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Purification, crystallization and identification by X-ray analysis of a prostate kallikrein from horse seminal plasma

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The purification, crystallization and identification by X-ray diffraction analysis of a horse kallikrein is reported. The protein was purified from horse seminal plasma. Crystals belong to space group *C*2 and the structure was solved by the MIRAS method, with two heavy-atom derivatives of mercury and platinum. X-ray diffraction data to 1.42 Å resolution were collected at the ESRF synchrotron-radiation source.

1. Introduction

Kallikreins are members of the mammalian serine protease family and are subdivided into two main groups: tissue (or glandular) and plasma kallikreins, found in glandular cells, neutrophils and biological fluids (Bhoola *et al.*, 1992). Kallikreins are involved in a significant number of physiological processes through the activation of specific peptides to their biologically active forms (Clements, 1989). These active forms (different types of kinins) can participate in processes such as blood clotting, fibrinolysis, regulation of vascular tone and inflammation (Bhoola *et al.*, 1992). The serine protease family includes other proteins, such as trypsin, chymotrypsin, tonin, thrombin and elastase, that share a high degree of structural conservation. Serine proteases cleave numerous substrates in a specific manner that involves three catalytic residues (catalytic triad). These residues, His57, Asp102 and Ser195 (chymotrypsinogen numbering, which is used throughout the text) are conserved in all serine proteases. Other features of this family include the overall fold of two β-barrel domains and the presence of four to six conserved disulfide bonds.

There are numerous reports on the identification and characterization of several members of the kallikrein family, but little is known about horse prostate kallikreins. In human prostate, two kallikreins have been identified: hK2 and PSA. PSA (prostate specific antigen) is a tumour marker currently used for prostate tumour diagnosis and monitoring. It is up-regulated by steroid hormones in human prostate cancer and its overexpression is associated with more aggressive forms of the disease (Yousef *et al.*, 2001). Unlike other kallikreins, PSAs have a serine residue, instead of an aspartate, at the bottom of the substrate-recognition pocket (residue 189) and are reported to have a chymotrypsin-like activity (Christensson *et al.*, 1990; Robert *et al.*, 1997; Akiyama *et al.*, 1987; Lilja *et al.*, 1989), although tryptic activity has also been detected in one case (Watt *et al.*, 1986). PSA is involved in the liquefaction of seminal plasma, cleaving semenogelin (Christensson *et al.*, 1990; Robert *et al.*, 1997; Peter *et al.*, 1998) and allowing sperm motility. The expression of hK2 is less than that of PSA and, since it has an aspartate instead of a serine residue at position 189, it is considered to be a trypsin-like protease (Schedlich *et al.*, 1987, 1988). Its substrate is unknown, but analysis of its primary sequence suggests that it may have an enzymatic preference for non-kininogen substrates (Qin *et al.*, 1991). The primary structure of hK2 shares 80% identity with that of human PSA (Clements, 1994). Though the structure of PSA would be a clue to understanding the functional properties of this important cancer marker, only three homology models based on structural alignments with other serine proteases have been proposed (Vihinen, 1994; Villoutreix *et al.*, 1994; PDB code 1pfa; Coombs *et al.*, 1998; PDB code 2psa). A model for hK2 has also been proposed (Vihinen, 1994).

In this study, we describe the preliminary X-ray diffraction analysis of a horse prostate kallikrein which was present as a contaminant in a sample of HSP-3 (Magdaleno *et al.*, 1997), a horse seminal protein thought to be involved in gamete membrane fusion (Ellerman *et al.*, 1998). Horse kallikrein was identified only after visualization of the experimental electron-density maps. The MIRAS map was of excellent quality and sequencing of several stretches of the density showed that it could not be HSP-3. A sequence-homology search showed that we had solved the structure of a

member of the kallikrein family. Biochemical characterization of this horse kallikrein is under way.

