Fitting of High-Resolution Structures into Electron Microscopy Reconstruction Images

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Summary

Dynamic macromolecular assemblies, such as ribosomes, viruses, and muscle protein complexes, are often more amenable to visualization by electron microscopy than by high-resolution X-ray crystallography or NMR. When high-resolution structures of component structures are available, it is possible to build an atomic model that gives information about the molecular interactions at greater detail than the experimental resolution, due to constraints of modeling placed upon the interpretation. There are now several competing computational methods to search systematically for orientations and positions of components that match the experimental image density, and continuing developments will be reviewed. Attention is now also moving toward the related task of optimization, with flexible and/or multifragment models and sometimes with stereochemically restrained refinement methods. This paper will review the various approaches and describe advances in the authors’ methods and applications of real-space refinement.

Introduction

In the last 15 years, there have been exponentially increasing opportunities to combine electron microscopic imaging of large complexes with high-resolution structure determination of the component parts. The potential for such investigations was first demonstrated in the structural biology of viruses and muscle, and they are now being used to understand protein translation, protein degradation, and many other cellular processes.

Research on adenovirus in the laboratories of Burnett and Fuller set the stage (Furcinitti et al., 1988; Stewart et al., 1993a, 1993b). By fitting crystallographic structures of known components to electron microscopic (EM) images of the whole virus, then subtracting their calculated density, it was possible to locate minor components. At about the same time, antibody-virus complexes were being visualized by cryo-EM (Prasad et al., 1990). With superimposed structures for the virus and antibody (homolog), the binding footprint was mapped (Wang et al., 1992). Similar studies in the laboratories of Baker and Rossmann revealed the molecular interactions of the common cold virus (human rhinovirus, HRV) with neutralizing antibodies and fragments of the virus’ cellular receptor (Olson et al., 1993; Smith et al., 1993a, 1993b). Collaborations between the groups of Raymond, Holmes, and Milligan in fitting crystallographic structures of actin and myosin domains to images of muscle protein complexes started to provide details of the molecular interactions in muscle contraction (Raymond et al., 1993a; Schröder et al., 1993). It was now clear that the constrained fitting of an atomic model to low-resolution electron microscopic images could yield “pseudo-atomic precision” in which model atoms could, it was proposed, be placed with an accuracy of 4- to 5-fold better than the nominal experimental resolution (Baker and Johnson, 1996; Rossmann, 2000). Thus, 4 Å detail could be interpreted from a map at 20 Å resolution. Justification was by analogy to crystallographic structure where ~0.5 Å model precision is attainable with ~3 Å data when the modeling is restrained/constrained to agree with standard stereochemistry determined from more detailed studies of model compounds. In the modeling of EM images, if the fitting is constrained to rigid motions of previously determined component structures, then there is relatively little freedom in the placement of individual atoms, leading to a precision well beyond the nominal resolution limit. With this realization came an explosion of joint EM/crystallographic studies in the mid-1990s (reviewed in Baker and Johnson, 1996). “Manual” modeling involved the use of interactive graphics to fit (subjectively) the known structures within the envelope (an isocontour) of the EM electron density. Soon, attempts were being made to make the fitting more quantitative and automated, borrowing from crystallographic technology.

Several challenges became apparent. At low (~20 Å) resolution, the electron density may not have sufficiently distinctive features for an unambiguous placement of a component. This was illustrated in the differences between parallel studies of poliovirus bound to fragments of its cellular receptor (Belnap et al., 2000; He et al., 2000). There was a need to search all possible configurations and quantify the fit to determine whether such ambiguities are resolvable. The last decade has seen the development of several computational algorithms for this “global search” that will be reviewed here. The article will also present methods for refining pseudo-atomic models and their application to several systems. There are common requirements of the global search and fine refinement, but distinctions that can lead to different algorithmic choices. They can be considered as separate but interdependent steps.

Global Search through Reduced Vector Representations

The assessment of model fit must be fast enough to support a search through a huge configurational space. The electron density maps, one experimental (ρexp) and one calculated from the model (ρcalc), are represented on finely spaced 3D grids requiring many-to-many comparisons for each point in a 6N-dimensional search for N rigid groups. The search has been accelerated.
through reduced real-space representations of the maps or through Fourier techniques.

SITUS, the popular program of Wriggers and colleagues, reduces the representation of maps to a handful of "codebook vectors" that describe the prominent features in skeletonized form (Wriggers et al., 1998; Wriggers and Birman, 2001; Wriggers and Chacon, 2001; Chacon and Wriggers, 2002). It is then tractable to search all possible pairings of experimental and model vectors to find the best mean square fit. The approach can be extended to the incorporation of plastic distortions to represent conformational changes that might have occurred as component structures are assembled, or as they change between functional states. A limitation is that the vector quantization applies to the entire electron density. If the model represents only part of the experimental density, a good match of codebook vectors cannot be expected unless the density is premasked, possibly leading to a prejudged outcome.

The Choice between Real- and Reciprocal-Space Target Functions

Direct real-space comparisons of electron densities are generally too inefficient for global searches. The speed can be dramatically improved through Fourier transformation so that the comparison is computed in reciprocal space. By Parseval's (Rayleigh's) theorem, there should exist equivalent real- and reciprocal-space formulations. Differences in the practical implementations mean that the real- and reciprocal-space methods are just first approximations of each other. The distinctions have been glossed over in the literature, resulting in a poor understanding of important differences.

