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Acta Crystallographica Section F
**Structural Biology and Crystallization Communications**
ISSN 1744-3091
Editors: **H. M. Einspahr and M. S. Weiss**

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*Acta Cryst.* (2012). F**68**, 1251–1254

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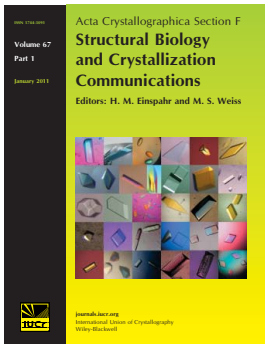


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# Purification, crystallization and preliminary crystallographic analysis of Kar9p from *Saccharomyces cerevisiae*

**Daniela Hüls and Dierk Niessing***

Institute of Structural Biology, Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany

Correspondence e-mail: niessing@helmholtz-muenchen.de

Received 30 May 2012
Accepted 20 August 2012

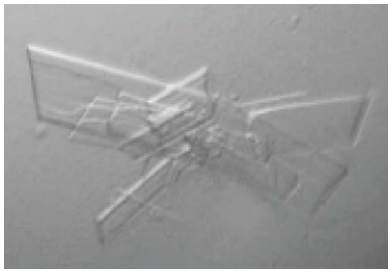
Kar9p is required for correct positioning of the mitotic spindle in *Saccharomyces cerevisiae*. The *in vivo* function of Kar9p is well understood, but no structural information is available. Additionally, molecular details of how Kar9p interacts with other proteins are scarce. Full-length Kar9p was expressed in *Escherichia coli*, purified and crystallized. Diffraction data were collected and processed at 7 Å resolution. One crystal showed diffraction to 3 Å resolution. The crystals that diffracted to 7 Å resolution belonged to space group *P*3, with unit-cell parameters $a = b = 195.02$, $c = 257.15$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The crystal that diffracted to 3 Å resolution belonged to space group *P*222, with unit-cell parameters $a = 46.37$, $b = 74.64$, $c = 133.63$ Å, $\alpha = \beta = \gamma = 90^\circ$.

## 1. Introduction

*Saccharomyces cerevisiae* undergoes asymmetric cell division by budding. To ensure the proper segregation of genetic material, the mitotic spindle has to be oriented correctly within the cell. This process is driven by the highly conserved type V myosin motor Myo2p, which interacts with the adapter Kar9p (karyogamy 9 protein). Kar9p itself also binds to microtubule-associated Bim1p (binding to microtubules 1 protein), thereby connecting both types of cytoskeleton (Hüls *et al.*, 2012).

At the onset of mitosis, Kar9p co-localizes with Bim1p to the spindle pole body (SPB; Liakopoulos *et al.*, 2003; Maekawa & Schiebel, 2004). Whereas Bim1p localizes to both SPBs, Kar9p localizes exclusively to the mother SPB destined for the daughter cell (Liakopoulos *et al.*, 2003; Maekawa *et al.*, 2003; Maekawa & Schiebel, 2004; Miller *et al.*, 2006). Localization of Kar9p is critical for proper spindle orientation and is tightly regulated. Being associated with the plus ends of dynamically growing microtubules, Kar9p encounters cortical Myo2p. The motor complex then moves Kar9p-decorated microtubules along actin filaments into the bud (Beach *et al.*, 2000; Yin *et al.*, 2000; Hwang *et al.*, 2003), resulting in the orientation of the mitotic spindle along the mother-bud axis.

Despite the availability of many functional studies on Kar9p (Miller & Rose, 1998; Moore & Cooper, 2010), no information is available on the three-dimensional structure of this protein. Kar9p is highly conserved within yeast and its functional homologue in higher eukaryotes is the adenomatous polyposis coli (APC) protein. Kar9p and APC share sequence homology at their C-termini. This C-terminal region in Kar9p and APC mediates binding to Bim1p in yeast and to EB1 in higher eukaryotes (Bienz, 2001). Structural information on Kar9p will help us to improve our understanding of its biological function and will promote the study of related proteins such as APC.



