Structural variations in the catalytic and ubiquitin associated domains of MARK1 and MARK2

Alexander Marx, Chanakya Nugoor, Jens Müller, Saravanan Panneerselvam, Thomas Timm, Matthias Bilang, Efstratios Mylonas§, Dmitri I. Svergun§#, Eva-Maria Mandelkow, and Eckhard Mandelkow

From the Max-Planck-Unit for Structural Molecular Biology, Notkestrasse 85, 22607 Hamburg, Germany, the §European Molecular Biology Laboratory, Hamburg Outstation, 22603 Hamburg, Germany, and the #Institute of Crystallography, Russian Academy of Sciences, Leninsky pr. 59, 117333 Moscow, Russia

Address correspondence to: E. Mandelkow, MPG-ASMB, c/o DESY, Notkestrasse 85, 22607 Hamburg, Germany, Phone: +49 40 8998-2810; Fax: +49 40 897168-22; E-mail: mand@mpasmb.desy.de

The MAP/microtubule affinity regulating kinase MARK/Par-1 phosphorylates microtubule associated proteins tau, MAP2, and MAP4, and is involved in the regulation of microtubule-based transport. Par-1, a homologue of MARK in Drosophila and C. elegans, is essential for the development of embryonic polarity. Four isoforms of MARK are found in humans. Recently, we reported the crystal structure of the catalytic and ubiquitin-associated domains of MARK2, an isoform enriched in brain (Panneerselvam et al., Structure (2006) 14, 173-183). It showed that the UBA domain has an unusual fold and binds to the N-terminal lobe of the catalytic domain. This is at variance with a previous low-resolution structure derived from small angle solution scattering (Jaleel et al., Biochem. J. (2006) 394, 545-555) which predicts binding of the UBA domain to the larger, C-terminal lobe. Here we report the crystal structure of the catalytic and UBA domain of another isoform, MARK1. Although the crystal packings of the two isoforms are unrelated, the overall conformations of the molecules are similar. Notably the UBA domain has the same unusual conformation as in MARK2 and it binds at the same site. Remarkable differences occur in the catalytic domain: at helix C, the catalytic loop, and the activation segment.

The Ser/Thr kinase MARK1 has been identified by the ability to phosphorylate tau at certain serine residues in the microtubule binding repeats (1). Phosphorylation of tau and other microtubule-associated proteins (MAP2, MAP4) at the KXGS motifs by MARK reduces the affinity to microtubules and leads to microtubule disassembly. Hyperphosphorylation of tau followed by aggregation to paired helical filaments (PHF) is one of the hallmarks of Alzheimer disease. MARK orthologues KIN1 in fission yeast and Par-1 in Drosophila and C. elegans are involved in the development of cell polarity (2,3). In neurons, MARK is required for neurite outgrowth and differentiation (4).

There are four isoforms of MARK in the human kinome which form a subfamily of the Snf1/AMPK family of kinases within the CAMK group (5). MARK kinases are relatively large, the longest isoform, MARK1, comprises 795 amino acids (Fig. 1). The catalytic domain is flanked by an N-terminal header of about 60 amino acids and a linker of about 20 amino acids which includes a four residue motif (adjacent to the catalytic domain) that may serve as a common docking site (CD domain) for regulatory binding partners in analogy to MAP kinases (6). The linker connects the catalytic domain to a bona fide UBA (UBiquitin Associated) domain, a globular domain of 40 amino acids consisting of three α-helices. The UBA domain is followed by a long spacer and a globular tail (NMR structures by Tochio et al.; PDB-ID: 1UL7) that comprises the KA1 domain (kinases associated domain 1) with the characteristic ELKL motif at the C-terminus. The
functions of the putative UBA and KA1 domains are not well understood. The fact that most of the AMPK-related kinases, including the yeast homologue Snf1, possess a UBA or UBA-like domain (7,8) suggests a conserved function in structural stabilization or regulation of kinase activity.

Like many other kinases, MARK is regulated by phosphorylation of the activation loop (T-loop). MARKK/TAO-1 (9) and the tumor suppressor kinase LKB1/Par-4 (10,11) activate MARK by phosphorylation of the T-loop. A fraction of MARK isolated from brain tissue is doubly phosphorylated at Thr208 and Ser212 (MARK2 numbering used throughout), phosphorylation of the second site, however, is inhibitory (9). There is a number of other mechanisms that seem to be able to regulate MARK, including phosphorylation of the spacer by atypical PKC (12,13) and binding to the scaffolding protein 14-3-3/Par-5 (14,15) or to PAK5, a member of the family of p21-activated kinases (16). Crystal structures of the MARK2 catalytic domain in combination with the UBA domain suggested a role of the latter in regulation of the enzyme (17). The catalytic domain seems to be locked in an open, inactive conformation through the action of the UBA domain, which – while being tethered via an extended linker to the C-terminus of the catalytic domain – binds firmly to the N-terminal lobe (N-lobe).

According to the crystal structures of MARK2, localization of the UBA domain (to the N-lobe) is directly opposite to a model derived from solution x-ray scattering (SAXS) experiments (8). Another surprising result was that the UBA domain adopts an unusual fold in the MARK2 crystals, raising the question, whether the unexpected conformation and localization of the UBA domain could be a crystal packing artifact rather than an intrinsic property of the kinase. To answer this question, we determined the structure of the MARK1 isoform (catalytic and the UBA domains). The two MARK isoforms differ by about 40 amino acids, half of them concentrated in the UBA domain. In addition, we measured the kinase activities of MARK constructs with and without UBA domain and compared the crystal structure with the conformation in solution by means of SAXS experiments. The crystal structure of MARK1 reveals significant differences in the conformation of the catalytic domain, especially in the catalytic loop and the activation segment. However, the overall arrangement of domains, including position and fold of the UBA domain, is the same for both isoforms. Biochemical assays showed that removal of the UBA domain has only a limited effect on the kinase activity in vitro, arguing against the alleged regulatory function. Nevertheless, the UBA domain could still play an important role in the regulation of MARK activity or in localization, assuming that it provides an additional template for the interaction with other binding partners. The results of the SAXS experiments are consistent with the crystal structures, indicating that the overall conformation observed in solution and in the crystals is the same.