The best-fit model should, in the least-squares sense be the one with minimal:

$$ R_{\text{lsq}} = \left( \frac{\rho_{\text{obs}}(x) - \lambda \rho_{\text{calc}}(x,m)}{\rho_{\text{calc}}(x,m)} \right)^2 d^2 x, $$

where $\lambda$ is a scaling function, $x$ is a position vector for points in the integration volume $X$, and $m$ is the vector of atomic parameters. Minimization of $R_{\text{lsq}}$ is equivalent to maximization of the linear correlation coefficient:

$$ C_{\text{lin}} = \int_X \rho_{\text{obs}}(x) \rho_{\text{calc}}(x,m) d^2 x $$

with the advantage that it may be scaling independent. Equation 2 assumes that the maps have been normalized so that the denominator of the correlation coefficient is unity. Both Equation 1 and Equation 2 have Fourier equivalents that are faster to calculate. For example, Equation 1 is equivalent to minimizing (with important caveats to be discussed later):

$$ R_{\text{lsq}} = \sum_h \left( F_{\text{obs}}(h) - \lambda F_{\text{calc}}(h,m) \right)^2 d^2 h, $$

where $F$ are the complex (phased) Fourier coefficients of the transformed experimental and model maps at each point, $h$, in reciprocal space. A summation has replaced the integral, as it will usually be computed through discrete Fourier transformation (DFT). Equation 3 is exactly the target used in "vector" refinement, a variant of the conventional reciprocal-space crystallographic least-squares residual:

$$ R_{\text{lsq}} = \sum_h (F_{\text{obs}}(h) - \lambda F_{\text{calc}}(h,m))^2 d^2 h $$

noting now that it is scalar, not complex, Fourier amplitudes that are compared. Equation 3 and Equation 4 provide a convenient way of using existing crystallographic software to optimize the fit of models to electron microscope reconstructions (Navaza et al., 2002). Equation 3 is preferred over Equation 4 for electron microscopy because it preserves the experimental phase information that is more reliable than in crystallography. In crystallography, Equation 3 is sometimes misleadingly described as real-space refinement, but for reasons to be discussed, it is at best pseudo-real-space refinement, achieving an effect in reciprocal space that is similar to fitting a model to a map in real space. Equation 3 includes both real and imaginary components of the Fourier coefficients. It may be transformed for the joint-explicit fitting of amplitudes and phase angles, allowing for weighting according to their respective errors. This approach was used by Hogle and Steven for EM analysis of poliovirus-receptor complexes (Belnap et al., 2000).

Superficial invocation of Parseval's theorem led many to think that these reciprocal-space formulations are equivalent to real-space fitting. In fact, Equation 3 is only equivalent to Equation 1 if the integration volume in Equation 1 includes the entire sample, or the entire (artificial) periodic unit cell implied in discretization of the Fourier transform. (Least-squares weighting can also lead to inequivalences.) Perhaps surprisingly, the integration volume is critical and can limit the utility of reciprocal-space approaches. The reciprocal-space methods are "global," fitting the model to the entire electron density. When fitting a component to the density of a larger assembly, it has been found that, at resolutions worse than 10 or 15 Å, the optimal value of the target function rarely corresponds to the true solution (Roseman, 2000; Wriggers and Chacon, 2001). There can be several reasons for this: (a) at low resolution, the optimum of the target function is often at the center of the experimental density; (b) there may be many (local) optima in the target function with similar numerical values; and (c) background effects, series termination, etc. may affect the (global) scaling of the model and experimental maps so that the true placement is not an optimum in the target function (Roseman, 2000; Wriggers and Chacon, 2001). From X-ray crystallography, we have also learned that refinements of a partial atomic model against diffraction from the whole molecule are highly susceptible to overfitting (Hodel et al., 1992). No model is fully complete, usually ignoring disorder, motion, and solvent. These unmodeled features will be larger components at low resolution (EM) than high (X-ray). Overfitting becomes a concern in EM refinements that allow for flexible or multiframe modeling.

Several approaches have been proposed to mitigate the errors of global refinement. One of several components can be better refined if the surrounding density is masked, or if difference density is used after subtract-
ing the calculated density of other components. This is easiest when some components have predefined positions. Thus, icosahedral viruses can be positioned by their symmetry and subtracted from the density of complexes to yield density for receptor or antibody fragments alone (Rossmann, 2000; Thouvenin and Hewart, 2000). More generally, difference methods can be iterated, refining one component after subtracting the density of approximated neighbors before cycling to the next component. This approach is embodied in the program COAN as applied to muscle protein complexes (Volkmann and Hanein, 1999, 2003; Volkmann et al., 2003). The limitation is that errors in the placement of masked/subtracted components, or in their scaling to the experimental density, impacts the refinement (Thouvenin and Hewart, 2000).

Overlap integral refinement targets can limit the tendency of components to move to the center of an assembly's density. Most target functions attempt to capture density variation within the assembly, even if this is less than the contrast between macromolecule and solvent (Baker and Johnson, 1996; Wriggers and Chacon, 2001). However, in EMFIT, the target function is the sum of experimental density values at each model atom position (Rossmann, 2000). This reaches a maximum as soon as the model fits within an envelope (isocontour) of the experimental map, and it is not further improved by moving the model to the center of the density.

