Helical structure of unidirectionally shadowed metal replicas of cyanide hydratase from *Gloeocercospora sorghi*

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Received 18 April 2007; received in revised form 26 September 2007; accepted 27 September 2007

Abstract

The helical filaments of the cyanide hydratase from *Gloeocercospora sorghi* have been reconstructed in three dimensions from freeze dried, unidirectionally shadowed specimens using iterative real-space helical reconstruction. The average power spectrum of all selected images has three clear reflections on different layer lines. The reconstruction is complicated by the fact that three possible indexing schemes are possible and reconstructions using the starting symmetries based on each of these indexing schemes converge on three-dimensional volumes which appear plausible. Because only one side is visible in shadowed specimens, it is necessary to examine the phases from a single filament by cryo-electron microscopy in order to make an unequivocal assignment of the symmetry. Because of the novel nature of the reconstruction method used here, conventional cryo-EM methods were also used to determine a second reconstruction, allowing us to make comparisons between the two. The filament is shown to have a left-handed one-start helix with $D_1$ symmetry, 5.46 dimers per turn and a pitch of 7.15 nm. The reconstruction suggests the presence of an interaction across the groove not previously seen in nitrilase helical fibres.

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Keywords: Cyanide hydratase; *Gloeocercospora sorghi*; Nitrilase; Helical reconstruction; Shadowing; Midilab; IHRSR

1. Introduction

Cyanide hydratase (CHT) is a substrate-specific member of the nitrilase family of enzymes. Nitrilases catalyse the hydrolysis of nitriles to amides or to acids and ammonia with a broad range of specificities ranging from hydrogen cyanide to various aliphatic and aromatic nitriles (Pace and Brenner, 2001). In general, their natural substrates have not been identified. Because of the inherent enantio-specific nature and selectivity of enzymatic catalysis, nitrilases are used in a variety of industrial processes (Brady et al., 2004). For instance, nitrilases are used to manufacture the active enantiomers, (R)-mandelic acid, (R)-3-chloromandelic acid (Brady et al., 2004), (S)-phenyllactic acid and (R)-3-hydroxy-4-cyano-butyric acid, an important intermediate in the synthesis of the cholesterol-lowering drug atorvastatin calcium (Banerjee et al., 2002; O’Reilly and Turner, 2003). Although microbial treatment of toxic industrial effluent is hampered by the presence of conditions that inhibit microbial growth (reviewed by Baxter and Cummings, 2006), the potential exists for the use of purified microbial nitrilases for on-site cyanide remediation since they may be engineered to tolerate varying levels of pH and temperature, which the organisms themselves
may be sensitive to (Jandhyala et al., 2003). CHTs have been identified in a variety of fungal species, but appear not to occur in bacteria or plants (Wang et al., 1999). They catalyse the conversion of cyanide to formamide with a small fraction (up to 0.4%, Nolan et al., 2003) being converted to formic acid. The different reaction pathways result from the breakdown of a different bond in the tetrahedral thioimidate intermediate formed with the active site cysteine (Jandhyala et al., 2003; reviewed by O’Reilly and Turner, 2003).

Gloeocercospora sorghi is a fungus that infects sorghum. When lesions are formed on sorghum, cyanoglycocides degrade to form hydrogen cyanide (HCN). Expression of CHT is induced in G. sorghi by HCN and this enzyme can account for up to 18% of the protein in the organism (Wang et al., 1992). It was therefore postulated that the purpose of the enzyme was to protect the pathogen in a high cyanide environment; however, expression of CHT is not necessary for infectivity and thus its role in the cell remains uncertain (Wang et al., 1999). G. sorghi CHT has about 30% identity to the bacterial cyanide dihydratases (cynD) from Pseudomonas stutzeri AK61 and Bacillus pumilus C1. Both of these enzymes form self-terminating, homo-oligomeric spirals of 14 and 18 subunits, respectively (Sewell et al., 2005). However, the CHT from G. sorghi has a significantly higher molecular weight (2–10 MDa) (Fry and Millar, 1972), despite having a similar subunit molecular weight of ~40.9 kDa (Wang et al., 1992).