Experimental Procedures

Protein preparation and crystallization
Fragments of MARK1 from human (GenBank No. Q9P0L2) were cloned and expressed in E. coli strain BL21 AI (Invitrogen, Karlsruhe, Germany) by using the manufacturer's protocol. The MARK1 fragment Asn45-Lys371 described here is similar to the MARK2 fragment Asn39-Lys364 (17) which was identified by limited proteolysis. The MARK fragments without the UBA domain range from Asn45 to Asp329 (MARK1) and Asn39 to Lys324 (MARK2). By sequence alignment, MARK1 has a single, one-residue insert (Thr51) relative to MARK2. Residue ranges N45-A50 and D52-K371 of MARK1 correspond to N39-A44 and D45-K364 in MARK2, respectively. In the following, we use the residue numbers based on the MARK2 sequence for both isoforms. The constructs include an N-terminal glycine left over from TEV protease cleavage of a His₆ or His₃₉₉ tag.

All proteins were prepared as described (9,17). Crystals of MARK1 were grown by vapor diffusion by mixing 2 µl of protein (20 mg/ml) with 2 µl of a reservoir solution containing 7-10 % PEG 3350, 0.1 M Bis-Tris pH 6.5, 0.2 M ammonium citrate at 4 °C. For SAXS analysis 1 mM DTT was added during the purification procedure and fresh DTT was added to a final concentration of 2 mM just before the SAXS measurement.
**Kinase assays**

The kinase assays were performed essentially as described (1,9). Kinase activity was determined with respect to the tau-derived peptide TR1 = NVKSKIGSTENLK, which contains Ser262 (underlined), the primary phosphorylation site of tau by MARK kinases. The kinases were activated by MARKK/TAO-1 as described (9), which phosphorylates a critical threonine (Thr208) in the activation loop of MARK kinases.

**Crystal data collection, phasing, and model building**

X-ray data were collected at the X13 Consortium beamline at DESY, Hamburg. Crystals were flash frozen at 100 K in cold nitrogen. The HKL data processing system V1.97.2 (18) was used for data reduction and analysis (Table 1). MARK1 crystallized in space group P2₁2₁2₁ with 8 molecules per asymmetric unit (Matthews coefficient 3.06). The structure was solved by molecular replacement with the catalytic domain of MARK2 as search model, using Phaser Version 1.2 (19). To reduce model bias, a composite omit map was calculated with CNS 1.1 (20) before starting cycles of manual model building with Coot (21) and refinement with Refmac5 (22). Noncrystallographic symmetry restraints were used until the R-factor dropped below 30%. The final model contains 2417 of 2624 residues in 8 independent molecules plus 210 water molecules. The model was checked with PROCHECK (23) and WHAT_CHECK (24).

**SAXS data collection and processing**

Synchrotron X-ray scattering data from solutions of MARK1, MARK2, and of T208E point mutant of the latter were collected at the X33 beamline of the EMBL (DESY, Hamburg) (25) using a MAR345 image plate detector. The scattering patterns were measured with 3-minute exposure time for multiple solute concentrations ranging from 0.5 to 6 mg/ml. To check for radiation damage two 2-minute exposures were compared; no radiation effects were observed. Using the sample-detector distance of 2.7 m the range of momentum transfer 0.09 < s < 0.5 Å⁻¹ was covered (s = 4π sin(θ)/λ, where 2θ is the scattering angle and λ = 1.5 Å is the X-ray wavelength).

The data were processed using standard procedures and extrapolated to infinite dilution by the program package PRIMUS (26). The forward scattering I(0) and the radii of gyration Rg were evaluated using the Guinier approximation (27) assuming that at very small angles (s < 1.3/Rg) the intensity is represented as I(s) = I(0) exp(-sRg²/3). The maximum dimensions Dmax and the interatomic distance distribution functions p(r) were computed using the indirect transform package GNOM (28). The molecular masses (MM) of the solutes were evaluated by comparison of the forward scattering with that from a reference solution of bovine serum albumin (MM = 66 kDa). The scattering from the high resolution models of MARK isoforms in the crystal was computed using the program CRYSOL (29). Given the atomic coordinates, the program fits the experimental intensity by adjusting the excluded volume of the particle and the contrast of the hydration layer to minimize the discrepancy

\[ \chi^2 = \frac{1}{N-1} \sum_j \left( \frac{I_{\text{exp}}(s_j) - cI_{\text{calc}}(s_j)}{\sigma(s_j)} \right)^2 \]

where N is the number of experimental points, c is a scaling factor, I_{\text{exp}}(s_j) and I_{\text{calc}}(s_j) are the experimentally determined and calculated intensities, respectively; and σ(s_j) the experimental error at the momentum transfer s_j.

**RESULTS**

**General organization of the MARK1 crystal structure**

MARK1 crystallized in space group P₂₁₂₁₂₁ with 8 molecules per asymmetric unit. The packing of the molecules is completely different from that of MARK2 molecules in the crystal form described previously (17). In the MARK1 crystal, the molecules do not form symmetric dimers as in the case of MARK2. The packing can be described by assuming two lozenge-shaped "tetramers", bent across the short diagonal (molecules A, B, C, D, and molecules E, F, G, H). Moreover, contacts between UBA domains of different tetramers are essential for the packing of the MARK1 "tetramers" (Fig. 2). Yet the general fold and organization of the subdomains of the individual molecules is the same as that of MARK2. The UBA domain has the same fold as observed for MARK2, with the third helix pointing in the
opposite direction compared to UBA domains of other proteins. Furthermore, the UBA domain of MARK1 binds at the same site as in MARK2, at the N-lobe and on the back side of the active site.

Conformational variability of MARK1 molecules

Although the general fold of the eight molecules in the MARK1 crystal is the same, different environments in the crystal lead to local and global variations. Because of this variability, NCS symmetry was not used for structure determination (except for the very first steps of refinement) at the expense of some parts of the molecules not being modeled. The following residues are not included in the final model (as deposited to the PDB): Ann-A45, A204-A212, Bnn-B46, B204-B212, Cnn-C47, C203-C210, Dnn-D45, D202-D230, D253-D257, D290-D291, D308-D311, Enn-E46, Fnn-F46, Gnn-G47, G203-G211, G219-G227, G290-G291, Hnn-H46, H203-H227, H252-H267 (nn: N-terminus; numbering according to MARK2; add +7 to convert to the MARK1 sequence numbering, in the entire range of residues that are visible in the MARK1 crystal structure, i.e. except the N-terminal part that is missing in the structure model).

Molecules E and F are almost complete, only 10 residues at the N-terminus are missing. Most affected by disorder are molecules D, G, and H. In molecule H, for instance, the C-terminal half of the activation loop and the following sequence up to the start of helix F is not modeled, as well as helix G and residues of the preceding loop. For a large part of the missing regions, electron density in the 2fo-fc map indicates the general trace of the polypeptide chain; these parts, however, were not included in the final model since completion of the model did not appreciably improve the quality of the model (by R factors and geometry). For the sake of clarity, some of the figures show such additional parts of the molecules (as indicated in the legends) using a more complete model. Since these regions are close to cavities in the crystal packing, the poor electron density is attributed to conformational disorder and multiple conformations with partial overlap. Omission of the doubtful regions in the final model may underline the fact that the observed variability reflects a property of the crystal structure and is not due to limitations of the data set.

