Structure of rhodocetin reveals noncovalently bound heterodimer interface

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Abstract

Rhodocetin is a unique heterodimer consisting of α - and β -subunits of 133 and 129 residues, respectively. The molecule, purified from the crude venom of the Malayan pit viper, *Calloselasma rhodostoma*, functions as an inhibitor of collagen-induced aggregation. Rhodocetin has been shown to have activity only when present as a dimer. The dimer is formed without an intersubunit disulfide bridge, unlike all the other Ca^{2+} -dependent lectin-like proteins. We report here the 1.9 Å resolution structure of rhodocetin, which reveals the compensatory interactions that occur in the absence of the disulfide bridge to preserve activity.

Keywords: platelet aggregation inhibitor; *Calloselasma rhodostoma*; heterodimer; C-type lectin-like protein; domain swapping

Many proteins with platelet aggregation and blood coagulation effects have been purified from snake venoms. These are a diverse group of molecules which differ in structure and mechanism of action. A group of nonenzymatic proteins from among them have structural homology to Ca^{2+} -dependent lectin-like proteins (CLPs) (Wang et al. 1999). These proteins are generally heterodimers of molecular weight ~30,000 with a disulfide bridge linking the subunits. Although CLPs share significant sequence and structural similarity, they display varying effects on blood coagulation and platelet aggregation (Wang et al. 1999). Some of them, for example, bind coagulation factors X and/or IX, such as ECLV IX/X-bp from *Echis carinatus leucogaster* venom (Chan et al. 1996), jararaca IX/X-bp from *Bothrops jararaca* venom (Sekiya et al. 1993), and habu IX/X-bp and IX-bp from *Trimeresurus flavoviridis* venom (Atoda et al. 1991, 1995). In contrast, bothraracarin from *Bothrops jararaca* venom (Zingali et al. 1993) is a specific inhibitor of thrombin.

Rhodocetin, a novel CLP platelet aggregation inhibitor, was purified from the crude venom of *Calloselasma rhodostoma* (Wang et al. 1999). Rhodocetin inhibited collagen-induced platelet aggregation in a dose-dependent manner with an IC_{50} of 41 nM (Wang et al. 1999). Like several other CLPs, rhodocetin is a heterodimer; the complete amino acid sequences of the α -chain (15,956.16 Da; 133 residues) and β -chain (15,185.10 Da; 129 residues) share 49% identity with each other and typically 29%–46% identity with other snake venom CLPs (Wang et al. 1999). Both reducing and nonreducing SDS-PAGE on the purified protein showed two bands consistent with the lack of an intersubunit disulfide bond. Therefore, unlike the other members of the CLP family, in which the subunits are held together by an interchain disulfide bond, the subunits of rhodocetin are held together only by noncovalent interactions. The cysteinyl residues forming the intersubunit disulfide bridge in all other known CLPs are replaced by Ser 79 and Arg 75 in the α - and β -subunits of rhodocetin, respectively (Wang et al. 1999). Interestingly, rhodocetin is functional only as a heterodimer; individually, neither the α - nor the β -subunit shows significant inhibition of aggregation (Wang et al. 1999).

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Here we present the 1.9 Å resolution X-ray structure of rhodocetin determined by molecular replacement. The structure reveals the intersubunit interface, which has compensatory interactions for forming the dimer in the absence of the disulfide bridge. This is the first structure of a CLP without an intersubunit disulfide and thus represents a novel molecule. Further, unlike other CLPs, rhodocetin does not require metal ions for its functional activity. However, like other CLPs, rhodocetin forms the heterodimer by domain swapping, in which the central looped region is swapped between the subunits.

Results and Discussion

X-ray structure determination

Rhodocetin was obtained from crude venom of *Calloselasma rhodostoma*. The protein (MW 31,140) was isolated using gel filtration, anion exchange, and hydrophobic interaction chromatography, and found to be homogeneous by mass spectrometry (data not shown). Crystals of the purified protein were obtained using the hanging drop vapor diffusion method and diffracted to 1.9 Å. Factor IX-bp (Protein Data Bank ID 1BJ3; Mizuno et al. 1999) has 46% identity to rhodocetin, and its structure was used to prepare a molecular replacement search model. A model consisting of the A chain of 1BJ3 lacking residues 1–6 and 69–102 was sufficient to obtain a molecular replacement solution which yielded excellent refinement statistics. Data collection and refinement statistics are given in Table 1.