Freeze-drying and unidirectional heavy metal shadowing are an established procedure for investigating biological macromolecular structures (Mes et al., 1990) allowing for unambiguous determination of the handedness. Micrographs of samples prepared using these methods display excellent signal to noise ratio, allowing direct observation of structures at resolutions down to ~20 Å (2 nm) (Hoeenger et al., 2000) without making replicas. The Midilab instrument in which the freeze-drying and shadowing occur in a chamber mounted on the microscope column allows optimal preservation of structural detail by maintaining the sample under vacuum and cryo-conditions during shadowing, transfer into the microscope and viewing (Gross et al., 1990).

Micrographs of unidirectionally shadowed material contain three-dimensional information in the form of pixel density, which is dependent on the angle between the sample surface normal, and the shadowing direction. The thickness of the metal film deposited is directly related to the topography of the sample when viewed from a direction other than the original evaporation source (Gross, 1987). The contrast in the resulting micrograph is therefore related to the surface topography (Guckenberger, 1985). This information, combined with image processing methods, has been used to derive three-dimensional surface reconstructions in the form of digital elevation maps (DEMs) by the method of surface relief reconstruction (Smith and Kistler, 1977; Smith and Ivanov, 1980).

The limitation of these methods is that the resulting map consists of a 2.5-dimensional (2.5D) representation of a three-dimensional object. This limitation has been overcome by applying conventional Fourier–Bessel methods to rotary shadowed filaments which produces a three-dimensional reconstruction of the metal cast, from which the filament can be extracted (Morris et al., 1994). More recently reconstructions have been produced by applying tomographic and single-particle approaches to rotationally shadowed samples (Lanzavecchia et al., 1998, 2005; Lupetti et al., 2005). This allows a complete reconstruction of the metal cast surrounding the object to be made. The advantage of this approach is that there is no topological constraint on the resulting reconstruction.

Although several superb visualizations of helical objects have been made with the Midilab instrument, no attempt has been made to reconstruct such images in three dimensions. Such reconstruction would require that the metal coating be sufficiently thin to allow proportionality between the thickness of the coating and the projected density. The coating applied in the Midilab instrument has a thickness of 0.3–0.4 nm and is thus thin enough to allow reconstruction as the linear relationship between the metal thickness and the absorbance is maintained. Since the shadowing is unidirectional, the amplitudes of the various layer lines are dependent on the orientation of the fibre relative to the shadowing direction. This means that Fourier–Bessel reconstruction cannot be used. However, since the fibres lie at all angles to the shadowing direction a reconstruction method based on averaging techniques could conceivably be used and this should result in a reconstruction of the metal component of the specimen. The ability to average successfully would require that there is sufficient overlap of information in fibres shadowed from different directions to allow for accurate alignment.

We have applied the iterative helical real-space reconstruction method (IHRSR) (Egelman, 2000) here to micrographs of unidirectionally shadowed G. sorghi filaments. IHRSR allows the determination of helical symmetry by iterative refinement. This method applies a single-particle approach, including reference-based alignment and back-projection to boxed segments of the continuous extended fibres. The advantage of IHRSR is that the method can deal with disordered or heterogeneous filaments and is unaffected by the problem of Bessel overlap (Egelman, 2000). Applying IHRSR to metal-shadowed data resulted in the determination of the low-resolution structure of G. sorghi CHT. We show here that the CH from G. sorghi forms extended left-handed helices, built on similar principles to those described for the cyanide dihydratases (Sewell et al., 2005). In parallel, for the purposes of comparison, we have used IHRSR to produce a low-resolution reconstruction from images of the CHT embedded in ice and recorded on a CCD camera. Comparisons between the two reconstructions give considerable insight into the nature of images made with Midilab.
2. Materials and methods

2.1. Expression and purification

The cyanide hydratase was recombinantly expressed in *Escherichia coli* BL21 pLysS by IPTG induction from the plasmid MB2313 (Jandhyala et al., 2005). The cells were pelleted by centrifugation at 4000 g at 4 °C for 20 min and resuspended in 50 mM Tris–HCl, pH 8.0 containing a protease inhibitor cocktail (Roche). Cells were disrupted by sonication (Misonix 3000, US) and the soluble fraction was clarified by centrifugation at 20,000 g at 4 °C for 30 min. The soluble lysate was subjected to ammonium sulfate precipitation at 50% saturation on ice. The resultant fraction was washed three times with 0.45-μm acetate filter (GE-Healthcare). Anion exchange chromatography was performed on a Hi-prep 16/10 Q XL Column (GE-Healthcare) equilibrated with 50 mM Tris–HCl, 100 mM NaCl pH 8.0 and eluted through a 0.45-μm acetate filter (GE-Healthcare). Anion exchange chromatography was performed on a Hi-prep 16/10 Q XL Column (GE-Healthcare) equilibrated with 50 mM Tris–HCl, pH 8.0 at 0.5 ml min⁻¹.

