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A copper-containing amine oxidase extracted from bovine serum (BSAO) and purified to homogeneity has been deglycosylated and crystallized. The crystals obtained belong to space group  $P2_12_12_1$ , with unit-cell parameters a = 77.68, b = 131.19, c = 134.00 Å, and diffract to at least 2.4 Å resolution. BSAO is the first mammalian amine oxidase to be crystallized.

oxidase from bovine serum

Crystallization and preliminary X-ray data of amine

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## 1. Introduction

The structural characterization of coppercontaining amine oxidases [amine-oxygen oxidoreductases (deaminating) (copper/TPOcontaining); EC 1.4.3.6] is of broad importance in order to understand the structure-function relationships of these enzymes, which play a variety of fundamental functions in the metabolism of cellular and extracellular amines. Copper-containing amine oxidases are a class of ubiquitous enzymes that catalyze the twoelectron oxidation of primary amines to the corresponding aldehydes, with reduction of molecular oxygen to hydrogen peroxide (Knowles & Dooley, 1994). These enzymes are homodimers, with a subunit size in the range 70-95 kDa. They are glycosylated, with their carbohydrate content depending on the enzyme source. Human plasma amine oxidase was found to be linked to pathologies such as diabetes (Yuen, 1987; Gronvall-Nordquist et al., 2001), congestive heart failure (Boomsma et al., 1997, 2000), atherosclerosis and other vascular endothelial damage (Boomsma et al., 1997; Magyar et al., 2001). Amine oxidases have been the subject of intense kinetic and spectroscopic investigations over many years (Klinman & Mu, 1994) and the nature of the covalently bound cofactor (2,4,5-trihydroxyphenylalanine quinone; TPQ), as well as the reaction mechanism, are well established (Padiglia et al., 2001). Conversely, despite the primary structures being well defined for about ten copper amine oxidases (Tipping & McPherson, 1995), crystal structures of these quinoenzymes are only available for the amine oxidases from Escherichia coli (Parsons et al., 1995), pea seedling (Kumar et al., 1996), Arthrobacter globiformis (Wilce et al., 1997) and Hansenula polymorpha (Li et al., 1998). To date, the three-dimensional structure of the enzyme from higher animals and, in particular, from mammalian organisms has not been determined. A crystalline form of 'highly purified' bovine and porcine plasma enzyme

was reported in an old paper (Yamada & Yasunobu, 1962), but no crystal structure has yet been solved. One reason for the difficulty in crystallization is probably the presence of the glycoside portion. Moreover, the deglycosylated enzymes have poor stability.

One of the most studied enzymes in this family is bovine amine oxidase (BSAO), the carbohydrate content of which accounts for about 4.3% of its weight (Watanabe & Yasunobu, 1970). In this paper, we report the production and preliminary characterization of crystals of deglycosylated BSAO, which currently represents the best model for the human enzyme.

## 2. Experimental and results

#### 2.1. Purification and deglycosilation

Bovine serum amine oxidase was purified according to the procedure reported by Vianello *et al.* (1992). The specific activity of the preparation was 0.36 U mg<sup>-1</sup> (Tabor *et al.*, 1954), where one enzyme unit (U) corresponds to the transformation of 1  $\mu$ mol of benzylamine per minute. The concentration of the purified enzyme was determined according to the method of Bradford (1976), assuming a molecular mass of 180 kDa.

Deglycosylation of the purified enzyme was carried out using a 'glycoprotein deglycosylation kit' from Calbiochem containing the following deglycosidases: N-glycosidase F from Chryseobacterium meningosepticum (activity 5000 U ml<sup>-1</sup>), endo- $\alpha$ -N-acetylgalactosaminidase from Streptococcus pneumoniae (activity 1.25 U ml<sup>-1</sup>),  $\alpha$ -2–3,6,8,9-neuroaminidase from Arthrobacter ureafaciens (activity  $5.3 \text{ U ml}^{-1}$ ) and  $\beta$ -1,4-galactosidase from *S. pneumoniae* (activity 1.66 U ml $^{-1}$ ). The deglycosylation was carried out in 50 mM sodium phosphate pH 7.0, incubating the samples at 310 K for 4 d, under the conditions (1 µg BSAO per microlitre of deglycosidase) suggested by Calbiochem.