Crosscorrelation between the Laplacian-filtered model and experimental densities can be used for the same effect (Chacon and Wriggers, 2002). The Laplacian is the diagonal of the curvature matrix of second derivatives. The optimization takes on features of edge matching, limiting movement of components to the center of the assembly. The target remains a global correlation coefficient, so fast calculation is possible in reciprocal space. Use of numerical second derivatives raises concerns about experimental noise. Simulations indicate that the method should be robust at resolutions better than 20 Å, but greater experience with the signal/noise of real maps is needed (Chacon and Wriggers, 2002).

Masking is the approach used in DOCKEM (Roseman, 2000). The only density considered is that within an envelope enclosing the part of the model being refined; hence, the method is referred to as “local correlation.” The envelope is updated automatically, and the method shares some of the advantages of local search methods (see below), but local rescaling is required, so acceleration through Fourier transformation is not possible.

None of the above-described methods account for the overlap in density between components that is substantial at low resolution. Bernie Brooks’ group has developed a weighting scheme that emphasizes the regions most internal to the EM density and component models, where the effects of altered conformation and overlapping density are likely to be least (Wu et al., 2003). It works best with Laplacian-filtered crosscorrelation (which is counterintuitive, because the Laplacian emphasizes the edges). The weighted target has been implemented in an extension of CHARMM (Brooks et al., 1983) that performs global searches efficiently with simulated data, but results with real images and multicompartment assemblies have not yet been presented.

Overall Strengths and Limitations of Existing Methods

The first objective in a multiresolution structural study is a unique and unambiguous approximate placement of the high-resolution component models within the assembly's density. Often this will involve an exhaustive 6N-dimensional search, where N is a small number of components, say three or less. The methods discussed above as implemented in SITUS (Wriggers et al., 1999), COAN (Volkmann and Hanein, 1999), EMFIT (Rossman, 2000), or DOCKEM (Roseman, 2000) (to be compared later) are giving good results, although the methods continue to be developed. Approximations invoked in making exhaustive searches tractable have sometimes compromised the final model precision, leaving a need for additional refinement methods.

The methods discussed so far are not suitable for large complexes with many free-floating domains, or for higher-resolution EM reconstructions in which flexibility might be modeled through fragmentation into subdomains. An example would be the 165 rigid fragments refined in dynamics studies of the ribosome (Gao et al., 2003). Overlap integrals and Laplacians cannot be expected to yield good results for components in the middle of the assembly. Multibody refinements are expected to be nonlinear, in the sense that the fit of one component depends on the configurations of its neighbors. The methods described above assume near linearity of optimization, in the sense that each component should dominate, at least locally, the experimental image, so that the placement of each component can be determined independently of other components. In multibody refinement, ill conditioning and poor convergence are to be expected. Of the methods discussed, local correlation (with iterative masking) should fare the best. With components much larger than the resolution limit, acceptable results should be obtained. With objects of size commensurate with the resolution, the effects of truncation error in smearing the electron density may mean that ~½ of the object’s density lies beyond its envelope, and that, within its envelope, neighbors combined may account for the majority of the overall density. A different approach is required to push the limits of interpretation in these multiresolution studies.

In addition to reviewing the several refinement methods available, this paper describes further development of the RSRef real-space method (Chapman, 1995; Chen et al., 2001, 2003; Gao et al., 2003). It gives robust results over a wide range of upper resolution limits from 70 to 11 Å in electron microscopic applications (and 4–1.2 Å in crystallographic applications). It is not sensitive to neighboring components that may be missing from the model. Flexibility can be accommodated by dividing the model into stereologically restrained rigid fragments. Finally, it has been possible to refine estimates of the microscope magnification and selected contrast transfer function parameters through optimization of the agreement of the model with the reconstructed EM image.
Results and Discussion

This section describes a “true” real-space refinement method and the results of several example applications. The algorithm is not fast enough for a global search, but the objective was precise refinement, assuming that an initial model could be built by interactive molecular graphics or the automated searches discussed above. Real-space refinement would support finer model fragmentation if the resolution allowed for analysis of the conformational changes upon assembly or between different functional states that had been imaged.

The forerunner was a crystallographic refinement method that was most useful at resolutions lower than usually needed, but when phase errors and therefore map quality were otherwise excellent (Chapman, 1995; Chapman and Rossmann, 1996). It was therefore natural to test whether the methodology could be extended to even lower EM resolutions. Several test cases have been examined with the then-current version of the crystallographic program. It is only recently that EM refinement has become an independent goal; so, although being used by a number of groups, development continues. This section will summarize the latest implementation and will discuss EM applications and experience from crystallographic use relevant to EM refinements.

Real-Space Target Function

As the size of the fitted fragments diminish and their numbers increase, the advantages of Fourier methods decrease. Direct calculation of the density avoids interpolation, because the model density can be calculated at the experimental map grid points. The density of the current model is calculated as the sum of contributing atoms. Each is calculated by numerical approximation to the Fourier integral of the spherically symmetric electronic scattering factor (Doyle and Turner, 1968; Cowley, 1995).

$$\rho_{\text{calc}}(r) = \frac{1}{(2\pi)^3} \int_{h_{\text{min}}}^{h_{\text{max}}} C(h) g(h)(\pi/2)/(2\pi rh)^{1/2} \cdot J_{3/2}(2\pi rh) dh \quad (5)$$

(adapted from Chapman, 1995), where $r$ is the distance from an atomic center; $i$ is contrast transfer correction (discussed later), which may optionally differ from unity; $g$ is the electron scattering factor, a function of the reciprocal-space distance, $h$; and $J$ is a $3/2$-order Bessel function. The reciprocals of the experimental resolution range ($h_{\text{min}} = 1/d_{\text{low}}$, $h_{\text{max}} = 1/d_{\text{high}}$) are used for integration limits, rigorously incorporating truncation effects. This is in contrast to the more usual use of ad hoc Gaussian real-space point spread functions (Diamond, 1971; Jones and Liljas, 1984) that are increasingly inaccurate at low resolution (Chapman, 1995).

