Expression, Purification, Crystallization, and Preliminary X-ray Analysis of Casein Kinase-1 from Schizosaccharomyces pombe*

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The catalytic domain of Schizosaccharomyces pombe casein kinase-1 (the product of the ckil gene) has been overexpressed in Escherichia coli, purified by chromatographic methods, characterized in vitro, and crystallized in the presence and absence of nucleotide substrate. The best crystals belong to the trigonal space group P3\(_2\)1 or its enantiomorph, have unit cell parameters \(a = b = 79\), \(c = 121\), and diffract x-rays to 2.0-A resolution. Kinetic characterization of the purified catalytic domain and other C-terminal deletion mutants of Ckil suggests that it is subject to two forms of regulation. One mechanism involves autophosphorylation, and results in a 4-fold decrease in the affinity for protein substrate. In contrast, truncation of intact Ckil results in a 3-fold activation in its catalytic rate. This activation may arise from the removal of an inhibitory domain present in the intact enzyme.

Casein kinase-1 (CK1)\(^1\) is a ubiquitous eukaryotic protein kinase active in the regulation of DNA repair pathways and cell morphology (Pinna, 1990; Tuazon and Traugh, 1991; DeMaggio et al., 1992; Robinson et al., 1993). Once considered a single entity, it is now known to consist of subspecies that together comprise a distinct branch of the eukaryotic protein kinase family (Rowles et al., 1991; Wang et al., 1992; Robinson et al., 1992). Family members identified to date consist of a highly conserved, \(\approx 290\) residue N-terminal catalytic domain, joined to a C-terminal region that is not conserved between family members and that varies in size from \(40\) to \(180\) amino acids.

The CK1 catalytic domain differs from that of most other protein kinases both structurally and enzymologically. First, it contains neither the peptide triplet Ala-Pro-Glu in subdomain VIII nor the Arg residue in subdomain XI that, in other protein kinases, interact to form a salt bridge (Knighton et al., 1991a; De Bondt et al., 1993; Hanks and Quinn, 1991). The conservation of this feature throughout the CK1 family suggests it contributes to an intrinsic biochemical property of CK1 family members, such as their ability to interact with substrates. Second, CK1 is unusual in that its substrate selectivity appears to be directed toward phosphate groups rather than unmodified amino acids. Although CK1 can phosphorylate synthetic peptide substrates containing glutamic or aspartic acids at positions \(-3\) or \(-4\) (relative to the position of a phosphorylatable Ser or Thr residue), substitution of those carboxylic acid residues with phosphoserine yields a dramatically superior substrate (Flotow et al., 1990; Meggio et al., 1991; Perich et al., 1992; Umpress et al., 1992). How CK1 recognizes phosphopeptides is unclear and may differ substantially from the well characterized interaction between the cAMP-dependent protein kinase and its inhibitor protein (Knighton et al., 1991b). Finally, CK1 catalytic domains interact selectively with two classes of inhibitors: the ribofuranosyl benzimidazoles (Meggio et al., 1990; Meggio et al., 1991) and the isoquinoline sulfonamides (Chijwa et al., 1989). Although both classes are competitive inhibitors of nucleotide substrate, the structural basis of their selectivity for CK1 over other protein kinases is not clear.

In contrast to the catalytic domain, little is known about the function of the C-terminal regions of CK1 homologs. Because they vary greatly in length and amino acid composition, these regions may promote differential subcellular localization of each isoform or direct their interaction with specific regulatory molecules (Wang et al., 1992).

To learn more about the unusual structural features of CK1, and the biological function of its individual isoforms, we turned to the lower eukaryote Schizosaccharomyces pombe to develop a system in which we could combine genetic and enzymological approaches. This organism contains four CK1 homologs encoded by \(c_{ki1}^{\ast}\), \(c_{ki2}^{\ast}\), \(h_{p1}^{\ast}\), and \(h_{p2}^{\ast}\). We have demonstrated that Ckil, the largest fission yeast CK1 homolog at 446 amino acids, can serve as a representative member of the CK1 family in that it retains the enzymological features of CK1, including substrate and inhibitor selectivities.\(^3\) We plan to establish the structural basis for this unusual ligand selectivity by determining the three-dimensional structure of Cki1. Toward that end, we define here the catalytic core of recombinant Ckil through C-terminal truncation analysis, and describe its crystallization in the presence and absence of nucleotide substrate.

