Identification and characterization of anion binding sites in RNA

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ABSTRACT

Although RNA molecules are highly negatively charged, anions have been observed bound to RNA in crystal structures. It has been proposed that anion binding sites found within isolated RNAs represent regions of the molecule that could be involved in intermolecular interactions, indicating potential contact points for negatively charged amino acids from proteins or phosphate groups from an RNA. Several types of anion binding sites have been cataloged based on available structures. However, currently there is no method for unambiguously assigning anions to crystallographic electron density, and this has precluded more detailed analysis of RNA–anion interaction motifs and their significance. We therefore soaked selenate into two different types of RNA crystals and used the anomalous signal from these anions to identify binding sites in these RNA molecules unambiguously. Examination of these sites and comparison with other suspected anion binding sites reveals features of anion binding motifs, and shows that selenate may be a useful tool for studying RNA–anion interactions.

Keywords: RNA structure; anion binding; ion–RNA interactions; X-ray diffraction; anomalous difference; selenate

INTRODUCTION

In crystal structures of RNA molecules, a variety of solvent and buffer components are observed to bind at discrete sites. This includes water molecules that form hydration spines in both the major and minor grooves (Auffinger and Westhof 1998; Sundaralingam and Pan 2002) and cations that interact directly with functional groups from the nucleic acid and indirectly through water-mediated contacts (Pyle 2002). The positive charge of these ions neutralizes the overall negative charge of the phosphates and is critical for stabilizing the folds of biologically functional RNAs (Draper 2004; Woodson 2005). Less well-characterized are the interactions of negatively charged anions with nucleic acids. Localized

binding of anions has been reported in many crystal structures. However, it can be difficult to identify anions unambiguously within the constellation of solvent molecules and metal ions that surround the RNA (Das et al. 2001). This is largely because many of the anions used in crystallization experiments have electron densities that are similar to cations and solvent molecules present in the crystals. It has been proposed that anion binding sites in RNA crystal structures may correlate with locations ideal for interactions with negatively charged protein side chains or with the phosphates of nucleic acids (Masquida et al. 1999; Auffinger et al. 2004).

A study of anion binding sites in nucleic acid crystal structures was published in 2004 by Auffinger et al. (2004). In this study, 18 structures were used to analyze the binding sites of chloride, phosphate, and sulfate anions and to identify several potential binding motifs. These diverse sites were formed by the amine, N1, N3, and N7 of the purine bases, the amine and N3 of the C base, and the 2'-OH of the ribose sugar. In some cases these sites were reported as anion binding sites in the original studies, but in other cases they were reported as cations; Auffinger et al. (2004) suggest that the latter may be mislabeled or misidentified sites. Misidentification of anion binding sites could occur because binding of the negatively charged anions to the highly negatively charged RNA is counterintuitive and

Abbreviations: CrPVd3, Cricket Paralysis Virus intergenic region internal ribosome entry site (IRES) domain 3 RNA; MPD, methylpentanediol; P4–P6, the P4–P6 domain of the *Tetrahymena* group I intron; SRP, signal recognition particle.

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because the identity of these anions generally must be deduced from their chemical environment and electron density. In rare cases, when resolution is high enough (<2.0 Å), sulfates or phosphates can be identified from their tetrahedral geometry. As most structures of complex RNA molecules are only determined at modest resolution, many anion binding sites within crystal structures may be missed or assigned as cations (Das et al. 2001). Direct methods to identify bound anions have not been reported.

Here, we report the direct identification of anion binding sites within folded RNA structures using X-ray diffraction and the anomalous signal from selenate anions. Anions are found bound to RNA under conditions of both high ionic strength and more biologically relevant ionic conditions. The location of these bound selenates reveals that many of the previously proposed anion binding motifs are authentic. We do observe, however, that most of the anion binding motifs involve multiple stacked bases. These results confirm that localized anion binding sites exist in RNA and that selenate may be a useful general tool to identify many of them.

