Crystallization and Preliminary Crystallographic Analysis of a Thermostable Mutant of Kanamycin Nucleotidyltransferase

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A thermostable mutant of kanamycin nucleotidyltransferase isolated by cloning and selection for kanamycin resistance in Bacillus steaothermophilus at 70°C has been crystallized in a form suitable for high-resolution diffraction analysis. This enzyme catalyzes nucleotidy group transfer from nucleoside triphosphates such as ATP to hydroxyl groups of various aminoglycosides, thus inactivating the antibiotic. The kanamycin nucleotidyltransferase gene, originally encoded on plasmid pUB110 from the mesophile Staphylococcus aureus, was transferred to the thermophile B. steaothermophilus via shuttle plasmids and the mutant carrying the substitutions D80Y and T130K was isolated from kanamycin-resistant colonies grown at 70°C. The thermostable enzyme was crystallized in two forms from solutions of polyethylene glycol 8000 (PEG8000) using batch and vapor diffusion methods. Type I crystals grown from 19% (w/v) PEG8000 and 200 mM NaCl belong to the orthorhombic space group C222₁, have unit cell dimensions of a = 128.4, b = 156.8, c = 155.8 Å, and diffract to at least 2.4-Å resolution. The type II form of the crystals were grown from 10% PEG8000, 200 mM KCl, and 3 mM CoCl₂, and belong to the tetragonal space group P4₁2₁2 or P4₃2₁2 with unit cell dimensions of a = b = c = 78.9, and c = 220.4 Å; these crystals diffract to at least 2.5-Å resolution. © 1992 Academic Press, Inc.

The use of mutant proteins to investigate the determinants of protein stability is well established (1–6). However, whereas it has been possible to genetically isolate variants of many proteins with reduced thermal stability (as “temperature-sensitive” mutants), there are fewer examples of variants with increased thermostability. Mutant proteins that are more stable than their wild-type complements have been isolated in several ways. They have been found fortuitously (7, 8), as second-site revertants of destabilizing mutations (9–11), and from experimental systems designed to recover mutants with enhanced thermostability. The latter include the preparation of variants of subtilisin (12) and T4 lysozyme (13). Still, because these systems rely on screens for the enzymatic activity at or following exposure to high temperature, they are not as powerful as methods based on direct selection.

A selection method for the identification and isolation of thermostable variants has been developed based on cloning the gene coding for a nonthermostable enzyme into a thermophile and then selecting for the enzymatic activity at the high growth temperatures of this organism (14, 15). This approach was tested with kanamycin nucleotidyltransferase (KNTase). Kanamycin nucleotidyltransferase (which is sometimes referred to as aminoglycoside-4'-nucleotidyltransferase, aminoglycoside antibiotics-4'-adenylyltransferase, 4'-O-aminoglycoside nucleotidyltransferase, ANT(4'), or AAD(4')) catalyzes the transfer of a nucleoside monophosphate group from a nucleotide to the 4'-hydroxyl group of aminohexose I of deoxystreptamine aminoglycoside (16) and thus inactivates the antibiotic (17–20). The KNTase gene encoded on the plasmid pUB110 from the mesophile Staphylococcus aureus was transferred to the thermophile Bacillus steaothermophilus via shuttle plasmid vectors. Wild-type

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²Abbreviations used: KNTase, kanamycin nucleotidyltransferase; PEG8000, polyethylene glycol 8000.
KNTase confers kanamycin resistance to *B. stearothermophilus* at up to 55°C. Thermostable mutants were isolated as kanamycin-resistant colonies at 61°C (15) and 63°C (14) and shown to carry missense substitutions of either the aspartic acid at position 80 to tyrosine (D80Y) or the threonine at 130 to lysine (T130K). The mutant carrying both changes was either isolated by plating at 70°C (14) or constructed by site-directed mutagenesis (21). The degree of stabilization in the double mutant (referred to as TK101) is qualitatively equivalent to the sum of the effects of each individual substitution (14, 21).

The full value of the kanamycin nucleotidyltransferase system for the isolation of thermostable variants has not been realized because the crystal structure of this protein has not been elucidated. Thus, while no structural information was originally required to obtain the thermostable variants, the sequence changes in these variants that cause the enhanced stability cannot be correlated with any changes within the architecture of the protein in the absence of knowledge of its structure, and the information inherent in these mutants on how stability may be increased by design in other proteins is unavailable. We therefore initiated a structural study of the kanamycin nucleotidyltransferase enzyme's subunits, and in this paper, we report the development of conditions for the crystallization of KNTase TK101 and present preliminary data on the diffraction properties of the crystals.

**MATERIALS AND METHODS**

Kanamycin nucleotidyltransferase TK101 was made in an *Escherichia coli* overproducing strain, BL21(DE3) (22), carrying the expression plasmid pX(T7)/TK101 (23). The enzyme was purified by chromatography on Q-Sepharose Fast Flow (Pharmacia LKB Biotechnology, Piscataway, NJ), then on a HPLC TSK phenyl-5PW column (Beckman Instruments, Inc., Alte Scientific Operations, Berkeley, CA), and finally on a HPLC Spheroegle TSK DRAE-5PW column essentially as described (14, 23, 24). The protein was stored in 10 mM Tris–HCl, pH 7.5, 0.5 mM EDTA at −70°C until required.

