An interesting question is whether this expanded state is reversible; if substrate were removed, would the majority of particles return to the standard conformation?

The expanded structure is highly deformed from the standard state, but the relatively modest resolution of the reconstructions (12–16 Å), due to unresolved heterogeneity in the dataset, means that interpretation of what exactly happens to the GroEL domains is unclear, although the apical and intermediate domains appear to expand outwards, and equatorial domains slide inwards. Until now, conformational changes due to nucleotide and GroES binding were characterized as rigid-body movements (albeit large ones) of the intermediate and apical domains. This expanded state shows that the GroEL molecule must be much more flexible than previously thought. Characterization of this conformation in terms of tertiary structure will require higher resolution cryo-EM reconstructions.

Another obvious question is whether such an expanded state exists in the double ring system. Trypsin digestion experiments (Song et al., 2003) suggest that the heterodimeric substrate used by Chen et al. is protected not only by SR GroEL/ES, but also by the DR system. The expanded state would seem unlikely to occur in the DR system, since all inter-ring contacts would be disrupted. A cryo-EM study of the substrate trapped in the DR system would provide definitive evidence, but it is also obvious that this is a much more difficult task, due to much higher heterogeneity in the population of particles. It would be useful to demonstrate expanded-state *cis* encapsulation using a large *monomeric* substrate, in order to simplify interpretation.

An important point to remember is that the 86 kDa heterodimeric substrate chosen for this study might be partially folded inside the GroEL cavity, since it is a stable folding intermediate (Wynn et al., 1998). Thus, its volume inside the cavity would be less than for an unfolded substrate of similar mass.

Finally, a very difficult problem in cryo-EM reconstruction methods is to differentiate between (1) views of different orientations of the same structure in a homogeneous population and (2) actual structural heterogeneity in a particle population. In this study, the authors knew from the outset that heterogeneity must exist, since some particles would carry substrate and others would not, even without the expectation of two different conformations. They successfully sorted out this mess by starting with initial references that were identical except for the addition of random noise, and continuing with multiple-reference alignment. This appeared to have been enough to allow for separation of the mixed images into more homogeneous subgroups. It is an intriguing and simple tool, and hopefully it can be used generally in other cases of heterogeneous populations of conformations.

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RecA Assembly, One Molecule at a Time

Several recent papers have applied optical methods to directly visualize the assembly of individual RecA and Rad51 filaments on DNA. The hope is that application of such methods will shed light on the many mysteries that still surround how these remarkable filaments function in genetic recombination.

Although the bacterial RecA protein has been actively studied for almost 30 years with techniques including X-ray crystallography, electron microscopy, spectroscopy, biochemistry and genetics, it is fair to say that

an understanding of the basic mechanisms by which this protein catalyzes the recognition of homology between two DNA molecules and effects a strand exchange reaction during homologous recombination still eludes us. The realization that the eukaryotic Rad51 protein, which is involved in many aspects of the maintenance of genomic stability, undoubtedly functions by similar mechanisms, has only increased the amount of interest in determining how RecA-like proteins function. Three recent papers (Joo et al., 2006; Prasad et al., 2006; Galletto et al., 2006) reported single-molecule experiments where the nucleation and growth of individual RecA-DNA or Rad51-DNA filaments could be visualized and followed in real time. What can we learn from these experiments, and what do we still not understand?

The first step in generating a detailed mechanistic picture of how a RecA filament functions is having a structure for the RecA-DNA filament. This has been quite problematic. The first RecA crystal structure (Story et al., 1992) was a remarkable achievement, and appeared 12 years after the protein was first crystallized. Since RecA forms a filament on DNA with $\sim$6 subunits per turn, and the protein in the absence of DNA crystallized as a 6_1 screw (a helix with exactly six subunits per turn), it was reasonably assumed that the helix in the crystal was the same as the active filament formed on DNA in the presence of ATP. Unfortunately, the putative DNA binding loops (L1 and L2) were not visualized in the crystal structure due to disorder, so there was no possibility of understanding how RecA binds to DNA, and both stretches it and untwists it. Subsequent RecA crystal structures have visualized these loops (Datta et al., 2003), but have not yielded significant new insight since DNA is not bound. A major breakthrough occurred with the determination of crystal structures for 6_1 filaments of archaeal Rada (Wu et al., 2004) and yeast Rad51 (Conway et al., 2004). These crystal filaments were both in a very different conformation from that found in the RecA crystal, and many indications suggested that their extended conformation was more similar to the active filament formed by all of these proteins on DNA (VanLoock et al., 2003). Despite the fact that DNA was present during crystallization of yeast Rad51, it was not visualized in the crystal.