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#### Figure 1

SDS-polyacrylamide gel electrophoretic pattern of deglycosylated BSAO. The gel, with 9% acrylamide concentration, was prepared according to Laemmli (1970). Lane 1, deglycosylated BSAO, 2 µg; lane 2, native BSAO, 2 µg; lane 3, molecular-weight markers, 2 µg.

The purity of the native BSAO and the degree of deglycosylation were assessed electrophoretically.

Denaturing SDS–PAGE was carried out according to the method of Laemmli (1970). The following proteins were used as molecular-weight markers: phosphorylase b (97 kDa) bovine serum albumin (66 kDa), ovoalbumin (45 kDa), glyceraldehyde-3phosphate dehydrogenase (36 kDa) and carbonic anhydrase (29 kDa).

## 2.2. Crystallization

Single crystals of BSAO were obtained at 295 K using the vapour-diffusion method with either hanging or sitting drops. Tests were performed with Crystal Screen 2 and PEG–Ion Screen from Hampton Research (Laguna Niguel, CA, USA) and Structure Screens 1 and 3D from Molecular Dimensions Ltd (Soham, UK). In a successful experiment, a 4  $\mu$ l drop was prepared by mixing 2  $\mu$ l of protein solution containing

2.0–2.3 mg ml<sup>-1</sup> BSAO in 10 m*M* HEPES, 10 m*M* NaCl pH 7.3 with an equivalent amount of the precipitant reservoir solution [PEG–Ion Screen solution No. 41; 0.2 *M* KH<sub>2</sub>PO<sub>4</sub>, 20%(w/v) PEG 3350 pH 4.7]. Small plate-shaped crystals of maximum dimensions 0.1 × 0.1 × 0.02 mm were obtained in a few days.

## 2.3. Data collection

Native diffraction data were collected at the diffraction beamline of the Elettra synchrotron (Trieste, Italy) at 100 K using a single crystal. Before measurement, the crystal was soaked for few seconds in a cryoprotectant solution prepared by mixing the precipitant solution with  $10\%(\nu/\nu)$ glycerol. During measurement, the ring was operated at 1.99 GeV, with a current ranging from 250 to 190 mA. A single wavelength of 1.2 Å was selected through the (111) face of a silicon double-crystal monochromator. Diffraction data were collected with a CCD

