

# Purification, characterization and crystallization of ERA, an essential GTPase from *Escherichia coli*

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**Abstract** ERA is an essential GTPase widely conserved in bacteria. Homologues of ERA are also present in higher eukaryotic cells. ERA is involved in bacterial cell cycle control at a point preceding cell division. In order to aid the functional investigation of ERA and to facilitate structure-function studies, we have undertaken the X-ray crystallographic analysis of this protein. Here, we report the purification and crystallization procedures and results. The purified ERA exhibits nucleotide-binding activity and GTP-hydrolytic activity. ERA is one of the very few multi-domain GTPases crystallized to date.

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**Key words:** ERA; GTPase; Purification; Crystallization; *Escherichia coli*

## 1. Introduction

ERA is a small GTPase (ca. 35 kDa in *Escherichia coli*) ubiquitously found in bacteria [1] and is essential for bacterial viability [2,3]. ERA homologues are also found in some higher eukaryotic organisms, such as *Antirrhinum* [4], mouse and human [5]. The sequences from polygenetic sources are highly homologous [6,5], e.g. the sequences from human and bacteria share nearly 30% identity and 53% similarity [5]. The sequence of the N-terminal GTPase domain is closely related to those found in Ras proteins [7,8], G $\alpha$  [9] and EF-Tu [10], while the C-terminal sequence is unique. The ability of ERA from other bacterial species to cross-complement ERA lethal mutants in *E. coli* [1,11] suggests common functions of the protein among genetically distant species.

Studies have shown that the expression of ERA in *E. coli* is positively correlated with the cell growth rate [5]. The ERA homologue, ERG, in *Antirrhinum* is most strongly expressed in dividing cells and metabolically active cells [4]. Limiting ERA expression in *E. coli* resulted in cells with two or four

nucleoids arrested in the cell cycle just before cytokinesis [12,13]. Similar cellular effects were observed for a mutant ERA with impaired GTPase activity [12]. Therefore, ERA is believed to play a role in cell cycle control and the coupling of cell growth rate with cell division [5,13]. Morphologically, ERA's action on cell cycle progression appears to be at a point after chromosome partitioning but before cytokinesis. This is supported by genetic studies that reduced ERA activity in *E. coli* suppresses normally lethal mutations in DNA replication and chromosome partitioning, but does not suppress temperature-sensitive lethal mutations in cell division [5]. However, the exact mechanism by which ERA signals growth and division remains unknown.

Recent sequence analysis indicates that ERA contains an RNA-binding KH domain in its C-terminal region (S. Mian, personal communication). Support that ERA may be directly involved in translation comes from the fact that the gene for 16S rRNA dimethyltransferase functions as a multicopy suppressor for a cold-sensitive mutant of ERA [14]. Mutations in either the N- or C-terminal domain cause severe defects in cell growth [11,15].

The lack of structural information of ERA has limited the progress towards unraveling its function. Since ERA is essential for viability in bacteria, including the pathogens, it is an important target for antimicrobial drug design, where structural information is crucial. In this study, we report the purification, characterization and crystallization of ERA from *E. coli*.

## 2. Materials and methods

### 2.1. Expression

Cloning of ERA from *E. coli* W3110 was previously described [16]. To over-express ERA from TAP106 (pCE31), a 5 ml overnight-grown L-broth culture containing 100  $\mu$ g/ml ampicillin was used to inoculate 500 ml of the same medium. The cells were grown with shaking at 200 rpm/min at 32°C until an OD<sub>600</sub> = 0.5 was reached. Then, the cells were transferred to 40°C and grown for another 2 h. The cells were quickly chilled in ice-water and harvested by centrifugation at 10 000  $\times$  g for 10 min. The pellet was washed with TMD buffer containing 20% sucrose before being frozen by dry-ice and stored at -70°C.