**EXPERIMENTAL PROCEDURES**

**Materials**—Casein (5% solution; partially hydrolyzed and dephosphorylated) for protein kinase assays and calibration proteins for electrophoresis and chromotography were from Sigma. Ni\(^{2+}\)-nitrotri-acetate-agarose was from Qiagen (Chatsworth, CA). The PET-18b expression vector was from Novagen (Madison, WI). Polyethylene glycol 4000 and 8000 for crystallization were from Fluka.

Cki1 Mutagenesis—A \(c_{ki1}\) cDNA that was modified by polymerase chain reaction (to introduce useful restriction sites) and isolated in phagemid vector pt7B (Carmel and Kuret, 1992) was prepared for mutagenesis by the method of Kunkel et al. (1987) as described previously (Kuret et al., 1988). All C-terminal deletions of the \(c_{ki1}\) coding

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\(\text{1}\) The abbreviations used are: CK1, casein kinase-1; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.


\(\text{3}\) N. Dhillon and M. Hoekstra, unpublished data.
sequence were prepared by loopout mutagenesis (Eghedatarzadeh and Henikoff, 1986) and were confirmed by DNA sequence analysis.

Escherichia coli Expression—The cDNAs for wild-type Cki as well as deletion mutants cki1A390 and cki1A348 were isolated as NdeI/BamHI fragments and ligated into the NdeI/BamHI sites of expression vector pET3b. Unlike pT7B, this derivative of the T7 expression system (Studier et al., 1990) drives the overproduction of proteins fused to an N-terminal, 20-residue peptide (Met-Gly-Ser-Ser-His-His-His-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-) that allows affinity purification on immobilized nickel columns (Hochuli et al., 1987). The final constructs (pET-15b/cki1, pET-15b/cki1A390, pET-15b/cki1A348, and pT7/cki1A298) were transformed into BL21(DE3) cells to create the strains used for Cki1 overproduction.

BL21(DE3) cells harboring any of the pET-15b- or pT7B-based plasmids described above were grown in Luria broth medium containing ampicillin (200 mg/ml) at 37 °C to an A600 of 1, at which point isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM. After 3 h of induction, cells were harvested by centrifugation (20 min at 3000 x g, 4 °C), washed with STE (10 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA), and stored at −70 °C until used. A 3-liter growth typically yields 8 g (wet weight) of cells.

Purification of Polyhistidine-tagged Cki1 Mutants—All steps were carried out at 4 °C. Frozen cells were thawed, resuspended in 5 volumes of lysis buffer (20 mM Tris, pH 7.5, 0.5% Brij 35, 50 mM imidazole, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.02% Brij 35), and ruptured by two passes through a French press operated at 1000 p.s.i. The resultant sonicate was centrifuged (10 min final spin) to make 0.1% Brij 35, and centrifuged (100,000 x g x 1 h) to yield a clear supernatant (crude extract).

Following filtration through a 0.45-μm filter, the extract was loaded directly onto a 4-mL Ni²⁺-nitrilotriacetate-agarose column pre-equilibrated in lysis buffer containing 0.1% Brij 35. The column was washed with 200 ml (50 bed volumes) of lysis buffer, and developed with sequential 20-ml steps of lysis buffer containing 0.05% Brij 35 and 10, 20, and 40 mM imidazole. Fractions containing casein kinase activity (eluting at 40 mM imidazole) were pooled, brought 75% saturation with solid (NH₄)₂SO₄, and then centrifuged (10 min final spin) to make 0.1% Brij 35, and centrifuged (100,000 x g x 1 h) to yield a clear supernatant (crude extract).

Analytical Methods—The concentrations of purified Cki1 preparations were quantified spectrophotometrically using absorbance coefficients calculated from amino acid content (Perkins, 1986).

