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Crystallization and preliminary X-ray analysis of the human vascular adhesion protein-1

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Human vascular adhesion protein-1 (VAP-1) is a membrane-bound multifunctional glycoprotein with both adhesive and enzymatic properties. The protein belongs to the copper-containing amine oxidase (CAO) family, which use 2,4,5-trihydroxyphenylalanine quinone as a cofactor. Here, the crystallization and preliminary X-ray analysis of a mammalian CAO, human VAP-1, is reported. The protein was expressed in Chinese hamster ovary cells as a full-length form with an N-terminal transmembrane region and multiple glycosylation sites. Hexagonal crystals with unit-cell parameters $a = b = 225.9$, $c = 218.7$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ were obtained using the vapour-diffusion method. Data from three different crystals were collected at 100 K using synchrotron radiation and were processed to 3.2 Å resolution with 95.9% completeness and an $R_{\rm merge}$ of 19.6%.

1. Introduction

Copper-containing amine oxidases (CAOs; EC 1.4.3.6) belong to the functionally diverse amine oxidase superfamily. They are also known as semicarbazide-sensitive amine oxidases (SSAOs) since their enzymatic activity can be blocked by the carbonyl-reactive compound semicarbazide (Buffoni & Ignesti, 2000; Dawkes & Phillips, 2001). They catalyse the oxidative deamination of primary amines to the corresponding aldehydes in a copper-dependent reaction in which molecular oxygen is consumed and hydrogen peroxide and ammonia are released. A characteristic feature of all CAOs is the use of 2,4,5-trihydroxyphenylalanine quinone (TPQ), a topaquinone, as a redox cofactor. TPQ is generated by post-translational modification from an intrinsic tyrosine residue within the highly conserved sequence Ser/Thr-*X*-*X*-Asn-Tyr(TPQ)-Asp/Glu-Tyr at the active site (Janes *et al.*, 1990; Mu *et al.*, 1992). CAOs have been isolated from several different organisms, including bacteria, fungi, plants and mammals. In plants, CAOs are involved for example in wound healing, whereas in prokaryotes CAOs allow the organism to utilize various amines metabolically as sources of nitrogen and carbon. In higher eukaryotes very little is known about the biological function of CAOs besides their role in the metabolism of biogenic amines.

Human vascular adhesion protein-1 (VAP-1) is a single-span membrane glycoprotein that functions as an endothelial cell-adhesion molecule and which has a large extracellular domain containing the CAO activity (Jalkanen & Salmi, 2001; Salmi & Jalkanen, 2001). Thus, VAP-1 is an ecto-enzyme. VAP-1 is biologically active in a 180 kDa dimeric form (Salmi & Jalkanen, 1992; Smith *et al.*, 1998). The cell-surface expression of VAP-1 is induced by inflammation and VAP-1 mediates leukocyte-subtype specific adhesion to endothelial cells (Salmi & Jalkanen, 1997; Tohka *et al.*, 2001). VAP-1 is heavily glycosylated and the sugar moieties are important for the adhesive function (Salmi & Jalkanen, 1996). The cDNA encoding the VAP-1 polypeptide was cloned in 1998 (Smith *et al.*, 1998) and revealed its function as a CAO in addition to being an adhesion molecule. Little is known of the natural substrates of VAP-1 *in vivo*, but the physiological amines methylamine and aminoacetone are VAP-1 substrates.

There is recent *in vitro* evidence suggesting that the physiological roles of the adhesive and enzymatic properties of VAP-1 are connected. As shown by Salmi & Jalkanen (2001), the catalytic activity of VAP-1 on primary endothelial cells appears to directly regulate lymphocyte rolling; VAP-1 might bind to a primary amino group presented on the lymphocyte surface followed by oxidative deamination of the bound group. This process results in the formation of a transient covalent bond between the two cell types. Other studies have shown that VAP-1 exhibits both short-term and long-term insulin-like effects on glucose and lipid metabolism (Enrique-Tarancon *et al.*, 1998, 2000; Marti *et al.*, 1998; Salmi *et al.*, 2002).

The crystal structures of CAOs from four different species have been solved: *Escherichia coli* (PDB code 1oac; Parsons *et al.*, 1995), *Pisum sativum* (PDB code 1ksi; Kumar *et al.*,

1996), *Hansenula polymorpha* (PDB code 1a2v; Li *et al.*, 1998) and *Arthobacter globiformis* (PDB code 1av4; Wilce *et al.*, 1997). All these homodimeric structures have a similar overall fold that can be divided into domains D1–D4, of which the D1 domain is found only in *E. coli*. Domains D2 and D3 contain ∼100 amino acids each and have an α/β-type fold, whereas the largest and C-terminal domain D4 is ∼400 amino acids in length and has a unique β-sandwich fold that is needed for dimerization. The active site, which is located in the D4 domain, is highly conserved within the CAO family. It is buried deeply within the protein and is only accessible *via* a long channel surrounded mainly by amino acids from the D3 and D4 domains. The amino-acid residues of the D3 domain are less conserved than the actual active site, suggesting that the cavity leading to the active site is of great importance in determining the substrate specificity of CAOs. Even though the structures of known CAO proteins are quite similar, the sequence identity at the amino-acid level is only 25–35%. The evolutionary relationship of VAP-1 to the structurally known members of the CAO family has not been characterized in detail, but the presence of a transmembrane domain at the N-terminus of VAP-1 suggests substantial divergence from the soluble CAOs.