For comparison of different molecules, a hierarchical approach for describing the variability seems appropriate: at the top level, the molecules are considered as consisting of three subdomains, N-lobe, C-lobe, and UBA domain, appearing as rigid bodies in first approximation. Least square superpositions of the molecules using each of the three subdomains in turn reveals (i) the displacement of the free subdomains relative to the fixed one, and (ii) the conformational variation within the fixed subdomain across the ensemble of the eight molecules (Fig. 3). For superpositions and calculation of relative displacements, it is convenient to define "core" structural elements for the N- and the C-lobe that may serve as a reference. Residues 52-84 (comprising β strands 1, 2 and 3) are chosen as reference for the N-lobe, residues 151-168 and 231-243 (helices E and F) for the C-lobe. Because of its small size and low variability, there is no need to select a core reference structure in the case of the UBA domain (residues 323-362).

Superposition of the molecules using any one of the subdomains (Fig. 3 a,b,c) as reference reveals considerable movements relative to each other. With the N-lobe fixed, the C-lobe rotates by up to 10° which results in a shift at the distal parts of about 7-8 Å (Fig. 3 a). Movement of the UBA domain relative to the N-lobe is more restricted, consistent with the tight interaction of these subdomains (up to 6°, corresponding to 2-3 Å at the periphery).

Two highly variable regions are found in the N-lobe (Fig. 3 a ). One of these regions comprises the N-terminus of helix C, the preceding loop, and the turn between β4 and β5. In fact, helix C pivots around its C-terminal end like a rigid rod. The other variable region is the loop between the fixed end of helix C and β4. This loop follows the movement of the C-lobe. It forms part of the hinge that links the N- to the C-lobe.

The most variable part of the C-lobe is the activation loop, its C-terminal anchor, and the following sequence up to the N-terminus of helix F (residues ~203-229). The activation loop is highly flexible, only two molecules (E and F) exhibit a well defined conformation of the entire activation loop (B-factors of the backbone atoms < 60). In another molecule (C) the activation loop could also be traced, but the result was unsatisfying (bad geometry, B-factors up to ~85), so it was omitted in the final model. Moreover, all three
conformations of the activation loop that have been traced are different from each other. In molecules E and F, the activation loops are stabilized by reciprocal intermolecular contacts (E with F, and F with E). This is reminiscent of the MARK2 crystal structure which consists of symmetric dimers with the activation loops at the center in close contact and even covalently linked by a disulfide bridge. However, in MARK1, the packing of the molecules is different. There is no special symmetry relation between E and F, and therefore there is no requirement for the activation loops of these molecules to be similar.

In molecules D, G, and H, the flexibility of the C-lobe affects a substantial part of the support structure of the activation loop, including the C-terminal anchor (P+1 loop), helix EF, the loop leading to helix F, as well as helix G, and the loop between helices I and H. These regions exhibit considerable shifts between the molecules or are untraceable due to conformational disorder or the presence of multiple conformations.

The UBA domains of all molecules are well defined. In fact, they rank among the best defined parts of the crystal structure in terms of root square deviations and B-factors. The linker (residues ~313-322) has to adapt to the displacements of the C-lobe and the UBA domain, nevertheless, its backbone trace and side chain conformations are well conserved in all molecules.

**Differences between MARK1 and MARK2**

Further discussion of the MARK1 structure will focus on molecules E and F, the best defined molecules in the MARK1 structure. These molecules differ mainly by the conformation of the activation loop between residues 202 and 214, and by relative movements of the subdomains as described above (with intermediate rotations, 6.3° for the C-lobe, 4.9° for the UBA domain; relative to the N-lobe). For comparisons with the MARK2 structure, the same approach is used as before, with the same core structural elements serving as a reference for describing the relative movement of the subdomains (Fig. 3). Unless otherwise noted, comparisons are between molecule E of MARK1 and molecule A of MARK2, using the coordinates of the selenomethionine derivative of the T208/S212 double mutant (PDB-ID: 1Y8G) which is the structure of highest resolution available for MARK2.

Superposition of the N-lobes or the UBA domains of MARK1 and MARK2 shows clearly that the UBA domain attaches to the catalytic domain at the same place and with the same orientation in both isoforms (Fig. 3, a, c). More generally, the relative movements of the subdomains (as defined by the core reference elements) are similar to those observed between the different molecules of the MARK1 structure, their amplitudes, however, tend to exceed the range covered by the MARK1 molecules. This is most obvious for the UBA domain in the superposition of the C-lobes (Fig. 3, b). Furthermore, large deviations between the two isoforms occur in the highly variable regions of the N- and the C-lobe and in the catalytic loop.

**N-lobe**

Helix C of MARK1 performs a movement relative to MARK2 that consists of a small shift in axial direction combined with a tilt rotation of about 5°. This movement also extends to the adjacent loop and to strands β4 and β5 (Fig. 3, a). Together with the overall rotation of the N-lobe, this movement leads to a substantial dislocation of helix C farther away from the C-lobe (Fig. 3 b, Fig. 4).

**C-lobe**

In MARK2, the N-terminal half of the activation segment (residues 193-219), starting with the DFG motif, is disordered and invisible; only the C-terminal half (residues 206-219) was traceable due to stabilization by direct interaction of the activation segments of the MARK2 dimers (17). In the MARK1 structure, the N-terminal anchor up to residue 201 is well defined and almost identical in all molecules, while the remaining part of the activation segment is more variable; it is clearly visible in two of eight molecules (E and F). The common part of the activation segment (residues 193-201) is nestled to helix C and the P-loop (β1-β2). Moving helix C to its position in MARK2 would result in a steric clash with the stable part of the activation segment (Fig. 4c).

The second half of the activation segment forms a severely distorted helix in molecule E. From Gly211 to the APE motif (217-219, which marks the end of the activation segment), the conformation of molecule E is very similar to that of MARK2. The APE motif, which is part of the
EF helix, is remarkably similar in all molecules of the MARK1 structure, inasmuch as they could be defined. The conformation of residues ~202 to 210 however is highly variable, indicating that this part of the activation segment (visible in molecules E and F, and to some extent in the other molecules) are at least partially determined by intermolecular contacts (Fig. 5).

