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Cobalt Activation of *Bacillus* BR449 Thermostable Nitrile Hydratase Expressed in *Escherichia coli*

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Abstract

Expression of nitrile hydratase enzymes utilized in a new "green" process for acrylamide production has proven difficult in *Escherichia coli* owing to lack of a cobalt transport system to introduce the required cobalt ion into this host. We describe the expression of a thermostable nitrile hydratase from a moderate thermophile *Bacillus* sp. BR449 in *E. coli* in which the cobalt required for enzyme activation is introduced by incubation of the apoenzyme in the presence of Co^{++} ion at 50°C, yielding active and thermostable enzyme.

Index Entries: Nitrile hydratase; cobalt; thermophile; acrylamide; enzyme activation.

Introduction

Hydration of acrylonitrile catalyzed by acid or mixed metals is a key step in the production of acrylamide, a commodity chemical utilized as polyacrylamide in waste treatment, secondary oil recovery, and textile applications. More recently, the utilization of nitrile hydratase enzyme catalysts to reduce catalyst costs and environmental impact has been initiated by Nitto Chemical in Japan *(1)*. Initial production by Nitto catalyzed by an Fe^{+++}-containing nitrile hydratase from *Rhodococcus* sp. N-774 has been replaced by a more effective Co^{+++}-containing nitrile hydratase from *Rhodococcus rhodochrous* J1 *(2)*. Investigations of the genes encoding the α- and β-subunits for both iron- and cobalt-containing nitrile hydratase enzyme families have revealed extensive sequence similarities and common participation of three cysteine or oxidized cysteine residues in the α-subunit, which are believed to position and stabilize the metal ion at the active site (reviewed in ref. *3*).

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Cloning of genes for nitrile hydratase into *Escherichia coli* desirable for obtaining increased enzyme content, eliminating contaminating amidases, and genetically improving enzyme characteristics has proven difficult owing to formation of insoluble inclusion bodies and difficulties in the provision and insertion of cobalt into nitrile hydratase *(4,5)*. Wu et al. *(6)* have described cloning of a cobalt-containing nitrile hydratase from *Pseudomonas putida* 5B in which expression of active enzyme in *E. coli* is aided by coexpression of a downstream gene encoding an "activator" protein. Nojiri et al. *(7)* achieved increased expression of the iron-containing nitrile hydratase of *Rhodococcus* sp. N-771 using a similar strategy. The function of the activator protein is not yet known but may be involved in the introduction of cobalt ion into the cell *(3)* or into the enzyme *(6,7)*. We previously described the cloning of genes encoding a thermostable nitrile hydratase from a moderately thermophilic *Bacillus* strain BR449 into *E. coli (8)*. Although growth at 32°C allowed high expression levels for both nitrile hydratase α- and β-subunits, activity levels were limited even when cobalt ion was provided in the growth medium and were not increased by coexpression of an open reading frame associated with the amidase/nitrile hydratase gene cluster. In this article, we describe effective activation of the overexpressed nitrile hydratase from BR449 in *E. coli* using enzyme incubation at an elevated temperature in the presence of Co^{++} ion. We also describe purification and initial characterization of the enzyme.

Materials and Methods

Bacterial Strain and Cultivation

Construction of the *E. coli* recombinant EC463, which contains the nitrile hydratase α- and β-subunit genes from the acrylonitrile-resistant moderate thermophile *Bacillus* sp. BR449 on a 2.4-kb insert has been described *(8)*. EC463 was grown in Luria Bertani broth containing 50 μg/mL of ampicillin at 32°C in a gyrorotatory water bath.

Enzyme Assay

Nitrile hydratase was assayed using formation of acrylamide from 0.5 *M* acrylonitrile in 0.05 *M* phosphate buffer, pH 7.5, at 50°C using high-performance liquid chromatography as described previously *(9)*. One unit is defined by 1 μmol/min formation of acrylamide under these conditions.

Enzyme Activation

Activated cell extracts were produced from EC463 cells grown to late exponential phase at 32°C. Following washing with 0.05 *M* potassium phosphate buffer and resuspension to a concentration of 5 mg/mL (dry weight), cell extracts were produced by sonication with a Cole-Parmer 4710 sonifier (Cole-Parmer, Chicago, IL) for 10 min (five 2-min bursts with 2-min cooling intervals on ice). The sonicate was centrifuged at 12,000*g* for 20 min

and the supernatant adjusted to 5 µ*M* $CoCl_2$ and incubated for 30 min at 50°C. For experiments measuring cobalt activation under anoxic conditions, extracts were sparged with helium for 15 min following cobalt addition and incubated at 50°C in a screw-cap vial with minimal head space. A similarly incubated tube containing unsparged enzyme extract served as the control.