2. Materials and methods

2.1. Protein purification, peptide mapping and N-terminal sequencing

Stallion ejaculates were collected and pooled from healthy reproductively active animals by means of an artificial vagina at Niedersächsisches Landesgestüt Celle (Germany). Spermatozoa were separated from seminal plasma by centrifugation at 1500*g* for 15 min at room temperature. The supernatant was further clarified by centrifugation at 13 000*g* for 10 min at room temperature. 20 ml of seminal plasma were diluted with an equal volume of 20 m*M* sodium phosphate buffer pH 7.4 and chromatographed on a 25 ml heparin-Sepharose CL-6B (Pharmacia) column equilibrated in the same buffer. Our initial goal was to purify and carry out structural studies on HSP-3, a non-heparin-binding protein thought to play a role in gamete membrane fusion at fertilization (Ellerman *et al.*, 1998). The protocol described by Magdaleno *et al.* (1997) was followed for purification of HSP-3. Apparent protein purity was assessed by SDS–PAGE. Gels were visualized by staining with Coomassie blue or electrotransferred to a nitrocellulose membrane and probed with a polyclonal monospecific anti-HSP-3 (Calvete *et al.*, 1994). After it became clear from crystallographic data that the crystallized protein was a contaminating kallikrein rather than HSP-3 (see §3), a method for the separation of the two proteins by hydrophobic interaction chromatography was developed. 70 mg of the protein mixture in 10 ml of 50 m*M* sodium phosphate buffer pH 7.2 containing 1 *M* $(NH_4)_2SO_4$ was loaded on a 40 ml butyl-Sepharose (Pharmacia) column (22 × 1.5 cm) equilibrated in the same buffer. The column was washed until the absorbance at 280 nm reached the baseline level and the bound proteins were separated eluting with a linear gradient of 75 ml of 50 m*M* sodium phosphate buffer pH 7.2, 1 *M* $(NH_4)_2SO_4$ (buffer *A*) and 75 ml 50 m*M* sodium phosphate buffer pH 7.2 (buffer *B*). Protein purity was assessed by N-terminal sequence analysis (using an Applied Biosystems 473 A instrument), amino-acid analysis (using a Beckman System Gold Amino Acid Analyzer after sample hydrolysis in 6 *M* HCl at 383 K for 24 h) and matrix-assisted laser-desorption ionization time-of-flight mass spectrometry using an Applied Biosystems DE-Pro instrument and α-cyano-hydroxycinnamic acid saturated in acetone as matrix. HSP-3, which has a blocked N-terminus, was identified by its molecular mass of 24 987 Da (Magdaleno *et al.*, 1997). Kallikrein, identified by Edman degradation (N-terminal sequence: IIGGWEXEKHSKPWQVAVY; Calvete *et al.*, 1994), had an isotope-averaged molecular mass of 26 762 Da. For internal peptide sequencing, 1 mg samples of purified seminal plasma kallikrein, dissolved at 2–5 mg ml^{-1} in 100 m*M* ammonium bicarbonate pH 8.3, 1.5 *M* guanidinium hydrochloride, were digested with trypsin or chymotrypsin (Sigma) at an enzyme: substrate ratio of 1:100(*w*/*w*) overnight at 310 K. Peptides were isolated by reverse-phase HPLC on an RP-100 Lichrospher (Merck) C_{18} column (25 × 4 mm, 5 µm particle size) eluted at 1 ml min^{-1} with a gradient of 0.1%(*v*/*v*) trifluoroacetic acid in water (solution *A*) and acetonitrile (solution *B*), first isocratically with 5% *B* for 5 min, followed by 5–40% *B* for 70 min and 40–70% *B* for 30 min. Peptides were detected at 220 nm, collected manually and subjected to N-terminal sequencing as described above.

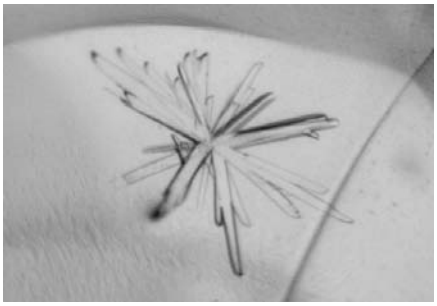

Figure 1
Native crystals of horse kallikrein, maximum dimensions ∼0.4 × 0.06 × 0.02 mm, grown for approximately two weeks using 10%(*w*/*v*) PEG 20 000 in the crystallization solution. The crystals are easily separated with a glass needle.