### 2.2. Cell lysis

The cell pellet was allowed to thaw on ice for 3 h. A lysis buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 10 mg/ml lysozyme, 1 mM RNase A) was added to the pellet in 3:1 (v/w) ratio. A protease inhibitor cocktail was added to reach a final concentration of 100  $\mu$ g/ml PMSF (Sigma), 40  $\mu$ g/ml Bestatin (Amresco), 10  $\mu$ g/ml E-64 (Amresco) and 1 mM EDTA (Sigma). The bacterial pellet was suspended by a brief sonication, and was incubated on ice for 30 min. The suspension was then sonicated again in ten 30 s pulses in ice-

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water. The lysate was centrifuged at  $15000\times g$  for 20 min and the supernatant containing soluble ERA was collected.

### 2.3. Polyethyleneimine (PEI) and ammonium sulfate (AS) precipitation

The following procedures were carried out at 4°C. 5% PEI solution (Sigma) (pretitrated to pH 8.0) was added drop-wise to the lysate with shaking to a final concentration of 0.35%. After centrifugation at  $15000\times g$  for 10 min, the soluble portion was isolated. Next, saturated AS solution (pretitrated to pH 8.0) was added drop-wise with shaking to the solution. The precipitate formed between 0 to 45% AS was collected.

### 2.4. Chromatographic purification

The chromatographic purification was performed on a BioCad/SPRINT chromatography system (PerSeptive) equipped with a Gilson fraction collector.

**2.4.1. Anion exchange chromatography.** The protein pellet from the AS precipitation was dissolved and diluted in the starting buffer (50 mM Tris-HCl, pH 8.0) and applied through a 50 ml Superloop (Pharmacia) to a MonoQ 16/10 column (Pharmacia). The column was washed with the starting buffer and the sample was eluted with a flat NaCl salt gradient (40–140 mM in 50 ml buffer) at a flow rate of 4 ml/min.

**2.4.2. Hydrophobic interaction chromatography.** The sample was diluted to match the salt concentration of the starting buffer (50 mM Tris-HCl, pH 8.0, 2.5 M NaCl) and loaded to the Superloop. The sample was applied to a phenyl Superose 10/10 column (Pharmacia) and washed with the 1.5 M NaCl before being eluted in a decreasing salt gradient from 1.5 M to zero salt in 12 ml buffer at a flow rate of 1 ml/min.

**2.4.3. Affinity chromatography.** A self-packed GDP-agarose (from Sigma, GDP immobilized onto beaded agarose through an 11-atom spacer to ribose hydroxyls) column (5 mm $\times$ 10 mm) was used. The sample was buffer exchanged to the starting buffer (50 mM Tris-HCl, pH 8.0) using an Amicon concentrator (10k, Amicon) and loaded to the column. A 0–1 M NaCl elution gradient in 50 ml buffer at a flow rate of 3 ml/min was used.

**2.4.4. Gel filtration chromatography.** The sample was concentrated to less than 200  $\mu$ l in the running buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, unless specified otherwise) and injected to a Superdex 75 10/30 column (Pharmacia). The sample was eluted isocratically using the running buffer. The column was calibrated using a SEC molecular weight standard kit from Bio-Rad. The calibration data fit to a straight line with  $r^2 > 0.999$ .

### 2.5. GTPase activity assay

Purified ERA (5  $\mu$ M) was incubated with varying concentrations of cold GTP in TMD buffer containing 100 mM NaCl, 0.5 mg/ml BSA in the presence of 0.43  $\mu$ M (0.4  $\mu$ Ci) [ $\alpha$ - $^{32}$ P]GTP (Amersham) for 30 min at room temperature. Aliquots (1  $\mu$ l) were spotted onto a PEI-cellulose TLC plate (Aldrich) and let run for 2 h in a buffer containing 0.5 M  $\text{KH}_2\text{PO}_4$  and 1 M NaCl. Spots for radioactive GTP and GDP on the plate were visualized and measured off the phosphor screen (after 16 h exposure) using a phosphor imaging system (Storm 820 from Molecular Dynamics). Cold GTP hydrolysis was calculated based on the ratio of hot GDP/GDP+GTP after subtracting spontaneous GTP hydrolysis from a negative control.