Fractions containing the cyanide hydratase were identified by performing the picric acid colorimetric assay. Twenty microliters of a 100 mM KCN solution (in 50 mM Tris–HCl pH 8.0) was added to 80 μl aliquots of fractionated protein and allowed to incubate at room temperature for 1 h. Equal volumes of 500 mM Na₂CO₃ and 1.5% picric acid in water were mixed and 80 μl added to the protein samples. Samples were placed into boiling water and after 5 min cyanide hydratase containing fractions turned a yellow colour. These fractions were pooled and concentrated using an amicon ultrafiltration membrane with a molecular weight cut off of 10 kDa (Millipore, USA). The concentrated protein fraction was separated on a Sephacryl S-300 HR gel filtration column (GE-Healthcare) equilibrated with 50 mM Tris–HCl, 200 mM NaCl pH 8.0 at 0.5 ml min⁻¹.

2.2. Electron microscopy

Micrographs of unidirectionally shadowed sample were obtained on the Midilab instrument (Gross et al., 1990). Briefly, a drop of purified sample was pipetted onto glow-discharged, carbon-coated grids and allowed to absorb for two to three minutes before being rinsed in distilled water, blotted and rapidly vitrified by plunging into liquid ethane. Electron microscopy was performed at low dose on a Phillips CM 200 FEG operated at 200 kV. Two hundred and twenty images were captured at a magnification of 38,000× using a Tietz 2k × 2k CCD camera (Fig. 1b). Calibration of the images was performed by scaling the calculated power spectrum to match the correctly calibrated Midilab micrographs, yielding a sampling of 3.40 Å/pixel.

2.3. Image processing

Helical segments (526) of 128 × 128 pixels with 92% overlap were selected and aligned using BOXER (Ludtke et al., 1999) from 49 Midilab helices. Four thousand five hundred and twenty-seven segments were selected from ~400 helices from the cryo-dataset. In both cases the segments were band-pass filtered between 270 Å and 25 Å and normalised to a mean of 0 and standard deviation of 1. Boxed helical segments were aligned in a reference-free procedure (Penczek et al., 1992) and averaged in order to improve the SNR; power spectra were calculated using Spider V9.05 (Frank et al., 1996). The IHRSR procedure as described by Egelman (2000) was initiated with a featureless cylinder, using starting values for the helical symmetry predicted by indexing. The cryo-dataset contained a large proportion of out-of-plane filaments; these were identified during reference-based alignment and eliminated. Images were excluded if they fell below a correlation threshold, which was increased every iteration. The final reconstructions were generated with 84 and 507 Midilab and cryo-helical segments, respectively.

Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen et al., 2004).

2.4. Resolution

The protocol for resolution estimation by Fourier shell correlation (FSC) was provided by E.H. Egelman. Briefly, two independent models were created from featureless cylinders by starting at different angles (φ₁ = −65.7°, φ₂ = −66.2°, z₁,₂ = 13 Å), the radial mask function was uncorrelated by using two different maximum helical diameters (90 Å and 93 Å, respectively). The two models were iterated 50 times and converged on the same stable solution. The two volumes were aligned in z and φ and windowed (x₁ = 128, y₁ = 128, z₁ = 110; x₂ = 128, y₂ = 128, z₂ = 116) and subsequently padded to the same volume (x = 128, y = 128, z = 128). The Fourier shell correlation was calculated between the two volumes with a ring size 1 × 10⁶ e⁻²/nm² at a magnification of 52,500× and sampling of 5.3 Å/pixel (Fig. 1a). Samples for cryo-microscopy were produced by pipetting a drop of 0.2–0.3 mg ml⁻¹ purified protein in buffer onto holey-carbon grids, blotted these with filter paper and plunging into liquid ethane. Electron microscopy was performed.
of 1 and plotted in Matlab V6.5 (©Mathworks Ltd.). For a discussion of the various issues involved with resolution determination by FSC see Yang et al. (2003). In the case of the cryo-reconstruction the image stack was split in half.