RESULTS AND DISCUSSION

Bound selenate and sulfate anions identified by anomalous diffraction

We recently solved the crystal structure of the Cricket Paralysis Virus intergenic region internal ribosome entry site domain 3 RNA (hereafter referred to as CrPvD3) by X-ray

crystallography (Costantino et al. 2008). Crystals of this RNA grow in high concentrations of Li_2SO_4 , which interferes with binding of many heavy atoms that are useful to derivatize RNA crystals for phase determination. As part of the strategy to solve this structure, the crystals first were grown in high concentrations of Li_2SO_4 (1.4–1.5 M) and then transferred to a solution containing lithium acetate (LiCH_3CO_2) and either cobalt (III) or iridium (III) hexammine. The manipulated crystals then were used to obtain phase information and refine the structure (PDB ID 3B31) (Keel et al. 2007; Costantino et al. 2008). The full rationale for this strategy is described elsewhere (Costantino et al. 2008). Subsequently, data collected from native, underivatized CrPvD3 crystals that were grown and cryoprotected in Li_2SO_4 were used to generate difference Fourier maps (Table 1). Examination of $1F_o - F_c$ difference Fourier maps from data sets obtained from two different sulfate-containing crystals (crystals CrPvD3 #1 and CrPvD3 #2) reveals four strong areas of density located near the RNA that we labeled sites 1–4 (Fig. 1A). Because these areas of density are not present in density maps from LiCH_3CO_2 -containing crystals but are visible in the maps generated from crystals cryoprotected with Li_2SO_4 , we hypothesized that these areas of density were bound sulfate anions, but the modest resolution of the diffraction data precluded verification based on geometry.

To unambiguously identify these areas of density, we replaced Li_2SO_4 with Li_2SeO_4 , either by directly growing the crystals in Li_2SeO_4 or by transferring crystals grown in Li_2SO_4 to Li_2SeO_4 . Selenium is in the same chemical family as

TABLE 1. Crystallographic data collection statistics

Crystal	P4–P6(ΔC209)	CrPvD3 #1	CrPvD3 #2	CrPvD3 #3	CrPvD3 #4
Anion	Selenate	Sulfate/native	Sulfate/native	Selenate	Selenate
Resolution range (Å)	20–2.9	42–2.2	49–1.9	42–3.0	50–3.1
Wavelength (Å)	0.9756	1.1271	1.1271	0.9786	0.9756
Space group	P2 ₁ 2 ₁ 2 ₁	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Unit cell					
a (Å)	75.12	58.94	59.28	59.21	59.63
b (Å)	125.4	58.94	59.29	59.21	59.63
c (Å)	145.2	99.06	99.72	99.70	99.35
α (degrees)	90	90	90	90	90
β (degrees)	90	90	90	90	90
γ (degrees)	90	90	90	90	90
Total observations	241,068	100,378	180,657	52,370	69,994
Unique reflections	30,965	9451	14,084	6953	3580
Intensity/s intensity	30.2	6.7	6.8	12.2	27.2
(last shell)	2.6	2.3	2.0	4.7	7.2
Completeness (%)	99.4	100	99.9	99.9	99.4
(last shell)	98.7	100	99.7	99.8	94.6
Rsym					
overall	6.4	22.2	22.3	9.6	15.0
(last shell)	53.2	83.0	86.2	31.6	41.0

R linear: $\text{SUM} [|\text{ABS}(I) - \langle I \rangle|] / \text{SUM}(I)$.

FIGURE 1. Identification of bound SO_4^{2-} and SeO_4^{2-} anions. (A) Two overlaid $1F_o - F_c$ difference maps generated using the structure of the CrPVd3 RNA (gray) solved using acetate-containing crystals and the diffraction data from two crystals containing SO_4^{2-} (cyan from crystal CrPVd3 #1 and red from crystal CrPVd3 #2). The four strong and distinct areas of density are labeled 1–4; they appear in both maps and, therefore, were preliminarily identified as SO_4^{2-} anions. Maps are contoured at 4.5σ and are rendered within $5\text{ \AA}$ of all RNA atoms. (B) Anomalous difference Fourier maps from two separate crystals containing $\text{Li}_2\text{SeO}_4^{2-}$ (red and yellow) overlaid on the structure of the CrPVd3. Areas of density unambiguously indicate bound SeO_4^{2-} . Map 1 is from crystal CrPVd3 #3 and map 2 is from crystal CrPVd3 #4. Sites corresponding to the four putative SO_4^{2-} sites in A are labeled. Map 1 is contoured at 6σ and map 2 at 5σ , and both are rendered within $5\text{ \AA}$ of all RNA atoms.

