Denaturation of replication protein A reveals an alternative conformation with intact domain structure and oligonucleotide binding activity

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Abstract

Replication protein A (RPA) is a heterotrimeric, multidomain, single-stranded DNA-binding protein. Using spectroscopic methods and methylene carbene-based chemical modification methods, we have identified conformational intermediates in the denaturation pathway of RPA. Intrinsic protein fluorescence studies reveal unfolding profiles composed of multiple transitions, with midpoints at 1.5, 2.7, 4.2, and 5.3 M urea. CD profiles of RPA unfolding are characterized by a single transition. RPA is stabilized with respect to the CD-monitored transition when bound to a dA15 oligonucleotide. However, oligonucleotide binding appears to exert little, if any, effect on the first fluorescence transition. Methylene carbene chemical modification, coupled with MALDI-TOF mass spectrometry analysis, was also used to monitor unfolding of several specific RPA folds of the protein. The unfolding profiles of the individual structures are characterized by single transitions similar to the CD-monitored transition. Each fold, however, unravels with different individual characteristics, suggesting significant autonomy. Based on results from chemical modification and spectroscopic analyses, we conclude the initial transition observed in fluorescence experiments represents a change in the juxtaposition of binding folds with little unraveling of the domain structures. The second transition represents the unfolding of the majority of fold structure, and the third transition observed by fluorescence correlates with the dissociation of the 70- and 32-kD subunits.

Keywords: denaturation; replication protein A; MALDI-TOF mass spectrometry; diazirine; methylene carbene; urea

Supplemental material: see www.proteinscience.org

Replication protein A (RPA) is a eukaryotic single-stranded DNA (ssDNA) binding protein that was first characterized as an essential component of the SV40 in vitro replication assay (Wobbe et al. 1987; Fairman and Stillman 1988; Wold and Kelly 1988). However, further experimentation has demonstrated central roles for RPA, not only in replication, but also in DNA repair and recombination (Heyer et al. 1990; Coverley et al. 1991, 1992; Moore et al. 1991). In these processes, RPA binds single-stranded regions of DNA

and interacts with other proteins that ultimately govern how the cell copies, fixes, and changes genetic information.

RPA is a heterotrimeric protein composed of 70-, 32-, and 14-kD subunits. Physical and biochemical studies of subunit fragments, as well as primary structure homologies, suggest the protein is composed of multiple domains (Bochkarev et al. 1997, 1999; Brill and Bastin-Shanower 1998; Jacobs et al. 1999; Mer et al. 2000; Bochkareva et al. 2001, 2002) as summarized in Figure 1. All but one are “oligonucleotide/oligosaccharide-binding” (OB) folds. These include characteristic five-stranded β -barrel structures that have been implicated in ssDNA and protein binding (Murzin 1993; Kerr et al. 2003). Variety exists within the fold family because of variation in the length of loops connecting secondary structural elements and the presence or ab-

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Figure 1. Schematic diagram of the three subunits of RPA. RPA 70 is composed of four folds that have been numbered 1–4. RPA 32 is composed of two structural folds, a central fold, and a C-terminal fold. RPA 14 is a single-domain subunit. The numbers beneath schematic representations of each peptide indicate approximate primary structure boundaries of each domain.

sence of ancillary α -helices between β -strands. The mechanism of ssDNA binding, however, is linked to interactions with OB folds and seems to be conserved. Studies of RPA fragments as well as other OB folds indicate that oligonucleotides lie in a groove created by the domain core, allowing conserved residues contained within the β -barrel and on connecting loops to interact with the oligonucleotide (Murzin 1993; Bochkarev et al. 1997). Aromatic amino acids provide particularly critical stacking interactions with substrate. High-resolution structures of all OB folds in RPA have been deduced by X-ray crystallographic and NMR analyses of proteolytic RPA fragments (Bochkarev et al. 1997, 1999; Jacobs et al. 1999; Mer et al. 2000; Bochkareva et al. 2001, 2002).

It seems likely that OB folds represent autonomous folding domains in the structure. Consistent with the independence of OB folds 1 and 2 (Fig. 1), NMR investigations find weak coupling between these folds (Daughdrill et al. 2001). Yet neither independent properties nor independent structures of several of these folds (3, 4 of the 70-kD subunit, the central 32-kD subunit fold, and the 14-kD subunit) have been reported. Furthermore, the structure of the entire protein has not been reported. The influence that one fragment exerts on another fragment in the total structure, therefore, remains a matter of conjecture.