Here, we report the crystallization and X-ray analysis of human VAP-1. No mammalian CAOs have previously been crystallized. The data collected and reported here will enable the determination of the crystal structure of VAP-1. The crystal structure of VAP-1 will broaden our understanding of the structural determinants that distinguish the members of the enzymatically diverse CAO family and may provide the means for the rational design of anti-inflammatory drugs targeted to VAP-1.

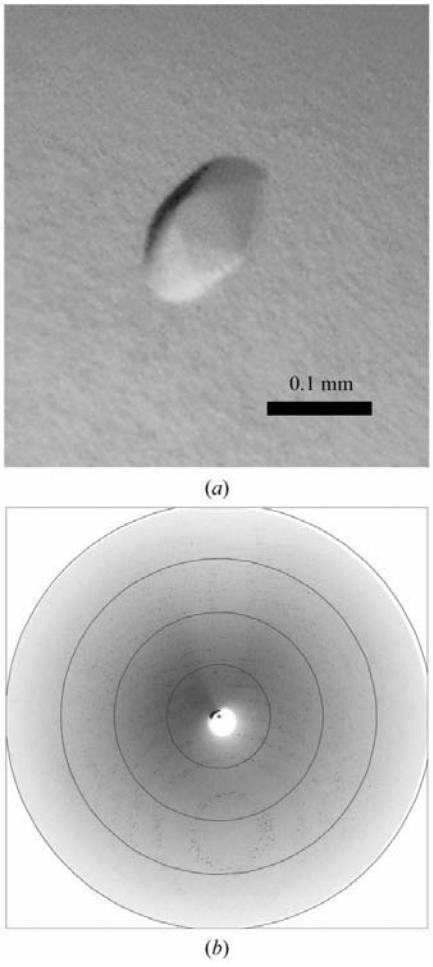

Figure 1
Hexagonal VAP-1 crystal (*a*) and a typical diffraction pattern (*b*). Resolution rings in (*b*) correspond to 2.9, 3.8, 5.7 and 11.5 Å resolution.

2. Materials and methods

2.1. Expression and purification

Recombinant VAP-1 protein was obtained from Chinese hamster ovary (CHO) cells stably transfected with full-length human VAP-1 cDNA which was generously provided by Professor Sirpa Jalkanen (Medicity Research Laboratory, Turku, Finland). The harvested cells were lysed using lysis buffer [150 m*M* NaCl, 10 m*M* Tris base pH 7.2, 1.5 m*M* $MgCl_2$, 1%(*v*/*v*) NP40]. Clarified cell lysate was diluted in a 1:1 ratio with 75 m*M* Tris–HCl pH 8.0 buffer (0.5% NP40 after dilution). The diluted cell lysate was used for the purification of VAP-1 based on an affinity chromatography column (HiTrap NHS-activated Sepharose, 5 ml; Pharmacia Biotech) armed with an anti-VAP-1 monoclonal antibody using the ÄKTA purifier system (Amersham Biotech) at a flow rate of 5 ml min^{-1}. VAP-1 was eluted with 0.1 *M* glycine–HCl pH 3.0 without detergent and protein-containing fractions were combined and rapidly neutralized by the addition of 1 *M* Tris base pH 9.0. We found that having the detergent in the final buffer decreased the aggregation of protein, but not significantly, and had no effect on the VAP/SSAO enzyme activity; however, it did disrupt the activity measurements. The buffer was changed using an Amicon concentrator (10 000 Da molecular-weight cutoff) to 10 m*M* potassium phosphate pH 7.2, 150 m*M* NaCl buffer. Finally, the VAP-1 was sterile filtered with a 0.2 µm filter and stored at 277 K. After purification, the presence of the VAP-1 protein (90 and 170–180 kDa bands) was confirmed by analysis on a silver-stained SDS–PAGE gradient gel (4–15%). Amine oxidase activity was measured using a peroxidase-linked spectrophotometric method (Holt *et al.*, 1997) in a 200 µl volume with 1 m*M* benzylamine as the substrate. The absorbance change was monitored in a Victor multi-label plate counter at 490 nm (Perkin–Elmer Life Sciences).