### 2.6. Protein N-terminal sequencing

Polypeptides were separated by preparative SDS-PAGE using a self-cast Tris-glycine gel (12%) with 10 mm $\times$ 2 mm wells. After being stained with Coomassie blue, polypeptide bands were transferred to a nylon membrane through standard technique [17]. Bands on the membrane were then cut out and used for N-terminal sequencing as previously described [16].

### 2.7. Crystallization and heavy atom derivative preparation

The hang-drop vapor diffusion technique was used. Initial crystallization screen as well as additive and detergent screens were performed using screen kits from Hampton Research. Heavy atom compounds used to prepare derivatives were from the following sources: ethylmercury phosphate, Noah Technologies; 2-amino phenylmercuric acetate, ICN Biochemicals; uranyl acetate, Aldrich. X-ray diffraction data were collected with a MarResearch imaging plate system

with mirrors using Cu K $\alpha$  radiation from a Rigaku rotating anode operated at 50 kV and 100 mA. Data collections were done at 100 K maintained by an Oxford Cryosystems. Diffraction data were processed with HKL2000 [18].

### 2.8. Other procedures

For mass spectroscopy purified ERA was analyzed using Hewlett-Packard 1100 series LC/MSD under the electrospray mode. ERA concentration was determined using Bradford [19] and BCA [20] methods with reagent kits from Pierce. Unless specified otherwise, all the SDS-PAGE analyses were carried out using precast tris-glycine gels from Novex. Pre-stained molecular weight marker and the silver-staining kit were also from Novex.

## 3. Results

### 3.1. Protein expression and purification

*E. coli* ERA was expressed under  $\lambda$  P<sub>L</sub> promoter with temperature induction. After a 2 h induction, the expressed ERA comprised  $\sim$ 30% of the total protein based on densitometry scan of the SDS-PAGE gel. When induced at 40°C, more than half of the expressed ERA was soluble (data not shown). Although the expression level of ERA in wildtype cells is closely related to growth rate [5], over-expression of ERA does not affect the cell growth.

The isolation of *E. coli* ERA from cells was confounded by serious protease degradation. Without protease protection, ERA was rapidly degraded in the cell lysate. Even after PEI and AS precipitation (see below), protease activity was still significant. Once degradation has occurred, the ERA fragments were difficult to separate by our chromatographic methods. PMSF alone or commercial protease inhibitor cocktails failed to eliminate degradation completely. To determine the protease cleavage sites, a preparative SDS-PAGE was performed using partially purified ERA (AS precipitation only) from unprotected cell lysate. As shown in Fig. 1A, two major degradation bands (D1 and D2) were observed. The larger band (D1) has a molecular weight of  $\sim$ 30 kDa. N-terminal sequencing of the two degradation products from the gel (Fig. 1A) revealed that both bands are missing the N-terminal 38 amino acids (cleavage between R38 and K39). If the N-termi-

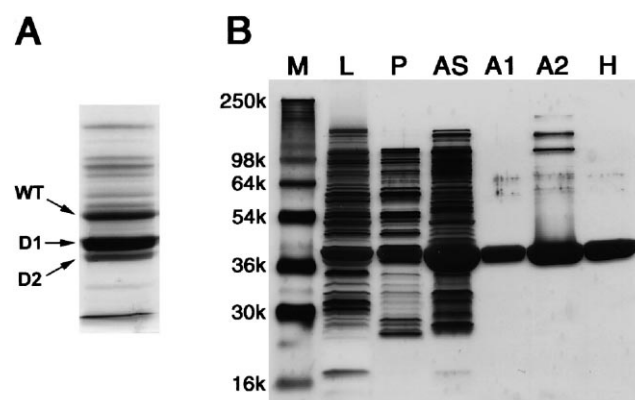


Fig. 1. A: SDS-PAGE (12% Tris-Gly) with Coomassie staining of ERA degradation products using an unprotected sample after the AS precipitation (see Section 2). The band labels are: WT, wild-type; D1, degradation band 1 (major); D2, degradation band 2 (minor). B: SDS-PAGE (12% Tris-Gly) with silver staining of ERA samples. Lane coding is: M, molecular weight marker; L, crude lysate; P, supernatant after PEI precipitation; AS, pellet after AS precipitation; A1 and A2, peaks A and B from AEC (Fig. 2A), respectively; H, after HIC purification.