The contributions from all atoms (Equation 5) are summed for calculation of a residual (Equation 1). Partial derivatives of the residual with respect to each atomic parameter are calculated analytically for gradient descent optimization. The integration volume $X$ (Equation 1) includes all observed map grid points within a certain distance $(r_{\text{ref}})$ of any refining atom. The contributions of neighboring atoms are included whether or not they are refining. Thus, unlike the other local correlation method (Roseman, 2000), there is no need to mask around a refining object, as smearing and density overlap are properly accounted for.

Several Implementations

Developmentally, the initial goal was to test the refinement target function, and there was no need to reinvent methods for molecular optimization or stereochemical restraint that were well developed by others. The real-space target function was therefore developed as a module for insertion within existing crystallographic refinement packages. The first implementation was for TNT (Tronrud et al., 1987) and supported stereochemically restrained least-squares optimization. The crystallographic real-space refinement (Chapman, 1995; Blanc and Chapman, 1997) was extended for EM with support for electronic scattering factors and contrast transfer functions with predefined parameters. This implementation was used in refinements of acto-myosin complexes (Chen et al., 2001) and the ribosome (Gao et al., 2003). A crystallographic real-space refinement was then implemented within X-Plor (Brünger et al., 1987, 1992) to test the impact of simulated annealing and torsion angle model parameterization (Rice and Brünger, 1994). This version, without EM-specific modifications, was used in tests of convergence and accuracy with simulated EM data (Chen et al., 2003). X-Plor has, in most circles, now been replaced by CNS (Brünger et al., 1998a). Our latest version consolidated and extended the EM-specific extensions within a CNS module, and they now support simulated-annealing molecular dynamics (Brünger et al., 1990, 1998b, 1999) in addition to least-squares optimizations, and torsion angle model parameterization (Rice and Brünger, 1994) as well as rigid-group optimization.

An advantage of adapting crystallographic refinement packages for EM refinement is that full panel of stereochemical restraints is available. This is important at low resolution, where the goal may be correction of poor contacts while remaining consistent with the experimental image (Chen et al., 2001). A disadvantage of the current real-space implementation is that calculations are atom based and unnecessarily inefficient for many-atom rigid objects. Large assemblies take several hours/cycle, although future improvements in computational efficiency are possible. Performance has been systematically evaluated with simulated EM data and the X-Plor implementation (Chen et al., 2003). At 20 Å resolution, the convergence radius is ~15 Å. With a signal/noise ratio of 1:4 (worse than expected), the precision was better than 1 Å at 20 Å resolution.

Refinement of Imaging Parameters

A recent focus has been a function $C(h)$ (Equation 5) and a magnification adjustment that allow for isotropic corrections for EM imaging effects. $C(h)$ incorporates an exponential envelope function to approximate the effects of beam incoherence, inelastic scattering and sam-
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Example Real-Space EM Refinements

This section describes some of the recent collaborative applications of the stereochemically restrained real-space refinement developed by our group. 70S E. coli Ribosome Dynamics

Two states functionally relevant to protein elongation were studied: the initiation-like state, and the elongation-like state. Challenges in evaluating EM refinement methods include the lack of: (1) EM images from assemblies of independently determined structures; (2) a gold-standard refinement against which new methods can be compared; (3) crossvalidated objective measures of model quality. It is sometimes helpful then to extrapolate to EM resolutions from better characterized X-ray refinements.

Mannose binding protein A (MBPA) was selected as a test system (Chen et al., 1999) due to high-accuracy phases and maps obtained through anomalous diffraction studies of a lanthanide-substituted sample (Burling et al., 1996). The starting point was an independent 2.3 Å structure (Weis et al., 1991). Test refinements were performed without “manual” adjustments and were compared to the “correct” 1.8 Å resolution structure that had been iteratively refined and manually adjusted (Burling et al., 1996). Evaluation was by root mean square deviation (rmsd) of coordinates, by the crossvalidated R_free (Kleywegt and Brünger, 1996; Brünger, 1997), and by ∆R R_free - R, a measure of overfitting (Kleywegt and Brünger, 1996; Brünger, 1997). Real-space refinement was superior even to maximum likelihood methods, which are not yet available for EM. Compared to conventional Fourier amplitude least-squares refinement, real-space refinement was 50% more precise (by rmsd), about 2% better in R_free, and showed no overfitting, compared to ∆R = 4% in Fourier amplitude refinement. Compared to “Vector” Fourier refinements (which are also applicable to EM), real-space refinement was 25% more precise and 2% less overfit.

These results illustrate how real- and reciprocal-space (or pseudo-real-space) methods are not strictly equivalent. Hodel et al. (1992) showed how overfitting results largely from model adjustments made in reciprocal-space refinement to correct for errors (or omissions) in remote parts of the model. All reciprocal-space methods are inherently susceptible, because each Fourier coefficient has contributions coming from all atoms simultaneously. Providing that the phases are not biased, real-space refinement avoids such overfitting because the refinement is local, meaning that atoms are being compared only to the neighboring density.