3. Results

The unprocessed, unidirectionally shadowed micrographs of G. sorghi nitrilase reveal that the enzyme forms a filament with a diameter of ~12 nm. The height of the fibre can be approximated from the length of the shadow cast by the fibre and the shadowing angle (Fig. 1a). The height is similar to the estimated diameter, suggesting that the fibre is not flattened. Prominent one-start left-handed helical striations as well as right-handed apparently four-start helical striations are visible in unprocessed images (Fig. 1a). The fibres seen embedded in vitreous ice in the holes of the carbon film were generally short and not all of them lay in the plane of the carbon film (Fig. 1b).

3.1. Symmetry determination

The power spectrum derived from unidirectionally shadowed helical segments was indexed by comparing the ratios of different principal maxima of different Bessel orders with the experimentally determined ratios obtained from the power spectrum. At the available resolution the data were consistent with three different indexing schemes; each predicting a different order for the $l = 3$ layer-line (Fig. 2). The IHRSR algorithm converged on a different reconstruction, when initiated from each of the three symmetries predicted by the indexing (Fig. 3) and thus failed to resolve the ambiguity. The correct indexing scheme, and therefore the correct reconstruction, was identified by calculating the phases and amplitudes of the Fourier transform of a single cryo-fibre. The uncertain layer-line arising from the front and back of the filament are in phase, indicating that the layer-line order is even (Fig. 4). The correct indexing is therefore the one that results from the unknown layer-line being of order four. The filament has an axial rise of ~13 Å and ~65° rotation per subunit.

Fig. 1. (a) Unprocessed micrograph of cryo-metal-shadowed G. sorghi nitrilase. The shadowing is at an elevation of 45°. The majority of the metal falls onto the front surface of the helix, allowing unambiguous determination of handedness (left-handed). Arrow indicates approximate shadowing direction. (b) Filtered and contrast-enhanced cryo-micrograph of G. Sorghi nitrilase. Asterisk indicates an end-on view of a short filament. Scale-bars indicate 50 nm.

Fig. 2. Power spectrum calculated from vertically aligned helical segments; layer lines arise from the front surface of the filament only, therefore phase information usually used to eliminate the ambiguity associated with indexing helical patterns is unavailable. Layer lines occur at 1/74 Å, 1/49 Å and 1/37 Å. Three indexing schemes are compatible with the data at the resolution available. Red spots indicate the resulting indexing scheme if the unknown layer-line is of order 5 (hence 13 dimers in 2 turns or 13:2), blue spots indicate the indexing scheme that results from the unknown layer-line being order 4 (hence 11:2) and green spots indicate the resulting indexing scheme if the layer-line is of order 3 (hence 9:2). The pitch is ~7 nm.
3.2. Iterative helical real-space reconstruction

The reconstructions converged on stable models, which closely approximated the predicted symmetries. Fig. 5 shows the convergence of the algorithm in the case of a starting model having 11 dimers in two turns for the Midilab dataset.

3.3. Analysis of the Midilab model

The model has an axial rise of ~13 Å and ~66° rotation per subunit (Fig. 5). The one-start helix has a pitch of 7.15 nm and there are 5.46 dimers per turn. The filament has a diameter of ~11.5 nm, a central channel of diameter ~6.2 nm. Sites of interaction homologous to those described in earlier reconstructions of helical and oligomeric nitrilases (e.g. Thuku et al., 2007) were identified (Fig. 6a). The filament has two dyad axes (Fig. 6b), the first passes through the A-surface, marked by a depression indicating the dimer interface, and the hole. The second, lies through the F- and C-surfaces. The C-surface is the site at which adjacent dimers interact. The F-surface represents an interaction occurring across the groove of the one start helix, in this case occurring between dimers \( n \) and \( n + 5 \) however; this interaction is in a different position to that normally seen in nitrilase fibres (Fig. 6a). The helical symmetry is \( D_{24}S_{5.5} \). The resolution was estimated to lie between ~33 Å and ~38 Å (Fig. 7).