2.2. Crystallization and preliminary analysis

Initial crystallization conditions for VAP-1 were screened at room temperature using the Wizard I random sparse-matrix crystallization screen (Emerald BioStructures Inc., USA) and the vapour-diffusion method. Small hexagonal crystals were obtained from a condition containing 1.0 *M* potassium/sodium tartrate, 100 m*M* imidazole pH 8.0 and 200 m*M* NaCl after several months of incubation. The hanging drops contained 2 µl of protein sample (1.0 mg ml^{-1}) in 10 m*M* potassium phosphate buffer pH 7.2 and 2 µl of well solution. After optimization, the best crystals were obtained using a reservoir solution consisting of 1.0 *M* potassium/sodium tartrate, 100 m*M* imidazole pH 7.8 and 150–250 m*M* NaCl as the precipitant. The crystals formed in a few days and grew to final dimensions of about 0.15 × 0.15 × 0.1 mm (Fig. 1). One crystal was mounted in a capillary and preliminary X-ray analysis was carried out in-house using a rotating-anode radiation source (Cu $K\alpha$ radiation, 50 kV, 150 mA) and a MAR 345 image-plate detector. However, the crystal only diffracted to 8 Å resolution and the space group could not be accurately determined. All further X-ray analysis, as well as data collection, were carried out using synchrotron radiation at beamline X11 at EMBL/DESY Hamburg, Germany equipped with a bent mirror, triangular monochromator and a MAR Research CCD detector. For data collection, the crystals were cryoprotected with 20%(*v*/*v*) glycerol and flash-frozen in a 100 K nitrogen stream. Diffraction data collected from three different crystals were processed with the program *XDS* (Kabsch, 1993). The solvent content and Matthews coefficient were calculated assuming a molecular weight of 90 kDa per monomer and using the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

For the first time, we have been able to crystallize a mammalian CAO, human VAP-1. The full-length protein with the

Table 1
Crystal and diffraction data statistics.

Values in parentheses refer to the highest resolution shell.

Space group	$P6_522$†
Unit-cell parameters (Å, °)	$a = b = 225.9$, $c = 218.7$, $\alpha = \beta = 90$, $\gamma = 120$
Matthews coefficient (Å^3 Da^{-1})	4.5‡
Solvent content (%)	72‡
Unit-cell volume (Å^3)	9665176
Molecules per asymmetric unit	2‡
Unique reflections	52367 (4588)
Observed reflections	739050 (61793)
Wavelength used (Å)	0.811
Resolution range (Å)	20–3.20 (3.30–3.20)
Completeness (%)	95.9 (97.2)
$R_{\rm merge}$ (%)	19.6 (46.4)
Average $I/\sigma(I)$	13.2 (6.0)
Redundancy	14.1 (13.5)

† The space group is assumed to be $P6_522$ based on preliminary molecular-replacement trials, but could also be a related space group. ‡ Assuming a 180 kDa dimer.

N-terminal transmembrane region was expressed in glycosylation-competent CHO cells. The protein was purified to homogeneity (>95%) using affinity chromatography and the size of one monomer was confirmed to be 90 kDa by SDS–PAGE analysis. The purified protein retained its CAO activity as determined using benzylamine as the substrate. The best-looking hexagonal crystals were obtained using 1.0 *M* potassium/sodium tartrate, 100 m*M* imidazole pH 7.8 and 150–250 m*M* NaCl as the precipitant. The crystals grew to typical dimensions of 0.15 × 0.15 × 0.1 mm (Fig. 1*a*), with unit-cell parameters $a = b = 225.9$, $c = 218.7$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. According to the diffraction data statistics (Table 1) the diffraction limit of the VAP-1 crystals was 3.2 Å, even though reflections corresponding to higher than 3.0 Å resolution were observed in some frames (Fig. 1*b*). The space group was assumed to be $P6_522$ based on preliminary molecular-replacement studies (data not shown), but this assumption needs to be verified when solving the phase problem. If we assume the presence of one dimer (180 kDa) per asymmetric unit, then the Matthews coefficient is 4.5 Å^3 Da^{-1} and the solvent content is 72%. The crystal parameters and diffraction data statistics are summarized in Table 1.

The data presented here provide the basis for determining the crystal structure of a membrane-spanning glycoprotein, human VAP-1, which would be the first solved mammalian CAO crystal structure. The crystal structure of VAP-1 would provide a means to better understand the relationship between the structural details of CAOs and their physiological role. Moreover, novel information about the sugar moieties attached to VAP-1 may be revealed, since the protein used in the crystallization experiments was expressed in CHO cells fully capable of glycosylation. The structure would also assist the development of small-molecule inhibitors of the CAO activity, which could be used to block the cell-adhesion function of VAP-1; thus, the structure of VAP-1 could be used to screen *in silico* potential anti-inflammatory drugs for treatment of inflammatory diseases such as rheumatoid arthritis. The structure determination of VAP-1 is in progress.

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