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Expression, purification from inclusion bodies, and crystal characterization of a transition state analog complex of arginine kinase: A model for studying phosphagen kinases

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Abstract

Phosphagen kinases catalyze the reversible transfer of a phosphoryl group between guanidino phosphate compounds and ADP, thereby regenerating ATP during bursts of cellular activity. Large quantities of highly pure arginine kinase (EC 2.7.3.3), the phosphagen kinase present in arthropods, have been isolated from *E. coli*, into which the cDNA for the horseshoe crab enzyme had been cloned. Purification involves size exclusion and anion exchange chromatographies applied in the denatured and refolded states. The recombinant enzyme has been crystallized as a transition state analog complex. Near complete native diffraction data have been collected to 1.86 Å resolution. Substitution of a recombinant source for a natural one, improvement in the purification, and data collection at *cryo* temperatures have all yielded significant improvements in diffraction.

Keywords: arginine kinase; crystals; expression; guanidino kinase; high resolution; phosphagen kinase; transition state complex

Arginine kinase (AK; EC 2.7.3.3) catalyzes the reversible transfer of a phosphoryl group between arginine and adenosine diphosphate (ADP):

$$\text{Arginine} - \text{P} + \text{MgADP} + \text{H}^+ \overset{\text{Arginine kinase}}{\rightleftharpoons} \text{Arginine} + \text{MgATP}. \quad (1)$$

This reaction and those of the rest of the guanidino (or phosphagen) kinase family, including creatine kinase (CK), enable ATP to be quickly regenerated from storage phosphagens during bursts of cellular activity. In addition to this "temporal" buffering, it has been proposed that these enzymes also function in "spatial" buffering, separating sites of cellular energy production from sites of use by a phosphagen shuttle with the guanidino kinases catalyzing opposite reactions at each end of the shuttle (Tombes & Shapiro, 1985; Wallimann et al., 1992).

AK is the only phosphagen kinase in arthropods as CK is in vertebrates (Morrison, 1973; Watts, 1973). Although the substrates are quite different, the enzymes are thought to share a common mechanism of direct, associative in-line γ-phosphoryl transfer (Hansen & Knowles, 1981). In spite of the diversity of substrates, the guanidino kinases are among the most conserved proteins with the most different sharing ~40% amino acid identity (Babbitt et al., 1986; Dumas & Camonis, 1993; Mühlebach et al., 1994; Suzuki & Furukohri, 1994). Subunits have molecular weights of about 40 kDa. Some arginine kinases are monomers (including that of horseshoe crab), and others are dimers like other invertebrate phosphagen kinases, and like mammalian cytosolic CK. Mammalian mitochondrial CK (Mi_b-CK) is octameric. It is widely believed that arginine kinase is the modern enzyme closest to the primordial form, based on sequence analysis, existence as monomers, and use of a freely available amino acid substrate.

A wealth of mechanistic data has been accumulated over the last 40 years through "classical" enzymology and spectroscopic techniques (reviewed in Kenyon & Reed, 1983). A stable complex can be formed containing creatine (or arginine), Mg^{++}, ADP, and the nitrate ion, which is thought to mimic the (missing) γ-phosphoryl

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Abbreviations: ADP, adenosine diphosphate; AK, arginine kinase; ATP, adenosine triphosphate; cDNA, complementary DNA; CK, creatine kinase; DNA, deoxyribunucleic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HEPES, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethansulfonic acid]); IEF, isoelectric focussing; MES, (2-[N-Morpholino]ethanesulfonic acid); Mi-CK, mitochondrial CK; PAGE; polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PDS, 4,4′-dithiopyridine; PEG, polyethylene glycol; PMSF, phenylmethylsulfonyl fluoride; psi, pounds per square inch; SDS, sodium dodecyl sulfate; TSA, transition state analog; UV, ultraviolet.

in a planar configuration during transfer (Milner-White & Watts, 1971). In the literature, this is referred to as a transition state analog (TSA) complex. When Mg^{++}, ADP and creatine are bound, CK undergoes conformational changes as indicated by EPR (Reed & Cohn, 1972), tryptic susceptibility (Lui & Cunningham, 1966), and X-ray scattering (Forstner et al., 1996). There is conflicting evidence as to whether it is the chemical step that is rate limiting or some other step, perhaps the conformational change (Engelborghs et al., 1975; Rao et al., 1976).