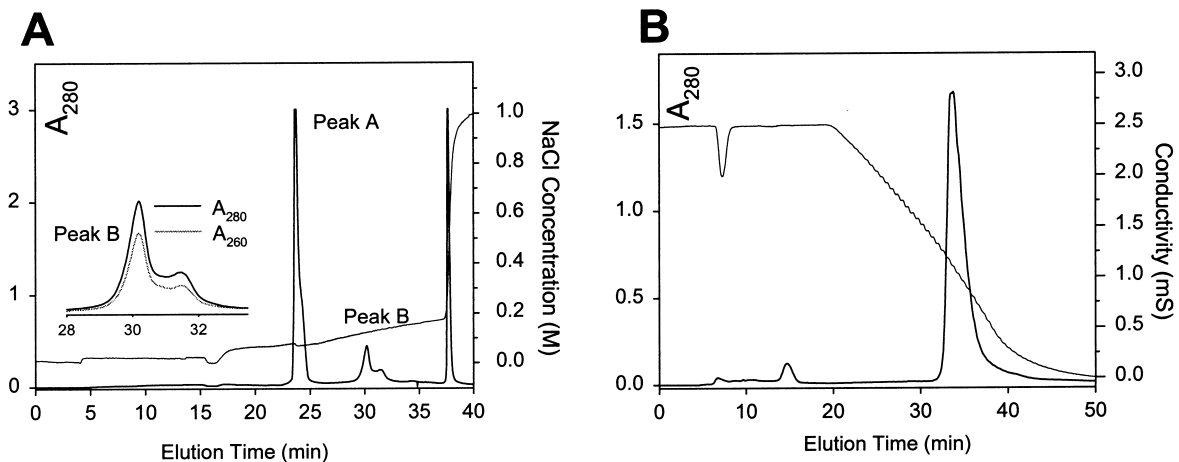


Fig. 2. A: Anion exchange chromatography of ERA. Both peak A and peak B are ERA (see text). Nucleic acid (last peak) is eluted at high salt. The inset shows an enlargement of peak B to show the relative scales of the  $A_{280}$  and  $A_{260}$ . B: Hydrophobic interaction chromatography of ERA. In both graphs, the salt gradient is indicated by the thin line with the scale on the right vertical axis.

nal cleavage is the only part missing in band D1, it would have a calculated molecular weight of 29 718 Da, very close to 30 kDa, therefore, the C-terminus of band D1 is probably intact, whereas the minor band D2 has been subjected to additional C-terminal degradation. N-terminal sequencing of the wildtype band WT indicated that only the N-terminal methionine was removed. Mass spectroscopy of purified ERA showed a molecular weight of  $33\,682 \pm 5$  Da, consistent with the calculated molecular weight of 33 678 Da. A protease inhibitor cocktail including PMSF, Bestatin, E-64 and EDTA was designed to inhibit a wide spectrum of proteases with each inhibitor prepared to achieve its effective concentration [21] (see Section 2). This cocktail proved to be effective in inhibiting protease activity to an undetectable level by the SDS-PAGE silver staining.

Only soluble ERA in the crude lysate was used for further purification; the pellet fraction was discarded. ERA was fractionated from the crude lysate by AS precipitation (0–45% saturation). The ERA recovered was associated with significant amounts of nucleic acid, which greatly interfered with later anion exchange chromatography (AEC). Prior precipitation of the lysate by PEI was able to remove most of these nucleic acids. A final concentration of 0.35% (v/v) PEI applied to the crude lysate largely removed nucleic acid without precipitating ERA. A sequential PEI separation and AS precipitation of ERA was used to prepare the sample for further chromatographic purification.