sulfur, positioned one row below it on the periodic table. Selenate and sulfate are isoelectronic, have similar bond lengths ($\sim 1.47\text{ \AA}$ for sulfate vs. $\sim 1.64\text{ \AA}$ for selenate) (Zalkin et al. 1964; Kolitsch 2002), and both anions interact with their ligands through tetrahedrally coordinated oxygen atoms. In these characteristics, sulfate and selenate are more similar than Mg^{2+} and Mn^{2+} , which have different ligand preferences and therefore can have overlapping but also distinctly different binding sites. We hypothesized that the chemical and structural similarity of selenate and sulfate would allow them to bind to the same locations in the RNA. Diffraction data were collected at a wavelength corresponding to the K edge of selenium in its oxidized SeO_4^{2-} state to maximize the anomalous diffraction signal (Table 1; Smith and Thompson 1998). These data were used with the refined structure (without modeled sulfate) to generate anomalous difference Fourier maps in which areas of density indicate selenium atoms. Maps from two separate data sets from two SeO_4^{2-} -containing crystals prepared using two different protocols were examined. These reveal unambiguous density in the locations of the four putative SO_4^{2-} binding sites, although neither map contained all four sites (Fig. 1B). Specifically, SeO_4^{2-} map 1 contains sites 1–3 and SeO_4^{2-} map 2 contains sites 2–4. The presence of anomalous difference density at these sites unambiguously confirms them as bound SeO_4^{2-} anions, and by extension, the corresponding sites in the maps calculated with SO_4^{2-} -containing crystals are assigned to bound SO_4^{2-} .

Binding of anions under low-salt conditions

Crystals of CrPVd3 grow in buffers that are extremely high in ionic strength, 1.4–1.5 M Li_2SO_4 (Costantino et al.

2008). These conditions are not physiological and anion binding to this RNA may be the result of these extreme buffer conditions. To investigate whether anion binding occurs in RNAs under low-ionic strength conditions, we explored anion binding to another RNA, the ΔC209 mutant of the P4–P6 domain of the *Tetrahymena* group I intron (P4–P6) (Juneau and Cech 1999; Juneau et al. 2001). This RNA crystallizes readily in physiologically relevant ionic strength conditions using methylpentanediol (MPD) as a precipitant. Crystals were soaked in a cryostabilization buffer containing 25 mM Na_2SeO_4 prior to freezing and data collection. Again, we used the ability to locate bound SeO_4^{2-} anions (and by extension, SO_4^{2-} binding sites) using anomalous diffraction. Anomalous difference Fourier maps contain several regions of density corresponding to bound selenate (Fig. 2A), suggesting that anions can bind to RNA under biologically relevant ionic strengths. These sites are present in both molecules in the asymmetric unit.

To summarize, the strategy of using SeO_4^{2-} coupled to anomalous X-ray diffraction analysis allowed us to assign the locations of bound SeO_4^{2-} anions unambiguously in two different crystallized RNAs. The concentration of SeO_4^{2-} present in these two crystals differed by more than an order of magnitude and similar binding sites were observed in both crystals. It is therefore unlikely that binding of these anions is an artifact of extremely high SeO_4^{2-} concentrations. Rather, these sites likely represent authentic areas of

FIGURE 2. Coupled cation and anion binding in the major groove. (A) Crystal structure of the P4–P6 $\Delta 206$ domain of the *Tetrahymena* group I intron (PDB ID 1HR2) (Juneau et al. 2001) in the presence of 25 mM SeO_4^{2-} and 25 mM Mg^{2+} . Bound Mg^{2+} cations are shown in green with coordinated waters in red. An anomalous difference Fourier map contoured at 5σ and rendered within $5\text{ \AA}$ of all atoms is overlaid in magenta. Two boxed regions show the locations of bound SeO_4^{2-} in the vicinity of bound Mg^{2+} (sites 7 and 8), and the identities of neighboring bases are labeled. (B) A portion of the hairpin ribozyme (PDB ID 2OUE) solved at $2.02\text{ \AA}$ resolution. A sulfate ion (red and yellow) is found bound in complex with a cobalt (III) hexamine ion (Salter et al. 2006). (C) A portion the structure of the *E. coli* SRP RNA bound to the M-domain protein (PDB ID 3LQX). Two cobalt (III) hexamine trivalent cations are shown in orange and blue, and an ion identified as Cl^- is shown in yellow. The Cl^- was identified on the basis of bond length, crystallographic temperature factor, and ligand identity. Bases that interact with the Cl^- are labeled.

local positive charge where the chemical environment is conducive to anion binding.