After many reports, over many years, describing crystals diffracting to ~3 Å resolution, the first guanidino kinase structure was reported recently (Fritz-Wolf et al., 1996). The 3 Å structures of apo-Mi–CK with and without bound ATP revealed subunits with a 112-residue α-helical N-terminal domain followed by a 277-residue domain with an eight-stranded antiparallel β-sheet flanked by seven helices. The active site is in the cleft between domains, but its details remain obscure, because of disorder and flexibility in the active site, and because the enzyme has been visualized before the substrate-induced conformational changes (Fritz-Wolf et al., 1996). The work described here is designed to complement the CK results with the development of an experimental system through which it will be possible to study the high resolution structure in its "active" state and perform mutational analysis.

Results

Purification of AK from a natural source (horseshoe crabs) and crystallization attempts

In spite of high yields (seven preparations of ~20 mg), and activities [220–240 (μmol arginine)/min/mg] (Strong & Ellington, 1993), crystallization of apo-AK purified from horseshoe crabs was unsuccessful. Overloaded silver-stained SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) suggested that proteolysis was an explanation with minor contaminant bands at about 36 and 38 kDa that had been shown to be AK (Strong, 1995). However, their intensity was proportional to the time boiled in SDS, i.e., they were due to chemical cleavage during SDS-PAGE. Slow accumulation of precipitate, partially (but not completely) alleviated by addition of β-mercaptoethanol or DTT and 10 mM salt, implicated conformational instability.

Prior formation of the TSA complex had a marked effect upon crystallization. Tiny aggregates were found in a matrix screen (Jancarik & Kim, 1991) with various salts or polyethylene glycol (PEG)/isopropanol/Mg^{++} mixtures. No progress was made with salts, but microcrystals appeared 1–3% below the precipitation points of various PEGs >1000 D: 15% w/v for PEG 6000 to ~30% for PEG 1000. Better crystals came with high purity commercial PEGs (Fluka or Hampton Research Inc.), but not with additional PEG purification (Ray & Puvathingal, 1985; Jurnak, 1986). Crystals were obtained at pH 7.5 and with 10 to 20 mg/mL protein at either 4 or 20 °C. The largest ($0.3 \times 0.1 \times 0.05$ mm^3), obtained by microbatch crystallization (Chayen et al., 1992) with 12% PEG 6000 or 23% PEG 1000, diffracted X-rays to ~$3\frac{1}{2}$ Å, similar to that reported for CK crystals (McPherson, 1973; Schnyder et al., 1990, 1991).

Over-expression and purification of AK

Parallel to the work on AK isolated from the horseshoe crab, AK was expressed in *E. coli* using a cDNA clone prepared as described previously (Strong & Ellington, 1996). Inclusion bodies were harvested, and AK was purified and solubilized. The yields and activities at various stages of the purification are shown in Table 1 and purity was examined with SDS-PAGE in Figure 1. The recombinant product was the purist enzyme with which we had worked. However, high-resolution isoelectric focusing (IEF) revealed two surprises (Fig. 2). First, the pI was shifted slightly consistent with a single non-conservative random E73G mutation that had occurred during PCR amplification (Strong & Ellington, 1996). Second, the recombinant enzyme showed IEF fine structure, albeit different from that isolated from natural sources. Test-

Table 1. *Yields and activities at stages in the purification of recombinant arginine kinase*

Step	Mass AK fraction (mg)	Specific activity[a]
Recovery of inclusion bodies (after French press and centrifugation)	~400	n/a
Washing of inclusion bodies	~300	n/a
Unfolding and filtering	220	n/a
Size exclusion chromatography (Sephacryl S-300)	183	n/a
Refolding	167	170
DEAE-Sepharose anion exchange chromatography	97	205
Size exclusion chromatography (Sephacryl S-100)	58	240

[a]Enzyme units (e.u.)/mg, where 1 e.u. = 1 μmol/min.