Systematic purification of ERA using combinations of chromatographic methods, including anionic exchange, size exclusion, affinity, hydrophobic interaction and cationic exchange chromatographies, was investigated. A combination of anionic exchange and hydrophobic interaction chromatographies turned out to be efficient and convenient.

The ERA pellet from sequential PEI and AS treatment was dissolved in a large volume of buffer A for AEC and the sample was loaded onto a MonoQ column (Pharmacia) without dialysis. A very shallow NaCl salt gradient separated the main ERA peak (peak A in Fig. 2A) from smaller ERA peaks (peak B in Fig. 2A). The main peak eluting at  $\sim 100$  mM NaCl is much purer than the ERA peaks eluting at higher NaCl concentrations as shown from SDS-PAGE in Fig. 1B. Peak B also contains relatively higher amount of nucleic acid

as indicated by an  $A_{280}/A_{260}$  ratio of 1.26 (Fig. 2A, inset), as compared to 1.80 of peak A. Peak B also appears to have more associated impurity proteins. Nevertheless, ERA samples from peaks A and B exhibit exactly the same molecular weight from mass spectroscopy. The fractions collected from AEC were diluted into high salt loading buffer for hydrophobic interaction chromatography (HIC) and loaded onto a phenyl-Superose column (Pharmacia) without dialysis. ERA eluted as a single peak (Fig. 2B) with a higher  $A_{280}/A_{260}$  ratio of 2.16, and was essentially pure as judged by SDS-PAGE silver staining (Fig. 1B). With the described purification procedures, 1 liter of cell culture typically yielded 15 mg of pure ERA.

### 3.2. GDP binding of ERA on affinity column

The binding of the purified ERA to GDP was shown by the retention of ERA in a GDP-agarose column. AEC-purified ERA was loaded onto the affinity column and eluted with a NaCl gradient from 0 to 1 M. Some ERA did not bind and came through as peak A in Fig. 3A, while the majority of the sample ( $\sim 73\%$ ) bound to the GDP-agarose gel and eluted as

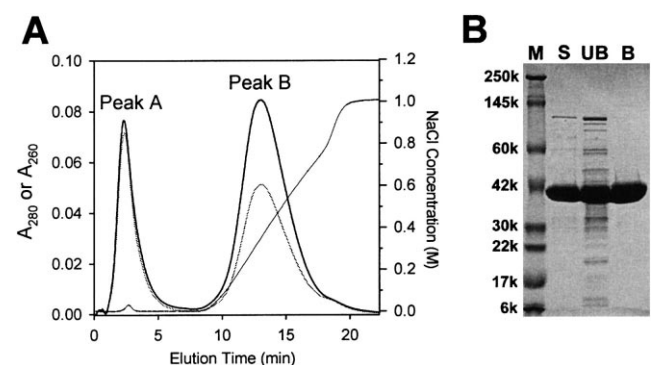


Fig. 3. A: Affinity chromatography of ERA by GDP-agarose column. The salt gradient is indicated by the thin line, while the  $A_{280}$  and  $A_{260}$  are shown in thick black and gray lines, respectively. B: SDS-PAGE (Coomassie staining) of ERA before and after the purification shown in A. Lane coding is: M, molecular weight marker; S, sample applied to the column; UB, unbound fractions (peak A); B, bound fractions (peak B).