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## 2. Materials and methods

### 2.1. Macromolecule production

*KAR9* was amplified by PCR from genomic yeast DNA and subcloned into the *Nhe*I and *Sac*I restriction sites of the vector pET-



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**Table 1**
Macromolecule-production information.

| | |
|---|---|
| Source organism | *S. cerevisiae* |
| DNA source | Genomic DNA |
| Forward primer | 5′-AAAAAGCTAGCATGGATAATGATGGACCC-AG-3′ |
| Reverse primer | 5′-AAAGAGCTCTCAATAAGTTGGGGTTTTATC-TAAACG-3 |
| Cloning vector | pET28a-TEV: modified pET-28a vector in which the original thrombin protease cleavage site was substituted by a TEV protease site 5′ to the *Nhe*I restriction site. The resulting sequence of the tag is MGSSHHHHHHSSGENLYFQSHMAS. |
| Expression vector | pET28a-TEV |
| Expression host | *E. coli* BL21 (DE3) Star |
| Complete amino-acid sequence of the construct produced | MGSSHHHHHHSSGENLYFQSHMASMDNDGPR-SMTIGDDFQENFCERLERIHNTLHSINDCNSL-NESTTSISETLLVQFYDDLENVASVIPDLVNK-K RLGKDDILLFMDWLLLKKYMLYQFISDVH-NIEEGFAHLLDLLEDEFSKDDQDSDKYNRFS-PMFDVIEESTQIKTQLEPWLTNLKELLDTSLE-FNEISKD HMDTLHKIINSNISYCLEIQEERFA-SPIRHTPSFTLEQLVKLLGTHTETTEPKVPKFS-PAEDILSRKFLNLKKNIPPIEKSLTDILPQRIV-QFGHRNITNITTLQTILQKKYELIMKDYRFM-NSEFRELKVELIDKRWNILFINLNHELLYILD-EIERLQSKLLTTKYTKDITIRLERQLERKSKT-VSKTFNIIYRALEFSLLDAGVASKTNELAQR-WLNIKPTADKILIKSSASNKIATSKKKIPKPKS-LGFGRPNSVIGTITQDFQERVAINEGDSNKT-PENSTTVALKGKKLGKALLQKMNIKPATSPN-SSNAINPFFDPESPNKGKLILSSVPPLPYDETD-ETTLRVSRGENEKSPDSFITSRHENKVQITET-PLMAKNKSVLDIEKDKWNHYRSLPSRIPIYK-DKVVKVTVENTPIAKVFQTPPTKITTPNSQV-WVPSTRRRTRLRPPTPLSQLLSPREGRLDKT-PTY |

28a-TEV. The plasmid was transformed into *Escherichia coli* BL21 (DE3) Star and cultured in LB medium. At an optical density (600 nm) of 0.3–0.4, the cultures were cooled to 291 K, expression was induced (0.25 m*M* IPTG) and the cultures were incubated for a further 16 h at 291 K. Cell pellets were resuspended in lysis buffer (50 m*M* $Na_2HPO_4$ pH 7.5, 500 m*M* NaCl, 5 m*M* imidazole, 1 m*M* DTT) and lysed by sonication. Cell debris was removed by centrifugation and the supernatant was loaded onto a His-Trap FF column (GE Healthcare). After washing with wash buffer (50 m*M* $Na_2HPO_4$ pH 7.5, 1 *M* NaCl, 50 m*M* imidazole, 1 m*M* DTT), the protein was eluted with elution buffer (50 m*M* $Na_2HPO_4$ pH 7.5, 500 m*M* NaCl, 500 m*M* imidazole, 1 m*M* DTT). Peak fractions were loaded onto a HiTrap Q column (GE Healthcare). The flowthrough was concentrated with centrifugal filter devices (Amicon Ultra, Millipore) and loaded onto a Superdex S200 16/60 size-exclusion chromatography column equilibrated with gel-filtration buffer (10 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT). Peak fractions were analyzed by SDS–PAGE and concentrated again. Further details are given in Table 1.

### 2.2. Crystallization

The protein solution was concentrated to 6 mg $ml^{-1}$ using centrifugal filter devices (Amicon Ultra, Millipore). Initial crystallization screens were set up with a nanolitre crystallization robot (Phoenix, Art Robbins Instruments) using several crystallization kits from Qiagen (The Classics Suite and The PEGs Suite) and Hampton Research (Index HT). A drop size of 0.5 µl and a 1:1 mixture of protein solution and crystallization solution were used for sitting-drop vapour-diffusion experiments in a 96-well plate format.

Crystallization conditions were manually refined by the hanging-drop vapour-diffusion technique using a drop size of 2 µl consisting of a 1:1 mixture of protein solution and crystallization solution and with a total reservoir volume of 500 µl in 24-well EasyXtal CrystalSupport plates (Qiagen). Further details are given in Table 2.

