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Crystallization Notes

Sharpening high resolution information in single particle electron cryomicroscopy

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ABSTRACT

Advances in single particle electron cryomicroscopy have made possible to elucidate routinely the structure of biological specimens at subnanometer resolution. At this resolution, secondary structure elements are discernable by their signature. However, identification and interpretation of high resolution structural features are hindered by the contrast loss caused by experimental and computational factors. This contrast loss is traditionally modeled by a Gaussian decay of structure factors with a temperature factor, or *B*-factor. Standard restoration procedures usually sharpen the experimental maps either by applying a Gaussian function with an inverse *ad hoc B*-factor, or according to the amplitude decay of a reference structure. EM-BFACTOR is a program that has been designed to widely facilitate the use of the novel method for objective *B*-factor determination and contrast restoration introduced by Rosenthal and Henderson [Rosenthal, P.B., Henderson, R., 2003. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J. Mol. Biol. 333, 721–745]. The program has been developed to interact with the most common packages for single particle electron cryomicroscopy. This sharpening method has been further investigated via EM-BFACTOR, concluding that it helps to unravel the high resolution molecular features concealed in experimental density maps, thereby making them better suited for interpretation. Therefore, the method may facilitate the analysis of experimental data in high resolution single particle electron cryomicroscopy.

1. Introduction

Single particle electron cryomicroscopy allows structure elucidation of unstained proteins and macromolecular assemblies at subnanometer resolution thanks to the technical and computational advances made during the last years (Henderson, 2004; Chiu et al., 2005; Frank, 2006). This experimental approach has made possible the structure determination of specimens with different symmetry levels in a resolution range of 4–9 Å, e.g. ribosome (Halic et al., 2006), groEL (Ranson et al., 2006; Ludtke et al., 2008), Bacteriophage T7 connector (Agirrezabala et al., 2005), and several icosahedral structures (e.g. Luque et al., 2007; Jiang et al., 2008; Zhang et al., 2008). There are exciting prospects that resolution levels adequate to build atomic models will soon be approachable with this technique (Henderson, 2004; Zhou, 2008).

The main objective to reach high resolution is driven by the fact that density maps determined at subnanometer resolution allow identification of secondary structure elements (SSE) by their signature (Chiu et al., 2005; Baker et al., 2007). In the range 6–10 Å, α-helices become visible as straight rods of density and β-sheets appear as continuous planes. Beyond 5–6 Å, loop densities may be discernable and the protein backbone may be traced. At resolutions higher than 4.5 Å, individual strands within the β-sheets may be observed. Eventually, resolutions better than 4 Å will allow the assignment of a sequence of aminoacid side chains into density features in the map (Henderson, 2004).

Identification and interpretation of the structural features in a map are, however, hampered by the loss of contrast at high resolution. This degradation of the image contrast is caused, in a great extent, by factors related to the experimental imaging process (e.g. specimen movement and charging, radiation damage, partial microscope coherence, etc.) (Henderson, 1992; Wade, 1992), as well as by factors related to the computational procedures for structure determination (e.g. inaccurate determination of the orientation parameters, etc.) (Conway and Steven, 1999; Rosenthal and Henderson, 2003; Henderson, 2004). The combined effect of all these factors has been traditionally modeled by a Gaussian amplitude decay of structure factors, given by $e^{-(B_{overall}/4d^2)}$ with an overall temperature factor $B_{overall}$ (also called *B*-factor) and where *d* denotes resolution (Å) (Glaeser and Downing, 1992; Conway and Steven, 1999; Rosenthal and Henderson, 2003). This amplitude fall-off significantly affects the high resolution components, thereby making the density map look apparently smooth and turning identification of SSE into a challenging task. Therefore, it is neces-

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sary to restore (also called sharpen) these components in order to bring out the structural features concealed in the map.

There have been several strategies to sharpen density maps in single particle electron cryomicroscopy. One common approach consists of correcting the amplitude fall-off by applying a *B*-factor in the form $e^{-(B_{restore}/4d^2)}$, where $B_{restore}$ is the negative of the estimated *B*-factor describing the contrast loss of the experimental data (e.g. Bottcher et al., 1997; Fotin et al., 2004). Other approaches use the X-ray solution scattering curve of the specimen (or a related homologue) to estimate the decay and correct the amplitudes accordingly (e.g. Gabashvili et al., 2000; Agirrezabala et al., 2005; Luque et al., 2007; Ludtke et al., 2008).

Rosenthal and Henderson (2003) introduced a method for estimating the structure factor amplitude decay by comparison with an approximate theoretical scattering curve. This method allows objective sharpening of density maps, with neither further needs of *ad hoc B*-factors, nor the availability of an X-ray scattering reference. Moreover, that work also showed that a weighting function accounting for the noise in the map is essential to avoid its amplification, while at the same time increasing the robustness against oversharpening.

Here, we introduce a program, EM-BFACTOR, that was developed to facilitate the general use of the method devised by Rosenthal and Henderson (2003). It allows automated determination of the *B*-factor for high resolution maps as well as noise-weighted sharpening of the density map to enhance features without noise amplification. Furthermore, the structure factor amplitudes of the map are also placed on an absolute scale. We have also further investigated on the sharpening method, and here, we describe its performance on several illustrative examples.