A major groove anion binding motif

In the CrPVd3 RNA, SeO_4^{2-} sites 1 and 3 are both within the major groove in the vicinity of tandem G–C pairs (both Gs on the same strand) and are positioned to interact with the two consecutive C bases (Fig. 3A). This binding position is similar to the CYT_CH site as defined by Auffinger et al. (2004); however, the authentic motif for these anion binding sites consists of more than one base or base pair rather than a single C as proposed by Auffinger et al. Major groove SO_4^{2-} binding sites similar to sites 1 and 3 also were observed recently in the crystal structures of mutated sarcin/ricin loops from 23S rRNA (PDB IDs 1Q93, 1Q96) (Fig. 3B; Correll et al. 2003), the hairpin ribozyme (PDB ID 2OUE) (Fig. 2B; Salter et al. 2006), and the ligand-binding domain of the preQ(1) riboswitch (PDB ID 3GCA) (Fig. 3C; Spitale et al. 2009). These RNAs, like the CrPVd3 RNA, crystallized in high concentrations of Li_2SO_4 or $(\text{NH}_4)_2\text{SO}_4$. These sites involve two C bases of consecutive G–C pairs as observed in the CrPVd3 RNA (Fig. 3A). A second class of site in which the sulfate binds in the major groove and interacts with the exocyclic amines on adjacent C–G and A–U base pairs are found in one structure of the sarcin/ricin loop (PDB ID 1Q93 but not 1Q96) (data not shown; Correll et al. 2003) and in the preQ(1) riboswitch (Fig. 3C).

The common feature among all of these sites is that they involve two bases and amine moieties in the major groove from these two adjacent bases, and this is consistent with

FIGURE 3. Details of the CrPVd3 RNA, sarcin/ricin loop, and preQ(1) riboswitch sulfate binding sites. (A) The structural details of sulfate/ SeO_4^{2-} binding sites 1–4 are shown. At *left*, the SO_4^{2-} is shown in a stick model in the context of the CrPVd3 RNA structure. At *middle* and *right*, the SO_4^{2-} anion is shown with just the nucleotides with which it interacts, viewed from two different orientations. The identity of interacting bases is labeled. For clarity, only the sulfur atom is shown as a sphere. Structures of (B) sarcin/ricin loop from 23S rRNA (PDB ID 1Q96) (Correll et al. 2003) and (C) the ligand-binding domain of the preQ(1) riboswitch (PDB ID 3GCA) (Spitale et al. 2009) with two SO_4^{2-} binding sites shown. The sulfates are depicted as spheres in the major groove. Interacting bases are indicated, with C bases in blue, G bases in green, a U base in red, and an A base in yellow.

the prediction that runs of consecutive C bases would be associated with anion binding sites (Auffinger et al. 2004). This is therefore likely a distinguishing feature of many RNA major groove anion binding sites and contrasts with major groove cation binding sites that often involve adjacent carbonyl groups of G and U bases (Cate et al. 1997; Kieft and Tinoco 1997; Masquida and Westhof 2000; Varani and McClain 2000; Keel et al. 2007).

Anion binding sites outside the major groove

Site 2 in the CrPVd3 is located in the minor groove where it interacts with the amine group of a G base (Fig. 3A). In this case the anion site appears to interact with a single base and is therefore similar to a GUA_S site previously described (Auffinger et al. 2004). Site 4 clearly involves only the Watson–Crick face of a single unpaired G base as no other base is positioned to interact with the anion (Fig. 3A). The anion can interact with both the amino and imino groups of the base; this type of anion binding motif is similar to the GUA_WC motif (Auffinger et al. 2004), and a similar site was observed in the mutant sarcin/ricin loop structure (data not shown; Correll et al. 2003).