2.2. Crystallization and preparation of heavy-atom derivatives

A lyophilized sample of (impure) HSP-3 isolated following the size-exclusion chromatography protocol of Magdaleno *et al.* (1997) was resuspended in 10 m*M* Tris–HCl buffer pH 7.5 at 10 mg ml^{-1} for a screening of crystallization conditions at 277 K and room temperature. The crystallization attempts included 80 factorial solutions adapted in house from the sparse-matrix method of Jancarik & Kim (1991) and Crystal Screen II from Hampton Research; the vapour-diffusion method was used. Each sitting drop contained 2 µl of protein solution and 2 µl of crystallization solution. Very small thin crystal needles grew at approximately 293 K from 20%(*w*/*v*) polyethylene glycol (PEG) 8000, 0.2 *M* magnesium acetate and 0.1 *M* sodium cacodylate pH 6.5. After a number of optimization experiments, crystals suitable for diffraction analysis (maximal dimensions of 0.40 × 0.06 × 0.02 mm within two weeks) could be obtained. The crystallization solution contained 10%(*w*/*v*) PEG 20000, 0.2 *M* magnesium acetate and 0.1 *M* sodium cacodylate buffer pH 6.5 and the protein solution had a concentration of between 25 and 50 mg ml^{-1}. Crystals were stored at 293 K in a harvesting solution similar to the crystallization solution, but with a slight increase in the PEG 20 000 concentration [15%(*w*/*v*)].

The crystals used for the heavy-atom derivatives search were prepared in the same way. The two successful derivatives were obtained by overnight soaking of the crystals in a harvesting solution prepared with 10 m*M* of the heavy-atom compounds K_2PtCl_4 and PCMBS (*p*-chloromercuribenzenosulfonate). A photograph of the horse kallikrein native crystals can be seen in Fig. 1.

2.3. Data collection and processing

For the diffraction experiments, we chose to flash-freeze the crystals in a stream of nitrogen gas at 100 K. A suitable cryoprotectant solution was found and consisted of 15% glycerol added to the harvesting solution. The crystals were transferred directly from the drop to the cryoprotectant solution and were allowed to equilibrate for approximately 1 min. After this, crystals were rapidly transferred to the Cryostream using a nylon loop. These crystals were resistant enough to survive annealing (back-transfer to the cryoprotectant drop) and sometimes a slight improvement in diffraction quality could be observed. The cryoprotected crystals diffracted to beyond 1.94 Å resolution on a MAR Research imaging-plate system using graphite-monochromated Cu *Kα* radiation from an Enraf–Nonius rotating-anode generator operated at 4.5 kW. The X-ray intensity data from the two heavy-atom derivatives were also collected at this X-ray source. After collecting a complete native data set of the best diffracting crystal using the in-house rotating-anode generator, the crystal was kept frozen in liquid nitrogen. A second native data set (to beyond 1.42 Å resolution) of this crystal was collected on a MAR Research CCD detector using X-rays from a

Table 1
Data collection and structure determination.

Values in parentheses correspond to the highest resolution shells.

Data set	Native		Derivatives	
Data set			K_2PtCl_4	PCMBS
X-ray source	Cu rotating anode	ID14-2, ESRF	Cu rotating anode	Cu rotating anode
Wavelength (Å)	1.5418	0.9326	1.5418	1.5418
Space group	*C*2	*C*2	*C*2	*C*2
Unit-cell parameters (Å, °)	*a* = 72.6, *b* = 79.1, *c* = 45.7, β = 98.3	*a* = 72.7, *b* = 79.1, *c* = 45.8, β = 98.2	*a* = 72.4, *b* = 78.4, *c* = 45.8, β = 97.7	*a* = 72.5, *b* = 79.0, *c* = 45.7, β = 98.2
Resolution (Å)	18.10–1.94 (2.01–1.94)	18.00–1.42 (1.47–1.42)	22.87–2.75 (2.85–2.75)	19.00–2.30 (2.38–2.30)
Total No. of reflections	125756	490556	63590	99636
No. of unique reflections	19040	48282	6287	11374
R_{sym}†(%)	9.2 (31.3)	7.4 (48.7)	12.9 (56.2)	10.0 (23.7)
Completeness (%)	96.0 (83.5)	99.4 (99.8)	95.3 (92.9)	99.4 (95.4)
Average $I/\sigma(I)$	10.4 (2.6)	17.9 (2.5)	8.4 (1.9)	13.5 (5.2)
Phasing power‡				
Centric	—	—	0.8	2.6
Acentric (iso/ano)	—	—	1.1/0.7	4.3/1.4
R_{Cullis}§	—	—	0.80	0.42
Mean figure of merit before/after solvent flattening	0.56/0.87			

