. The hemes are numbered I, III and IV in keeping homology with well-studied tetra-heme cytochromes *c*₃ [10].

One of the sequence differences between PpcA and *Dac*₇ is residue 58 [2], which is a non-polar methionine in PpcA but is a polar serine in *Dac*₇. In PpcA structure, Met58 is located such that it is expected to limit the exposure of heme III to solvent (Fig. 1). Surface exposure of hemes is one of the factors that are known to affect heme reduction potentials. In order to examine the role of methionine at this position in determining the reduction potential of heme III in PpcA, we designed and studied single site mutants: M58S, M58N, M58D and M58K. The reduction potentials of the three hemes in the mutants will be reported elsewhere (Morgado, L. et al. Bioscience Reports, in press). In the present paper, we report the crystal structures of the four mutants, and the surprising structural changes observed in the mutants compared to the native protein are discussed in light of the crystal packing effects.

Materials and methods

Site directed mutagenesis

For mutagenesis, QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used in accordance with the manufacturer's instructions. Oligonucleotides were synthesized by

MWG Biotech (High Point, NC, USA). PpcA expression vector pCK32 [11] was used as a template. The presence of desired mutations was confirmed by DNA sequencing performed by MWG Biotech.

Expression and purification of PpcA mutants

E. coli strain BL21(DE3) containing the plasmid pEC86 [12] was co-transformed with the expression vector containing the PpcA mutants (M58S, M58N, M58D, M58K). Transformed *E. coli* cells were grown in 2xYT medium containing 34 µg/mL chloramphenicol and 100 µg/mL ampicillin. Protein expression was induced with 10 µM IPTG. PpcA mutants were purified by cation-exchange chromatography followed by gel-filtration chromatography, as described for the wild-type cytochrome [2, 11]. The purity of the proteins was evaluated by SDS-PAGE (15 %), stained with Coomassie blue. The correct heme incorporation in the mutants was confirmed by determination of molecular mass by electrospray ionization mass spectrometry performed in the HHMI Biopolymer Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Crystallization

All PpcA mutants reported here were crystallized in the same conditions as the native protein in presence of the additive deoxycholic acid [2] by hanging-drop vapor-diffusion method at room temperature. The precipitant solution was high concentrations (3.5–4.0 M) of ammonium sulfate (pH adjusted with ammonium hydroxide). Protein concentrations were 30 mg/mL for M58S, 60 mg/mL for M58D, 50 mg/mL for M58N, 50 mg/mL for M58K. Precipitant solutions were as follows: M58S: 3.8 M ammonium sulfate pH 6.0; M58N: 4.0 M ammonium sulfate pH 6.0; M58D: 3.6 M ammonium sulfate pH 6.5; M58K: 4.0 M ammonium sulfate pH 5.7. In each case, X-ray diffraction quality crystals were obtained in presence of 0.25 % deoxycholic acid in the drop.

Cryoprotection was achieved by transferring the crystals to the reservoir solution containing 28 % (w/v) sucrose for 20–30 s prior to plunging directly into liquid nitrogen. X-ray diffraction data sets on each mutant to a resolution of about 2 Å were collected at the Structural Biology Center's 19BM beam line at the Advanced Photon Source (Argonne National Laboratory, Lemont, IL). Data sets were processed with HKL2000 [13].

Structure determination and refinement

Structures of M58 mutants were determined by rigid body refinement of the native structure against the mutant diffraction data using the program CNS [14]. Inspection of the electron density maps and model adjustments were performed with the program Chain [15] and refinement was accomplished initially with the program CNS and with Refmac 5 [16] in the final stages. The data collection and refinement parameters are summarized in Table 1.

Results and discussion

M58 mutants had similar expression yields (3 mg/L of culture) and identical UV–visible spectra in both oxidized and reduced forms when compared with the native protein (data not shown). All M58 mutants required deoxycholate for crystallization; they produced well-

diffracting crystals that were isomorphous with the native crystals with a maximum observed deviation in the *c*-axis of about 0.8 % (see Table 1). Therefore, the mutant structures were determined by rigid-body refinement of the native coordinates (PDB code: 1OS6, with the side chain of 58 truncated at CB) against the respective mutant data set.