		25	20	10 I.	40		00		80		100		120		140	
BSAO :						REEGGVO	SSEEGVGKQCHE	SLPPRCPSI	RSPSDQPWTHP	DQSQLFAD	REPLTTVMSF	LTQQLGPDLV	DAAQARPSD	CVFSVE	EPKARA :	92
QALEC :	HMVPMDK	TLKEFGAD	VQWDDYAQLFTI	LIKDGAYVK	VKPGAQTAIVN	GQPLALQVI	PVVMKDNKAWVS	DTFINDVF	SGLDQTFQVE	KRPH LNA I	ADDIKÇAVET	VKASA	DEKEN. 7	RFTEIS	DKE V :	131
A2VHp :									PA	RPAH:LDP:S	TADIKAATNT	VKSY	FAGKK.J	SENTVICE	ARK Y :	47
AVKAg :									MTPSTI	QTAS FRLAS	AGOISEVQGI	LRTAG	ILGFE	RIAYLGVLE	ARG G :	52
KSIPs :										.VQH LDP T	KEDFLAVQTI	VQNKY	PISNNRI	AFHYIG	EKDHV :	46
															5	
		*	160	•	180		200	•	220	•	240		260	*	280	
SAO :	LAHLDRG	SPPPAREAL	LAIVFFGGQPQI	PNVTELVVG	PLPQPSYMRDV	TVERHGGPI	LPYYRRFVLLRE	YLDIDQMI	IN ELPOAAGV	LHHCCSYKQG	GQKLLTMNSA	PRGVQSGDRS	TWFGIYYNIT	KOGPYL	G.LELL :	231
QALEC :	WAFALEN	KPVDQPRK	ADVIMLDG. KH	IIEAVVDLQ	NNKLLSWQPIK	DAHGMVLLI	DFASVQNIINN	SEEFAAAVI	KK GITDAKKV	ITTPLTVG	YFDGK	DGLKQDARLI	KVISYLEVG.	DGNYWA	ENLVAV :	262
A2VHp :	IQWKEQG	GPLPPRLA	YYVILEAGKPG	VKEGLVDLA	SLSVIETRALE	TVQPILTVE	DLCSTEEVIRM	DPAVIEQC	LSGIP. ANEM	HKVYCDPW	TIGYD	ERWGTGKRLC	QALVYYRSDE	DDSQYS	DF :	175
AVKAg :	SEAEDRR	FRVF	IHDVSGARPQE	VTVSVTNGT	VISAVELDTAA	TGELFVLE	EFEVVEQLIAT	DERWLKAL	ANLD. VSKV	RVAPLSAG	VFEYA	EER GRRII	RGLAFVQDF	EDSAWATEV	DGLVAY :	178
IKSIPs :	LRYETHP	TLVSIPRK	IFVVALINSQT	HEILINLRI	RSIVSDNIHNG	YGFPILSVI	DEQSLAIKLELE	YPPFIDSVE	KERGLN. LSET	VCSSFTMG	WFGEE	KNVRT	VRLDCFMKES	TVNIYVREI	TGITIV :	173
									-					-		
		*	300	*	320	*	340	*	360		380	*	400	*	420	
SAO :	VEHKALD	PADWTVQK	VFFQGRYYENL	AQLEEQFEA	GQVNVVVIPDD	GTGGFWSLI	SQVP GPT PI	OFHEQEPR	SVOC . NRVAS	SLTSFELG	AFSEPRVFDV	RFQGE	RLAYEI	AGAVYCGN	TPAAML :	364
QALEC :	V LEQKK	IVKIEEGP	VVPVPMTARPFI	DGR			DRVA AVK MQ	IIESECKN	TITE . DMIN	RN D HLSMN	SVEPMISTV	TYN NGTKR.	. KVMYEG LO	GMIVEYCDE	DIGWYF :	373
A2VHp :	CPIVDTE	EKKVIFID	IPNRRRKVSKH	KHANFYPKH		MIEK	GAMR EAP IN	VTQLECVS	KMTC . NVME	SNFKCHICFN	Y ECIVLSDV:	SYN HGNVR.	. PIFHRICLS	<b>EMIVEY</b> CSE	EFPHQR :	297
AVKAg :	VEVVSKE	VTRVIDTG	VFPVPAEHGNY	TDP		EL/	<b>IGPLRTTQK</b> IS	ITQUECPS	TVICGNHIE	EKSLDVEFD	VEEGVVLHNI	AFR GDRLR.	. PIINRA	<b>BMVVEX</b> GDE	SPIRSW :	293
KSIPs :	A LDLMK	IVEYHDRD	IEAVPTAENTE	vovs			SPPFG KQHSLT	SHQEQCPG	QING . HSVS	ANKHISFD	V ACIVISLA	SIY LEKHKS	RRVLYKGYIS	BLFVPYQDE	TEEFYF :	290