† $R_{sym} = \sum(|I - \langle I\rangle|)/\sum\langle I\rangle$, where I is the measured intensity of each reflection and $\langle I\rangle$ is the intensity averaged from multiple observations of symmetry-related reflections. ‡ Phasing power = $\langle F_H/\mathrm{LOC}\rangle$, where LOC is the lack of closure. § $R_{Cullis} = \big||F_{PH} \pm F_P| - F_H\big|/|F_{PH} - F_P|$ for centric reflections.

synchrotron source (λ = 0.9326 Å) at beamline ID14-2 at the ESRF (Grenoble, France). All data were processed and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL* suite of programs (Otwinowski & Minor, 1997). The native crystals belong to space group *C*2 (unit-cell parameters *a* = 72.7, *b* = 79.1, *c* = 45.8 Å, β = 98.2°) with one molecule in the asymmetric unit; V_M = 2.74 Å^3 Da^{-1} with 54.7% solvent content (Matthews, 1968). The R_{sym} of the native data was 7.4%, 48.7% in the last shell (1.47–1.42 Å). The data-collection statistics for the native crystal (collected at two different X-ray sources), together with the statistics for the two heavy-atom derivatives, are presented in Table 1.

2.4. Structure solution and identification

The heavy-atom data sets were quite isomorphous to 2.3 Å resolution for the PCMBS derivative and to 3.3 Å for the PTCL derivative. The scaling of the derivative data to the native data and the analysis of the Patterson maps, as well as the cross phasing of the difference Fourier maps, were all performed with version 0.9 of *CNS* (Brunger *et al.*, 1998) and confirmed using the automated Patterson search methods implemented in *SOLVE* (Terwilliger & Berendzen, 1999). Five sites were found for mercury and three sites for platinum. The anomalous signal was included and the heavy-atom coordinates were refined using *SHARP* (de La Fortelle & Bricogne, 1997). The initial MIRAS phases with a mean figure of merit of 0.56 were further improved by solvent flattening (solvent content of 50%) using *SHARP*'s interface with the *CCP*4 program *SOLOMON* (Abrahams & Leslie, 1996). The resulting electron-density map, with an overall figure of merit of 0.87, was of excellent quality and was readily interpretable. The initial MIRAS electron-density map was visualized and immediate identification of some characteristic residues such as Trp allowed the sequencing of several fragments of the polypeptide chain, the largest being 16 residues long (see electron-density map in Fig. 2). These sequenced polypeptides showed that the protein could not be HSP-3 but surprisingly was an unknown protein which happened to be copurified. A sequence-homology search with the BLAST server revealed significant and clear homology to several kallikreins, members of the serine protease family (see alignment in Fig. 3).

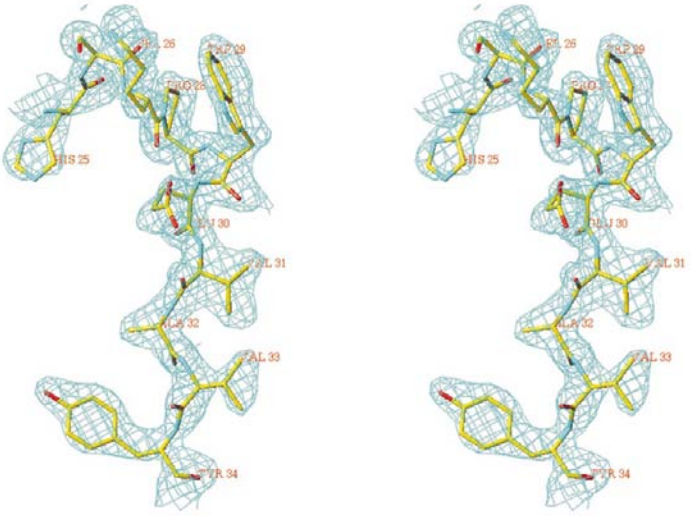

Figure 2
Stereo picture of the initial 1.94 Å MIRAS electron-density map around fragment His25–Tyr34 (map contour level: 1.2σ), showing the high quality of the initial map that allowed sequencing and protein identification.