Purification of Enzyme

Nitrile hydratase enzyme from EC463 cell extracts was purified to electrophoretic homogeneity utilizing cobalt-activated EC463 cell extract. Forty milliliters of cell extract was adjusted to 1 *M* ammonium sulfate in 50 m*M* potassium phosphate buffer, pH 7.5, and applied to a column containing 70 mL of Phenyl-Sepharose (Pharmacia, Uppsala, Sweden) preequilibrated with 1 *M* ammonium sulfate in 50 m*M* potassium phosphate buffer, pH 7.5. Following removal of unbound protein using elution with this buffer, nitrile hydratase was eluted using a 200-mL gradient of 1 to 0 *M* ammonium sulfate in 50 m*M* potassium phosphate buffer, pH 7.5, at a flow rate of 2 mL/min. Fractions containing active enzyme determined by assay were concentrated by ultrafiltration and dialyzed against 50 m*M* potassium phosphate buffer, pH 7.5.

Protein Electrophoresis

Protein purification was monitored and subunits were examined using a Bio-Rad mini-Protean gel II electrophoresis (Bio-Rad, Hercules, CA). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed using a 4% stacking gel and 12% separating gel according to the manufacturer's directions. Native protein electrophoresis utilized a 4% stacking gel and 12% separating gel. SDS and native electrophoresis protein standards were obtained from Sigma (St. Louis, MO).

Visible and Ultraviolet Spectrum of Nitrile Hydratase

Spectral data for holoenzyme and apoenzyme forms of BR449 nitrile hydratase were measured using a Beckman DU7500 spectrophotometer (Beckman, Fullerton, CA) at a concentration of 20 mg/mL for the visible range and 1 mg/mL for the ultraviolet (UV) range.

Results

Activation of Nitrile Hydratase with Co^{++}

After initial discovery of cobalt stimulation of intact cells and cell extracts, conditions for optimization of cobalt activation were determined. As seen in Table 1, stimulation of enzyme activity in the presence of 5 µ*M* Co^{++} was optimal with incubation at 50°C. An incubation time of 30 min was optimal with longer times or increased cobalt concentrations not improving activation. Attempts to utilize other ions including Fe^{++},

Table 1
Cobalt Activation of BR449 Nitrile Hydratase at Varied Temperatures[a]

Temperature (°C)	NHase activity (U/mg protein)
20	7
30	6
40	58
50	327
60	2

[a]Activation time was 30 min at the temperature shown followed by standard assay.

Table 2
Heat Activation of Nitrile Hydratase with Co^{++}

	Unheated	Heated separately	Heated combined	Heated combined (anoxic)
NHase activity (U/mg protein)	6	15	307	310

Table 3
Purification of BR449 Nitrile Hydratase

Purification step	Volume (mL)	Protein concentration (mg/mL)	Protein (mg)	Specific activity (U/mg)	Purification (fold)
Cell extract	50	2.3	115	271	1.0
Phenyl-Sepharose	1	21.0	21	852	3.1

Ni^{++}, Mn^{++}, and Cu^{++} instead of cobalt ion for enzyme activation resulted in inactive extracts (data not shown). Separate incubation of cobalt and apoenzyme at 50°C followed by cooling and mixing at 18°C prior to assay indicated that activation required incubation of combined reactants at elevated temperature, and that incubation under anoxic conditions did not affect the activation at this elevated temperature (Table 2).

Purification of Nitrile Hydratase

Purification of BR449 nitrile hydratase from extracts of EC465 was accomplished in a single-step, Phenyl-Sepharose affinity purification as shown in Table 3, allowing a threefold purification. The specific activity of the purified BR449 enzyme compares favorably with those reported for other nitrile hydratase enzymes *(3,10)*, although differences in temperature and other conditions of assay preclude exact comparison. The high specific

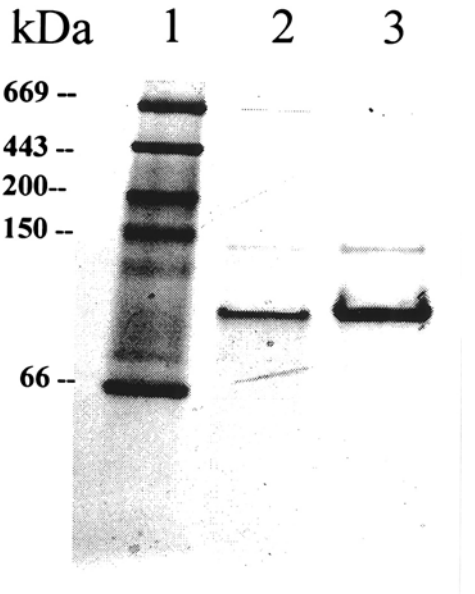

Fig. 1. Comparison of BR449 nitrile hydratase holoenzyme (lane 2) and apoenzyme (lane 3) molecular weights. Electrophoresis molecular weight markers are shown in lane 1.

activity obtained for purified enzyme suggests extensive, if not complete, activation of the enzyme. SDS slab gel electrophoresis of the purified nitrile hydratase showed the expected equal amounts of 25-kDa α- and 27.5-kDa β-subunits described previously using the *Bacillus* parent *(8)*.