Casein kinase activity was assayed as described previously (Vancura et al., 1993). The standard reaction (40 μl) contained 25 mM MOPS, pH 6.5, 50 mM NaCl, 15 mM MgCl₂, 2 mM mg casein, 2 mM EGTA, and 10 μM [γ-³²P]ATP (100–400 cpm/pmol). Initial velocity measurements were carried out in duplicate under these conditions with caseins (0.6, 1.0, 2.0, and 4.0 mg/ml) as the varied substrate, Kcat, Vmax, and their standard errors were calculated as described by Wilkinson (1981).

Results and Discussion

C-terminal Deletions of Cki1—On the basis of its primary structure and subcellular location, Cki1 is the fission yeast homolog of the YCK gene products from Saccharomyces cerevisiae. Its structural organization is shown schematically in Fig. IA. Like Yck1p and Yck2p, it consists of a typical Cki1 catalytic core (residues 8–296) followed by a 12-residue segment (residues 299–310) that is conserved among Yckp and Cki proteins but not other forms of Cki1. This in turn is connected to a putative C-terminal prenylation site (residues 396–439) via a hydrophilic, 85-residue segment that is rich in Pro and Ser residues and that is predicted to contain the most flexible region of the molecule. We have referred to this region as the hydrophilic tether, because it links the catalytic domain to the C-terminal localization signal (Wang et al., 1992). Like all forms of Cki1 isolated to date, Cki1 is constitutively active in vitro.

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Mass Spectrometry—Positive ion electrospray mass spectrometry was performed on a triple quadrupole instrument (Finnigan MAT TQS-700). Purified protein samples were precipitated with 6% trichloroacetic acid, washed twice with neat acetone, dried under vacuum, and stored at −20 °C until they were analyzed. Dried samples were dissolved in Buffer D (50% methanol, 5% acetic acid, ~1% hexafluoroisopropanol), brought to an 10-μl final volume, and applied to a MALDI matrix (1 μl/min) with a Harvard syringe pump. Data from over the mass-to-charge range 400–2000 (16 x 5-s scans) was used to calculate mass and its standard deviation. Spectra were calibrated with equine myoglobin (18,551.5 Da; Beavis and Chait (1990)) and deconvoluted as described by Mann et al. (1991).

Crystallography—Crystallization conditions were sought for 10 mg/ml Cki1A298 (peak I) in the presence and absence of 6 mM ATP, 1.5 mM MgCl₂ at 16 °C using the sparse matrix screening method of Janzen and Kim (1991). Hanging drops (10 μl) containing equal volumes of protein or protein/nucleotide solution and precipitant were mixed, placed on top of siliconized microscope covergrips, and equilibrated over 1 ml of precipitant solution in 24-well tissue culture plates (ICN). The growth of large single crystals was optimized by varying precipitant concentration and pH. For data collection, single Cki1A298 crystals were harvested and mounted as described previously (Kuret and Pfugrath, 1991).

Nomenclature—Cki1 truncation mutants are designated by the symbol Δ followed by a number that indicates the point of C-terminal deletion. Proteins that are fused to an N-terminal polyhistidine tag are indicated by the letter h. Thus, the enzyme Cki1A390h consists of polyhistidine-tagged Cki1 residues 1–390.

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To better define the amino acid residues that comprise the catalytic core of Cki1, a series of three C-terminal truncation
mutations were prepared by oligonucleotide-directed mutagenesis. These are illustrated in Fig. 1A. The first truncation, CkilΔ390, eliminates the C-terminal 56 residues of Ckil that may be responsible for localization of Ckil to the plasma membrane. The second truncation, CkilΔ348, eliminates the C-terminal 98 residues of Ckil, including approximately half of the tether region. The final truncation, CkilΔ298, contains only those residues that are conserved in all known forms of Ckil. To determine the effect of truncation on catalytic efficiency, each truncation mutant was expressed in E. coli and purified as described below.