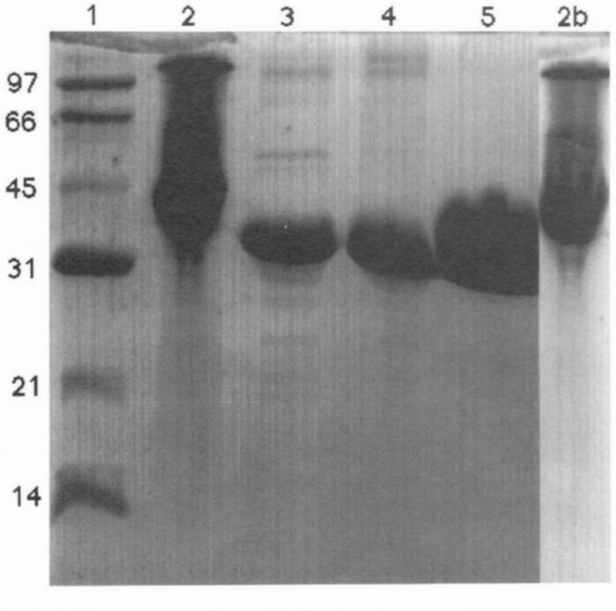

Fig. 1. SDS polyacrylamide gel following the steps of AK purification. The gel is heavily overloaded (to show impurities) and stained with Coomassie blue. Lanes: (1) markers [molecular weights (kDa) are shown on left]; (2) recovered inclusion bodies (repeated on right as lane 2b at lower contrast); (3) washed inclusion bodies; (4) following S300 size exclusion chromatography in denaturing conditions; (5) following S100 size exclusion chromatography in native conditions.

ing the effects of acid and alkaline phosphatases and cysteine reagents, only one of the eight bands could be accounted for, and was attributed to phosphorylation. The other seven fine bands, separated by about 0.05 pH units, are of unknown origin (see Discussion). Apparently, this microheterogeneity can be tolerated in crystallization.

Crystallization of recombinant AK

Crystals of the recombinant protein grown under the microbatch conditions that were optimized for the natural protein, were of similar size and isomorphous to the non-recombinant crystals, but diffracted to 2.6 Å (cf. 3.5 Å). Further optimization and change to the vapor diffusion method led to a ~10-fold increase in crystal volume and some diffraction visible to 1.7 Å (Fig. 3). During the optimization it was found that (1) morphology was improved at 4° cf. 23 °C; (2) that the PEG molecular weight affected the appropriate concentration, but not crystal quality; (3) that substituted PEGs (Brzozowski & Tolley, 1994; Patel et al., 1995), detergents or organic solvents did not improve the crystals; (4) that slight improvements were found (a) through fine-filtering (300 kDa membrane) (Blow et al., 1994) and (b) addition of either 50–200 mM NaCl or $MgCl_2$.

Cryoprotection, crystal characterization, and preliminary data collection

Gradual loss of high-resolution diffraction was avoided with cooling to near liquid nitrogen temperatures, using cryoprotectants to prevent destructive ice formation. Substitution of PEG 400, a common cryoprotectant, for the PEG 6000 crystallizing agent, failed. Serial transfer of crystals through increasing glycerol concentrations (Rodgers, 1994) proved successful, but only in the presence of TSA components. In their absence, crystals dissolved, circumstantially confirming that the crystals were of the TSA complex.