2. Methodology and implementation

A Guinier plot shows the natural logarithm of the spherically averaged structure factor amplitudes of a map as a function of the resolution $(1/d^2)$. The low resolution region (to about 10 Å) is determined by the shape of the protein and the solvent contrast and shows a steep curve that gets the maximum scattering amplitude at zero, rapidly decaying afterwards. Beyond 10 Å, the amplitude depends on the specific structural features of the protein. In that range, the average scattering amplitude is determined by the random position of atoms in the protein and remains steadily constant with resolution, according to Wilson (1942). For an in-depth theoretical background, refer to (Rosenthal and Henderson, 2003).

The approach by Rosenthal and Henderson (2003) for *B*-factor determination relies on the fact that the high resolution contrast loss in an experimental map may be observed in a Guinier plot as the decay of its spherically averaged amplitude (F) compared to the relatively flat theoretical scattering profile corresponding to the Wilson regime. In order to better estimate the *B*-factor, the noise-weighted amplitude $C_{ref}F$ is used as it represents more properly the average signal in the experimental map. The term C_{ref}, which is computed by resolution shells from a smoothed version of the FSC (Fourier Shell Correlation) as $\sqrt{2\mathrm{FSC}/(1+\mathrm{FSC})}$, gives a measure of the average signal-to-noise ratio (SNR) in the reciprocal space and, hence, of the reliability in the structure factors.

Our procedure for automated *B*-factor determination calculates the line that best fits the decay of the spherically averaged amplitude $C_{ref}F$ by a least squares procedure. For the fitting, a range of resolution is used, typically [10–15Å, R_{max}], where R_{max} is the maximum resolution in the map as assessed by means of the FSC. The *B*-factor affecting the experimental map is then determined from the slope of the line fitted. The *B*-factor for sharpening ($B_{restore}$) is thus set as the negative of the *B*-factor affecting the data.

Contrast restoration is carried out by applying the *B*-factor in the form $e^{-(B_{restore}/4d^2)}$. This type of restoration amplifies signal and noise regardless of the SNR, which may be especially dangerous at high resolution where the signal is weak. To attenuate the amplification of high resolution noise, a common solution is based on low-pass filtering. Rosenthal and Henderson (2003) devised an approach based on a down-weighting of resolution shells where noise is dominant by means of C_{ref}. This noise-weighting has been shown to avoid noise intensification, increase the robustness against oversharpening and reduce the sensitivity to the resolution cutoff of the map. Therefore, the amplitudes F are restored in the form $C_{ref}Fe^{-(B_{restore}/4d^2)}$. On the other hand, the experimental scattering amplitudes may be placed on an absolute scale by setting the zero angle scattering equal to $0.28 \times N_{atoms}$ and the average amplitude in the high resolution region (Wilson regime) to $\sqrt{N_{atoms}}$. N_{atoms} denotes the number of carbon atom equivalents corresponding to the molecular mass of the protein, and 0.28 is the solvent contrast factor.

The program EM-BFACTOR has been developed for automated *B*-factor determination and sharpening of high resolution density maps as described above. Application of a specific value for *B*-factor is also possible. The program accepts 3D maps in any format common in EM (e.g. MRC, Spider, PIF, EM, Xmipp, etc.) by using the Bsoft library (Heymann and Belnap, 2007). EM-BFACTOR accepts FSC curves in the format of most common packages for single particle electron cryomicroscopy (Frealign, Spider, EMAN, Xmipp, Bsoft). The output sharpened map, placed on an absolute scale, can be written in any format common in EM. The program also generates a Guinier plot with the average structure factor amplitudes of the original map, the noise-weighted map and the sharpened map. The zero angle scattering and Wilson statistics used for scaling the map are also indicated. This plot, which is generated in Postscript file and in text format, can be used for assessment of the sharpening. An output report is shown on console with the progress of the program, the parameters for the least squares fitting and the *B*-factor that is finally found out. The command line user interface follows the Unix-style and the options follows the conventions of Bsoft (Heymann and Belnap, 2007). Examples of commands and the output report are shown in Appendix. EM-BFACTOR and a comprehensive documentation will be available for public use at `http://www.ual.es/~jjfdez/SW/embfactor` and through the wikipedia "Software tools for molecular microscopy".

3. Illustrative examples

EM-BFACTOR was tested on experimental 3D maps of several specimens to further investigate the method for automated *B*-factor determination and sharpening, and for illustrative purposes. First, we worked on two specimens whose structure has been solved at subnanometer resolution in our laboratory: Infectious bursal disease virus T = 1 subviral particle (IBDV T = 1 SVP) (Luque et al., 2007) and Bacteriophage T7 connector (Agirrezabala et al., 2005), solved at 7.2 and 8.0 Å resolution, respectively. Resolution was assessed by the standard technique based on the Fourier shell correlation (FSC) calculated between maps obtained from independent halves of the raw dataset at a 0.5 cutoff. IBDV T = 1 SVP was solved by X-ray crystallography and electron cryomicroscopy (Coulibaly et al., 2005; Luque et al., 2007), and it is thus adequate for objective assessment of the proposed contrast restoration methodology. The T7 connector map was sharpened according to the decay of the X-ray scattering amplitude of a homologous specimen, the Bacteriophage ϕ29 connector (Agirrezabala et al., 2005). Therefore, this represents a complementary analysis to evaluate the performance of that sharpening strategy and whether it is possible further improvement. Second, we focused on several maps