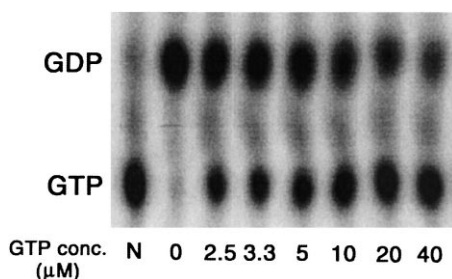


Fig. 4. Hydrolysis of GTP to GDP by the purified ERA. The TLC separation of  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  and  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$  is shown. The initial cold GTP concentration for each reaction is indicated at the bottom. A negative control (N) without ERA is shown in the first lane.

a large peak at  $\sim 0.05\text{--}0.6\text{ M NaCl}$  (peak B). ERA from peak A, probably already GDP-bound, showed a low  $A_{280}/A_{260}$  ratio of 1.07, and was not able to be retained by the affinity column, while peak B is presumably a GDP-free form of ERA. Peak A also contains more impurities than peak B (Fig. 3B). The relatively large fraction of the putative GDP-bound form (peak A) may comprise endogenous ERA-GDP complex as well as complex formed from the association of ERA with leached GDP from the GDP-agarose gel during loading.

### 3.3. The purified ERA is GTPase-active

The GTPase activity of the purified ERA was demonstrated by the hydrolysis of  $[\alpha\text{-}^{32}\text{P}]\text{GTP}$  to  $[\alpha\text{-}^{32}\text{P}]\text{GDP}$ . GTP and GDP were separated by TLC and corresponding spots were measured off the phosphor imaging plate (Fig. 4). The apparent turnover rate ( $k_{\text{cat}}$ ) of ERA GTPase activity is estimated to be  $>0.006\text{ min}^{-1}$  from the last lane in Fig. 4, where the substrate concentration ( $40\text{ }\mu\text{M}$ ) is much larger than that of ERA ( $8\text{ }\mu\text{M}$ ). This value is comparable to that reported previously [16]. The  $k_{\text{cat}}$  value is probably close to those seen in Ras proteins [22] and less than that of  $G_{\alpha s}$  of heterotrimeric G-proteins [23], which are known to exhibit an internal GTPase-activating (GAP) activity [23]. The low  $k_{\text{cat}}$  value of ERA may be an intrinsic necessity for this protein to maintain GTP-bound as Ras proteins.

### 3.4. The purified ERA is partially GDP-bound

Freshly purified ERA showed a single peak at  $\sim 35\text{ kDa}$  from size exclusion chromatography (SEC) (Fig. 5, line a). Aged samples (stored at  $-20^\circ\text{C}$  for 4 months) showed a small peak with high  $A_{260}$  absorbance at a low molecular weight position in SEC (Fig. 5, line b), which coincides with the elution time of a GDP control (line c), indicating that GDP was released from the aged ERA. The difference in the elution times of GDP (line c) and GTP (line d) controls was well resolved by the SEC. Since there was no GDP or GTP added during the preparation, the released GDP appears to be endogenous. Larger amount of nucleotide or nucleic acid could be released by increasing the salt concentration in the buffer to  $1\text{ M Li}_2\text{SO}_4$  (line e) (the chromatography deviated from the conventional SEC mechanism by showing elongated retention times due to the high salt concentration). Sulfate ion competes with phosphate groups of nucleotides for binding to proteins and can replace a bound nucleotide (unpublished results). The ERA-GDP form of ERA present in the crude cell lysate appears to become unligated gradually during AEC and HIC, as