Molecular Weight of Enzyme

The molecular weight of the BR449 holoenzyme was estimated using gel electrophoresis at 100 kDa (Fig. 1). This suggests that the holoenzyme is composed of two α- and two β-subunits as found for the nitrile hydratase of *R. rhodochrous* J1. Molecular weight determination of the apoenzyme utilizing the same purification but without cobalt activation yielded an inactive apoenzyme of 100-kDa molecular weight (Fig. 1). This indicates that cobalt ion is not required for subunit assembly.

Enzyme Absorption Spectrum

The visible and UV absorption spectrum of the purified nitrile hydratase apoenzyme and holoenzyme were compared (Fig. 2). In addition to the similar UV absorption bands below 300 nm expected from aromatic amino acids, the holoenzyme shows a weak absorption band at 340 nm, which is not observed for the apoenzyme. Similar weak absorption bands have been observed for Co^{+++}-containing nitrile hydratases from *R. rhodochrous* J1 *(11)* and *Pseudomonas putida (10)* and attributed to ligand:metal charge transfer.

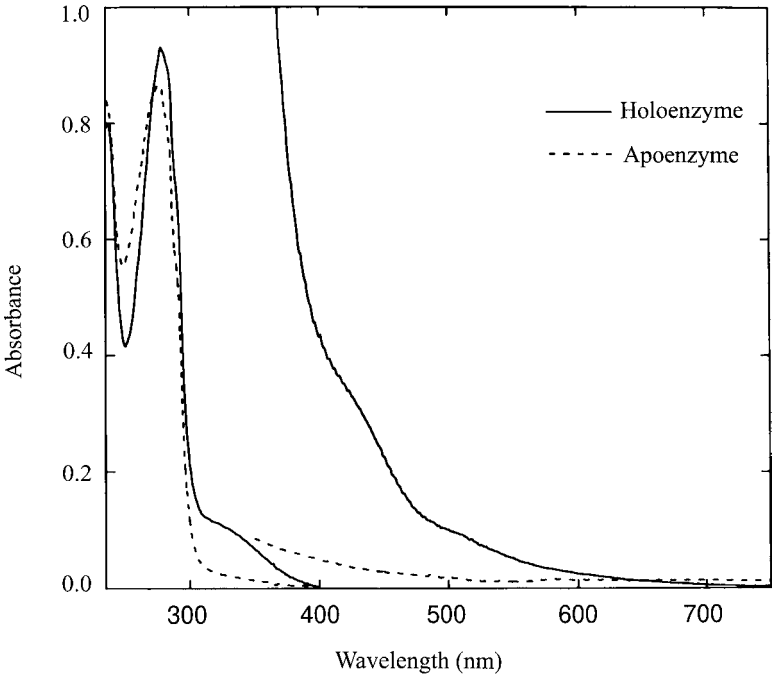

Fig. 2. Comparison of BR449 nitrile hydratase apoenzyme (dashed line) and holoenzyme (solid line) visible and UV absorption spectra. Visible spectra utilized an enzyme concentration of 20 mg/mL and UV spectra utilized a concentration of 1 mg/mL.

Discussion

The ability to activate BR449 nitrile hydratase to high activity levels following overexpression using Co^{++} ion is unexpected, because the active-site cobalt in nitrile hydratases has been reported to be tightly bound in the Co^{+++} state *(11,12)*. Although the nature of the activation is not yet understood, it requires incubation of combined Co^{++} and apoenzyme at elevated temperature. If the cobalt ion oxidizes to Co^{+++} during insertion into the active site as suggested by the spectral data, the undiminished activation under anoxic conditions would indicate participation of an oxidant other than oxygen. Consistent with this interpretation, Nojiri et al. *(13)* recently reported partial activation of a cobalt-substituted Fe-type nitrile hydratase with the chemical-oxidizing agent potassium hexacyanoferrate. Although details of the chemistry remain to be elucidated and are under study, the simple and effective nitrile hydratase heat activation procedure described in this article is of value in circumventing the requirement for cobalt introduction into *E. coli* during growth and expression, and the complexities associated with coexpression of activation genes. It remains to be seen whether the procedure can be utilized with nitrile hydratase enzymes of mesophilic origin.

As described previously, the BR449 nitrile hydratase demonstrates good thermostability in buffer but is rapidly inactivated by acrylonitrile,

probably reflecting the proclivity of acrylonitrile for protein alkylation *(14)*. The ability to express high levels of active enzyme in *E. coli* offers the opportunity for utilization of new genetic techniques such as directed evolution or site-directed mutagenesis to improve alkylation resistance of this nitrile hydratase in industrial applications.

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