Extrapolating from crystallography, refinements are expected to be increasingly susceptible to overfitting at lower resolution, and/or as the number of model parameters increases (Kleywegt and Brünger, 1996; Brünger, 1997). Overfitting is unlikely to be severe if EM refinement is limited to the rotations and translations of a few rigid bodies. However, with more detailed interpretations possible with flexible or many-body refinements, it is important to perform the refinement with a local real-space method.
Figure 1. Optimization of the Imaging Parameters for Heavy Meromyosin and the 70S Ribosome

The heavy meromyosin is shown with light shading, and the 70S ribosome is shown with dark shading.

(A) Magnification. Relative to that initially assumed, the figure shows that a significant improvement could be made for the 70S ribosome.

(B and C) Envelope function $d$ parameter (attenuation) showing that, in both cases, the fit to the experimental density could be improved.

(D) Defocus parameter. The search for heavy meromyosin failed to indicate any change in average defocus ($\delta$), and, for the ribosome, it was insignificant (a change of 0.015 Å), as the search started close to the maximal correlations.

In both refinements, the assembly was broken into rigid fragments, starting with each protein and RNA domain as a single rigid group and with standard restraints against overlap. Iteratively, the model was subdivided into regions of poor fit or stereochemical overlap, breaking about $\frac{1}{4}$ of the proteins into their constituent domains and allowing hinges in the single-stranded parts of the RNA. This was a subjective process, but conservative in that rigid groups were only subdivided in regions where the number of poor contacts indicated that subdivision would be required to obtain a good fit to the density. Subdivisions were made only in the loops between protein domains and in regions of nucleic acid that were outside the secondary structures. By the end, the RNA had been divided into 70 rigid groups, which, with the proteins, led to a total of 165 fragments. For the initiation-like state, the correlation coefficient increased from 0.53 to 0.71. Equally important, the number of poor van der Waals contacts fell from 10,000 to 1,900 (Table 1). For the EF-G-GTP bound state, the correlation coefficient was increased from 0.37 to 0.67 with a reduction in poor van der Waals contacts to 1,200. The refinement is robust, with tests indicating that it is possible to converge on the correct model starting from the other state. The largest changes are seen in some of the protein subunits (Figure 2), with translations of up to 15 Å and rotations of up to 19°, though most of the changes are more subtle (3–4 Å). The biological implications of the refinement were to localize most of the conformational changes to the protein components that had been previously viewed as passive structural elements, and to characterize the ratchet-like motions that help to ensure unidirectionality of peptide elongation (Gao et al., 2003).

More recent efforts have explored further improvement with optimization of imaging and refinement parameters using the newer CNS implementation of RSRef. For expediency, tests have focused on the 30S subunit, refined against the 11.5 Å experimental density for the entire 70S ribosome initiation-like complex described above. Refinements started with the manually built 30S model as a single rigid group during which the
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Table 1. Example EM Refinements by RSRef

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<th>Structures</th>
<th>Imaging/Resolution (Å)</th>
<th>RSRef Implementation</th>
<th>Fragments: Number/Average Size (kDa)</th>
<th>Shift, rmsd (Å)</th>
<th>Real-Space R Factor</th>
<th>Correlation Coefficient</th>
<th>Number of Bad Contacts</th>
</tr>
</thead>
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<tr>
<td>E. coli Ribosome (70S) initiation-like</td>
<td>Ice-embedded single-particle/11.5</td>
<td>TNT</td>
<td>165/13</td>
<td>10</td>
<td>0.29</td>
<td>0.23</td>
<td>0.53</td>
</tr>
<tr>
<td>state (Gao et al., 2003)</td>
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<tr>
<td>E. coli Ribosome (70S) EF-G/GTP complex (Gao et al., 2003)</td>
<td>Ice-embedded single-particle/12.3</td>
<td>TNT</td>
<td>165/13</td>
<td>10</td>
<td>0.32</td>
<td>0.24</td>
<td>0.37</td>
</tr>
<tr>
<td>E. coli Ribosome 30S subunit</td>
<td>70S ice-embedded single-particle (see above)/11.5</td>
<td>CNS</td>
<td>90/9</td>
<td>6.5</td>
<td>0.39</td>
<td>0.31</td>
<td>0.71</td>
</tr>
<tr>
<td>Acto-myosin from sectioned muscle (mean statistics from 11 class-average images) (Chen et al., 2001)</td>
<td>Fixed, plastic-embedded, negative-stained, tomograms/40–70</td>
<td>TNT (+ restraints between fragment ends)</td>
<td>37/4</td>
<td>0.46</td>
<td>0.46</td>
<td>0.66</td>
<td>0.67</td>
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<tr>
<td>Heavy meromyosin (smHMM) (Liu et al., 2003)</td>
<td>2D arrays on lipid monolayers, unstained cryo-tomography/20.0</td>
<td>TNT</td>
<td>5/70</td>
<td>6.5</td>
<td>—</td>
<td>—</td>
<td>0.69</td>
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<tr>
<td>Myosin 10 S heterohexamers (Liu et al., 2003)</td>
<td>As above/20</td>
<td>CNS</td>
<td>5/70</td>
<td>5.5</td>
<td>0.37</td>
<td>0.30</td>
<td>0.57</td>
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<tr>
<td>α-Actinin (Liu et al., 2004)</td>
<td>As above/20</td>
<td>TNT</td>
<td>10/20</td>
<td>6.6</td>
<td>—</td>
<td>—</td>
<td>0.6</td>
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</table>

crosscorrelation improved from 0.57 to 0.68. A lowering of the assumed magnification factor by 1% led to an improvement from 0.68 to 0.70. A 4D grid search through the CTF parameters led to adjustment of the defocus and envelope function (d) parameters (Equation 6) and an improvement in the correlation coefficient from 0.70 to 0.74, but no changes in spherical aberration or wavelength were indicated. Fragmenting the RNA into domains while retaining the proteins as single rigid groups led to an improvement in correlation coefficient to 0.77. Although not yet exhaustive, this refinement shows that optimization of imaging parameters can lead to a significantly improved fit.