3.4. Comparison of the models

The metal shadowed- and cryo-reconstructions correlated to a resolution of 38 Å (FSC = 0.5) (Fig. 7). This value approximates the resolution at which the highest frequency layer-line is visible (37 Å) as well as the estimated resolution of the Midilab reconstruction (33 Å). This similarity is immediately apparent when the aligned metal-shadowed images are compared to the Midilab and cryo-reconstructions (Fig. 8). The helical striations and features, as well as the diameters of the two reconstructions closely resemble the aligned shadowed images as well as each other. In cross-section, the metal component of the Midilab
reconstruction is visible as a hollow cylinder (Fig. 9). When superimposed, the regions of highest protein density of the cryo-reconstruction correlate well with the highest density regions of the metal-shadowed reconstruction (Fig. 9). The reconstructions differ in some important respects however; the diameter of the central channel is smaller in the case of the cryo-reconstruction and the individual subunits of the metal-shadowed reconstruction are less distinct than the equivalent features of the cryo-reconstruction (Figs. 8 and 9).

4. Discussion

4.1. Reconstruction strategies

Surface relief reconstruction (Guckenberger, 1985) provides a 2.5D digital elevation map of the structure of metal-shadowed surfaces. This method has been successfully applied (e.g. Rockel et al., 2000; Walz et al., 1996; Dimmeler et al., 2001) to specimens of appropriate topology. However, the majority of biological structure is incompatible with the digital elevation map (Lupetti et al., 2005). The alternate method is to treat metal-shadowed surfaces as two-dimensional projections of the metal cast. Lanzavecchia et al. (1998) used the method of random conical tilt to reconstruct projections of rotationally shadowed metal replicas. Lanzavecchia et al. (2005) applied the method of conical-tilt tomography to rotationally shadowed specimens of integral membrane proteins. This method allows proteins to be studied, at high resolution, in the cellular milieu. Lupetti et al. (2005) applied a similar method to study dynein arms in situ.

The power spectra of metal-shadowed helices resemble those obtained from negative-stain- and cryo-reconstructions and Fourier–Bessel helical reconstruction methods have been applied to rotationally shadowed actin filaments (Morris et al., 1994). However, the same approach could not be taken in this case because the contrast arising from each fibre provided only partial structural information, as a function of the shadowing direction. In a shadowing experiment, the fibres are randomly oriented on the grid and the relative shadowing direction is different in each case. This
means that different fibre segments do not strictly represent equivalent views. Contrast arises exclusively from the metal coating (Walz et al., 1996), which is on the front side of the filament: this allows unambiguous determination of the handedness of the helix, but provides only half the necessary information required to reconstruct the filament.

4.2. Iterative helical real-space reconstruction

The IHRSR algorithm, in which views of the helix that have been shadowed from different directions are averaged and helical symmetry is imposed during reconstruction, overcomes these limitations. Instead of physically rotating the source; images of the filaments, which have been unidirectionally shadowed from all possible directions, are combined and averaged in the computer. Back-projection of these symmetry related views produces a reconstruction of the average metal distribution surrounding the entire filament—or more accurately, an asymmetric unit within the filament. This, however, requires that the helical segments can be aligned correctly with the reconstruction during projection matching. As evidenced by the similarity of the Midilab reconstruction to the cryo-reconstruction, the method appears to be successful.

It is important to consider what the basis of the apparently successful alignment of filaments from different shadowing directions really is. It can be seen from reference-free alignment of filaments shadowed from different directions (Fig. 10), that even though shadowing from different directions accentuates different features, some common information is preserved, this information is clearly sufficient for the alignment of orthogonal views. A possible alternative explanation is that the protein component of the filament facilitates alignment. However, this seems unlikely because the calculated power spectrum of the metal-shadowed images show that the contrast arises exclusively from the metal covered parts, reflecting the typically asymmetric density distribution expected from unidirectional shadowing i.e. only helical reflections arising from one side of the filament are visible.

In an ideal shadowing experiment, the distribution of metal clusters differs as a function of shadowing direction.