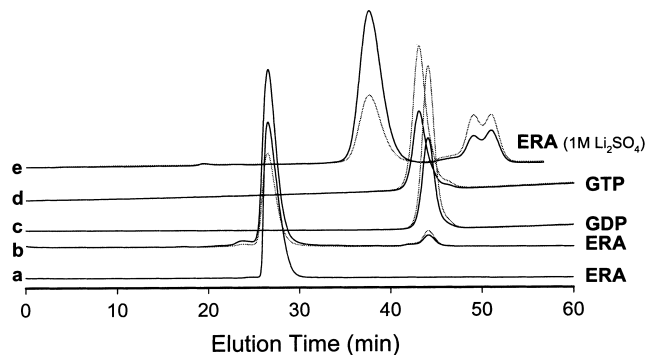


Fig. 5. SEC chromatograms of ERA and guanine nucleotide under different conditions: a, ERA freshly purified; b, purified ERA after storage at  $-20^\circ\text{C}$  for 4 months; c and d, standard GDP and GTP; e, ERA after treatment with  $1\text{ M Li}_2\text{SO}_4$ . The typical SEC buffer used for a–d contains  $0.15\text{ M NaCl}$ , while in e,  $\text{NaCl}$  was replaced with  $1\text{ M Li}_2\text{SO}_4$ . The  $A_{280}$  absorbance is shown in dark lines and the  $A_{260}$  absorbance where shown is in gray lines.

indicated by the increasing  $A_{280}/A_{260}$  ratio for the ERA peaks during the purification.

### 3.5. Crystallization of ERA

After ERA was purified by AEC, it was crystallized using condition no. 16 ( $0.1\text{ M}$  sodium HEPES, pH 7.5,  $1.5\text{ M}$  lithium sulfate) of the Crystal Screen I from Hampton Research. Crystals grew in about 10 days from the 1:1 mixture of concentrated protein ( $8\text{ mg/ml}$ ) and the screening solution. However, the crystals were clusters of microcrystals, unable to be used for X-ray diffraction. Varying the protein concentration or the precipitant concentration had little effect on the crystal morphology. Single crystals were not obtained from additive and detergent screens. Further purification of the sample with HIC and affinity chromatography improved the morphology of the crystal slightly but still not suitable for diffraction data collection. Single crystals were eventually obtained by combining AEC with HIC and the addition of  $0.8\text{ M NaCl}$  to the crystallization mixture (Fig. 6). The typical starting equilibrium contained  $0.1\text{ M}$  Tris-HCl, pH 8.0,  $0.8\text{ M}$  lithium sulfate,  $0.8\text{ M}$  sodium chloride and  $8\text{ mg/ml}$  ERA. The high  $\text{NaCl}$  concentration may help to eliminate non-specific ionic interactions in the crystal packing. Single crystals were grown to near millimeter size using the micro-seeding technique. However, irrespective of the crystal size, they diffracted to at best  $3.2\text{ }\text{\AA}$  resolution. The crystals, in space group  $P2_1$  with cell

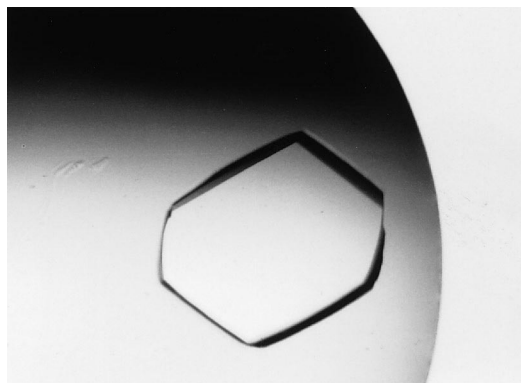


Fig. 6. The monoclinic crystal of ERA.

Table 1  
Summary of heavy atom derivatives of ERA crystal

Heavy atom compound	Soaking concentration	Soaking time	Sites	Diffraction resolution (Å)
EMP (Tris)	saturate	24 h	4	3.5
EMP (HEPES)	1/100 satur.	30 s	8	2.95
APMA	saturate	24 h	4	4.0
UA	10 mM	1 week	2	3.2

EMP, ethyl mercury phosphate; APMA, 4-aminophenylmercuric acetate; UA, uranyl acetate.

dimensions of  $a = 86.8$  Å,  $b = 67.6$  Å,  $c = 87.3$  Å and  $\beta = 115.8^\circ$ , contains 65% solvent ( $V_m = 3.5$  Å<sup>3</sup>/Da, assuming two molecules in the asymmetric unit). Diffraction of the crystal was greatly improved to  $\sim 2.4$  Å by an overnight solvent exchange with a solution containing 30% sucrose (0.1 M Tris-HCl, pH 8.0, 1.2 M, Li<sub>2</sub>SO<sub>4</sub>, 0.5 M NaCl, 30% sucrose). Complete diffraction data were collected at 100 K to 2.4 Å resolution with an  $R_{\text{sym}}$  of 0.056. Cocrystallizations of ERA with GDP or non-hydrolyzable GTP analogues (in concentrations of 1–10 mM) were also attempted. The crystals from cocrystallizations showed no difference in morphology and cell constants from the apo crystals.