3. Results and discussion

Initially, we sought to determine the crystal structure of HSP-3 (Magdaleno *et al.*, 1997), a stallion seminal plasma protein thought to play a role during gamete membrane fusion at fertilization (Ellerman *et al.*, 1998). The protein was isolated by size-exclusion chromatography of the non-heparin-binding fraction of stallion seminal plasma (Magdaleno *et al.*, 1997). Crystals were grown by the vapour-diffusion hanging-drop method using polyethylene glycol 20 000 in the crystallization solution. Since the primary structure of HSP-3 (Schambony *et al.*, 1998) does not display discernible sequence similarity to any proteins of known three-dimensional structure which could serve as a search model for solving the structure of the stallion protein, isomorphous heavy-atom derivatives had to be prepared. PCMBS turned out to be a very successful derivative (five mercury sites). Two useful native data sets of the same crystal were collected using Cu $K\alpha$ X-ray radiation (1.94 Å resolution data) and synchrotron radiation (1.42 Å resolution data). The structure was solved by the multiple isomorphous replacement with anomalous scattering (MIRAS) technique using the X-ray data collected in-house. The

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                19                  25                  34                  209                 221
horse_kall . . . . G W E C X A H S K P W E V A V Y . . . . H E G I T A W G H V S C G . . . .
Q29474     . . . . G W D C T K N S Q P W Q A A V Y . . . . L Q G I T S W G H V P C G . . . .
P00752     . . . . G R E C E K N S H P W Q V A V Y . . . . L Q G I T S W G H T P C G . . . .
P15945     . . . . G F N C E K N S Q P W Q V A L Y . . . . L H G I T S W G P S P C G . . . .
P12323     . . . . G Q E C A R D S H P W Q A A I Y . . . . W Q G L T S W G D S P C G . . . .
P20151     . . . . G W E C E K H S Q P W Q V A V Y . . . . L Q G I T S W G P E P C A . . . .
M24543     . . . . G W E C E K H S Q P W Q V L V A . . . . L Q G I T S W G S E P C A . . . .
P33619     . . . . G W E C E K H S Q P W Q V L V A . . . . L Q G I T S W G S Q P C A . . . .
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Figure 3
Amino-acid sequence alignment of two of the crystallography-derived segments (horse_kall) with deposited primary sequences of several members of the kallikrein family: Q29474, canine pancreas kallikrein; P00752, porcine pancreas kallikrein; P15945, mouse liver kallikrein; P12323, guinea pig prostate kallikrein; P20151, human prostate kallikrein hK2; M24543, human PSA (prostate specific antigen); P33619, Rhesus monkey PSA.

1.42 Å resolution synchrotron data will be employed in the final refinement. The MIRAS electron-density map, calculated using the 1.94 Å data, was of excellent quality, allowing the assignment of stretches of amino-acid sequence. For example, the amino-acid sequence GWECXAHSKPWE-VAVY was unambiguously read from the initial electron-density map. This sequence was surprisingly not present in HSP-3; a sequence-similarity search showed extensive identity with kallikrein sequences (Figs. 2 and 3).

Reverse-phase HPLC analysis of the protein material used for crystallization and of the crystallized protein showed that the former consisted of 85% HSP-3 and 15% seminal plasma kallikrein, whereas kallikrein was the only protein in the crystals. These results were confirmed by mass-spectrometric analysis of both the initial HSP-3 preparation used for crystallization and the protein recovered from the crystals and clearly indicated that seminal plasma kallikrein contaminating the HSP-3 preparation was selectively crystallized. The similar molecular masses of HSP-3 and kallikrein (24 987 and 26 762 Da, respectively) easily explain the failure of size-exclusion chromatography to separate the two proteins. On the other hand, the rather low percentage of kallikrein in the protein sample used for crystallization experiments explains the need to further concentrate the protein in order to obtain good-quality crystals. No previous crystallographic structure of a prostate kallikrein has been reported, although kallikrein crystal structures from other tissues are available in the Protein Data Bank: 1ao5, kallikrein-13 from mouse submandibular gland (Timm, 1997); 1hia, kallikrein from porcine pancreas complexed with hirustasin (Mittl *et al.*, 1997); 2kai, kallikrein A from porcine pancreas complexed with bovine pancreatic trypsin inhibitor (Chen & Bode, 1983); 2pka, kallikrein A from porcine pancreas (Bode *et al.*, 1983).

Sequencing of the purified protein, as well as model building and refinement of the three-dimensional structure, are under way. The primary structure of the protein will be determined by combination of peptide mapping and Edman sequential degradation of selected peptides and molecular cloning of prostate cDNA coding for a partial amino-acid sequence. The primary sequence is also being derived by analysis of the improved electron-density maps. So far, we have identified the catalytic triad conserved residues, as well as five conserved disulfide bridges.