### 2.3. Data collection and processing

Crystals were cryoprotected by adding different cryoprotectants (glycerol, ethylene glycol or PEG 400) to the reservoir solution at different concentrations and with a variety of incubation times. Diffraction data were collected from crystals at the ESRF, Grenoble

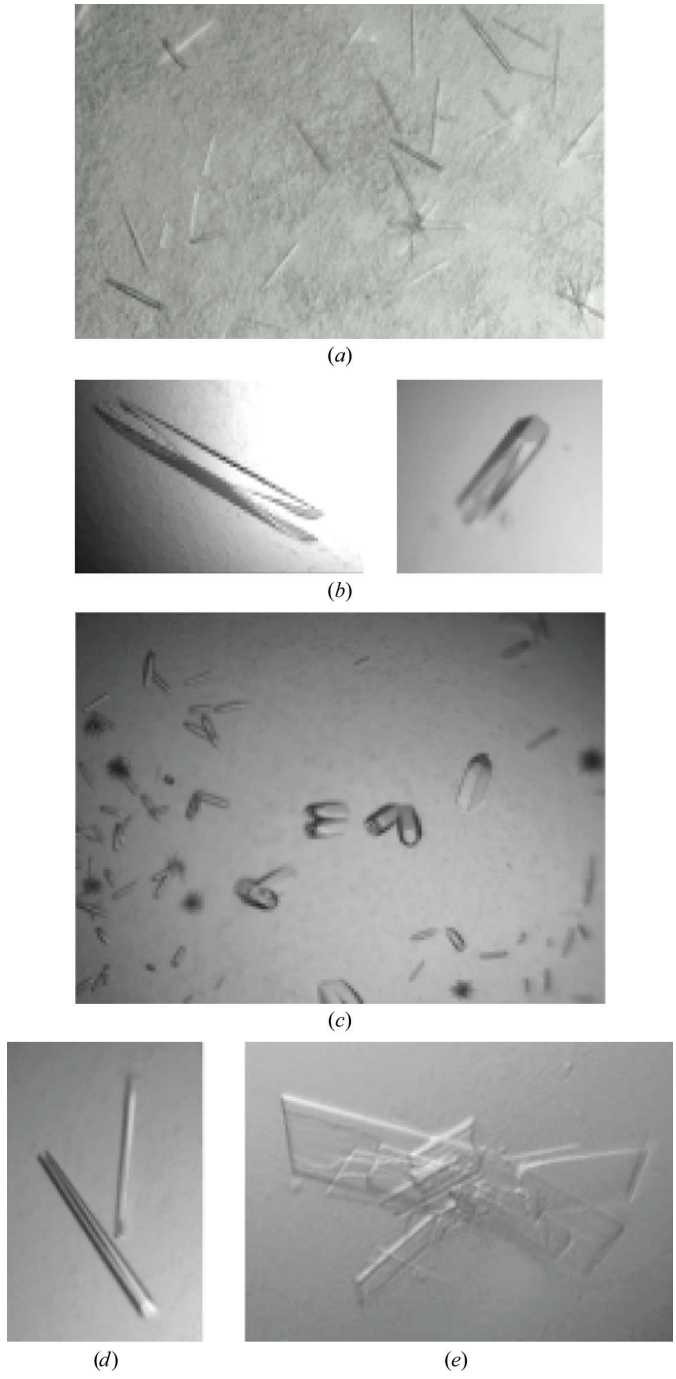


**Figure 1**
Crystals of Kar9p. (*a*) Crystals from the initial crystallization screens. (*b*) Crystals grown after manual refinement of the crystallization conditions in 0.1 *M* HEPES pH 8.0, 21% PEG MME 2000, 125 m*M* serine. (*c*) Crystals of Kar9p grown at 277 K using the same conditions as in (*b*). (*d*, *e*) Crystals of methylated Kar9p. These crystals failed to show detectable X-ray diffraction.



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and the crystals showed diffraction to up to 7 Å resolution. Data sets were processed and scaled with *XDS* (Kabsch, 2010). The data-collection statistics are summarized in Table 3.

## 3. Results and discussion

Full-length Kar9p was expressed as a His-tagged protein in *E. coli* and purified. Kar9p was stable for several days and could be used for crystallization.