							_					-				
		*	440	•	460	•	480	*	500	•	520	*	540	*	560	
SAO :	TRYM	. FOMEYFA	IP IR VO PY	LATYMDWHF	VVESQTERTLH	DFOVEDQ	KCLPLRRHHSE	FLS. HYFG	SVAQTV FRS	VSTMLXYDDV	WDMV YPNCA	EVKLHA CY	ISSAFLEGA.		RRY NQ :	493
QALEC :	KAYLNS	DY MCTLT:	SPIARCKCAPSI	NAVILLNETI	ADYTEVEMEIP	R IAVEDRY	ACPEYK	Q PNV3	STERREDVVRW	ISTVGXXDVI	FDIIBHENGT	IGIDAGA	EAVKGVKAK	MHDETAKDD	TRYSTL :	509
A2VHp :	KHALIT	EY⊂A€YMT	NPISLCODOKG	VIHYLDAHF	SDRACDEITVK	N-V-IHPEI	DCLLFK SDFF	DNF. ATSL	TRATK	IFLANCE	LY V MQDCA	RLDIRL	LNTYILGDD.	EEA	GPWSTR :	429
AVKAg :	QNYF To	EYLVSQYAI	NS ELCODIGI	DITYLSPVI	SDAFENEREIR	NGIOMHDEI	W ILAK SDL	SGINY	TRRNRRM ISF	FTAIGNYDYG	FYYILLDGT	JEFEAKA (CV	VFTSAFFEG.	GSE	DNISQ :	421
KSIPs :	KTFF SC	EFEFCLST	VS IPNRDOPPI	HAQFIDTYV	HSAN TEILLK	NIVEDQ	CON IMWR TENG	IPNESIEE	RTEVN IVRT	IVAVGXXDNV	IDEKASES	IKPSIALSCI	LEIKGTNIK.	HKDEIKE	DLHEKL :	427
							-									
		*	580	*	600	*	620	*	640	*	660	*	680	*	700	
SAO :	VGEHTLG	PUTTISAH	IKVDLDVGCLE	WVWAEDMA	FVPTAIPWSPE	HQIQRLQV	TRKQLETEEQAA	FPLGGASP	YLYLAS . KQS	KWG.H RG	RIQTVSFAGG	PMPQNSPMEP		GRYQLAI	RKET P :	625
QALEC :	IDHNIVG	TTHOHIYN	FRIDLDVDGEN	SLVAM	VKPNTAG G	PRTSTMQVI	QYNIGNEQDAA	QKFDFGTI	LLSNENKE	RMG.N VS	QIIPYAGGTH	<b>FVAKGAQFAE</b>	DEWIYHELSE	MDKQLWUAF	SHFG R :	643
A2VHp :	VYPNVNA	HNHOHLFSI	LRIEPRICOG	SAAAC AK	SSPYPLSPEN	MYGNAFYSI	EKTTFKTVKDSI	TNYESATO	SWDIFNENKV	PYSGKEPS	KLVSTQCPPL	LAKEGSLVA.	KAP	ASHSVNVVE	KENRL :	563
AVKAg :	LAPGLGA	PRIO IFS	ARL MAI OFT	RVEEE VV	RQTMGP N	ERGNAFSRI	RTVLTRESEAV	READARTG	TWIISNEESK	RLN.E VG	KLHAHNQPTL	LADPGSSIA.		ATKOLW	ADD R :	551
KSIPs :	VSANSIG	INDEFYI	YYLDFDIDETH	SFEKTSLK	TVRIKD S	SKRKSYWT	retqtaktesda	KITIGLAP	ELVVVNENIK	TAVG.NEVG	RLIPAIPAHP	LLTEDDYPQ.	I GAN	TNYNVWVTA	NRT K :	557
					-				_				-	-		
		•	720	•	740	•	760	٠	780	•	800	•	820			
SAO :	SSSSVEN	QNDFWTFT	VDFSDFINN	TAGKEL	AVACTIN	HAN DENT	TVGNGVGFF	EYNTEDOD	SMDSADSIYF	REGODAGSCE	INFLACLEQA	ATCAPDLEVE	SHGGYPEY :	746		
QALEC :	FPECKYP	NRSTHD	TGLOQYSKD. N	ESLONTON	VOMOTETTIVA	RADEWE	TEWVHTLD P	EWNEADET	TIGALKKD					721		
LA2VHp :	YPSODHV	POWSGDGVI	RGMREWIGDGS	ENCONTOIL	FFHAFCITHE	APP FE	AEPITIM	GRHONTEN	G DIQPSYAM	TTSEAKRAV.				655		
1 AVKAG	VPTODEV	NOUSAG	AGT.PSYTAO DI	DOGONT	VOHOFCLOUP	RUNAN	MEVDTVGFK	REGEDERS	VIDVPANPSO	SASHCHA				638		