Interestingly, the activation loops of molecules E and F are in close contact to each other, as they are in the MARK2 dimers. At the first glance, molecules E and F form dimers similar to MARK2 (see overlay in Fig. 5 a). However, if one of the molecules is overlaid by best fit to a molecule of the MARK2 dimer, the other one is rotated by an angle of about 75° compared to the MARK2 structure. Still, the overall shape of the "EF dimer" is roughly conserved since the rotation is around the long axis of the molecule. Furthermore, the structural elements involved in interdimer contacts remain at the contact interface.

By analogy to structures of active kinases, the structural elements supporting both the substrate and the substrate binding part (P+1 loop) of the activation segment are the short helix EF at the end of the activation segment (residues ~218-221), the following loop up to the start of helix F, as well as helix G and the preceding loop. In molecules A, B, C, E, and F of the MARK1 structure, these regions are fairly well defined and similar to each other, but in molecules D, G, and H they are disordered and hardly traceable. Both these regions are well defined in the MARK2 structure. In fact, they are the key structural elements responsible for tight interaction between the molecules of the MARK2 dimer. Thus, large displacements in these regions, especially in the vicinity of the short helix EF (~3-4 Å), are probably related to the formation of dimers in the MARK2 structure.

The catalytic loop in MARK2 (res 171-183) is very similar both in conformation and position to that of Snf1, the yeast orthologue of AMPK and close relative of MARK (30), and it is also quite similar to more distant kinases, both in active and inactive states, like Aurora-A and B, PKA (max. displacement ~2.5 Å), or CDK2. In the MARK1 structure on the other hand, the catalytic loop assumes a distinctly different fold, resulting in a shift of ~6 Å in the CA position of His173. Since the conformation of the catalytic loop is well defined and virtually identical in all 8 molecules (in spite of different crystal contacts), the change in conformation is unlikely to be a crystallization artifact. It seems to be the consequence of a single amino acid exchange, Phe170 in MARK2 to Tyr in MARK1. This residue Tyr/Phe170 seems to be the only amino acid exchange of the two MARK isoforms that has a noticeable effect on the conformation of the catalytic domain. All the other exchanges are either (conformationally) conservative or they are located in regions of high flexibility. Phe170 (MARK2) locates at the end of helix F and the start of the catalytic loop. Its side group points to the exterior of the molecule (Fig. 6). In MARK1, the corresponding residue Tyr170 is flipped towards the interior. The hydroxyl group of Tyr170 is engaged in a hydrogen bond with the carbonyl oxygen of Phe194 in the DFG motif (part of the N-terminal anchor of the activation loop; this part of the activation segment is disordered in MARK2.) Flipping of Tyr170 induces other conformational changes in the catalytic loop, including the inverse flip of His173. This allows the N-terminal anchor to fill in the space left by the outward movement of the catalytic loop. Thus, it seems that the single exchange of Phe170 to tyrosine causes many, if not most of the differences between MARK1 and MARK2 catalytic domains: (i) the unusual conformation of the catalytic loop, (ii) the stabilization of the N-terminal anchor and (eventually) the activation loop, (iii) the upward shift and stabilization of the P-loop, as well as the displacement of helix C by repulsion through the N-terminal anchor, and thus, possibly, (iv) the shift/rotation of the N-lobe relative to the C-lobe.

UBA domain

Most amino acid differences between MARK1 and MARK2 concentrate in the UBA domain (residues 323-364). No compensatory exchanges can be identified in the N-lobe. Only one exchange in the N-lobe, Arg/Lys77, comes close to the binding site of the UBA domain and may have an effect on the interaction of UBA and catalytic domain. But even this effect appears minor, as Arg77 in MARK1 interacts with the terminal amino acids of the construct (residues 362-364), leading to stabilization of the N-terminus by polar interactions and hydrogen bonds with Arg363 (Tyr363 in MARK2). This explains why the terminal residues are better defined in
MARK1 than in MARK2. The overall docking of the UBA domain, however, is not much different.

Of the 18 amino acid exchanges in the UBA domain, only one (Ile/Leu359) is located in helix \( \alpha_3 \), two more (Asp/Asn352 and Arg/Tyr363) are situated at the N- and C-terminal ends of \( \alpha_3 \). Since the binding of the UBA domain to the N-lobe is mainly due to hydrophobic interactions with helix \( \alpha_3 \), there is virtually no difference between the two MARK isoforms in this respect. Similarly, the interior of the UBA domain is also unchanged, as the other residues that are different in the two isoforms have their side chains mostly directed to the outside. As a consequence, the main chain conformation of the UBA domain is almost the same in both isoforms. The rms difference in CA positions by superposition of the UBA domain (residues 323-362) is 0.41 Å for MARK1 chain E and MARK2 chain A, while the rms difference for all combinations of the eight MARK1 chains ranges between 0.19 and 0.36 Å. If a significant difference between the UBA conformations exists at all, this is probably due to the interactions with other molecules.

**Intermolecular contacts**

In the MARK2 crystal structures, dimers are arranged in helical tubes. Interaction of the dimer along the tube axis consists in UBA – UBA contacts. Two slightly different modes of such contacts between UBA domains were observed, resulting in the occurrence of two crystal types, that differ by the length of the c-axis (parallel to the tube axis) by about ~6 Å with the overall packing of the molecules being conserved. This means that the interaction between UBA domains in MARK2 is of minor importance for the crystal packing. In MARK1, the UBA domains of one tetramer participate in two-fold pseudo-symmetric interactions with UBA domains of adjacent tetramers, including contacts to the N-lobes (residues ~74-77 at the turn between \( \beta_2 \) and \( \beta_3 \)). The UBA-UBA interactions mainly involve Thr334 and Met335 of the MGY/F motif. Contacts to the N-lobe involve several residues at the outer surface of helix \( \alpha_1 \) (Thr326, Lys327, Asp330, Ile331) as well as Thr334 and Met335. Except for Met335, these residues are different in MARK1 and MARK2 (Pro326, Arg327, Glu330, Leu331, Ser334 in MARK2).

Molecules A and F, as well as molecules B and E of adjacent tetramers exhibit another type of local, two-fold symmetry based on contacts of the loops between helices H and I on the one hand, and the N-terminal part of the catalytic loop around Tyr170 on the other hand. Thus, it appears that the inward flip of Tyr170 (compared to Phe170 in MARK2) is not only responsible for the internal conformational differences between MARK1 and MARK2 molecules, but could also directly affect the crystal packing. The reverse argument is not true: the unusual conformation of the catalytic loop in MARK1 is not due to this special type of crystal contacts, since the catalytic loops of molecules D, H, and G make different contacts to adjacent molecules, and the catalytic loop of molecule C is not engaged in any crystal contacts at all. Yet the conformation of all eight molecules in the MARK1 structure is the same. Furthermore, an overlay of MARK2 to MARK1 shows that the conformation of MARK2 is not far from being compatible with two-fold symmetric packing involving the catalytic loop. The latter observation, however, also suggests that the conformation around Phe/Tyr170 has only limited effects on the crystal packing.