A data set 98% complete to 1.86 Å has been collected with the following statistics: R_{merge} = 4.7%, $I/\sigma(I)$ = 17 (overall), 3.0 (at 1.86 Å), 232,917 observations of 35,102 unique reflections in $P2_12_12_1$. Unit cell dimensions are a = 70.9 Å, b = 80.4 Å, c = 65.4 Å, $\alpha = \beta = \gamma = 90°$. This gives a V_M = 2.24 Å/Da^3 (Matthews, 1968), with one subunit per asymmetric unit.

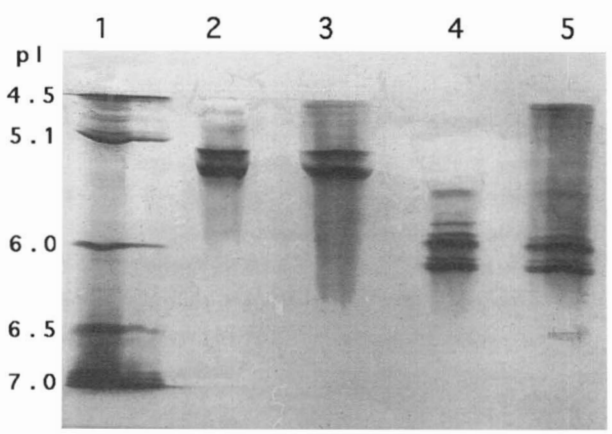

Fig. 2. Isoelectric focusing gel comparing AK isolated from natural sources and recombinant AK. Lanes 1: markers, annotated with pI; 2 and 3: AK purified from horseshoe crab; 3: after treatment with alkaline phosphatase; 4 and 5: recombinant AK—note the different fine structure and the shift consistent with the E73G PCR mutation; 5: after alkaline phosphatase treatment, showing that one of the bands disappears; the remaining fine bands are separated by about 0.05 pH units and are consistent with transamination modifications (see Discussion).

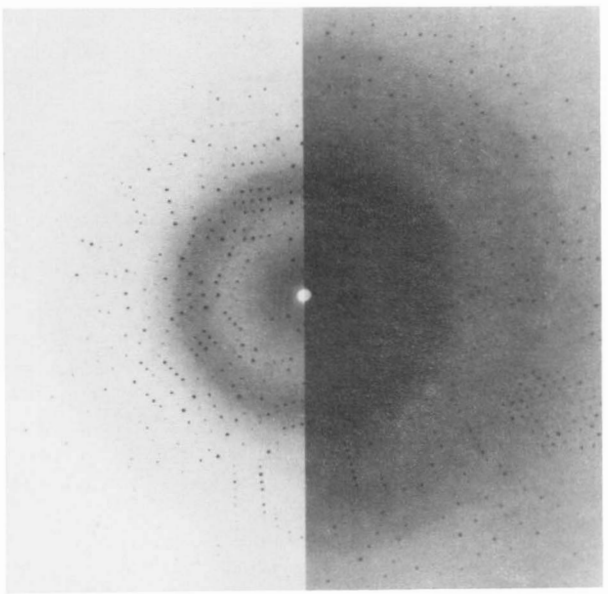

Fig. 3. Diffraction image of recombinant arginine kinase. The image is split with different gray scales: left with high saturation to show the low resolution diffraction; and right with low saturation to show the weaker high resolution reflections. The edge of the background "water-ring" is at ~3.8 Å, and the edge of the image plate is at 1.8 Å resolution. Some reflections can be seen in the corners at up to 1.7 Å. The "Still" image was taken with a 15-min exposure.

Discussion

For the recombinant AK, all steps in the purification give acceptably high yields, except the step immediately following refolding. A 40% loss occurs by either anion exchange or size exclusion chromatographies, whichever follows the refolding. With anion exchange chromatography, the lost protein elutes with the 1 M salt wash, and SDS-PAGE shows that it is AK. Size exclusion chromatography shows two peaks, the first corresponding to large aggregates of presumably mis-folded AK. Attempts to improve the efficiency of refolding by using temperature leaps (Xie & Wetlaufer, 1996) were unsuccessful. The high yield of crude inclusion bodies and the efficiency of the other steps contribute to the success of the protocol in spite of the low efficiency of refolding.