Crystals of Kar9p were grown at 294 K by hanging-drop vapour diffusion using a 1:1 mixture of protein solution (1–6 mg ml$^{-1}$) and crystallization solution consisting of 0.1 *M* HEPES pH 8.0, 21% PEG MME 2000, 125 m*M* serine or 0.1 *M* potassium citrate, 20% PEG 3350, 125 m*M* serine. Needle-shaped crystals with cracked ends appeared within 1–2 d (Figs. 1*a* and 1*b*). Cryoprotection was achieved with various cryoprotectants (glycerol, ethylene glycol and PEG 400) using differing concentrations and incubation times.

**Table 2**
Crystallization.

| | |
|---|---|
| Method | Hanging drop |
| Plate type | Qiagen EasyXtal 24-well plates |
| Temperature (K) | 294 ± 2 |
| Protein concentration (mg ml$^{-1}$) | 1–6 |
| Buffer composition of protein solution | 10 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT |
| Composition of reservoir solution | 0.1 *M* HEPES pH 8.0, 21% PEG MME 2000, 125 m*M* serine or 0.1 *M* potassium citrate, 20% PEG 3350, 125 m*M* serine |
| Volume (µl) and ratio of drop | 1, 1:1 |
| Volume of reservoir (µl) | 500 |

Diffraction data were collected from crystals showing diffraction to 6 Å resolution (Fig. 2*a*). Processing of the data sets showed that the crystals yielded high-quality diffraction to 7.0 Å resolution and