### 3.6. Heavy atom derivative preparations

To facilitate the structural determination of ERA using multiple isomorphous replacement (MIR) method, potential heavy atom derivatives of the crystal were screened. ERA crystals were soaked in a protection solution (0.1 M Tris-HCl, pH 8.0, 1.5 M lithium sulfate, 0.8 M sodium chloride, 25% sucrose) containing varying concentrations of heavy atom compounds (Table 1). Most heavy atom compounds have very limited solubility ( $< 0.1$  mM) in the protection solution. Ethyl mercury phosphate (EMP) in either the Tris buffer or HEPES buffer resulted in very good Hg derivative. 4-Aminophenylmercuric acetate and uranyl acetate also provided reasonable derivatives. Complete data sets of the derivatives have been collected.

## 4. Discussion

Since ERA is an essential protein for every bacterium tested to date, no null mutations can be isolated, making the study of its function difficult. Structural studies of ERA are expected to provide insight into its function, particularly the structure of the unique C-terminal domain, for which no global sequence similarity has been identified.

In this study we successfully purified *E. coli* ERA in large quantity to near homogeneity. More purification steps were introduced compared to that reported previously [16], and this facilitated the crystallization. The purified ERA is active in hydrolyzing GTP to GDP with a specific activity comparable to previous results [16]. The purified ERA also has GDP affinity and is partially GDP-bound. However, under the crystallization condition, ERA is probably nucleotide-free, due to the presence of high concentrations of sulfate ion and NaCl. Sulfate ion may compete effectively with the phosphate groups of GDP or GTP for binding to the protein. So far, no sulfate-free crystallization conditions have been found despite extensive efforts.

ERA has recently been suspected to be an RNA-binding protein based on the presence of an RNA-binding KH domain in its C-terminal region (S. Mian, personal communication). This is supported by the observation that ERA is asso-

ciated with substantial amounts of nucleic acid after ammonium sulfate precipitation if PEI precipitation is not performed. The associated nucleic acid is degraded by RNase A but not DNase I (data not shown). The addition of RNase A during cell lysis helped to lower the nucleic acid contamination in AEC. The AEC shown here resolved the main peak of ERA from the second peak much better than the previous study [16]. We imagine that during purification from crude extracts some ERA remain bound to RNA, especially if RNase is not added. Both RNA-bound and RNA-free ERA bind to the anionic exchange column with the RNA tightly bound and eluted at very high salt ( $\sim 1$  M). During the salt gradient elution, free ERA elutes first in the main peak and RNA-bound ERA elutes at higher salt concentrations leaving most of the column-bound RNA behind. Thus, two peaks of ERA were detected, which have exactly the same molecular weight from mass spectroscopy and were indistinguishable in subsequent analyses. We have always used the first/major peak in all studies.

*E. coli* ERA is highly susceptible to protease degradation. The major cleavage site, according to sequence alignment with p21<sup>Ras</sup>, is around the switch I region of the GTPase domain. The cleavage totally eliminates the G1 region, and presumably renders the degradation product unable to bind guanine nucleotides. Small amounts of degraded ERA are usually undetectable by Coomassie-stained SDS-PAGE (data not shown), and therefore special precautions should be taken by applying protease inhibitors during all the purification.

A GTP hydrolysis-coupled conformational switch plays a wide variety of roles in biological systems. However, there are very limited number of GTPases in bacteria that have been structurally characterized. The structural solution of ERA is expected to add a new member to this short list and in addition, provide new insight into the functions of this widely conserved cell cycle regulator.

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