**Conformation of MARK in solution**

A unique feature of all MARK isoforms and constructs solved so far is the attachment of the UBA domain to the N-lobe of the kinase domain, tethered to the C-lobe by an extended peripheral linker (Fig. 3). UBA domains are thought to be involved in regulation by interaction with poly-ubiquitin chains, but on the other hand the presumptive ubiquitin interaction site (deduced from other UBA-ubiquitin structures, e.g. (31)) is occluded in the MARK-UBA interface (17). We therefore considered the possibility that the UBA domain might detach from the body of the kinase domain in solution, at least in certain states of regulation. Thus we studied the structures of the catalytic and UBA domains of MARK1 and MARK2 in solution by small-angle X-ray scattering (SAXS). The processed scattering patterns from the two isoforms in Fig. 7 yield an effective molecular mass of about 36 ± 3 kDa, in agreement with the value predicted from the sequence, indicating that the proteins were monomeric in solution. The experimental \( R_g \) and \( D_{max} \) values of MARK1 (23.4 ± 0.6 Å and 75 ± 5 Å, respectively), and those of MARK2 (23.2 ± 0.6 Å and 75 ± 5 Å, respectively) are very close to each other. Moreover, the entire scattering
patterns of MARK1 and MARK2 coincide with each other within the experimental errors. These integral parameters of MARK1 and MARK2 agree with those computed from the crystal structures by CRYSOl accounting for the hydration shell (about $R_g = 22.9$ Å, $D_{max} = 73$ Å). Even more conclusive, scattering patterns computed from the crystallographic models of MARK1 and MARK2 neatly fit the experimental curves with discrepancy $\chi = 1.3$ and 1.5, respectively (Fig. 7, curves 1 and 2). These results indicate that the overall crystallographic structure of the MARK isoforms remains preserved in solution, i.e. the UBA domain remains attached to the N-lobe of the catalytic domain even free of the constraints of crystal packing.

Extending this approach, we asked whether the conformation of the UBA domain was related to the state of activity. We therefore mutated the critical Thr residue (Thr208) in the activation loop into Glu, creating a pseudo-phosphorylated state. As shown previously (9), this point mutation is able to enhance the specific activity of MARKs up to 4-fold, comparable to phosphorylation by upstream kinases such as MARKK. However, the SAXS pattern yielded the $R_g$ and $D_{max}$ (23.7 ± 0.7 Å and 77 ± 6 Å, respectively) virtually identical to those of the wild type MARK2. The scattering from the mutant can be neatly fitted by the curve computed from the crystallographic model of MARK2 with $\chi = 1.7$ (Fig. 7, curve 3) indicating that the UBA domain remains attached to the N-lobe. Summarizing the SAXS results, the solution structure of the catalytic plus UBA domain of all MARKs is consistent with the crystal structures, and there is thus far no evidence of a major conformational change in response to the state of activation.

**Role of UBA domain in kinase activity**

The structural data show that the UBA domain interacts with the N-lobe of the kinase domain in MARK1 and MARK2. The crystal structures also show the cleft between the N-lobe and C-lobe in a widely open conformation, consistent with an inactive state. These features suggested the possibility that the UBA domain pulls the N-lobe "back" (to the left in Fig. 3), thus keeping it in an inactive state. We therefore wanted to test whether the presence of the UBA domain had an influence on the activation of the kinase. We compared the catalytic plus UBA domain with the catalytic domain alone and with the full-length protein, either before or after treatment with the activating kinase MARKK/TAO-1 which phosphorylates the conserved Thr208 in the activation loop (9). As a readout we used the 13-mer peptide TR1, derived from the sequence of tau protein. It harbors the residue Ser262 which represents the main target of MARK kinases and strongly reduces the affinity of tau to microtubules. The results (Fig. 8) show that unphosphorylated MARK1 constructs with and without UBA domain and full-length MARK1 and MARK2 have similar activities, around 0.01 – 0.05 min$^{-1}$. The basal activity of the MARK2 construct without UBA domain is a little higher (~0.1 min$^{-1}$). Phosphorylation by MARKK increases the activity of the full-length proteins and all constructs, however, the activation levels are different. The activity of the full-length kinases increases to about 0.25 min$^{-1}$. Activation by MARKK is highest for the constructs without UBA domain (0.9 min$^{-1}$ for MARK1, 0.6 min$^{-1}$ for MARK2). For the constructs with UBA domain, the activities are intermediate (0.5 min$^{-1}$, MARK1) or close to that of the full-length kinase (~0.15 min$^{-1}$, MARK2). Compared to the constructs without UBA domain, the activities of the constructs comprising the UBA domain are reduced by a factor of 2 (MARK1) to 4 (MARK2). Thus, the UBA domain has a moderate but significant inhibitory effect on the kinase activity of MARK1 and MARK2 after phosphorylation by the activator kinase MARKK.

**DISCUSSION**

**Effect of amino acid exchanges**

The MARK1 and MARK2 constructs used for crystallization are of the same size, both comprising the catalytic domain and the UBA domain. The isoforms differ by 37 amino acids and a single amino acid insertion in the N-terminal leading sequence (not resolved in the crystal structures). Of the amino acids differing in both isoforms, 19 are clustered in the UBA domain, thus, almost 50% of the UBA residues are different in MARK1 and MARK2. Most of the exchanges in the UBA domain are conservative and directed to the outside. The result is that they affect neither the folding of the UBA domain, nor its interaction with the catalytic domain. The different crystal packing could however be due to the amino acid
exchanges in the UBA domain, considering that UBA-UBA interactions are different for the two isoforms and more important for the MARK1 crystal structure.

The remaining 18 exchanges are scattered across the rest of the molecule, mostly the catalytic loop. These differences lead to significant changes in the conformation of the catalytic domain. It seems that a single exchange of tyrosine to phenylalanine in residue 170 has the greatest impact on the conformational changes of the catalytic domain. Remarkably, residue 170 is different in all four human isoforms (Tyr, Phe, Arg, and Asn in MARK1-4, respectively). In MARK1, Tyr170 induces an unusual conformation of the catalytic loop. The side group of Tyr170 is buried and forms a hydrogen bond with the DFG motif, thus stabilizing the N-terminal anchor of the activation loop. So far, it is unclear whether this amino acid has a significant effect in vivo. It is conceivable that this residue does not fundamentally change functions and mechanisms of MARK, but has a modulating effect, by changing the barriers for activation/inactivation.