**Overexpression and Purification of Ctk1 Truncation Mutants**—Unlike most protein kinases, Ctk1 expresses solubly in E. coli, where it can accumulate to 3–6% of the total soluble protein. Nonetheless, its purification is tedious, in part because it elutes broadly from ion-exchange chromatography columns. As described below, this behavior probably results from extensive autophosphorylation of the enzyme. The problem was overcome by expressing Ctk1 tagged at its N terminus with polyhistidine, which allows rapid and efficient purification of the resultant fusion protein after affinity chromatography over a nickel-chelate column. Because the site of fusion is well away from the active site of Ctk1, addition of the polyhistidine tag should not affect enzyme activity in vitro. To confirm this hypothesis, kinetic constants for purified recombinant Ctk1 were estimated by assaying its phosphotransferase activity at various concentrations of protein substrate as described under “Experimental Procedures.” The results, summarized in Table I, show that Ctk1h is a very active casein kinase, with apparent $V_{\text{max}}$ and $K_m$ values that are within the standard error of those measured for nonfusion recombinant Ckil. Analysis of the preparation by automated Edman degradation gave the N-terminal sequence Gly-Ser-Ser-, which corresponds to the polyhistidine tag sequence minus its initiating formylmethionine residue. Thus, the final preparation consists of Ctk1 fused to a 2032-Da nonadecapeptide (the polyhistidine tag). We conclude that addition of this polyhistidine tag to Ctk1 confers rapid affinity purification without affecting Ctk1 catalytic activity, and that it is possible to directly compare the activity of polyhistidine-tagged Ctk1 mutants to that of nonfusion Ctk1.

In addition to full-length Ctk1, truncation mutants Ctk1Δ390 and Ctk1Δ348 were expressed as polyhistidine tag fusions and purified to near homogeneity. As shown in Fig. 1B, the migration of Ctk1h and Ctk1Δ390h during SDS-polyacrylamide gel electrophoresis is 16–18% slower than predicted from the calculated molecular masses of these fusion proteins. The anomaly does not result from the polyhistidine tag epitope, because a similarly slow migration was observed with nonfusion Ctk1

![](https://i.imgur.com/3G3G3G.png)

**Fig. 1. Design and purification of Ctk1 C-terminal truncations.** Panel A, schematic diagram of four forms of Ctk1 created by C-terminal truncation. The size of each mutant is shown alongside a scale reflecting protein length in amino acid residues. Also shown is the relative positions of the highly conserved, ~290-residue catalytic domain (black), a 12-residue segment that is conserved among the YCK and cki gene products (white), and the 51-residue region that includes a putative prenylation site (shaded). Panel B, this Coomassie Blue-stained 10% SDS-polyacrylamide gel contains in lane M, molecular mass standards; lane 1, Ctk1h; lane 2, Ctk1Δ390h; lane 3, Ctk1Δ348h; lane 4, Ctk1Δ298 (peak 1); and lane 5, Ctk1Δ298 (peak 2). Approximately 0.5 μg of each protein was loaded on this gel.

![](https://i.imgur.com/4G4G4G.png)

**Fig. 2. Cation exchange chromatography resolves multiple forms of recombinant Ctk1Δ298.** Fraction 4 (MonoQ flow-through) was chromatographed on MonoS as described under “Experimental Procedures.” $A_{280}$ and the salt gradient (- -) are shown versus elution volume. Major peaks I and II were pooled as indicated by the horizontal bars.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}^{a}$</th>
<th>$K_m^{a}$</th>
<th>$k_{\text{cat}}/K_m^{a,b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctk1h</td>
<td>7.37 ± 0.73</td>
<td>5.81 ± 0.81</td>
<td>1.71 ± 0.29</td>
</tr>
<tr>
<td>Ctk1Δ390h</td>
<td>7.26 ± 0.59</td>
<td>3.90 ± 0.53</td>
<td>2.92 ± 0.35</td>
</tr>
<tr>
<td>Ctk1Δ348h</td>
<td>20.1 ± 1.6</td>
<td>7.40 ± 0.84</td>
<td>3.67 ± 0.42</td>
</tr>
<tr>
<td>Ctk1Δ298 (peak 1)</td>
<td>16.5 ± 1.7</td>
<td>4.90 ± 0.83</td>
<td>4.54 ± 0.77</td>
</tr>
<tr>
<td>Ctk1Δ298 (peak 2)</td>
<td>18.7 ± 0.8</td>
<td>1.55 ± 0.22</td>
<td>16.3 ± 2.4</td>
</tr>
</tbody>
</table>

$^{a}$ Values ± S.E. of the estimate.