Subsequent inspection of the resulting $2F_o - F_c$ map allowed unambiguous tracing of 99% of the amino acid resi-

Figure 1. Electron density map. A portion of $2F_o - F_c$ map after the final refinement demonstrating the quality of the structure.

dues in the α -subunit and 96% of the amino acid residues in the β -subunit (Fig. 1). However, the side chain of N-terminal residue Asp 1 of the α -subunit and the side chains of Arg 65 and Pro 128 of the β -subunit were omitted owing to poor interpretable density, and these residues were modeled as alanines. For the same reason, side chains of Trp 104, Glu 105, and Lys 107 of the β -subunit were modeled as glycines. The C-terminal residue of Arg 133 of the α -subunit and residues Asp 1, Asp 62, Lys 63, Gln 64, and His 129 of the β -subunit were omitted from the model due to poor density.

The Ramachandran plot shows that 92.1% of the residues fall in the most favored region and the rest in the additional allowed region, except for Thr 7 in the β -subunit, which falls in the generously allowed region. In total, the α -subunit (132 residues), the β -subunit (124 residues), and 168 solvent molecules were built into the high-quality electron density map. The structure was refined at 1.9 Å to an R-factor of 18.9% and an R-free of 23.1%.

Overall fold

Though the α - and β -chains of rhodocetin share only 49% identity with each other, their structures superimpose almost perfectly except for the extended loop and the loop region following the first α -helix: The RMSD between their C_α atoms is 1.04 Å. In the α -subunit, the loop region (Gln 33–Lys 35) following the first α -helix forms a γ -turn. However, the corresponding loop region in the β -subunit (Ala 36–Gly 39) forms a β -turn. Each subunit contains three intrasubunit disulfide bonds: Cys 2–Cys 13, Cys 30–Cys 127, and Cys 102–Cys 119 in the α -subunit, and Cys 4–Cys 15, Cys 32–Cys 123, and Cys 98–Cys 115 in the β -subunit. The disulfides in each subunit are found in similar 3D positions in both subunits (Fig. 2). The structure of rhodocetin comprises a globular unit with an extended loop and forms a CLP fold. The α -subunit contains eight β -strands and two

Table 1. Data collection and refinement statistics

Data collection	
Space group	$P2_12_12_1$
Unit cell parameters (Å, °)	$a = 46.87, b = 65.93, c = 118.84,$ $\alpha = \beta = \gamma = 90.0$
Resolution range (Å)	30.0–1.90
No. of observed reflections	184,500 (6507)
No. of unique reflections	28,995 (2304)
Redundancy	6.36 (2.82)
Completeness (%)	97.20 (78.7)
R_{sym} (all reflections)	0.060 (0.307)
Model refinement	
No. of protein atoms	2104
No. of water molecules	167
R-factor	0.189
Rfree (test set of 5%)	0.231
Data cutoff in σ units	none
RMS bond length	0.020
RMS bond angle	1.672
RMS chiral	0.175

Values for parameters in the highest resolution shell, 1.97–1.90 Å, are parenthesized and in italics.

Figure 2. Stereo view showing superposition of subunits. The disulfides in each subunit are found in similar positions in the 3D structure. The α -subunit is in red, and β -subunit, in blue. Disulfide bonds are colored yellow.

α -helices, with seven strands forming two antiparallel β -sheets, while the β -subunit contains seven β -strands, six forming two antiparallel β -sheets, and two α -helices. It is important to note that the β -strand spanning four residues from Asn 80 to Ser 84 of α -subunit runs antiparallel to the β -strand spanning four residues from Gly 71 to Arg 75 of the β -subunit to form a two-stranded β -sheet which is a critical secondary structure element for dimerization (Fig. 3). In addition to this β -sheet, the extended loops also form interactions. The backbone carbonyls of Gly 69, Asn 93, and Trp 114 in the α -subunit form hydrogen bonds with the

Figure 3. Novel interface of rhodocetin. Residues from Asn 80 to Ser 84 of the α -subunit run antiparallel to the β -strand spanning four residues from Gly 71 to Arg 75 of the β -subunit to form a two-stranded β -sheet. This is a critical secondary structure element for dimerization. Hydrogen bonds are shown in yellow. The α -subunit is in green and β -subunit in blue.

backbone amides of Thr 80, Trp 110, and Tyr 91 in the β -subunit, while the backbone amides of Tyr 95 and Trp 114 in the α -subunit form hydrogen bonds with the backbone carbonyls of Trp 110 and Asn 89 in the β -subunit.