**Role of helix G in intermolecular interaction**

The crystal structure of MARK2 is built up from NCS dimers arranged in helical tubes. The MARK2 dimer exhibits approximate two-fold symmetry. The catalytic sites face each other in the center of the dimer. In contrast to MARK2, the MARK1 crystal consists of tetrameric complexes of elongated shape which are interconnected by UBA-UBA contacts. Within each tetramer, two pairs of molecules exist that interact in a similar way as the molecules of the MARK2 dimer. Although these pairs of molecules have lost two-fold symmetry and transformation of the real MARK2 dimer into the form of the MARK1 "dimer" requires substantial distortions, the structural elements participating in intermolecular contacts are mostly the same (Fig. 5 b, c). Helix G and the preceding loop plays the leading part in these interactions.

The crystal structure of the AMP-activated protein kinase Snf1 (30) contains NCS dimers very similar to the MARK2 dimers. In another structure of Snf1 (32), symmetry-equivalent molecules form dimers of exact two-fold symmetry. Interestingly, these dimers are significantly different from the NCS dimers of the other crystal form. Nevertheless, the general organization of the dimers with the active sites in the center is preserved. In particular, helix G plays the most important role in the interactions. Another example of the same type of dimer formation is the gamma subunit of phosphorylase kinase (33), a more distant member of the CAMK group of kinases. The details of the interactions might be different, but in all these cases helix G and the preceding loop form a structural motif that serves as a plug which inserts into the cleft between the N- and the C-lobe of the partner molecule. The positional variability of this motif which is obvious from the comparison of the 8 molecules in the MARK1 crystal structure allows the "helix G plug" to adapt to various geometries. It seems that helix G and the preceding loop at the bottom of the C-loop functions as a flexible docking site that is capable to interact with molecules of the same type and perhaps with other interacting partners.

**Conformation of MARK in solution**

The binding of the UBA domain to the N-lobe of the catalytic domain, and the known role of UBA domains in regulating protein-protein interactions, especially with different forms of poly-ubiquitin (34,35), made it tempting to speculate that the UBA domain could influence the activity of MARK by a conformational rearrangement, for example by detaching from the catalytic domain or changing its position in order to allow interactions with other partner molecules. In addition we were concerned about the possibility that the position of the UBA domain in the crystal structures might be constrained by the packing in the crystal lattice.

However, the speculations about a conformational change of the UBA domain are not supported by the data: the attachment site remains unchanged in different crystal lattices with different contacts between neighbors, arguing that the attachment site is determined by intrinsic properties (hydrophobic surface patches). There is no sign for an UBA rearrangement, even when the molecule is in solution and is not constrained by crystal contacts. In fact the solution structure and the crystal structure are in very good agreement with each other. Furthermore, the dimensions of the molecule remain unchanged even when the kinase activity is turned on by pseudo-phosphorylation of the T-loop (Thr to Glu mutation at residue 208). These observations
exclude any gross conformational changes intrinsic to the catalytic plus UBA complex.

The data reported here are at variance to recent conclusions by Jaleel and coworkers (8). These authors reported SAXS experiments on the catalytic plus UBA domains of MARK2, comprising 345 residues, similar in length to our constructs (326 residues). They reported $R_g$ values of 26.5 Å in the non-activated ground state, and 22.7 Å in the activated state after phosphorylation by the LKB1:STRAD:MO25 kinase complex. The high value of 26.5 Å for the ground state is not compatible with the crystal structures we have observed so far. Furthermore, we do not observe the compaction of the molecule to 22.7 Å after activation, neither in the crystal structures nor by solution scattering. In fact, the "active" structure according to Jaleel and coworkers is in good agreement with our observed crystal and solution scattering dimensions. A possible explanation of this discrepancy could be the influence of interference effects. Indeed, in our SAXS experiments, samples at growing solute concentrations (3-6 mg/ml) systematically yielded elevated $R_g$ values up to 27 Å, pointing to a tendency of MARK proteins to form aggregates at higher concentrations. On the other hand, all the samples at lower concentrations (less than 2 mg/ml) consistently provided lower $R_g$ values, which could be reliably extrapolated to infinite dilution yielding the scattering patterns fully compatible with the compact crystal structures. Finally, we note that the position of the UBA domain in the model of Jaleel and coworkers, is at the bottom of the C-lobe and thus diametrically opposite to the position in the crystal structure.

### Kinase activity vs. UBA domain

We determined the enzymatic activities of different kinase variants to see if the presence of the UBA domain had an influence. The results show that activation by MARKK is possible in either case, whether the UBA domain is present or not. However, in the presence of the UBA domain, the activation is less pronounced, consistent with a moderate inhibitory effect of the UBA domain. Our results are in contrast to those of Jaleel and coworkers (8) who reported that the UBA domain strongly enhanced the activation of MARK2. The reasons for the discrepancy are currently unclear. The constructs used by these authors are similar to ours. They used a different readout parameter (phosphorylation of the AMARA peptide), however, in our hands this does not differ significantly from the results on the TR1 peptide used here. More significant may be the use of the activating kinase complex LKB1:MO25:STRAD rather than MARKK. It is possible that the kinases phosphorylate not only the critical threonine in the T-loop, but other sites in MARK which may modulate the activity and response to the UBA domain. These issues will require more detailed analysis.

### Regulatory functions of the UBA domain

In summary, our solution scattering data are consistent with the structures derived by analysis of both, MARK1 and MARK2 crystals, which are virtually identical in spite of the fact that they are packed in completely different crystal lattices. This suggests that the UBA domain is tightly bound to the N-lobe. In fact, deletion of the UBA domain has a strong impact on solubility and crystallizability of the construct. Thus, it is not surprising that the UBA domain plays an important role for MARK as for MELK (7) and other AMPK-related kinases (8). As the UBA domain binds at the distal side of the catalytic domain and since it is tethered by a straight linker to the C-lobe, we proposed an autoinhibitory function – by enforcing an open, inactive conformation of the catalytic domain – for the UBA domain, analogous to what has been found for Src kinases (36). A similar function in autoinhibition has been proposed for the C-terminal extension of Aurora B (37). However, we found only a moderate effect of the UBA domain after activation by MARKK. How can this be explained?