Examination of the recombinant enzyme by high-resolution IEF revealed surprising fine structure (Fig. 2). One band is the product of phosphorylation, but the other seven fine bands that are separated by about 0.05 pH units are of unknown origin. Although N-terminal modification cannot be ruled out, it is likely that the fine structure is similar to that found for other expressed phosphagen kinases (Wood et al., 1995) for which Fourier transform mass spectrometry shows isoforms differing in mass by 1 Dalton, consistent with transamination. The microheterogeneity has not been exhaustively examined, because it does not appear to interfere with the formation of high-resolution crystals.

Improvement of the crystals was a text book case. Although diffraction grade crystals of apo-AK (Berthou et al., 1975), cyto-

solic apo-CK (McPherson, 1973), and apo-Mi-CK (Schnyder et al., 1990, 1991) have previously been obtained from other sources of enzyme, it was necessary to form the TSA complex for horseshoe crab AK. Formation of the complex is likely stabilizing a flexible protein (Lui & Cunningham, 1966; Reed & Cohn, 1972; Forstner et al., 1996) into a single conformation. This was critical for horseshoe crab AK, as it has been with other enzymes (McPherson, 1982). Increase of crystal quality came with the increase of purity and the elimination of the inherent genetic variability of phosphagen kinases (Hershenson et al., 1986), possible with purification from an insoluble, denatured cloned source. Stabilization of the high-resolution diffraction, as with other proteins (Rodgers, 1994), came with cooling crystals to *cryo*-temperatures.

Although no direct evidence can be offered, instability of the crystals in the absence of the TSA components is strong circumstantial evidence that it is the complexed form that is crystallized. Arginine kinase is therefore likely to join a select handful of enzymes (Lolis & Petsko, 1990) for which high-resolution structures are available for a transition state analog complex. Attempts are underway to determine phases by isomorphous replacement, but it is also hoped that it will be possible to determine the structure by the molecular replacement method (Rossmann, 1972) using the recent Mi-CK structure (Fritz-Wolf et al., 1996), when the coordinates become available.

Materials and methods

Crystallization of AK purified from horseshoe crabs

Crystallization was first attempted with apo-AK, purified from *Limulus polyphemus* muscle following Strong and Ellington (1993) using ion exchange and either Cibacron blue Sepharose affinity or hydroxyapatite chromatography. A year-long search for crystallization conditions was unsuccessful, testing general conditions (Jancarik & Kim, 1991) and those similar to ones successful for other phosphagen kinases (McPherson, 1973; Berthou et al., 1975; Burgess et al., 1978; Gilliland et al., 1983; Hershenson et al., 1986; Schnyder et al., 1990, 1991), with and without common additives (McPherson et al., 1986).

The transition state analog (TSA) complex (Milner-White & Watts, 1971) was formed by dialysis against $MgCl_2$ (5 mM), ADP (4 mM), KNO_3 (50 mM), and arginine (20 mM), conditions analogous to those that inhibit CK (Gross et al., 1994). A reducing agent (DTT, 1 mM) and an antibacterial (sodium azide; 0.05%) were added. Protein concentration was measured by Bradford assay (Biorad Inc.), calibrated against apo-AK (which could be measured by UV, in the absence of ADP). Crystallization trials started with hanging drop vapor diffusion (Ducruix & Giegé, 1992) and matrix screens (Jancarik & Kim, 1991). Crude optimization was by: (1) vapor diffusion/incomplete factorial trials (Carter, 1992) for combinations of PEG/isopropanol/Mg^{++}; (2) binary search (Stewart & Khimasia, 1994) and grids using the microbatch method (Chayen et al., 1992) and 1–2 μL drops of concentrate protein (>20 mg/mL) with which buffer (various: pH 6.5–10) and crystallizing agent were mixed.