Overall protein structure

The overall structure of the native protein, PpcA is shown as a ribbon drawing of the C_α carbons (Fig. 1). The three hemes together with a few hydrophobic amino acids form the hydrophobic core of the protein. PpcA contains an N-terminal two strand β-sheet followed by helices and loops, and also a deoxycholate molecule required for crystallization [2, 3]. The site of mutation, methionine 58 is rendered as a stick model in Fig. 1. The side chain of M58 in PpcA was well defined in the electron density and was found to form van der Waals interactions with heme III. In the observed conformation, the sidechain of methionine 58 limits the surface exposure of heme III. To examine the consequences of mutation at this site, we replaced it with serine (the amino acid found at the equivalent position in *Dac7*; see Ref. [2]), and with asparagine and aspartic acid (same size but one is neutral and the other is negatively charged), and a positively charged lysine.

Comparison of native and mutant structures

The overall structure of the mutant proteins was essentially the same as the native with the root mean square deviations (RMSD) of all C_α carbons of 0.1, 0.2, 0.2, 0.2 Å for M58S, M58D, M58N and M58K, respectively. Comparisons of the molecular structures between the native and mutant proteins revealed, in each case, two regions with significant structural deviations, labeled region 1 and 2 (see Fig. 1). Region 1 is at or adjacent to the mutation site, residues 57–60 and region 2 consists of residues 25–27. See Table 2 for deviations and Fig. 2 for an illustration of changes observed between the native and M58D structures. Surprisingly, except in the case of M58S, the changes in region 2 are larger than the changes observed at the mutation site (region 1). At first glance, because of the reasonable proximity of regions 1 and 2 in the protein, one could conclude that the changes observed in region 2 are caused directly by the changes at the mutation site (i.e., region 1). This interpretation would have the erroneous inference that a mutation near heme III has influenced the properties of heme I.

Crystal packing effects on molecular structure

Closer examination of the neighboring molecules in the crystal revealed that region 1 of one molecule and region 2 of another molecule related by a crystallographic twofold axis are closer than regions 1 and 2 within the same molecule (Fig. 3). The contact distance between C_α of residue 58 and C_α of residue 25[#] is 6 versus 12 Å in the same molecule. In other words, region 1 and region 2 are closer across a crystal contact than they are within the same molecule. The close packing of the two molecules is shown in Fig. 4. Any change caused by mutation at residue 58 will have an impact on the conformation of the symmetry-related molecule due to close packing. The changes at or near the mutation site within the molecule lead to slight adjustment of the surface residues in contact (regions 1 and 2, in particular) with the neighboring molecule for crystal production. Even though the original cause of the

changes in protein structure is the mutation (in region 1), the observed deviations between the native and mutant structures (in both region 1 and region 2) are derived from the new crystal packing. The crystal structure of the mutant protein results when the mutant protein crystallizes in the best possible crystal packing arrangement, starting with changes within the protein molecule at region 1, near the mutation site. Therefore, the final observations are affected by both the original change in structure caused by the mutation and the changes due to optimization of the new interface between the molecules in contact within the crystal. In the M58S mutant, the smaller serine residue had a smaller effect on region 2 residues than did the other mutations with longer side chains at M58 (see Table 2).

Implications for the non-expert

In the present case of M58 mutants of PpcA, insufficient thought could lead to the erroneous conclusion that the changes observed in region 2 (which affect the heme binding residues of a distant heme and cause shifts in that heme position) are linked directly to the changes at the mutation site (region 1). In reality, the deviations observed in both regions 1 and 2 upon comparison of crystal structures result from a combination of the original changes at the mutation site and the effects of slight rearrangement of the crystal packing. Interestingly, the side chain of mutated residue M58 is not directly in the crystal contact. It is likely that there may not be any significant changes in region 2 of the mutant proteins in solution.

Conclusion

Evaluation of the influence of a mutation on the structure of a protein molecule as derived from crystal structures requires consideration of effects on the whole crystal lattice. The mutation can influence not only the neighboring residues in the molecule (primary effect), but also residues of neighboring molecules in the crystal (secondary effect). The structural changes caused by the primary effect can cause secondary effects; together, they result in a new minimum energy conformation for the crystal. It is therefore impossible to isolate the effects of the mutation when structural changes occur at or near the interfaces of molecules in the crystal. In case of the M58 mutants of PpcA, the crystals are essentially isomorphous with the native protein crystal. If the primary and secondary effects of the mutation are large, then the mutant protein mostly likely will not crystallize in the same lattice as the native protein. Erroneous conclusions could be drawn without a careful consideration of this fact, especially by non-specialists.