#### Figure 2

Sequence alignment of bovine serum AO with enzymes from other sources whose crystal structure is known. BSAO, amine oxidase from bovine serum; 1A2VHp, amine oxidase from *H. polymorpha*; 1AVKAg, amine oxidase from *A. globiformis*; 1KSIPs, amine oxidase from pea seedlings.

LLSTSFE

1KSIPS : WAGELYVDHSRGD. DTLAVWTKQ.NRE

VIKTLSPRDVAWPGC...... 642

## crystallization papers

#### Table 1

X-ray data-collection and processing statistics.

Values in	parentheses	refer	to	the	highest	resolution
shell, 2.50-	-2.37 Å.					

1.2	Wavelength (Å)
MAR CCD	Detector
$P2_{1}2_{1}2_{1}$	Space group
a = 77.68, b = 131.19,	Unit-cell parameters (Å)
c = 134.00	
0.5	Mosaicity (°)
25-2.37 (2.50-2.37)	Resolution range (Å)
47368 (4684)	No. of unique reflections, $I \ge 0$
93.7 (77.3)	Completeness (%)
3.5 (3.4)	Redundancy
10.1 (2.1)	$\langle I/\sigma(I) \rangle$ of measured data
0.106 (0.390)	$R_{\rm sym}(I)$ †
$\begin{array}{c} c = 134.00\\ 0.5\\ 25-2.37 \ (2.50-2.37)\\ 47368 \ (4684)\\ 93.7 \ (77.3)\\ 3.5 \ (3.4)\\ 10.1 \ (2.1)\\ 0.106 \ (0.390) \end{array}$	Mosaicity (°) Resolution range (Å) No. of unique reflections, $I \ge 0$ Completeness (%) Redundancy $\langle I/\sigma(I) \rangle$ of measured data $R_{sym}(I)^{\dagger}$

 $\dagger R_{\text{sym}}(I) = \sum_{hkl} \sum_{i} |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_{i} |I_{hkl,i}|$ , where  $\langle I_{hkl} \rangle$  is the mean intensity of the multiple  $I_{hkl,i}$  observations of symmetry-related reflections.

detector (MAR Research) positioned at a distance of 180 mm from the sample, with oscillations of  $0.7^{\circ}$ . Data processing and scaling was performed with *MOSFLM* (Leslie, 1991) and *SCALA* (Collaborative Computational Project, Number 4, 1994). Processing statistics are summarized in Table 1.

#### 3. Results and discussion

# 3.1. Protein preparation and deglycosylation

All the BSAO preparations used for crystallization, both of the native and of the deglycosylated form, were pure to homogeneity (>95%), displaying a single band under denaturing conditions. According to SDS–PAGE, the deglycosylated enzyme is characterized by a reduction of the molecular mass of about 4% (from ~93 to ~89 kDa; Fig. 1), apparently corresponding to the carbohydrate content reported by Watanabe & Yasunobu (1970). Furthermore, the deglycosylation of BSAO does not affect its kinetic characteristics; the deglycosylated enzyme shows a specific activity

equal to that of the native enzyme within experimental error.

#### 3.2. Crystallization results

Crystals belong to the orthorhombic space group  $P2_{1}2_{1}2_{1}$ , with unit-cell parameters a = 77.68, b = 131.19, c = 134.00 Å. Assuming the presence of one dimer in the asymmetric unit, the  $V_{\rm M}$  crystal packing parameter is 2.06 Å<sup>3</sup> Da<sup>-1</sup>, corresponding to a solvent content in the unit cell of 39% (Matthews, 1968). The crystal used diffracted to a maximum resolution of 2.4 Å, as reported in Table 1.