**Acto-Myosin Structures from Sectioned Muscle**

This example provided an opportunity to test the refinement in a very different regime. Force production in muscle is derived from the interaction of myosin and actin. Crystallographic structures are available for actin and myosin subfragments, but not for the complex relevant to force generation.

EM samples were obtained by sectioning plastic-embedded insect flight muscle, followed by negative staining. 3D images were obtained by tomography at 40 Å resolution (Chen et al., 2001), but after averaging within 11 different classes, the resolution was sometimes as low as 70 Å. Refinement was potentially challenging, not only due to the low resolution, but also the negative staining. A crude approximation for negative staining was implemented through “difference scattering factors,” in which atoms of the model replaced stain atoms of occupancy chosen to mimic the background. Error-free simulations indicated that the method was capable of refining rigidly displaced models, improving the crosscorrelation from <0.2 to >0.99 with residual errors of <1 Å, even at 50 Å resolution. Of course, the real images were far from error free.

For each class average with its distinctive crossbridge configuration, models were built from five actin molecules and an S1 myosin fragment homolog, remodeled to account for missing pieces (Chen et al., 2001). Assemblies based on an earlier 40 Å acto-S1 cryo-EM reconstruction (Rayment et al., 1993a) were rebuilt for the in situ muscle images through repositioning the components with the S1 heavy chain split into four fragments. Manual fitting within the density envelope led to ~1,400 poor van der Waals contacts between fragments. Least-squares refinement with the TNT implementation of RSRef (Chapman, 1995) included added restraints to limit the separation between cut fragment ends to 3.4 Å, thereby constraining them to be hinge points. The fit to the density could not be improved, but the fragments were rearranged to resolve 70% of the poor contacts without loss of fit (Table 1). Some residual overlap was expected, because the side...
Figure 2. Refinement of the E. coli Ribosome in Its Initiation-like State with RSRef Real-Space Refinement
See Gao et al., 2003.

(A–C) Parts of the 11.5 Å resolution electron density near the initial (unrefined) models of protein subunits: (A) S4, (B) S5, (C) S11.

(D–F) The model components after rigid group refinement. (Model covered by density appears dimmer.) The figure was prepared with PyMol (DeLano, 2002).

Chains were built by homology modeling, and because flexibility in subunit association is not accounted for in rigid-fragment refinement.

The implications of this study from a technical perspective were: (1) that pseudo-atomic refinement could be applied to a broad range of EM imaging techniques and resolutions; (2) that stereochemical restraints could be very useful in building plausible models that were consistent with experimental data, even at very low resolution (70 Å); and (3) that partial models could be refined in the absence of components whose structures were not then known (tropomyosin, troponin, ubiquitin). Biologically, the study enabled the visualization of the range of S1 configurations in leading/rear crossbridges with one or two S1 heads, an important step in progressing from purified complexes to what occurs naturally in muscle (Chen et al., 2001).

10S Smooth Muscle Myosin and Heavy Meromyosin
Myosin is the ATP-driven molecular motor that interacts with actin filaments and converts chemical energy into muscle force and movement. Myosin II isoforms are hexamers with two heavy chains and two pairs each of the essential light chain (ELC) and the regulatory light chain (RLC). Phosphorylation of the RLC is required for activity. Regulation requires the presence of both S1 head domains as in the heavy meromyosin (smHMM) fragment. The 10S form of myosin is soluble in contrast to the 6S filament-forming form. smHMM undergoes a conformational change upon dephosphorylation of the RLC that is required for ATP-dependent filament disassembly (Liu et al., 2003).

2D arrays of smHMM showed asymmetric placement of the S1 heads in which actin binding of one S1 was blocked by juxtaposition to the other S1 (Wendt et al., 2001). There was a question as to whether the asymmetry was due to crystal packing. Subsequently, 2D arrays were obtained on lipid monolayers for dephosphorylated smooth muscle myosin and imaged at 20 Å resolution (Liu et al., 2003), on to which the smHMM density could be superimposed. Models were built based on known atomic structures of the smooth muscle myosin domain and the ELC (Domínguez et al., 1998), and a homology model based on a skeletal RLC (Rayment et al., 1993b). The published refinements of both smHMM and smooth muscle myosin used the TNT implementation of RSRef, with fragments defined as for actomyosin (above). (Subsequent optimization of the CTF parameters with the CNS implementation has already been described.) For smHMM, the correlation coefficient improved from 0.69 to 0.79 and the poor van der Waals contacts were reduced from 450 to 80 (Table 1). For myosin, the correlation coefficient improved from 0.63 to 0.75, and poor contacts were reduced from 390 to 30 (Figure 3) (Liu et al., 2003). All poor contacts could
Refinement of EM Models

Figure 3. Refinement of the Myosin 10S Complex with RSRef Real-Space Refinement
See Liu et al., 2003.