$^{b}$ Calculated assuming the molecular mass of casein is 22.5 kDa.
Fig. 3. Mass spectrum of CkilA298.
Peak II enzyme was subjected to mass spectrometry as described under “Experimental Procedures,” using equine myoglobin as mass standard.

Physical Characterization of CkilA298—Analysis of major peaks I and II by automated Edman degradation gave identical amino acid sequences corresponding to residues 2 through 8 of Ckil: Ser-Gly-Gln-Asn-Asn-Val-Val-. Thus, the initiator for-mylmethionine of CkilA298 is absent in both peaks I and II, and the differential migration of CkilA298 peaks I and II seems during cation exchange chromatography is not the result of N-terminal modification.

To determine the structural relationship between peaks I and II, samples of each were subjected to electrospray mass spectrometry as described under “Experimental Procedures.” The mass spectrum of peak II enzyme shows the preparation is composed principally of a single species of molecular mass 34,217.7 ± 19.6 Da (Fig. 3). This value is within 1 S.D. of the predicted mass of Ckil residues 2–298 (34,200.0 Da), and suggests the preparation consists of unmodified protein. It is possible, however, that the −18 Da difference between the measured and calculated masses is significant and results from oxidation of one of the 7 methionine residues in CkilA298 to the sulfoxide during enzyme preparation or storage (Allen, 1981). In contrast, the mass spectrum of peak I enzyme is complex and suggests it consists of a heterogeneous mixture. The masses of its two most abundant components are summarized in Table II, along with the absolute mass difference between each of them and peak II enzyme. The results show that each form of peak I enzyme is larger than peak II by a multiple of ~80 Da, or the mass of a single phosphate group, and that peak I consists mostly of mono- (~60%) and tri- (~30%) phosphorylated CkilA298. We conclude that the two peaks of CkilA298 are related through the presence (peak I) or absence (peak II) of multiple phosphate groups. This conclusion was confirmed by assaying both peaks for phosphate content as described under “Experimental Procedures.” While peak II contains trace amounts of covalently bound phosphate, peak I contains on average 1.5 mol of phosphate/mol of protein. The addition of negatively charged phosphate is consistent with the earlier elution of peak I relative to peak II during cation exchange chromatography.

Because CkilA298 is expressed solubly in E. coli, and is active catalytically, the multiple forms of CkilA298 observed probably result from autophosphorylation occurring in vivo during bacterial expression. Recombinant Ckilh, CkilA390h, and CkilA348h also contain covalently bound phosphate, and exhibit average stoichiometries of 9.1, 4.8, and 4.0 mol of phosphate/mol of enzyme, respectively. We suspect that the
broad elution pattern observed during ion-exchange chromatography of these three enzymes results from the copurification of multiply phosphorylated forms.

Kinetic Properties of CK1 Truncation Mutants—To determine the effect of Cki1 C-terminal deletion on catalytic efficiency, kinetic constants were estimated for each of the Cki1 truncations by measuring phosphotransferase activity at varying concentrations of casein as described under “Experimental Procedures.” Results are presented in Table I. Full-length recombinant Cki1h phosphorylates casein with a V_max of 8.41 ± 0.83 μmol of mg⁻¹ min⁻¹ (k_cat = 7.37 s⁻¹) and a K_m of 5.81 ± 0.81 mg/ml (k_cat/K_m = 1.71 μM⁻¹ min⁻¹). Comparison of these kinetic values with those of the truncation mutants shows that, although Cki1 is constitutively active, deletion of its C-terminal region results in a modest increase in its k_cat/K_m for casein. The increase in k_cat/K_m comes in part through a ~3-fold increase in catalytic rate. Indeed, the turnover numbers observed for Cki1A348h and Cki1A298h (16–20 s⁻¹) are the highest reported to date for any CK1 homolog. Examination of the data in Table I points to the removal of residues located between Ser348 and Gly349 as being responsible for the increase in catalytic rate. It will be of interest to determine whether this activation phenomenon is a general feature of the CK1 family. Like other protein kinases, CK1 may bind a pseudosubstrate or other motif that inhibits the catalytic site until removed by proteolysis in vitro or through interaction with an activator in vivo (e.g. Knighton et al. (1992)). Evidence for the latter mechanism has been presented for the δ form of mammalian CK1 (Cki8; Graves et al. (1993)). This 428-residue CK1-homolog increases its k_cat/K_m for peptide substrate 5-fold in response to heparin. Deletion of the C-terminal region of Cki8 eliminates heparin-mediated activation.