Structure comparison with C-type lectins

As mentioned earlier, rhodocetin is homologous to other CLPs, in particular snake venom CLPs (up to 46% identity; see Fig. 6, below). A structure similarity search using the DALI server (Holm and Sander 1993) revealed 18 known structures that belonged to the C-type lectin superfamily which had similarity to the α - and β -subunits of rhodocetin with Z scores >2.0 . In particular, IX/X-bp (Mizuno et al. 1997), lithostathine (Bertrand et al. 1996), CRD-4 (Feinberg et al. 2000), and tetranectine (Nielsen et al. 1997) were most similar, with Z scores $>0.13.0$. Superposition of IX/X-bp, lithostathine, CRD-4, and tetranectine with rhodocetin revealed that major differences in their structure reside in the central loop region. Lithostathine and tetranectine are monomeric C-type lectins, whereas IX/X-bp and CRD-4 are dimers. In monomeric C-type lectins, the central loop folds back into the body, while in dimers it forms an central loop extended away from the body, as seen in IX/X-bp and CRD-4 (Mizuno et al. 1997) and rhodocetin. Furthermore, superposition of IX/X-bp subunit A with rhodocetin α -subunit shows that the significant deviation in structure is due to a rotation of the loop by $\sim 90^\circ$. The rotated loop spans 27 residues, from residue 73 to residue 100 (Fig. 4).

A comparison of the 3D structures of coagulation factor IX/X-bp and botrocetin shows that only local differences exist between them, which are mainly due to insertions/deletions of residues. In addition, all of the heterodimers are linked by a disulfide bond (Mizuno et al. 1999), unlike rhodocetin. In both factor IX/X-bp and X-bp, the interchain disulfide bond occurs between Cys 79A and Cys 75B, while in botrocetin, it occurs between Cys 80A and Cys 75B. The residues in homologous positions to these Cys residues are Ser 79 and Arg 75 in the α - and β -subunits of rhodocetin, respectively (Wang et al. 1999). Moreover, rhodocetin has a unique interface, as it lacks the canonical intersubunit disulfide bridge. This novel interface of rhodocetin allows noncovalent interactions to compensate for the missing disulfide bond. There is an ordering of the loop regions from Asn 80 to Ser 84 of the α -subunit and from Gly 71 to Arg 75 of the β -subunit to create an extra β -sheet not previously observed in other CLPs at the interface, in which the backbone carbonyls of Asn 80A and Glu 82A form hydrogen bonds with the backbone amides of Arg 75B and Thr 73B, whereas the backbone amides of Glu 82A and Ser 84A participate in forming hydrogen bonds with the backbone carbonyls of Thr 73B and Gly 71B.

Figure 4. Superposition of subunits A in IX/X-bp (red), IX-bp (green), and botrocetin (yellow) with rhodocetin α -subunit (blue) shows that the loop in rhodocetin spanning 27 residues is rotated by $\sim 90^\circ$ with respect to the similar loop in IX/X-bp.