Each panel shows the electron density at 20 Å resolution superimposed on the (A) initial and (B) refined models. Statistically, the improvement in cross-correlation of 0.63–0.75 is moderate, but improvements of the fit throughout the model can be found, some of the largest being highlighted with arrows. (Model covered by density appears dimmer) Although the improved fitting brought subunits closer, the stereochemical restraints helped to avoid overlap, and, in fact, there was a 10-fold reduction in the number of poor van der Waals contacts. The figure was prepared with PyMol (DeLano, 2002).

be removed by subsequent energy minimization that moved atoms by an rms of 0.2 Å. Both refinements involved rms C\textsubscript{α} changes of ~6.5 Å.

The similarity of the smHMM and myosin structures (rmsd = 2.2 Å, excluding S2), especially in the asymmetric positioning of the S1 head, indicates that the asymmetry is not an artifact of crystal packing. The new model is more consistent with available mutational data than prior models (reviewed in Liu et al., 2003) and provides a plausible model of how interactions between the S1 heads can mediate regulation of actin binding.

Outlook—Model Parameterization
Our refinements of the ribosome and muscle protein complexes confirm that it is possible to extract quite detailed information about conformational rearrangements from relatively low-resolution EM images. The crystallographically based optimizers are powerful, allowing full model flexibility. The chief concern is to avoid overfitting. Currently, the onus is on the investigator to be conservative in dividing the structure into a modest number of rigid groups.

To allow for flexible fitting with minimal degrees of freedom, Brooks and colleagues have explored refinements limited to shifts along the principal normal mode directions, with the rationale that the model should move only in directions of low-frequency vibrations that do not greatly increase the (calculated) energy (Tama et al., 2004a, 2004b). Ad hoc fragment designations are avoided, but concerted changes throughout the model are allowed with few degrees of freedom (typically 16–36), thereby limiting overfitting. In practice, the improvement in fit is limited unless the process is iterated. Shifts along the initial normal modes are modest to avoid geometrical distortions, then the normal mode calculation is repeated for the next set of shifts. With simulated data, the quality of fit obtained is similar to rigid fragment refinement by SITUS (Wriggers et al., 1999; Wriggers and Birmanns, 2001), but perhaps with physically more reasonable conformational changes (Tama et al., 2004a). With experimental data, applied after rigid fragment fitting, correlation coefficients of 0.9 are attainable at 11–28 Å resolution with shifts of 7–11 Å.

In summary, there are several methods for global searches of the overall configuration and other methods for refining the model. The resolution of EM reconstructions often permits the (conservative) modeling of conformational changes within domains. There are several ways that this can be parameterized: (a) elastic deformations (Wriggers and Birmanns, 2001; Tama et al., 2002); (b) normal mode refinement (Tama et al., 2004a; 2004b); or (c) rigid subfragments linked with stereochemical restraints (Gao et al., 2003, and this paper). All are achieving good results. It is not yet clear which approach is most appropriate with different resolutions or imaging techniques. This may have to await more objective and independent tests of model quality and overfitting. Below, we summarize some of the factors pertinent to the choice of method for a particular application.

The Most Popular Search and Refinement Methods
Among the array of programs for modeling of EM reconstructions there are similarities and important distinctions relevant to different applications. Acknowledging that it is not possible to be both expert and disinterested, a much-simplified comparison is presented, with apologies to any whom, out of ignorance, we may have done an injustice!
Global Search for Initial Configuration

The model generated may suffice, but these methods are optimized for a large convergence radius rather than final precision, and they do not include stereochemically restrained refinements.

1. SITUS: Features – (a) reduced vector representations for fast global searches; (b) variety of correlation-based fitting targets that are Fourier accelerated for fast global searches; (c) support of elastic distortions to model conformational change (with or without an atomic model). Limitations – (a) Global correlations become less reliable below 10 or 15 Å resolution, and improvements through Laplacian filtering may be sensitive to experimental noise; (b) final precision might be limited. References: (Wriggers et al., 1999; Wriggers and Birmanns, 2001; Wriggers and Chacon, 2001; Chacon and Wriggers, 2002; Kovacs and Wriggers, 2002; Tama et al., 2002).

2. COAN: Features – (a) fast measures of correlation; (b) fast global search methods; (c) families or sets of acceptable solutions from which the errors of fitting may be estimated; (d) difference mapping to fit each component in turn; (e) discrepancy mapping to highlight conformational changes; (f) biochemically derived distance restraints/constraints can be incorporated. Limitations – (a) final precision might be limited; (b) limited number of rigid groups. References: (Volkman and Hanein, 1999, 2003; Volkman et al., 2003).

3. DOCKEM: Features – (a) local crosscorrelation target; (b) uses only electron density close to the search probe/refining model; (c) 6D exhaustive search. Limitations – (a) considers one molecule at a time – potential overlap is checked post facto; (B) limited number of rigid groups. References: (Roseman, 2000).

Final Refinement

These methods are optimized for final precision, but they may have limited convergence radius or be too slow for global searches. An approximate starting model is needed from the search methods described above or manual modeling.

1. URO: Features – (a) optimization of correlation coefficient; (b) fast; accelerated through Fourier transformation. Limitations – (a) global refinement in reciprocal space is more susceptible to overfitting and other problems. References: (Navaza et al., 2002).

2. NMFF-EM: Features – (a) density fitting along normal modes of vibration; (b) allows for large conformational changes without overfitting; (c) no need for ad hoc definitions of fragments; (d) local cross-correlation fitting; (e) calculation of model density from Gaussian atoms with ad hoc smearing at low resolution; Limitations – (A) stereochemical distortions need to be corrected by energy minimization. References: (Tama et al., 2004a, 2004b).