Over-expression of AK

The complementary DNA (cDNA) for *L. polyphemus* AK had previously been cloned into *E. coli* and sequenced (Strong & Ellington, 1995). Expression from a pET-22b–based vector, p22b-AK17 (Strong & Ellington, 1996) had yielded ~3 mg of soluble, active protein per liter of culture, but this proved difficult to purify in the quantities needed for structural analysis. Here, purification from the majority insoluble component in inclusion bodies is described.

Following induction of expression by standard methods (Strong & Ellington, 1996), the *E. coli* cells were spun down at 6,000 × *g* for 15 min using a Beckman JA10 rotor, and resuspended in 40 mL lysis buffer (50 mM Tris pH 8.0, 7 mM DTT, 1 mM PMSF). The suspension was French pressed at 1500 psi with two passes, and then centrifuged to remove cell debris, using a Beckman JA12 rotor at 10,000 × *g*. The inclusion bodies were washed as follows to remove lipids, DNA, and some of the contaminating proteins: the pellet was alternately (re)suspended in 80 mL of washing buffer "W" (50 mM Tris pH 8.0; 2% Triton X-100; 10 mM EDTA; 1 M urea) and spun down at 5,000 × *g* in a Beckman JA20 rotor [1 M urea is sufficient to denature some proteins, but not AK, as determined by a difference absorption urea titration (Copeland, 1993), which showed a sigmoidal unfolding transition between 2 and 4.5 M, agreeing with data that was subsequently reported by others (France & Grossman, 1996).] On the final, fourth, cycle of washing, AK was resuspended in storage buffer, "S" (10 mM Tris·HCl pH 8.0; 1 mM EDTA; 1 mM DTT; 10 mM KCl, and 0.02% w/v NaN_3). It was then unfolded with a one-hour incubation in 8 M urea (in buffer S) at room temperature. The solution was clarified by centrifugation at 10,000 rpm (8,000 × *g*) for one hour in JA20 rotor, and filtered through 0.22 μm membrane filter. AK was partially purified in the unfolded state by size exclusion chromatography (2.5 × 120 cm Sephacryl S-300 column) in 6 M urea/buffer S. [Crystals of urea sometimes formed with 8 M solutions, and 6 M is above the unfolding transition (see above).] AK was diluted to 0.25 mg/mL and refolded by sequential dialysis against 4 M, 2 M, 0.5 M, and 0 M urea in refolding buffer "R" (50 mM Tris·HCl pH 8.0; 1 mM EDTA; 100 mM β-mercaptoethanol) with four-hour incubations at 4 °C. Unsuccessful attempts were made to increase the yield of refolding by using temperature leaps during incubation (Xie & Wetlaufer, 1996). Purification following refolding was by anion exchange and size exclusion chromatographies. Overall, it makes little difference which is first, but explaining the low yield by mis-folded aggregates (see Discussion) suggests that size exclusion chromatography should be first, the opposite of all but our last preparations. Size exclusion chromatography was with a 2.5 × 120 cm Sephacryl S-100 column, gravity fed, and monitored with an in-line UV monitor. Anion exchange chromatography followed extensive dialysis and concentration by pressure ultrafiltration, and was with a 1.5 × 10 cm DEAE-sepharose CL-6B column, eluting with a 10 to 60 mM KCl gradient in a 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, 1 mM DTT, and 0.02% NaN_3. The product was concentrated by ultrafiltration starting with a large volume pressure system, and then down to ~1 mL by centrifugal filters. The TSA complex (Milner-White & Watts, 1971) was formed by dialysis against 5 mM $MgCl_2$, 4 mM ADP, 50 mM KNO_3, and 20 mM arginine using the membrane from a 10 kDa centrifugal microconcentrator. Activities were measured by the enzyme linked assay (see above). Concentrations prior to TSA complex formation were measured by UV absorption, as above, except that an extinction coefficient of 0.60 mL/cm/mg was assumed for unfolded AK.