Dimerization by 3D domain swapping

The structure of rhodocetin shows an exchange of the extended loop region between the α - and β -subunits (residues within Leu 81–Leu 94 in the α -subunit and residues within Leu 77–Leu 90 in the β -subunit), indicating that dimerization is likely accomplished through 3D swapping. The phenomenon of 3D domain swapping has been studied by examining (1) bona fide 3D domain-swapped proteins, the structures of whose monomeric and oligomeric forms have been characterized by X-ray diffraction or NMR; (2) pairs of proteins whose structures mimic monomeric and 3D domain-swapped oligomers, but whose amino acid sequences differ; and (3) intertwined oligomers that are reminiscent of 3D domain-swapped proteins, but for which no monomeric form is known (Schlunegger et al. 1997). If mannose-binding protein (Weis et al. 1991) is taken as the reference monomer as described for IX/X-bp (Mizuno et al. 1997), rhodocetin falls into the second category—as a quasidomain-swapped dimer. Domain swapping in the C-type lectin superfamily is dominated by characteristic hydrophobic interactions. These specific interactions between the loop and the body of the adjoining subunit form a C-interface (Sen et al. 2001). In rhodocetin, the C-interface involves Leu 81, Trp 83, Ile 89, Tyr 91, and Leu 94 on the swapped loop of the α -subunit, and Ile 44, Ala 47, Leu 72, Phe 99, and Trp 110 on the body side of the β -subunit (Fig. 5). Likewise, the other C-interface consists of Leu 77, Trp 79, Val 85, Tyr 87, and Leu 90 on the swapped loop of the β -subunit and the Ala 48, Leu 70, Ile 72, Phe 103 and Trp 114 on the body side of the α -subunit. In addition to the hydrophobic interactions, hydrogen-bonding interactions are also observed in the C-interface: The α -subunit carbonyl Asn 93 and amide

Tyr 95 interact with the amide of Trp 110 and the carbonyl of Trp 110 of the β -subunit, respectively. Similar structural features occur between the loop of the β -subunit and the body of the α -subunit.

The characterization of rhodocetin by gel filtration showed that it is a dimer in solution (Wang et al. 1999). As mentioned earlier, the integrity of the dimer is essential for the activity of rhodocetin; neither the α -subunit nor the β -subunit in isolation inhibits platelet aggregation (Wang et al. 1999). Topological comparison of the CLPs IX/X-bp, IX-bp, lithostathine, E-selectin, tetranectin, and mannose-binding protein showed that the C-terminal side of the hinge loop in the dimers is six or more residues shorter than that of the monomeric proteins. If a hinge loop is shortened by a deletion, then the closed monomer structure may no longer be possible in geometry, and the resultant open monomer may be unstable because of the exposure of residues normally buried in the C-interface. Domain-swapped dimers would then be favored (Bennett et al. 1995; Mizuno et al. 1999). Comparison of the rhodocetin C-terminal side hinge loop with mannose-binding protein revealed that the C-terminal side hinge loop of rhodocetin is six residues shorter than that of the monomeric protein. As a result, the central loop is swapped and subsequently a functional heterodimer is formed.

Lack of metal binding site

Calcium ions are essential to the function and for the crystallization of IX-bp protein. There are only a few examples of proteins crystallized without calcium ions that require Ca^{2+} to function. Intrinsic fluorescence analysis of IX/X-bp in the presence of calcium ions indicated that Ca^{2+} binding induces conformational changes which stabilize the structure of the protein (Mizuno et al. 1999). In contrast to these proteins, inhibition of platelet aggregation by rhodocetin was shown to be independent of any metal ligand (Kong

Figure 5. One of the C-interfaces of rhodocetin. Residues on the body side are indicated in blue, and residues located in the swap loop are in green.

2002). Moreover, this protein can crystallize without metal ions. This indicates that rhodocetin does not need metal ligands to fold into an ordered structure in order to preserve its function.

A structural comparison of IX-bp, IX/X-bp, and botrocetin Ca^{2+} -binding residues with rhodocetin revealed some interesting features. Each subunit of IX-bp and IX/X-bp has a Ca^{2+} -binding site. In botrocetin, only subunit B has a bound divalent metal ion, and in rhodocetin, neither subunit has the ability to bind Ca^{2+} ions. The loss of the metal ion-binding site in subunit A of botrocetin is due to the replacement of residues Glu 43 and Glu 128 in IX/X-bp and IX-bp with Lys 43 and Lys 128 (Sen et al. 2001). In IX-bp and IX/X-bp, residues Ser 41, Glu 43, Glu 47, and Glu 128 in subunit A, and residues Ser 41, Gln 43, Glu 47, and Glu 120 in subunit B coordinate the binding of Ca^{2+} ions (Mizuno et al. 1999). The corresponding residues in rhodocetin are Ser 41, Glu 43, Glu 47, and Lys 128 (α -subunit) and Ser 41, Gly 45, Glu 47, and Lys 124 (β -subunit) (Fig. 6). It is likely that the substitutions of α -chain Lys 128 and β -chain Lys 124 in rhodocetin for A-chain Glu 128 and B-chain Glu 120 in IX-bp, respectively, introduce positive charges in the Ca^{2+} -binding sites and hence prevent Ca^{2+} ions from binding rhodocetin by charge-charge repulsion (Fig. 7). Furthermore, Lys 128 of the α -subunit forms a salt bridge with Glu

49, while Lys 124 of the β -subunit forms a salt bridge with Glu 47. These salt bridges could stabilize the fold in the absence of Ca^{2+} -binding site, and hence form the functional CLP heterodimer.