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References

Abrahams, J. P. & Leslie, A. G. W. (1996). *Acta Cryst.* D**52**, 30–42.

Akiyama, K., Nakamura, I., Iwanaga, S. & Hara, M. (1987). *FEBS Lett.* **225**, 168–172.

Bhoola, K. D., Figueroa, C. D. & Worthy, K. (1992). *Pharmacol. Rev.* **44**, 1–80.

Bode, W., Chen, Z., Bartels, K., Kutzbach, C., Schmidt-Kastner, G. & Bartunik, H. (1983). *J. Mol. Biol.* **164**, 237–282.

Brunger, A. T., Adams, P. D., Clore, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.

Calvete, J. J., Nessau, S., Mann, K., Sanz, L., Sieme, H., Klug, E. & Töpfer-Petersen, E. (1994). *Reprod. Dom. Anim.* **29**, 411–426.

Chen, Z. & Bode, W. (1983). *J. Mol. Biol.* **164**, 283–311.

Christensson, A., Laurell, C.-B. & Lilja, H. (1990). *Eur. J. Biochem.* **194**, 755–763.

Clements, J. A. (1989). *Endocr. Rev.* **10**, 393–419.

Clements, J. A. (1994). *Mol. Cell. Endocrinol.* **99**, C1–C6.

Coombs, G. S., Bergstrom, R. C., Pellequer, J. L., Baker, S. I., Navre, M., Smith, M. M., Tainer, J. A., Madison, E. L. & Corey, D. R. (1998). *Chem. Biol.* **5**, 475–488.

Ellerman, D. A., Brantúa, V. S., Martínez, S. P., Cohen, D. J., Conesa, D. & Cuasnicú, P. S. (1998). *Biol. Reprod.* **59**, 1029–1036.

Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.

La Fortelle, E. de & Bricogne, G. (1997). *Methods Enzymol.* **276**, 472–494.

Lilja, H., Abrahamsson, P. & Lundwall, A. (1989). *J. Biol. Chem.* **264**, 1894–1900.

Magdaleno, L., Gasset, M., Varea, J., Schambony, A. M., Urbanke, C., Raida, M., Töpfer-Petersen, E. & Calvete, J. J. (1997). *FEBS Lett.* **420**, 179–185.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Mittl, P. R., Di Marco, S., Fendrich, G., Pohlig, G., Heim, J., Sommerhoff, C., Fritz, H., Priestle, J. P. & Grutter, M. G. (1997). *Structure*, **5**, 253–264.

Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.

Peter, A., Lilja, H., Lundwall, A. & Malm, J. (1998). *Eur. J. Biochem.* **252**, 216–221.

Qin, H., Kemp, J., Yip, M. Y., Lam-Po-Tang, P. R. & Morris, B. J. (1991). *Hum. Hered.* **41**, 222–226.

Robert, M., Gibbs, B. F., Jacobson, E. & Gagnon, C. (1997). *Biochemistry*, **36**, 3811–3819.

Schambony, A., Gentzel, M., Wolfes, H., Raida, M., Neumann, U. & Töpfer-Petersen, E. (1998). *Biochem. Biophys. Acta*, **1387**, 206–216.

Schedlich, L. J., Bennetts, B. H. & Morris, B. J. (1987) *DNA*, **6**, 429–437.

Schedlich, L. J., Catanzaro, D. F. & Morris, B. J. (1988). *J. Hypertens. Suppl.* **6**, 395–398.

Terwilliger, T. C. & Berendzen, J. (1999). *Acta Cryst.* D**55**, 849–861.

Timm, D. E. (1997). *Protein Sci.* **6**, 1418–1425.

Vihinen, M. (1994). *Biochem. Biophys. Res. Commun.* **204**, 1251–1256.

Villoutreix, B. O., Getzoff, E. D. & Griffin, J. H. (1994). *Protein Sci.* **3**, 2033–2044.

Watt, K. W. K., Lee, P., M'Timkulu, T., Chan, W. & Loor, R. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 3166–3170.

Yousef, G. M., Scorilas, A., Jung, K., Ashworth, L. K. & Diamandis, E. P. (2001). *J. Biol. Chem.* **276**, 53–61.