Tyrosine kinases of the Src family contain SH2 and SH3 domains (Src homology domains) N-terminally to the catalytic domain, which are responsible for targeting the kinase to specific sites containing phosphotyrosines and proline helices, respectively. The SH2 and SH3 domains are also involved in regulation of the kinase activity. Src kinases like c-Src and Hck are regulated by phosphorylation of tyrosine residues in the activation segment (Tyr416 in chicken c-Src) and the C-terminal tail of the catalytic domain (Tyr527 in c-Src, (38,39)). Phosphorylation of the T-loop is activating, while phosphorylation at the tail inhibits kinase activity. The inhibitory effect depends on binding of the SH2 domain to the phosphotyrosine in the tail. This results in a
clamped configuration of the kinase with the SH2 and SH3 domains tightly bound at the distal side of the catalytic domain (Fig. 9). The SH3 domain is firmly attached to the N-lobe (via an intermediate segment linking the SH2 domain to the catalytic domain) in a similar position as the UBA domain in MARK1 and MARK2. Together, the SH2 and SH3 domains form a rigid strut at the rear side of the catalytic domain that prevents breathing movements which are required for substrate phosphorylation. Essential for inhibition is a short segment of the linker between the SH2 and the SH3 domains which acts as a "snap lock" (36). Replacement of this connector by glycine residues or detachment of the SH3 domain by interaction with Nef (a high-affinity ligand for SH3, (40)) leads to activation.

The inhibitory mechanisms described for Src kinases and confirmed by extensive molecular dynamics calculations (36) can serve as an illustration for the hypothetical regulation of MARK by the UBA domain proposed in this and the previous paper (17). In spite of the intriguing possibility that the UBA domain and the UBA linker in MARK function in analogy to the SH3 domain and the SH3-SH2 connector, Fig. 9 also illustrates an important difference between MARK and Src kinases: the SH2 and SH3 domains form a solid structure able to clamp the molecule in the open conformation even in solution, without stabilizing crystal contacts. They serve as lever arms amplifying the torque applied by the connector ("snap lock"). In MARK, on the other hand, the connector (corresponding, by function, to the UBA linker and the CD domain) is closer to the hinge, and the maximum torque it can exert is much lower, probably, than in Src kinases. This is consistent with the kinase activities we observe for our MARK constructs in solution. In the crystal lattice, the open "clamped" conformation is further stabilized by crystal contacts, especially by the contacts leading to the formation of symmetric dimers (in MARK2) or similar pairs of molecules (MARK1). Without these additional interactions, the UBA linker may be too weak to efficiently inhibit kinase activity. Nevertheless, the mechanism of kinase inhibition exemplified by Src kinases could also be relevant for MARK in vivo, where the kinase domain is complemented with other extra-catalytic domains and with upstream or downstream interaction partners. For instance, binding of a regulatory molecule to the UBA linker or to the CD domain could lend enough rigidity to the "connector" to efficiently suppress breathing movements of the kinase and, thus, reduce kinase activity.

In conclusion, the comparison of the presently available MARK structures points to some regions of conformational variability which are likely to be important for the mechanism (catalytic loop, T-loop), regulation (helix C, G-loop), and interaction (helix G plug) of MARK. The most prominent feature, however, is conserved in all MARK crystals structures known so far, as well as in solution: the UBA domain with its atypical fold (helix α3 inverted) is tightly bound to the N-lobe and tethered via an extended linker to the C-lobe of the catalytic domain. This special configuration of the two domains suggests a role of the UBA domain in autoinhibition by restraining internal motions of the catalytic domain that may be required for enzymatic activity. Since the effect of the UBA domain that we observed in vitro was rather weak, the UBA domain alone seems not sufficient for efficient down-regulation, but may be assisted by regulatory binding partners of MARK in vivo.

REFERENCES


**FOOTNOTES**

*We thank the X13 consortium (University of Hamburg and EMBL Outstation Hamburg) for synchrotron beam time, and in particular Dr. M. Perbandt for helpful discussions and Dr. Gunter Stier (EMBL Heidelberg) for the gift of TEV protease expression plasmid.*

1The abbreviations used are: MARK = MAP-microtubule affinity regulating kinase; UBA = ubiquitin-associated domain; NCS = non-crystallographic symmetry

**FIGURE LEGENDS**

**Fig. 1.** Sequence and domain organization of MARK1 and MARK2. a: Sequence comparison of the N-terminal half of MARK1 (M1, Swiss-Prot entry Q9P0L2) and MARK2 (M2, Q7KZI7), including the catalytic domain and the UBA domain. The constructs used for crystal structure analysis in this and the preceding work (17) correspond to the region outlined with thin lines. Within this region, the catalytic domain and the UBA domain are outlined in thick lines. b: Domains of the full-length kinases with residue numbers according to the sequence of MARK1 (above) and MARK2 (below). Throughout the text, MARK2 numbering was used for both isoforms.

**Fig. 2.** Crystal packing. a: Front and side views (by 90° rotation) of tetramers formed by molecules A, B, C, D and E, F, G, H. b: Stacking of tetramers along the c-axis; only half the number of tetramers in the unit cell is shown. c: The same stack of tetramers as in (b), after rotation of 90°, together with a translationally equivalent stack (both in grey) and one representative tetramer of another stack (yellow), illustrating the linkage of the grey tetramers by the remaining tetramers. Note that the linkage relies heavily on UBA-UBA interactions. In all panels, UBA domains are highlighted in red. CA traces in (b) and (c) include structural elements that have been modeled tentatively (see main text and legend to Fig. 3).

**Fig. 3.** Conformational variability of MARK1 in comparison with MARK2. Overlay of CA traces of MARK2 (in red; 1Y8G, molecule A) with the eight molecules of MARK1 (molecule E in green, other molecules in grey and yellow; yellow indicates the amino acids that are different from MARK2); superposition by RMS minimization of deviations in CA positions of core structural elements of (a) the N-lobe (β1 to β3), (b) the C-lobe (helix E and helix F), and (c) the UBA domain (complete). For this overlay, a model of the MARK1 crystal structure is used, which includes some ill defined regions that still could be
traced, at least tentatively (A: res. 204-212, D: res. 211-230, 253-291, G: res. 225-227, 290-291, H: res. 213-227, 252-267; these residues are not considered part of the final model; see text).

**Fig. 4. Helix C and activation segment of MARK1 (chain E) and MARK2 (chain A).** Ribbon diagrams of MARK1 and MARK2 shown in the same orientation after superposition of the N-lobes (calculated by a fit of the CA atoms of helices E and F), (a) MARK1, (b) MARK2, (c) overlay of MARK1 and MARK2. Side groups of helix C and the N-terminal part of the activation segment, as well as the side chain of Lys82 in β3 are drawn as stick model (Lys82 and Glu100 colored by atom type). In MARK1, the catalytic cleft is even more open than in MARK2, and helix C is pushed up and to the right by the activation segment. The distance between atom NZ of Lys82 and OE1 of Glu100 is 5.2 Å in MARK1 (6.6 Å in MARK2). Thus, these residues do not form a hydrogen bond as required for stabilization of ATP in active kinases. By contrast to Cdk2, however, it seems that a hydrogen bond could be formed without substantial reorientation of helix C.