A survey of possible crystallization conditions was conducted at room temperature and at 4°C using both the hanging drop and sitting drop vapor diffusion methods (for a review of crystallization techniques, see Ref. (25)). The following parameters were examined during the search for crystals of KNTase TK101: pH (ranging from 5 to 8), NaCl and KCl concentration, polyethylene glycol 8000 (PEG8000) concentration, temperature (ambient or 4°C), the presence of 2-methane-2,4-pentanediol, dimethyl sulfoxide, MgCl₂, CoCl₂, Ba(OAc)₂, VOSO₄, SrCl₂, β-octyl glucoside, or kanamycin, and (NH₄)₂SO₄ as the precipitant. (All chemicals were from Sigma Chemical Co., St. Louis, MO, unless otherwise noted.) Protein treated with iodoacetamide was also tested. Once promising conditions were identified, the batch method was employed in order to grow larger crystals. Two crystal forms, both of which diffract well, were obtained.

To determine the number of molecules of KNTase per asymmetric unit, measurements of crystal density were made using a bromobenzene: toluene density gradient column calibrated with droplets of potassium iodide solutions of predetermined density (26).

For X-ray diffraction experiments, the crystals were sealed in thin-walled quartz capillary tubes (Charles Supper Co., Natick) and air-cooled at 4°C. Precession photographs were recorded with nickel-filtered copper Kα radiation from a Rigaku RU200 rotating anode X-ray generator with a 0.02-mm focal spot, operated at 50 kV and 40 mA (Figs. 2 and 3, respectively). The crystal-to-film distance was 100 mm, and the exposure time was 24 h.

**RESULTS AND DISCUSSION**

The first crystals (type I) were grown from a solution that contained 19.0% (w/v) PEG8000, 200 mM NaCl, 50 mM Hepes, pH 8, and 10 mM sodium azide at a final protein concentration of 8 mg/ml. Crystals grew as long rods to typical dimensions of 0.1 × 0.2 × 1.0 mm in a month.

A second crystal form (type II) was obtained by a microbatch method in the presence of cobalt chloride. A protein solution at 4 mg/ml in 20 mM Tris–HCl, pH 7.5, 1 mM EDTA was mixed by gentle vortexing with an equal volume of a solution of 20.0% PEG8000 (Baker Chemical Co., Fairlawn, NJ), 400 mM KCl, 50 mM Hepes, pH 8 (Research Organics Inc., Cleveland, OH), 6 mM CoCl₂, and 5 mM sodium azide. After mixing, the solution was clarified by centrifugation in a microfuge for 15 min prior to being placed under a layer of mineral oil in a deep well depression slide. Crystals grew as square rods with typical dimensions of 0.2 × 0.2 × 1.0 mm in a month (Fig. 1).

The type I crystals grown in the absence of divalent cations belong to the orthorhombic space group *C222₁*, and have unit cell dimensions of *a* = 128.4 Å, *b* = 156.8 Å, and *c* = 155.8 Å. The measured density of a crystal, 1.19 g/cm³, is consistent with six molecules in the asymmetric unit for which the calculated density would be 1.20 g/cm³ (27). Assuming a molecular weight of 28,765 for the protein, the volume/unit molecular weight in the unit cell (*V*/M) is 2.27 Å³/Da. This lies within the normal range (1.68–3.53 Å³/Da) observed for globular proteins (28). Diffraction spots at the edge of the precession circle correspond to 3.4 Å resolution, and as can be seen there is little falloff in intensity at this resolution (Fig. 2). Based on

![FIG. 1. Typical type II crystals of KNTase of dimensions 0.2 mm × 0.2 mm × 1.0 mm. The crystals were grown at 4°C from 10% PEG8000, 200 mM KCl, 50 mM Hepes, pH 8, and 3 mM CoCl₂, and belong to either space group *P*₄₃₂₂ or *P*₄₂₁₂.](image-url)
An interesting relationship between the unit cell lengths of the two crystal forms was noted by one of the referees. The unit lengths of \(b\) and \(c\) of the type I crystal are twice that of \(a\) and \(b\) of the type II crystal, and \(c\) of the type II crystal is the square root of 2 times the \(b\) and \(c\) of the type I crystal. These relationships suggest a higher symmetry that may simplify the structure determination for the large unit cell size. However, since the densities of the type I and II crystal forms are different the relationship between the packing of the molecules in these lattices cannot be a simple rotational or sliding transformation. In addition a comparison of the precession photographs of the principal zones of these two crystal forms does not reveal an obvious relationship between their packing. These observations will be explained once the structure for both crystal forms has been determined.

KNTase is a relatively small (253 amino acid residues), soluble, monomeric, easily assayed and purified enzyme, and it has an activity that is selectable in a variety of bacterial species. This enzyme thus constitutes an ideal basis for a system to study protein stability and folding. Accumulation of diffraction data and a search for isomorphous heavy atom derivatives for both types of crystals of the thermostable KNTase mutant TK101 is underway. In addition, a survey of crystallization conditions for mutant enzymes with single site substitutions and the wild-type protein is in progress. A detailed X-ray study of the crystals of this series of enzymes that differ only in their thermostability will yield valuable information regarding the structural basis of the enhanced thermostability in these mutants. Moreover, elucidation of the active site of KNTase will provide insight into the mecha-

![FIG. 2. 13° precession photograph of the 0kl zone of the type I crystal. The photograph was recorded as described under Materials and Methods.](image)

![FIG. 3. 13° precession photograph of the hkl zone of the type II crystal grown with cobalt chloride. The photograph was recorded as described under Materials and Methods.](image)
nism of nucleotide phosphate transfer, possibly serving as a model for the reaction catalyzed by more complex polymerases.

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