The catalytic activities of Cki1A298 peak I and peak II enzymes point to another potential mechanism of regulation. Peak I enzyme phosphorylates casein with a V_max of 28.9 ± 3.0 μmol of mg⁻¹ min⁻¹ (k_cat = 16.5 s⁻¹) and a K_m of 4.90 ± 0.83 mg/ml (k_cat/K_m = 4.54 μM⁻¹ min⁻¹). Under identical conditions, peak II phosphorylates casein with a V_max of 32.8 ± 1.3 μmol of mg⁻¹ min⁻¹ (k_cat = 18.7 s⁻¹) and a K_m of 1.55 ± 0.22 mg/ml (k_cat/K_m = 16.3 μM⁻¹ min⁻¹). Thus, while its catalytic rate remains essentially unchanged, autophosphorylation of Cki1A298 results in a ~4-fold increase in its apparent K_m for peptide substrate. As shown in Table I, the combination of truncation and dephosphorylation of Cki1 results in an overall activation of nearly 10-fold.

Crystallization of Cki1A298—Because of its ease of preparation, high specific activity, and absence of post-translational modification, Cki1A298 peak II was selected for crystallization trials. Crystallization conditions for Cki1A298 (peak II) were sought in the presence and absence of MgATP as described under “Experimental Procedures.” Results are summarized in Table III and illustrated in Fig. 4. We note that it is not possible to crystallize Cki1A298 peak I under either the conditions described below or by seeding with existing crystals of the peak II enzyme.

In the absence of substrate, Cki1A298 crystallizes in 8.5% (w/v) polyethylene glycol 4000, 8.5% (v/v) isopropanol alcohol over pH range 8.5–9.5. The crystals grow as rhombohedral prisms up to 0.3 x 0.3 x 0.3 mm in size and diffract to 3.2 Å resolution. Assignment to space group P2₁ 2₁ 2₁ was made on the basis of systematic absence of reflections along the h00, 0k0, and 00l axes.

In the presence of MgATP, Cki1A298 crystallizes in two different crystal forms. The first form (binary 1) emerged from 3%
(w/v) polyethylene glycol 8000, 20 mM (NH₄)₂SO₄ at neutral pH. Although crystals grown under these conditions are frequently twinned, single rods that have dimensions 1.0 x 0.1 x 0.1-mm and that diffract to 2.7-Å resolution are obtainable. Again, assignment to space group P2₁2₁2₁ was made on the basis of systematic absences in the diffraction data. The secondary binary form emerged from 1.55 m (NH₄)₂SO₄, 50 mM sodium citrate, pH 5.6. These crystals grow to dimensions 0.8 x 0.5 x 0.5-mm and diffract to ~2.0-Å resolution. Assignment to space group P3₂1 (or its enantiomorph) was made on the basis of precence photography (Fig. 5). Although the 00l zone shows reflections along the 00l axis when l = 3n (where n is an integer), which is consistent with a hexagonal crystal system, the upper level 3n symmetry in the hkl zone confirms P3₂1 or its enantiomorph as the true space group. Six trigonal crystals were used to collect a native data set that is 94.2% complete between 20.0 and 3.07 Å, 90.1% between 3.07 and 2.44 Å, 76.2% between 2.44 and 2.13 Å, and 33.6% between 2.13 and 1.94 Å. A total of 142,178 measurements with 24,212 unique reflections (to 0.75 Å) and solvent contents of 50–55% for the three crystal forms described above. These values, which were confirmed by crystal density measurements, are typical of protein crystals (Matthews, 1968). Because CK1 is so distantly related to other protein kinases in primary structure (Wang et al., 1992), successful use of the molecular replacement technique for phase determination (Aguilar et al., 1993) using established structures as search models may be difficult. Therefore, we plan to solve the phase problem by multiple isomorphous replacement of the trigonal crystals, and a search for useful heavy atom derivatives is underway.

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