At present, the three-dimensional structures of four different amine oxidases (from E. coli, pea seedling, A. globiformis and H. polymorpha) are known. Their sequence alignment is reported in Fig. 2; it shows that a relatively high homology can only be detected in the central part of the protein, between amino-acid residues 290 and 700 approximately. A partial model of the dimer comprising only those amino acids was built with the ExPASy modelling server (http:// www.expasy.org). Using this partial model, a preliminary molecular-replacement solution was obtained using the program AMoRe (Navaza, 1994). Refinement and the model building of the remaining part of the model is in progress in our laboratory.

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#### References

Boomsma, F., van-Dijk, J., Bhaggoe, U. M., Bouhuizen, A. M. & van-den-Meiracker, A. H. (2000). Comput. Biochem. Physiol. C Toxicol. Pharmacol. 126, 69–78.

- Boomsma, F., van-Veldhuisen, D. J., de-Kam, P. J., Man-in't-Veld, A. J., Mosterd, A., Lie, K. I. & Schalekamp, M. A. (1997). *Cardiovasc. Res.* 33, 387–91.
- Bradford, M. M. (1976). Anal. Biochem. 72, 248–254.
- Collaborative Computational Project, Number 4 (1994). Acta Cryst. D50, 760–763.
- Gronvall-Nordquist, J. L., Backlund, L. B., Garpenstrand, L., Ekbolm, J., Landin, B., Yu, P. H., Oreland, L. & Rosenqvist U. (2001). J. Diabetes Complications, 15, 250–256.
- Klinman, J. P. & Mu, D. (1994). Annu. Rev. Biochem. 63, 299–344.
- Knowles, P. F. & Dooley, D. M. (1994). Metal Ions in Biological Systems, edited by H. Sigel & A. Sigel, Vol. 30, pp. 361–403. New York: Marcel Dekker.
- Kumar, V., Dooley, D. M., Freeman, H. C., Guss, J. M., Harvey, I., McGuirl, M. A., Wilce, M. C. J. & Zubak, V. M. (1996). *Structure*, 4, 943–955.
- Laemmli, U. K. (1970). Nature (London), 227, 248–254.
- Leslie, A. G. W. (1991). Crystallographic Computing V, edited by D. Moras, A. D. Podjarny & J. P. Thierry, pp. 50–61. Oxford University Press.
- Li, R., Klinman, J. P. & Mathews, F. S. (1998). *Structure*, **6**, 293–307.
- Magyar, K., Meszaros, Z. & Matyus, P. (2001). Pure Appl. Chem. 73, 1393–1400.
- Matthews, B. W. (1968). J. Mol. Biol. 33, 491-497.

- Padiglia, A., Medda, R., Bellelli, A., Agostinelli, E., Morpurgo, L., Mondovì, B., Finazzi-Agrò, A. & Floris, G. (2001). *Eur. J. Inorg. Chem.* 1, 35– 42.
- Parsons, M. R., Convery, M. A., Wilmot, C. M., Yadav, R. D. S., Blakeley, V., Corner, A. S., Philips, S. E. V., McPherson, M. J. & Knowles, P. F. (1995). *Structure*, **3**, 1171–1184.
- Tabor, C. W., Tabor, H. & Rosenthal, S. M. (1954).*J. Biol. Chem.* 208, 307–315.
- Tipping, A. J. & McPherson, M. J. (1995). J. Biol. Chem. 270, 16939–16946.
- Vianello, F., Di Paolo, M. L., Stevanato, R. & Rigo, A. (1992). Protein Expr. Purif. **3**, 362–367.
- Watanabe, K. & Yasunobu, K. T. (1970). J. Biol. Chem. 245, 4612–4617.
- Wilce, M. C. J., Dooley, D. M., Freeman, H. C., Guss, J. M., Matsunami, H., McIntire, W. S., Ruggiero, C. E., Tanizawa, K. & Yamaguchi, J. (1997). *Biochemistry*, **36**, 16116–16133.
- Yamada, H. & Yasunobu, K. T. (1962). J. Biol. Chem. 237, 1511–1516.

Navaza, J. (1994). Acta Cryst. A**50**, 157–163.

Yuen, C. T. (1987). Br. J. Dermatol. 116, 643-649.