Fig. 8. (a) 2D classified average of the metal-shadowed helical segments, the left-handed one-start helix is indicated by black lines, white lines indicate the right-handed four-start helix. (b) The metal-shadowed- and (c) cryo-reconstructions, thresholded and oriented to approximate (a). Corresponding features can be identified between all three images.

Fig. 9. Successive transverse slices along the reconstructions; each slice represents 5.3 Å. The metal-shadowed reconstruction is contoured in grey. Corresponding slices through the cryo-reconstruction are superimposed and shown in blue. The protein component of the cryo-reconstruction and metal component of the Midilab reconstruction correlate well.
In reality, however, metal may accumulate at preferential nucleation sites on the surface of the protein; this effect is referred to as “decoration” (Weinkauf et al., 1991). Because the position of nucleation sites is independent of shadowing direction, the contrast arising from decoration would presumably aid in alignment. However, with the amount of metal used here, the pure decoration component is likely to be very small. The calculated power spectra (Fig. 10) from individual fibres show a layer-line amplitude dependence on shadowing direction, meaning that the data are predominately unidirectional and precluding Fourier–Bessel type reconstruction strategies.

4.3. Interpretation of the Midilab reconstruction

This is the first attempt to reconstruct unidirectionally metal-shadowed data in three dimensions. Perhaps the most surprising feature of the resulting reconstruction is the unexpected similarity between the Midilab- and cryo-volumes. Rather than surrounding the filament with a thin film, the metal appears to be localized within the protein. There are a number of possible explanations for this observation. One is that it may be an artefact of the process; the thin layer of metal deposited on the surface of the filament is convoluted with a Gaussian function arising from resolution limitations and alignment errors, leading to an apparent thickening of the metal layer. If this were the case it would be expected that the highest density regions of the Midilab reconstruction would correspond to the outside edge of the cryo-reconstruction. This is obviously not the case (Fig. 9). Furthermore, the diameter of the Midilab reconstruction corresponds closely to the diameter of the classified average (Fig. 8).

An alternative explanation is that metal atoms partially penetrate the surface of the protein matrix during the shadowing process and that contrast arising from these atoms allows visualisation of the protein volume. This hypothesis is consistent with the observation that contrast arises exclusively from the front of the filament and yet in cross-section the metal density of the Midilab reconstruction appears to encroach on the protein density of the cryo-reconstruction. This mechanism would maintain the relationship between the sample surface normal and the corresponding pixel density used to calculate the digital elevation map. A further alternative explanation is that the internal density is an artefact of the back projection algorithm. In any case, the surface structure is preserved.

4.4. Biological Interpretation

The interaction occurring across the groove, termed the D-surface in the cyanide dihydratases (Sewell et al., 2003; Jandhyala et al., 2003) and Rhodococcus rhodochrous J1 nitrilase (Thuku et al., 2007) is found in a different location in the G. sorghi cyanide hydratase metal shadowed- and cryo-reconstructions. The position of this interaction, termed the F-surface here, has implications for the stabilization of the filament; an important consideration in industrial applications. In purely geometric terms; this change in interaction position is necessary to accommodate the decreased twist of the G. sorghi filament which has 5.6 dimers per turn compared to 4.9 dimers per turn in the nitrilase from R. rhodochrous J1. Both of these interactions occur between dimers n and n + 5. We have shown here that the G. sorghi cyanide hydratase is unambiguously left-handed.

5. Conclusions

The helical structure of G. sorghi CHT has been determined at a resolution of ~33 Å by applying the IHRSR algorithm to freeze-dried unidirectionally shadowed filaments. These filaments have been unambiguously determined to be left-handed. The reconstruction is plausible, but so are those which converge on the incorrect helical symmetry. Therefore considerable care needs to be taken
when indexing the power spectrum. Surprisingly, the resulting reconstruction closely resembles that obtained using conventional cryo-EM methods. The final reconstruction suggests interactions across the groove of the one start helix in the case of the CHT differ from those previously seen in negative stain reconstructions of the cyanide dihydratases from P. stutzeri and B. pumilus and the nitrilase from R. rhodococcus J1.

Acknowledgments

We thank Heinz Gross and Peter Tittmann for generously giving us access to the Midilab instrument; Edward H. Egelman for his considerable assistance with IHRSR; the National Research Foundation and the Carnegie Corporation of New York for their financial support.

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