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Residue Y70 of the Nitrilase Cyanide Dihydratase from *Bacillus pumilus* Is Critical for Formation and Activity of the Spiral Oligomer

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Nitrilases pose attractive alternatives to the chemical hydrolysis of nitrile compounds. The activity of bacterial nitrilases towards substrate is intimately tied to the formation of large spiral-shaped oligomers. In the nitrilase CynD (cyanide dihydratase) from *Bacillus pumilus*, mutations in a predicted oligomeric surface region altered its oligomerization and reduced its activity. One mutant, CynD Y70C, retained uniform oligomer formation however it was inactive, unlike all other inactive mutants throughout that region all of which significantly perturbed oligomer formation. It was hypothesized that Y70 is playing an additional role necessary for CynD activity beyond influencing oligomerization. Here, we performed saturation mutagenesis at residue 70 and demonstrated that only tyrosine or phenylalanine is permissible for CynD activity. Furthermore, we show that other residues at this position are not only inactive, but have altered or disrupted oligomer conformations. These results suggest that Y70's essential role in activity is independent of its role in the formation of the spiral oligomer.

Keywords: Nitrilase, cyanide dihydratase, cyanide, bioremediation, oligomerization surface, quaternary structure

Introduction

Cyanide is the simplest but also most hazardous nitrile compound. Nonetheless, it is used extensively in industries ranging from textile production to gold mining [8]. The cyanide-degrading nitrilase cyanide dihydratase (CynD) from *Bacillus pumilus* (GenBank No. AF492815.1) is being engineered for use in detoxifying cyanide-laden industrial waste [7, 17]. Presently, the lack of fine structural knowledge of nitrilase enzymes is the major hurdle to rationally manipulating these enzymes [15].

Limited structural insights can be gleaned from a conserved dimer structure that is seen in crystal structures of enzymes from the larger superfamily [10]. The conserved dimers have a basic αββα-αββα architecture [5, 11] and a C-E-E-K active site [4]. From electron microscopy, we know that CynD expands on the basic dimer building block to form large 18-subunit left-handed spiral oligomers [3]. Similar spiral structures have been seen in all active nitrilase enzymes examined so far [15].

Modeling and fitting of CynD's dimer to the 3D shell created from electron microscopy data have led to the prediction of the primary interface between dimers, the C-surface. Two regions were predicted to participate in this C-surface interaction, as they stand out as relative insertions compared with nonspiral-forming superfamily members [12–15].

We have previously tested the participation of these regions in spiral formation by scanning these two regions in CynD with cysteine substitutions to identify residues critical for activity and proper oligomer formation [12]. These cysteine substitutions revealed multiple residues in the CynD region 55–72 that influence the size and activity of CynD oligomers. Two of these mutants, R67C and Y70C, had no detectable activity. The mutant R67C blocked proper CynD oligomerization, resulting in a heterogeneous

mixture of partially formed oligomer fragments. This indicates a critical role in oligomerization at the C-surface and emphasizes the dependence of activity on proper oligomer formation. On the other hand, the inactive mutant CynD Y70C forms homogeneous oligomers, as detected by size exclusion chromatography, intermediate in size between the 18-subunit CynD from *B. pumilus* and the 14-subunit CynD from *P. stutzeri* (GenBank No. D82961.1) [13]. Unlike CynD Y70C, other mutants in this region that had oligomers of intermediate size retained partial activity. We hypothesized that Y70 may play an additional role in CynD's activity or oligomerization beyond its effect on oligomer size [12].

In this work, our aim was to test one simple hypothesis: does the mutation Y70C have an effect on quaternary structure because of a disruptive effect of the cysteine residue, or does the mutation result from loss of the tyrosine residue at this position? The latter would support our hypothesis that Y70 plays a specific role in modulating oligomer size. Here, we performed site saturation mutagenesis at position 70 and examined the effect of all other amino acids at this position on protein activity. A subset of mutants was selected and examined for changes in their quaternary state.