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Abbreviations

Da	<i>Desulfuromonas acetoxidans</i>
Gs	<i>Geobacter sulfurreducens</i>
NMR	Nuclear magnetic resonance
PDB	Protein Data Bank
PpcA	Periplasmic cytochrome A
RMSD	Root mean square deviation

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Fig. 1. Structure of PpcA showing protein as a cartoon of C_α tracing along with the heme cofactors and the deoxycholic acid moiety required for crystallization. Side chain of methionine 58 is shown in *pink* as a *stick* representation. The two regions of change (region 1 and 2) are also indicated. The figure was prepared by using the program PyMOL

Fig. 2.

Native (carbons in *green*) and M58D (carbons in *gray*) mutant structures. The two structures were overlapped using all C_{α} carbons. For both structures, O atoms are in *red*, N atoms are in *blue* and S atoms are in *yellow*. Note the higher deviations at Asp26 compared to the changes at 58, the site of mutation. The figure was prepared by using the program PyMOL

Fig. 3.

The two protein molecules related by a crystallographic twofold axis in the native PpcA crystal. The molecule generated by crystallographic symmetry is shown in *gray*. The contacts between residues 58 and 25 are indicated by *broken lines* with the corresponding distances shown in Å. The regions 1 and 2 are indicated. The figure was prepared by using the program Chain [15]

Fig. 4.

Close-up view of the interactions between the symmetry-related molecules of the native protein crystal as shown in Fig. 3. The symmetry-related molecule is at the *top*. The C atoms for the symmetry-related molecule are in *cyan*; C atoms for the molecule in the asymmetric unit are in *green*. For both molecules, O atoms are in *red*, N atoms are in *blue* and the S atoms are in *yellow*. Note the close packing of the two molecules. Any changes caused by mutation at residue 58 will have an impact on the symmetry-related molecule due to close packing. The figure was prepared by using the program PyMOL

Table 1

Crystallographic parameters of M58 mutants

	M58S	M58N	M58D	M58K
<i>Crystal parameters</i>				
a = b (Å)	32.4	32.4	32.5	32.5
c (Å)	178.1	177.5	177.0	177.6
Space group	P4 ₃ 22	P4 ₃ 22	P4 ₃ 22	P4 ₃ 22
<i>Data collection</i>				
Resolution range (Å)	50–2.0	50–2.1	90–1.9	50–1.9
Completeness (%)	93.8 (70.6)	95.0 (70)	94.2 (72.5)	95.0 (72.3)
Mean I/σ(I)	52 (16)	55 (18)	41 (8)	34 (6)
R-merge	0.041 (0.072)	0.055 (0.130)	0.052 (0.094)	0.068 (0.246)
Redundancy	7 (3)	11 (8)	7 (2)	6 (3)
<i>Refinement</i>				
R-factor	0.193	0.180	0.190	0.189
R-free	0.220	0.223	0.215	0.222
<i>No. of atoms [mean B-factor (Å²)]</i>				
Protein	530 (18.4)	532 (18.4)	532 (18.6)	533 (16.5)
Heme	129 (14.1)	129 (15.0)	129 (14.1)	129 (12.2)
Deoxycholate	28 (16.9)	28 (16.4)	28 (16.3)	28 (13.9)
Water	68 (28.0)	56 (27.1)	65 (30.8)	65 (25.5)
Sulfate	15 (32.6)	15 (33.5)	15 (33.0)	15 (34.1)
<i>RMS deviations from ideal geometry</i>				
Bond lengths (Å)	0.015	0.016	0.014	0.015
Bond angles (°)	1.5	1.6	1.5	1.5
PDB code	3SJ0	3SEL	3SJ1	3SJ4

For comparison purposes, native unit cell parameters are a = b = 32.4 Å and c = 178.4 Å taken from Ref. [2]

Table 2

Deviations in C_{α} positions ($\text{\AA}$) observed in regions 1 and 2 between the native and the M58 single mutant structures

Protein	M58D	M58N	M58K	M58S
Overall RMSD	0.2	0.2	0.2	0.1
Region 1				
57Glu	0.3	0.3	0.3	0.4
58Xxx	0.4	0.4	0.3	0.4
59Lys	0.4	0.4	0.3	0.4
60Lys	0.4	0.4	0.3	0.3
Region 2				
24Val	0.1	0.1	0.1	0.04
25Pro	0.5	0.5	0.5	0.1
26Asp	0.6	0.6	0.7	0.2
27Cys	0.3	0.3	0.3	0.2