While it is hoped that a cocomplex of DNA with either RecA or Rad51 will eventually be solved at atomic resolution, we also need to obtain information using many other techniques to develop a picture of how the extended nucleoprotein filament functions in homologous genetic recombination. The recent single-molecule solution studies are very encouraging, as they show in real time how the nucleation, polymerization, and depolymerization of RecA-like proteins occurs on individual DNA molecules. Three very different methods were used. Ha and colleagues (Joo et al., 2006) used single-molecule fluorescence resonance energy transfer (FRET) assays to quantitatively look at the separation between fluorescent donor and acceptor labels located at different positions within DNA substrates as a function of RecA binding and filament formation. Kowalczykowski and colleagues (Galletto et al., 2006) used a fluorescently labeled RecA protein that bound to an optically trapped DNA molecule within a flow chamber, enabling them to visualize the RecA filament nucleation and extension in real time. Greene and colleagues (Prasad et al., 2006) used fluorescently labeled DNA molecules whose extension could be monitored in a flow chamber to measure the stretching of double-stranded DNA that takes place when Rad51 binds in the presence of ATP.

One interesting difference that emerges in a comparison of these experiments is that Rad51 nucleates quite readily on double-stranded DNA, and nucleation is not rate limiting in the polymerization reaction (Prasad et al., 2006). In contrast, spontaneous nucleation of RecA is much less likely, and is strongly favored to take place on a region of single-stranded DNA (although subsequent polymerization onto an adjacent double-stranded region takes place quite readily) (Galletto et al., 2006; Joo et al., 2006). This difference likely reflects evolutionary differences: RecA activation within a bacterium occurs when single stranded DNA is seen by the protein, as might occur when replication encounters a lesion, while Rad51 binding to DNA is presumably controlled in eukaryotic cells by other proteins, such as BRCA2 and Rad52. This suggestion is consistent with what we know about other eukaryotic protein polymers. Consider actin, which can be the most abundant single protein in a cell. A large number of actin-binding proteins have been characterized, and quite a few, such as cofilin, ADF, ARP2/3, formin, and gelsolin, are involved in the nucleation and depolymerization of actin filaments (Blanchoin et al., 2000). As far as we know, there are no actin filaments in a cell that are spontaneously nucleated, and it is quite likely that the same situation will hold true for Rad51. In the simpler bacterial repair and recombination pathways, self-nucleation of RecA on regions of single-stranded DNA is all that is needed for activation.

The single molecule studies provide an elegant means of monitoring DNA binding by RecA-like proteins since they are sensitive to the actual conformation of the filament formed on DNA, and can distinguish between compressed filaments that do not extend the DNA and active filaments where the DNA length is increased by 50%–60%. This approach can therefore be used to look at the effect of mutations in ways that may not be possible by bulk binding studies. For example, it was shown that mutations in the Rad51 L1 loop abolished formation of extended filaments on DNA while mutations in the L2 loop did not (Prasad et al., 2006), consistent with other observations suggesting that the RecA L1 forms the primary DNA binding site (Wang and Adzuma, 1996; Cazaux et al., 1998).

As elegant as these studies are, they still do not provide us with all the details we crave about how RecA and Rad51 filaments can perform the remarkable reactions involved in transferring strands of DNA between homologous molecules. Realistically, no single experiment or technique will provide such a picture, but each new approach generates more information that can be incorporated into a model for protein-mediated homologous recombination. The fact that RecA-like filaments are found to exist over huge evolutionary distances tells us that understanding how these filaments function will be a monumental step in understanding the evolution of life itself.

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