Materials and Methods

Bacterial Strains and Plasmids

All substitution mutants were constructed in pMB5006, derived from pBC KS+ with *B. pumilus cynD* (GenBank No. AF492815.1) as an XbaI-XhoI insertion using Phusion High Fidelity DNA polymerase master mix (NEB;USA). *Escherichia coli* strain MB3436 (Δ*endA thiA hsdR17 supE44 lacI*^{q}*ZΔm15*) was used for cloning, mutant construction, and expression of pBC-based plasmid constructs. Select mutants were independently reconstructed in pMB4407, a pET28a-based plasmid, for protein production in *E. coli* BL21(DE3)(pLysS) as previously described [1, 12]. Bacteria were grown in LB broth with antibiotics as previously described [12].

Site Saturation Mutagenesis

Mutants were constructed by site-directed mutagenesis following the QuickChange protocol (Stratagene, USA) using mutagenic primers and Phusion DNA polymerase. Primers were designed to have a semi-random codon to mutate Y70 to all possible amino acids. Not all mutations were obtained from this reaction; the remainder were individually constructed using specific primers (Table 1). Mutagenic primers were designed using the program PrimerX (http://www.bioinformatics.org/primerx/). Mutants were identified and confirmed by sequencing.

Protein Expression

Activity from each pBC-derived construct was measured in MB3436. Overnight cultures were diluted (200 µl into 3 ml of media) and grown at 37°C to OD_{600} between 0.5 and 0.7, at which time they were induced for expression with IPTG at 1 mM and transferred to 37°C for another 1–2 h. CynD activity was tested immediately after the induction period. His-tagged purified CynD protein produced from pET28a-derived constructs was purified and examined by gel filtration as previously described [12].

Activity Assay

CynD activity from whole cells and purified protein was measured by picric acid assay to measure cyanide [17] as described in our previous work [12].

Chromatography

Purified protein samples in 0.1 M MOPS (pH 7.7) were separated on a Superdex 200 10/300 GL column (Amersham Biosciences, Sweden) as described previously [12]. The column was calibrated using a Sigma-Aldrich gel filtration marker kit.

Results

Activity of Y70-X Substitutions

In our previous work, cysteine scanning mutants across

Table 1. Primers used in this study.

Primer	Sequence
Y70 (random)	GGT CAT CCA GAA TAT ACG AGA AAG TTC NNK CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70 (K,N,Q,H,D,E)	GGT CAT CCA GAA TAT ACG AGA AAG TTC VAK CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70I	GGT CAT CCA GAA TAT ACG AGA AAG TTC ATT CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70F	GGT CAT CCA GAA TAT ACG AGA AAG TTC TTT CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70G	GGT CAT CCA GAA TAT ACG AGA AAG TTC GGT CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70E	GGT CAT CCA GAA TAT ACG AGA AAG TTC GAA CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC
Y70K	GGT CAT CCA GAA TAT ACG AGA AAG TTC AAA CAT GAA TTA TAT AAA AAT GCC GTT GAA ATC CC

Forward sequences are shown; reverse primers used are reverse compliments of the forward sequences.

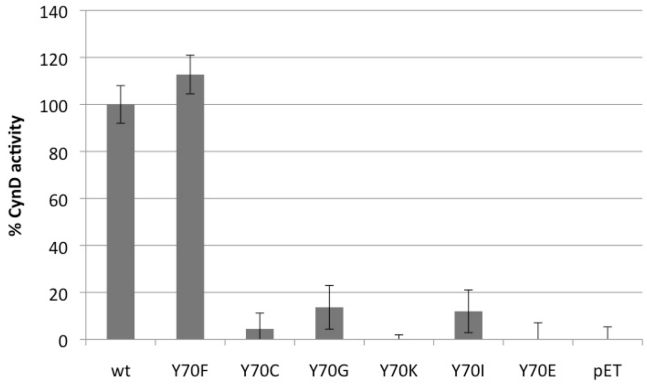

Fig. 1. Activity of purified mutant CynD proteins.
Cyanide was measured, and the CynD activity is shown relative to the wild type, which was set as 100%. Error bars show the standard deviation from triplicate assays.

the entire predicted C-surface region identified mutants with reduced or abolished activity, which were mostly correlated with a significant change in mobility, suggesting a change or disruption of oligomer size or conformation. The mutant CynD Y70C was unusual and unique in that it showed only a small change in mobility (migrating intermediate between the 18-subunit and the 14-subunit controls) and retained its oligomer formation, yet had no significant activity [12]. To test if position 70 is critical for activity in CynD, we individually constructed all 19 amino acid substitutions at this position. Expression of each mutant protein was verified by western blot assay and all mutants had reasonable protein levels (data not shown). From the activity tests in whole cells of all possible amino acid substitutions, only phenylalanine and the native tyrosine at residue 70 were tolerated for activity.