Characterization of the expressed AK by IEF

The phosphorylation states of isolated and recombinant AK were examined by IEF following either of the following treatments: (1)

5 mg/mL AK was incubated with 625 units/mL of alkaline phosphatase for two hours at 30 °C in a buffer of 5 mM Tris/HCl pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 10 mM spermidine; (2) 5 mg/mL AK was incubated with 60 units/mL of acid phosphatase for two hours at 30 °C in a buffer of 50 mM MES pH 6.0, 10 mM DTT. Potential cysteine modification was explored by the reaction of free cysteines with 4,4′-dithiopyridine (4-PDS): AK and PDS were mixed to make a 1 mL solution of 0.4 mg/mL AK and 50–100 μM PDS (measured using an extinction coefficient of 16 cm^{-1} mM^{-1} at 248 nm) in a 50 mM phosphate buffer, pH 7.0, and 1 mM EDTA. The oxidized cysteine concentration was measured at 324 nm using an extinction coefficient of 19.8 cm^{-1} mM^{-1}. IEF was run using a Bio-Rad mini apparatus, and ampholites with a pI range of 5.1 to 6.9. Gels were stained with Coomassie.

Optimization of crystallization for recombinant AK

The following were compared in controlled trials: (1) the microbatch (Chayen et al., 1992) and vapor diffusion methods (Ducruix & Giegé, 1992); (2) temperatures of 4° and 23 °C; (3) PEG molecular weights of 3,000 to 12,000 D; (4) Monomethyl ether substituted PEGs (Brzozowski & Tolley, 1994; Patel et al., 1995) and unsubstituted PEGs; (5) salt additives at 50, 100, and 200 mM–NaCl, $MgCl_2$, $Na_3C_6H_5O_7$, Li_2SO_4, $Mg(C_2H_3O_2)_2$ $(NH_4)_2SO_4$, NH_4Cl, $(NH_4)_3C_6H_8O_7$, all adjusted to pH 7.5 with isomolar solutions of the conjugate acid/base; (6) sample filtration—none compared to use of 0.22 μm and 300 kDa membrane filters (Blow et al., 1994); (7) detergent additives (McPherson et al., 1986): up to 0.1% w/v β-octyl glucoside, nonyl-β-glucopyranoside or decyl maltoside; (8) organic co-solvents: 5% ethanol, isopropanol, or hydroxyacetone.

The optimized protocol for crystallization uses a grid of 8 μL hanging drops with starting concentrations of 10 mg/mL AK, 53 mM $MgCl_2$, 2 mM ADP, 25 mM KNO_3, 10 mM arginine, 0.5 mM DTT, 2.5 mM sodium azide, 25 mM HEPES (pH 7.5), and 11, 12, or 13% w/v PEG 6000. The drops are equilibrated against a reservoirs of 15, 16, 17, 18, 19, or 20% PEG 6000 and incubated for 7 to 14 days at 4 °C.

Cryoprotection, crystal characterization, and data collection

Crystals were cooled to 100 K in a nitrogen stream from an Oxford Cryosystems apparatus. Crystals were mounted in rayon loops (0.7–1.0 mm diameter; Hampton Research Inc.) (Rodgers, 1994), which were mounted on the goniostat while the cold stream was deflected. Cryoprotection was optimized by microscopic inspection for clear vitreous ice, and through determination of the crystal mosaic spread by diffraction methods. In the optimized protocol the crystal was transferred every 20–30 min between glycerol solutions, each increasing in concentration by 5%, to a final concentration of 25% w/v. It was essential that the glycerol solutions contained the components of the TSA complex to avoid cracking and dissolution of the crystal.

The diffraction data were collected from a crystal with dimensions of $\sim 1.0 \times 0.3 \times 0.25$ mm^3 using 1.5418 Å X-rays from a Rigaku RU200 HB generator run at 40 kV × 100 mA with a graphite monochromator and a 0.3 mm collimator. Fifteen-minute exposures were collected with the R-Axis II imaging plate system 90 mm from the crystal, and processed with the programs BioTex (Molecular Structure Corp.), XDisplayF (Minor, 1993) and Denzo (Otwinowski, 1990).

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