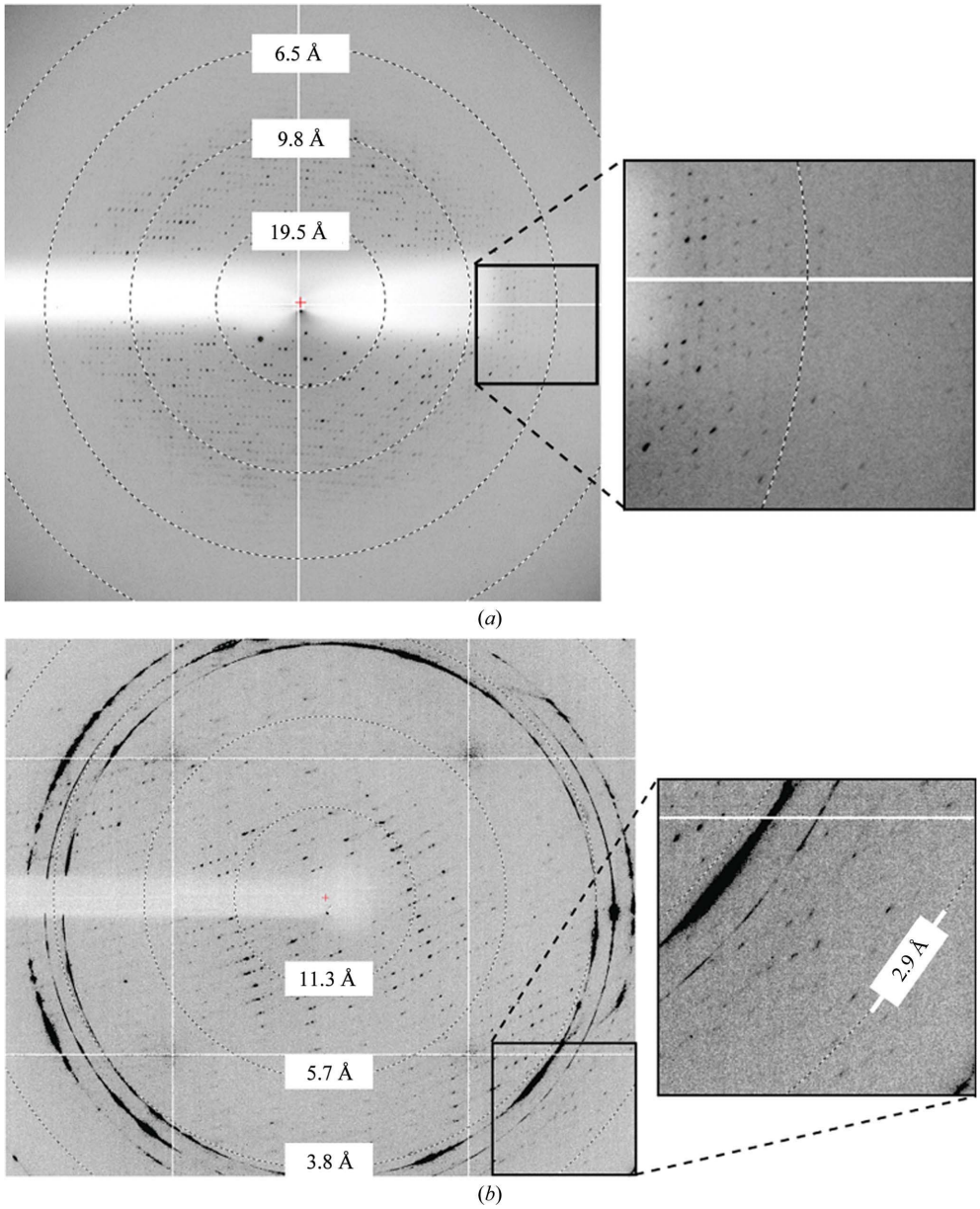


**Figure 2**
X-ray diffraction images collected from crystals of Kar9p. (*a*) Diffraction image of crystals with a resolution of up to 7 Å. (*b*) Diffraction image of the crystal with a resolution of up to 3 Å. The presence of several ice rings in the diffraction pattern caused additional problems during processing of the data set.



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**Table 3**
Data collection and processing.

Values in parentheses are for the outer shell.

| | |
|---|---|
| Diffraction source | ID14-1 |
| Wavelength (Å) | 0.934 |
| Temperature (K) | 100 |
| Detector | ADSC Quantum Q210 |
| Crystal-to-detector distance (mm) | 437.28 |
| Rotation range per image (°) | 1 |
| Total rotation range (°) | 200 |
| Exposure time per image (s) | 1 |
| Space group | *P*3 |
| Unit-cell parameters (Å, °) | $a = b = 195.02$, $c = 257.15$, $\alpha = \beta = 90$, $\gamma = 120$ |
| Mosaicity (°) | 0.447 |
| Resolution range (Å) | 30–7.0 |
| Total No. of reflections | 104433 |
| No. of unique reflections | 17015 |
| Completeness (%) | 98.5 |
| Multiplicity | 6.1 |
| $\langle I/\sigma(I)\rangle$ | 7.9 (2.4) |
| $R_{\rm meas}$ | 0.184 |

belonged to space group *P*3, with unit-cell parameters $a = b = 195.02$, $c = 257.2$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Using diffraction data to 7 Å resolution, a resolution-normalized Matthews coefficient (Kantardjieff & Rupp, 2003) of $V_{\rm M} = 7$ Å$^3$ Da$^{-1}$ suggested an average of 5.4 molecules per asymmetric unit and a solvent content of over 80%. This high solvent content offers a potential explanation for the limited diffraction quality of these crystals. One crystal grown in 0.1 *M* HEPES pH 7.5, 29% PEG MME 2000, 125 m*M* serine using 20% glycerol as a cryoprotectant showed diffraction to 3 Å resolution (Fig. 2*b*). However, the data set was incomplete as only 180° of data were recorded and some smeared spots were obtained that could not be used for data processing. The presence of several ice rings in the diffraction pattern caused additional problems and prevented processing to a resolution better than 3.4 Å. Processing of the data set with *XDS* showed that this crystal belonged to space group *P*222 and has a smaller unit cell, with unit-cell parameters $a = 46.37$, $b = 74.64$, $c = 133.63$ Å, $\alpha = \beta = \gamma = 90^\circ$. Despite much effort, this crystal form could not be reproduced.

Multiple attempts to slow crystal growth (the use of oil to cover the reservoir solution and crystal growth at 277 K; Fig. 1*c*) and to find protein fragments that were better suited for crystallization were unsuccessful. Crystals obtained from a fragment consisting of amino acids 1–398 showed some additional diffraction spots up to 4–5 Å resolution. However, in comparison to full-length Kar9p the diffraction patterns mainly consisted of smeared less defined spots. Other attempts such as crystallization in the presence of proteases or methylation of the protein prior to crystallization also failed to improve the crystal diffraction properties (Figs. 1*d* and 1*e*).

In summary, it can be concluded that Kar9p tends to crystallize but does not readily form well diffracting crystals. Our study outlines efforts to overcome hurdles during crystallization and represents a starting point for future studies. However, since multiple attempts to improve the conditions failed to result in well diffracting crystals, this might not be an easy task.

We acknowledge support from the crystallization facility of the Max Planck Institute of Biochemistry and the European Synchrotron Radiation Facility for the provision of synchrotron-radiation facilities.

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