Anion binding sites often occur near cation binding sites

In the P4–P6 ΔC209 RNA, two strong SeO_4^{2-} binding sites are observed, both within the P5abc subdomain (Fig. 2A). There are two molecules in the asymmetric unit of these crystals, and a bound SeO_4^{2-} ion is observed at the same sites in both molecules. These two sites share two features. First, in both sites the SeO_4^{2-} is positioned to interact with the C in a G–C pair. Second, each of these anions is bound in close proximity to a bound magnesium cation in the major groove. These observations show that anion binding in RNA structures can be proximal to cation binding in the major groove. While we do not observe cations bound in the CrPVd3 structure, these crystals contained >4 M lithium. These lithium ions do not possess sufficient electron density to be visible, and they likely displaced bound divalent cations. However, in the P4–P6 structure, the presence of 25 mM MgCl_2 and lower monovalent concentration allows the divalent cations to bind in discrete locations and to be observed crystallographically.

Additional evidence for cations and anions binding together in the RNA major groove is found in the aforementioned hairpin ribozyme structure, where the bound sulfate is accompanied by a bound trivalent cobalt (III) hexamine cation (PDB ID 2OUE) (Fig. 2B; Salter et al. 2006). Also, the structure of the *E. coli* SRP RNA bound to the M-domain protein (PDB ID 3LQX) contains a chloride ion bound between two cobalt (III) hexamine trivalent cations (Fig. 2B; Batey et al. 2000; Batey and Doudna 2002). The identity of the chloride was inferred by the presence of chloride in the

crystallization solution, the low crystallographic B-factor (compared with water), and the chemical environment (R Batey, pers. comm.). Specifically, six amine groups are positioned within 3.2–3.5 Å from the putative chloride, including one from a C base and one from an A. The position of this chloride is likely dictated by these base functional groups, and the strong positive charge of the two proximal cations: It is attracted to both cations and likely facilitates the close approach of these two cations. Similar “electrostatic sandwiches” are seen in some protein active sites (Jiang et al. 2002). Hence, it is likely that anion and cation binding are coupled in this site; our data suggests this may be a theme that applies to many other ion–RNA interactions in the major groove. If true, the combined effects of cations and anions may be important for stabilizing the fold of complex RNA structures.

Selenate as a tool

The ability to observe the anomalous signal from SeO_4^{2-} anions bound to RNA is a potentially useful tool for studying RNA structure. By soaking SeO_4^{2-} into RNA crystals and collecting anomalous diffraction data, anion binding sites potentially can be distinguished from water or cation binding sites, and putative anion binding sites can be identified. This can reduce ambiguity in assigning electron density in X-ray structures. In the case of the P4–P6 crystals, SeO_4^{2-} binding was observed at only 25 mM SeO_4^{2-} concentration and, hence, the high levels of SO_4^{2-} or SeO_4^{2-} present in the other structures (CrPVd3, preQ(1) riboswitch, sarcin/ricin loop) may not be necessary to induce binding of anions to authentic sites. In addition, we observed SeO_4^{2-} binding at motifs previously reported to bind phosphate (GUA_WC), sulfate (GUA_WC, CYT_CH, GUA_S), and chloride (GUA_WC, CYT_CH, GUA_S) anions. Thus, selenate may be a useful general probe for sites capable of binding other anions (Auffinger et al. 2004), although binding sites specific for certain anion species may also exist.

Unlike selenium, phosphorus and chloride do not have X-ray absorption edges at wavelengths that are readily observable by most synchrotron light sources; however, they are potentially detectable by several means. First, these anions are expected to have ligand geometries that differ significantly from those of water and magnesium ions. Second, these anions are expected to have a preponderance of nitrogenous ligands from the RNA. Third, when modeled as a water, these ions will often have an unrealistically low B-factor. Fourth, although the signal is significantly weaker than selenium, phosphorus and chlorine can be observed by anomalous scattering using a diffraction data set obtained at longer wavelengths (at 8000 eV, f'' equals 0.71e and 0.44e for chlorine and phosphorus, respectively). Finally, bromide, like selenate, has an X-ray absorption edge that can be used to identify binding sites for halide ions using anomalous difference Fourier methods. Unfortunately, attempts to

identify bromide binding sites in the P4–P6 $\Delta 206$ domain yielded ambiguous results (E Chase and BL Golden, unpubl.).

Conclusions

Anions bind to RNA. Although localized bound anions are often found in crystal structures in the presence of very high anion concentrations, we unambiguously observe SeO_4^{2-} bound at lower concentrations as well. This suggests that anions can bind in solution at physiological concentrations. Binding sites are found at positively charged regions: exocyclic amines of A and C and metal ions. The biological relevance of these anions remains unclear, however. They may work in concert with cations to help stabilize RNA structure, they may indicate interaction sites for protein side chains or phosphates, and they could modulate intermolecular interactions (Auffinger et al. 2004). The use of SeO_4^{2-} to identify anion binding sites provides a useful tool for future explorations of anion–nucleic acid interactions, anion–protein interactions, and may even be a tool to obtain phases to solve RNA crystal structures.