**Fig. 5.** MARK2 dimer compared with MARK1 molecules E and F. a: Overlay of the MARK2 dimer (red) and molecules E and F of MARK2 (green). b: Close-up view of the activation segment (magenta) and the surrounding elements in molecule E of MARK1 (dark colors), with nearby elements of molecule F shown in pale colors. c: As in (b), but with molecule F (dark colors) in the same orientation as molecule E in panel (b); pale colors for elements belonging to molecule E. All superpositions by fit of the C-lobes using helices E and F as reference structures. Similar interactions as between molecules E/F exist between MARK1 molecules A/B, C/D, and G/H.

**Fig. 6.** Effect of the amino acid exchange Tyr170 to Phe170 on the conformation of the catalytic domain. Surroundings of Tyr170/Phe170 at the beginning of the catalytic loop shown as CA traces, with helices C, E, and F indicated by cylinders (MARK1 green, MARK2 red); backbone and side chains are drawn for selected residues in the catalytic loop (including the end of helix E) and in the activation segment (olive, starting with the DFG motif; disordered and invisible in MARK2). In MARK1, the side group of Tyr170 is buried and forms a hydrogen bond with the carbonyl oxygen of Phe194 within the DFG motif, thus stabilizing the N-terminal anchor of the activation segment. In MARK2, the side group of the corresponding Phe170 is exposed. The backbone flip induced by the side chain of Tyr/Phe170 results in an inverse flip of His173 and in a maximum displacement of the backbone by about 6 Å.

**Fig. 7.** SAXS patterns from MARK1 and MARK2 isoforms. Plots (1-3) correspond to MARK1, wild type MARK2 and its T208E mutant, respectively (the latter mimics the phosphorylated active form of MARK2). The experimental data are displayed as dots with error bars, the fits from the crystallographic models computed by CRY SOL (29) are displayed as solid lines. The logarithm of the scattering intensity (I) is plotted as a function of the momentum transfer s; the successive fits are appropriately displaced along the logarithmic axis for better visualization.

**Fig. 8.** Kinase activities of constructs with and without UBA domain compared to full-length MARK1 and MARK2. All constructs have basal activities comparable to the full-length MARKs and can be activated by phosphorylation with MARKK. Compared to the constructs without UBA domain, activation by MARKK is significantly reduced if the UBA domain is present (long constructs and full-length kinases). Activities (expressed in mols of product per minute and mols of kinase) are averages of three independent measurements, error bars indicate s.e.m. values. Proteins were adjusted to the same molar concentrations by comparison with the full-length kinases. The coomassie-stained SDS gel below the bar diagram shows the amount of kinases used for the activity assay. Bands in lane 3 and 6 appear more heavily stained because the full-length proteins are two to three times larger than the kinase domain with or without UBA domain.

**Fig. 9.** Inhibition by extra-catalytic domains in c-Src and MARK. Catalytic domains in blue with activation segments (ASeg) in yellow, hinge regions marked by white circles. All molecules are shown in
the same orientation as determined by least squares fitting of helices E and F of the C-lobes. (a) Restrained (inactive) conformation of human c-Src (PDB-ID: 2SRC, (38)). The SH3 and SH2 domains of c-Src are N-terminal to the catalytic domain. The SH3 domain and the linker between SH2 and catalytic domain bind together to the N-lobe and are shown in the same color (red). The SH2 domain binds to the tail of the catalytic domain via interaction with phosphotyrosine Tyr527. The connector between the SH3 and the SH2 domain ("lock", shown in green) is essential for inhibition of c-Src as it efficiently restricts breathing movements of the catalytic domain (36). (b) MARK, represented by molecule E of the MARK1 crystal structure. (c) Hypothetical active conformation of MARK, with activation segment and orientation of the N-lobe (and UBA domain) relative to the C-lobe modeled by comparison with Aurora-A, active conformation (PDB-ID: 1OL5; (41)). Rotation of the UBA domain in synchrony with the N-lobe is accomplished by unfolding the base of the linker (labeled "CD" in analogy to CD domains of MAP kinases; (42)). According to this model, efficient damping of the catalytic domain's breathing movements requires stabilization of the CD domain by binding of another interaction partner.

Table 1

Data reduction and refinement statistics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁,2,2₁</td>
</tr>
<tr>
<td>Unit cell [Å]</td>
<td>a = 110.7, b = 116.5, c = 285.7</td>
</tr>
<tr>
<td>Data collection</td>
<td></td>
</tr>
<tr>
<td>Resolution limits [Å] overall (last shell)</td>
<td>50 – 2.60 (2.64 – 2.60)</td>
</tr>
<tr>
<td>Number of observations &gt; 1σ</td>
<td>537334</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>112949</td>
</tr>
<tr>
<td>Completeness, overall (last shell)</td>
<td>99.4% (98.5%)</td>
</tr>
<tr>
<td>Rsym, overall (last shell)</td>
<td>8.2% (47.3%)</td>
</tr>
<tr>
<td>(</td>
<td>I</td>
</tr>
<tr>
<td>Refinement</td>
<td></td>
</tr>
<tr>
<td>Resolution limits [Å] overall (last shell)</td>
<td>10 – 2.60 (2.667 – 2.604)</td>
</tr>
<tr>
<td>Number of reflections, working set (test set)</td>
<td>107900 (2786)</td>
</tr>
<tr>
<td>Number of atoms</td>
<td>19579</td>
</tr>
<tr>
<td>R, overall (last shell)</td>
<td>21.2 (28.9)</td>
</tr>
<tr>
<td>Rfree, overall (last shell)</td>
<td>29.0 (41.0)</td>
</tr>
<tr>
<td>Average B Factor [Å²]</td>
<td>48.6</td>
</tr>
</tbody>
</table>
Figure 1
Figure 4
Figure 5

(a) MARK1MARK2

(b) αC αG

(c) αC αG
Figure 6
Figure 8
Figure 9

(a) SH3, linker, lock, and SH2 domains are labeled in the structure of c-Src.

(b) UBA, linker, CD, and ASeg domains are labeled in the inactive MARK structure.

(c) UBA, linker, CD, and ASeg domains are labeled in the active MARK structure.