3. RSRef: Features – (a) direct calculation of model image, rigorously accounting for resolution; (b) wide applicability from ~1 to 80 Å resolution, unstained or stained specimens; (c) local method – fitting to density near selected components; (d) full stereochemical restraints; (e) several optimization methods, including least-squares and simulated annealing; (f) refinement of EM magnification and selected contrast transfer parameters. Limitations – (a) slow algorithm that is unsuitable for global searches. References: (Chapman, 1995; Chen et al., 2001, 2003; Gao et al., 2003, and this paper).

Methods Bridging between Search and Refinement

These methods commonly invoke ad hoc approximations in an attempt to capture features most essential to an accurate model with an algorithm fast enough for a global search.

1. EMFIT: Features – (a) a variety of target functions, including overlap integrals and number of steric clashes; (b) difference mapping to highlight individual components; (c) refinement of EM magnification. Limitations – (a) overlap integrals fit the density envelope, but not the density variation (Baker and Johnson, 1996); (b) final refinement in reciprocal space can be susceptible to overfitting. References: (Rossmann, 2000; Rossmann et al., 2001).

2. SITUS: – see above.

3. CHARMM extension: Features – (a) weighting emphasizing internal parts of components that likely have least density overlap and conformational change in assemblies; (b) direct crosscorrelation or Laplacian-filtered correlation searches; (c) grid-threading search that efficiently combines a coarse-grained search over all translational/orientational parameters with a Monte Carlo optimization. Limitations – (a) results only from simulated error-free data, to date. Reference: (Wu et al., 2003).

Experimental Procedures

This section describes the implementations of the RSRef real-space refinement whose results are discussed above.

The TNT, X-Plor, and CNS implementations of RSRef are fundamentally similar, but they differ in details and in some of the options available. Each program suite calculates the residual of an overall target function to be minimized and its partial derivatives with respect to each of the atomic coordinates. The total residual and gradient are weighted sums of components to be jointly refined. The most important components involve fit to the experimental data and agreement with standard stereochemistry, but other terms, such as consistency with molecular symmetry, can also be included. In each case, residuals and gradients calculated from RSRef replace conventional (reciprocal-space) experimental terms. In the TNT implementation, RSRef is provided as a subroutine that, in the customized package, can add RSRef terms to the residuals and gradients stored in memory. The distribution package includes a source for the added fortran/C subroutines and a patch that makes minor changes to X-Plor or CNS ready for recompilation.

In practice the user’s choice of implementation is largely governed by familiarity with the host program suite (TNT, X-Plor, or CNS). TNT offers rapidly converging least-squares methods that use second derivatives (Tronrud, 1992), although this option has
been little used with EM refinements. The X-Pilor and CNS implementa-
tions have usually been used with least-squares Cartesian coordinate optimizations (just like the TNT version), but simulated annealing and reduced torsion angle model parameterizations are also available (Brünger et al., 1990; Rice and Brünger, 1994; Brünger and Rice, 1997). These options require all-atom refine-
ment, which should be used only with extreme caution until im-
proved methods of EM model validation are available to monitor overfitting. It is the CNS version that is being most actively de-
volved.

Modest extensions to the X-ray version of RSRRef have been made to support EM refinement. The TNT and CNS implementa-
tions support electronic scattering factors (Doyle and Turner, 1968; Cowley, 1995). The TNT implementation supports application of a CTF to the model density by using predefined parameters (Kenney et al., 1992). The CNS version fully implements Equation 6, thereby supporting searches for improved CTF parameters. Several sup-
porting programs are also provided. These are mostly to adapt the host packages from periodic crystalline structures’ local refinement (of an isolated structure). SelectCoord and Reexpand surround the refining part of the model with neighboring atoms and ensure cor-
responding changes to symmetry-equivalent parts of the model (if applicable). An extension for the TNT version allows distance re-
strictions to be applied such that fragments are treated as hinged, rigid segments, a feature that is available through CNS itself.

RSRRef has two parameters that affect the speed and accuracy of the refinement. The model density is calculated by Fourier trans-
formation of the salient atomic scattering factors, leading to a reso-
lution-dependent truncation ripple that becomes smaller farther from atom centers. The parameter r_{\text{cutoff}} is a cut-off beyond which the electron density contribution of any atom is considered to be zero. The crosscorrelation coefficient between experimental and model density can be used to choose an optimal value. For the refinement of the 30S ribosome subunit into the 70S density at 11.5 Å, the optimal value was 12 Å. This is consistent with experi-
ence in crystallographic refinement in which the optimal values are commensurate with the experimental resolution limit. The param-
eter r_{\text{cutoff}} is a cut-off that restricts the calculation of correlation coefficients and refinement derivatives to grid points within this distance of any refining atom. Experience with crystallographic re-
finements (Chapman, 1995) has shown that refinement is degraded with fewer than 20 grid points contributing to the refinement of each atom. In practice, with the map grid spacings usually used, this corresponds to about half the numerical value of r_{\text{cutoff}}.

A value of 6 Å was used for the refinements against the ~12 Å resolution 70S ribosome maps. Similar determinations with the smooth muscle myosin reconstruction at 20 Å resolution gave r_{\text{cutoff}} = 22 Å and r_{\text{cutoff}} = 10 Å, indicating that the prior guidelines for these parameters are holding true for extended resolution ranges now seen in EM refinement.

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