MATERIALS AND METHODS

Crystallization and data collection

The transcription, purification, and crystallization of CrPVd3 RNA is described in detail elsewhere (Keel et al. 2007; Costantino et al. 2008). To generate native, sulfate-containing crystals of this RNA, a solution of 5 mg/mL RNA, 10 mM K-HEPES (pH 7.5), 2.5 mM MgCl_2 , and 0.5 mM spermidine-HCl was heated to 65°C for 3 min and allowed to cool on the bench. This was combined at a 1:1 ratio (1 μL + 1 μL) with 1.4 M Li_2SO_4 , 40 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 50 mM HEPES-NaOH (pH 7.5) in a hanging-drop/vapor diffusion experiment at 30°C. Crystals appeared in a few days, and then grew over the course of several weeks to maximum dimensions. To cryoprotect, the well solution was replaced with 2.4 M Li_2SO_4 and the well was resealed and allowed to equilibrate for at least 24 h. The crystals CrPVd3 #1 and CrPVd3 #2 were harvested from the drop and cooled directly in liquid nitrogen. Data were collected at beamline 8.2.1 at the Advanced Light Source (Table 1).

The SeO_4^{2-} -containing crystal CrPVd3 #3 was grown as above except the Li_2SO_4 was replaced with 1.8 M Li_2SeO_4 and was cryoprotected by replacing the well solution with 2.4 M Li_2SO_4 as described above. CrPVd3 #4 was grown in 1.4 M Li_2SO_4 and then transferred into saturated Li_2SeO_4 . Crystals were harvested and flash-cooled in liquid nitrogen. Data for crystal CrPVd3 #3 were collected at beamline 4.2.2 of the Advanced Light Source and data for crystal CrPVd3 #4 were collected at beamline 22-ID at the Advanced Photon Source (Table 1).

Crystals of the P4–P6 $\Delta 206$ domain were produced essentially as previously described (Juneau et al. 2001). A 1–2 μL vol of a solution containing 4 mg/mL RNA, 10 mM NaCl, 25 mM MgCl_2 , and 5 mM HEPES-KOH (pH 7.5) was mixed with a 1–2 μL vol of precipitant containing 50 mM K-cacodylate pH 6.5, 0.5 mM spermine, and 18%–20% MPD. The resulting drop was equilibrated against the precipitant in a hanging-drop/vapor diffusion

experiment at 18°C. Crystals were soaked in 25% MPD, 20% isopropanol, 50 mM potassium cacodylate (pH 6.5), 1 mM spermine, 25 mM MgCl₂, 25 mM Na₂SeO₄ for 2 h prior to flash-cooling in liquid nitrogen. Crystallographic data were collected at beamline 14-ID-B at the Advanced Photon Source (Table 1).

Diffraction data processing, molecular replacement, and refinement

Diffraction data were integrated and scaled using either d*trek (Pflugrath 1999) or HKL2000 (Otwinowski and Minor 1997). The structures of CrPvD3 #1, CrPvD3 #2, CrPvD3 #3, and CrPvD3 #4 were solved by molecular replacement using Phaser (McCoy et al. 2007) with a search model that consisted of PDB entry 3B31 with water and ions removed (Costantino et al. 2008). The solution was refined with CNS (Brunger et al. 1998) by energy minimization. Sulfates or selenates were added as their binding sites were identified, and additional refinement was conducted. The final R/R_{free} of CrPvD3 #2 was 24.2/27.6, of CrPvD3 #3 was 23.2/28.4, and of CrPvD3 #4 was 22.7/26.3 (CrPvD3 #1 data was not used in refinement). Data from the P4–P6 crystals was integrated and scaled with HKL2000 (Otwinowski and Minor 1997). The selenium anomalous difference map was calculated with CNS (Brunger et al. 1998) using phases from the model 1HR2 (Juneau et al. 2001). Coordinates have been deposited in the Protein Data Bank with accession numbers 3MJB, 3MJa, and 3MJ3.

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