The activities of wild-type CynD and CynD Y70F were compared using purified protein, and the activity of CynD Y70F was not significantly different to the wild-type enzyme (Fig. 1). The activities of five other purified mutants (Y70C, G, I, K, E) were not measurably different to the negative control (pET). The apparent residual activity was within the error of the assay due to spontaneous cyanide loss.

Size-Exclusion Chromatography

To test if other Y70 mutants were altered in mobility, suggesting changes to their oligomeric structures, a subset of mutants (Y70C, G, I, K, E, F) were selected to be purified and examined by size-exclusion chromatography (Fig. 2) as previously described [12]. These mutants represented a range of amino acid characteristics. The active Y70F eluted as a single peak similar to the "wt" 18-subunit oligomer

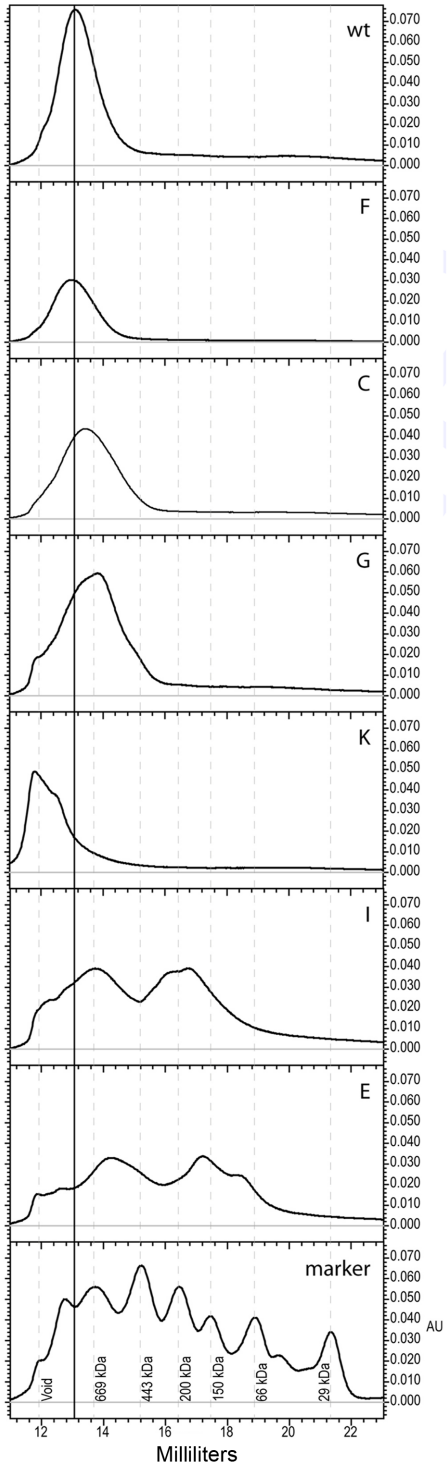

Fig. 2. Size-exclusion chromatography of CynD and Y70x mutants.
Wild-type CynD, mutants Y70F, Y70C, Y70G, Y70K, Y70I, and Y70E, and Sigma-Aldrich gel filtration size standards (marker) are shown in each panel. The solid vertical line highlights the peak of the wt CynD 18-mer. The faint vertical dotted lines highlight the following marker peaks: void peak, ~669, 443, 200, 150, 66, and 29 kDa from left to right.

(Fig. 2, wt, F). CynD Y70C and Y70G also eluted as single peaks, but were slightly delayed relative to the normal 18-mer, migrating with a size intermediate to the 18- and 14-mer controls (Fig. 2, C, G). The mutant Y70K was eluted from the column in the void fraction, indicating aggregation or extended non-terminated spirals (Fig. 2 K). Two mutants (Y70I and Y70E) blocked normal oligomer formation and eluted as multiple peaks. The peaks from Y70I resemble possible 14-, 10-, and 8-subunit oligomers (Fig. 2, I). CynD Y70E eluted slower with a range of 10-12-, 6-, and 2-subunit complexes (Fig. 2, E).