Differences from the Eble rhodocetin

We have also clearly established that the rhodocetin described in this paper as well as that of Wang et al. (1999) is a distinct entity from the rhodocetin reported by Eble et al. (2001). The rhodocetin reported by Eble et al. was in fact shown to be a dimer under nonreducing denaturing SDS-PAGE conditions, but fell apart into two subunits under reducing SDS-PAGE conditions. Additionally, there are differences in the N-terminal amino acid sequence of the Eble rhodocetin. We checked the electron density in particular for residue 10 in the β -subunit, and found that it corresponded to an Ala (consistent with our rhodocetin sequence), and not Met (in the rhodocetin reported by Eble et al. 2001). The Eble rhodocetin had integrin-binding activity which was not observed with our rhodocetin. Therefore these molecules should be regarded as distinct entities. A structural comparison of the two rhodocetins may help to pinpoint regions in these proteins critical to their different activities.

Figure 6. Sequence alignment of IX/X-bp, IX-bp, and botrocetin with rhodocetin. (A) Alignment of A-chain. (B) Alignment of B-chain. The potential Ca^{2+} -binding residues are highlighted in red. Noncalcium binding residues in homologous positions are in blue.

Figure 7. Geometry around the Ca²⁺-binding site in CLPs. (A) Subunit A. (B) Subunit B. Superposition of the Ca²⁺ ion coordination site in IX/X-bp (green) with the corresponding site in rhodocetin (blue). Glu 128A and Glu 120B of IX/X-bp are replaced by Lys 128 in the α -subunit and Lys 124 in the β -subunit, respectively. This substitution could prevent the metal ion from binding rhodocetin.

Materials and methods

Protein crystallization and data collection

Well diffracting rhodocetin crystals were grown by vapor diffusion from hanging drops in 0.7 M NaH₂PO₄, 0.7 M KH₂PO₄, 0.1 M Na HEPES (pH 7.5) at 21°C. Native crystals were soaked in mother liquor containing 25% glycerol and flash-frozen at 100 K. Native data from a crystal were collected at the National Synchrotron Light Source (NSLS) beam line X8C at one wavelength (0.978569 Å). The crystal diffracted to 1.9 Å, belonged to the orthorhombic space group *P*2₁2₁2₁, and had unit cell dimensions *a* = 46.875, *b* = 65.935, *c* = 118.841, and $\alpha = \beta = \gamma = 90.0$. The data were processed and scaled using DENZO and SCALEPACK from the HKL2000 suite of programs (Otwinowski and Minor 1997).

Structure solution and refinement

The structure was solved by molecular replacement using the program MOLREP (Vagin and Teplyakov 1997). The A chain of coagulation factor IX-bp protein (PDB 1BJ3; Mizuno et al. 1999) was chosen as a search model as it had the highest sequence identity (46%) to rhodocetin. The six N-terminal residues as well as residues 69–102 were deleted from the search model, and two monomers were searched for in the asymmetric unit. A top peak was obtained after translation, with a correlation coefficient of 27.5%. The resulting electron density map revealed distinct secondary structure elements. The phases obtained by molecular replacement were used directly in ARP/wARP (Morris et al. 2002) for automated main chain tracing, resulting in the building of six continuous fragments with connectivity index 0.95 that contained 245 residues. The rest of the model and side chains were fitted

manually using XtalView (McRee 1999). Refinement was carried out with REFMAC5 (Murshudov et al. 1999) using a resolution range of 30.0–1.9 Å, and water molecules were identified using ARP/WARP later in the refinement. The quality of the final model was verified with PROCHECK (Laskowski et al. 1993). The atomic coordinates and structure factors have been deposited in the PDB as entry 1SB2.

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