Discussion

In the oligomeric nitrilases examined to date, the formation of a spiral oligomeric structure is needed for activation of the enzyme [15]. It is hypothesized that upon oligomerization, the interactions at the C-surface region influence the conformation of the active site [4, 6]. The residue Y70 in CynD is predicted to lie at this interface by its location within the loop region 55–72, which is an insertion relative to the nonspiral-forming nitrilase homologs [13, 14]. A nearby residue identified by the mutation R67C has been shown to abolish oligomerization and disrupt activity [12].

Saturation mutagenesis at position 70 reveals that only tyrosine or the similar phenylalanine is tolerated for activity of CynD. This strongly supports the hypothesis that the presence of tyrosine (or phenylalanine) at position 70 is necessary for activity, and it is not merely disruption by cysteine causing the activity defect in Y70C. Analysis of other predicted nitrilase sequences by BLAST homology searching was surprising and showed that Y70 is strictly conserved at this position in all sequences with an overall identify of 55% or higher, and only F70 was found among the more dissimilar sequences. This further supports the key role that residue 70 plays.

The Y70F mutant was not altered in the size of the CynD oligomer, which eluted consistently with the wild-type 18-mer (Fig. 2, F). By comparison, all five of the inactive mutants examined by size exclusion differed in their elution profile relative to the wild type (Fig. 2, C–E). The most dramatic changes were seen with CynD Y70I and CynD Y70E. These mutants eluted as multiple peaks, although the estimated size of the different oligomers present differed between them (Fig. 2, I, E). Similar to CynD R67C, these mutants were blocked for proper oligomerization, indicating that position 70 is critical for oligomerization as well as activity [12].

CynD Y70K eluted in the void fraction during size-exclusion chromatography (Fig. 2, K). The upper limit of our chromatography column was about 1 MDa, and therefore we were unable to distinguish between extended non-terminating oligomers and large protein aggregates. Although non-terminating CynD oligomers are seen at pH 5.4 or result from other mutations [3, 17], they retain activity, unlike CynD Y70K. Given the disruptive pattern of other mutations at this position, it is more likely that CynD Y70K is forming protein aggregates.

The two inactive mutants Y70C and Y70G had less dramatic alterations to the oligomer, similar to the numerous cysteine scanning mutants in C-surface regions 55–71 and 221–235 [12]. In that work, we showed that CynD Y70C eluted with a delay relative to the wild-type *B. pumilus* 18-mer and was intermediate between that and the *P. stutzeri* 14-mer, indicating an altered or shorter oligomer (Fig. 2, C). CynD Y70G was even further delayed and eluted at a position corresponding to the 14-mer (Fig. 2, G). However, the shortening of the spiral is insufficient to explain the loss of activity in Y70C and Y70G, as other mutants with similarly reduced oligomer size retained activity (F57C, G61C, T66C, and H71C) as does the native 14-mer of *P. stutzeri* [12]. However, other than R67C, the other cysteine scanning mutants retained activity. Therefore, only the mutants at position Y70 retained an ability to form oligomers (albeit altered) but had no activity.

This leads to us to hypothesize that Y70 is playing a role in activation of the oligomeric spiral, as well as participating in the formation of the oligomer itself [12]. Other wild-type nitrilases have oligomeric sizes similar to this mutant, and other mutants retain activity, leading us to conclude the defect in Y70C is not solely due to a change in oligomeric state.

Abundant evidence [4, 6, 9, 16] suggests that dimers are inactive, and only upon assembly to the oligomer is the enzyme activated. Oligomerization may cause a change in the conformational change of the active site, possibly transmitted upon assembly through Y70. However, the loss of proper oligomer formation in both CynD Y70I and Y70E (Fig. 2, I, E) indicates that position 70 is critical to oligomer formation, or alternatively these specific residues at this position inhibit assembly. Other mutants (Y70C, Y70G, and possibly Y70K) are able to form oligomers, but all differ from the native CynD 18-mer and remain inactive.

Taken together, these observations lead to the hypothesis that Y70 (or F70) is at the oligomerization interface, and changes at Y70 prevent activation by blocking a needed conformational change. By virtue of its location at the

oligomer interface, certain mutants can also disrupt oligomer formation or alter the final structure depending upon the properties of the residue introduced. A definitive determination of the role of this critical residue Y70 awaits an experimentally derived high-resolution structure that has yet to be solved for any similar nitrilase.

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