Mechanisms of ssDNA binding likely involve structural rearrangements, perhaps primarily changes in the juxtaposition of OB folds. The following observations are pertinent. An ssDNA-binding mechanism involving multiple conformations and several different folds has been proposed for long strands of ssDNA. Glutaraldehyde cross-linking experiments and crystallographic studies suggest that the central two OB folds of RPA 70, thought to contain the highest-affinity binding site, interact with ssDNA (Bochkarev et al. 1997). This binding event occludes eight nucleotides of ssDNA and is thus referred to as the 8-nt mode (Blackwell

and Borowiec 1994). Perhaps, following initial binding at this location, an auxiliary fold interacts with the ssDNA to occlude a total of ~13–14 nt (13–14-nt mode; Lavrik et al. 1999). These transient conformations ultimately give way to a stable mode (Blackwell and Borowiec 1994; Kim and Wold 1995), which occludes 30 nt and may involve the binding of additional OB folds (Bastin-Shanower and Brill 2001). Comparisons of proteolytic digests of RPA and RPA–ssDNA complexes suggest that the different binding modes involve different protein conformations (Gomes et al. 1996). This contention is supported by electron microscopy examination. Without ssDNA, RPA appears as spots in electron micrographs. When bound to long stretches of ssDNA (30 nt), the RPA–ssDNA complex appears linear. Intermediate lengths of ssDNA (8 nt) result in partially extended structures (Blackwell et al. 1996).

We explore the unfolding of RPA for several reasons. We anticipate multiple energetically accessible conformations are important for RPA function. These conformations would be characterized by intact local structures/folds. In addition, the problem we address for RPA is one likely to be common to several medium and large proteins. Structural details are frequently more accessible for fragments of proteins' structures than the whole protein owing to size constraints associated with physical methods or ease of crystallization (Feher and Kam 1985; Wider and Wuthrich 1999). Finally, computational analyses of protein structures are usually hierarchical in design, involving the deconvolution of a protein primary structure into fragments (e.g., Schultz et al. 1998; Bateman et al. 2002) that are then analyzed in detail. Constraints on the independence of domains are important for judging the utility of these views and approaches.

In this study, we have supplemented CD and fluorescence methods for monitoring unfolding with chemical reactivity using methylene carbene modification methodologies (Richards et al. 2000). In combination with limited proteolysis, the latter method can assess the folding properties of at least some of RPA's purported folding domains. This reagent is reactive with all amino acid side chains (Kirmse 1971; Richards et al. 2000), and its reactivity is proportional to amino acid exposure (Craig et al. 2002), which changes with protein unfolding. In fact, using α -lactalbumin (a small protein), the latter investigators showed a close correlation between denaturation curves monitored by changes in CD spectroscopy and by changes in carbene modification using the reagent, diazirine (DZN). We have used this approach in studies reported here.

Results

Denaturation of replication protein A

The urea-induced unfolding of RPA was monitored using peptide backbone CD spectroscopy and intrinsic protein

fluorescence (Fig. 2). Changes in secondary structure were followed by measuring ellipticity changes at 215 nm. Denaturation profiles were fit as a single unfolding transition with a midpoint of 3.3 M urea. Qualitatively similar results characterized guanidine HCl-induced unfolding (data not shown). Denaturant concentrations at CD transition midpoints and values of a parameter that quantifies steepness of each transition (M ; see Materials and Methods) are summarized in Table 1.

The ratio of fluorescence intensities (F_{340}/F_{304}) was also used to monitor unfolding. The high-fluorescence contribution by the tyrosine residues, specifically in the denatured state of RPA, made it necessary to normalize the fluorescence intensity measured at 340 nm (tryptophan and tyrosine fluorescence), to fluorescence intensity measured at 304 nm (predominately tyrosine fluorescence; Lakowitz 1983). Denaturation profiles indicate at least four transitions. A steep transition with a midpoint at 1.5 M urea was followed by a large transition with a midpoint of ~ 2.7 M urea and smaller amplitude transitions with midpoints at 4.3 M and 5.3 M urea, respectively. Additional transitions,

which are transparent to our analysis, may also occur. Although amplitudes of transitions 1, 3, and 4 were relatively small, they were reproducible and larger than the experimental error in the fluorescence-monitored denaturation experiment. The transitions were well enough separated that plateau regions were identifiable. Values of m , which quantifies cooperativity for equilibrium transitions, and M for nonequilibrium transitions, as well as the transition midpoints, were calculated and are summarized in Table 1.

In view of the complex denaturation profile we observed by fluorescence spectroscopy, we characterized the association state of RPA at the end of each transition. This was done by performing HPLC gel exclusion chromatography at appropriate urea concentrations and identifying the subunits of RPA present in the various fractions by SDS-PAGE (Fig. 3). The experiment conducted at the end of the first transition (2.0 M urea) showed a major peak and also a shoulder. SDS-PAGE analysis indicated that the major peak contains all three subunits, indicating intact RPA. The shoulder contained RPA 32 and RPA 14. The elution volume of the smaller peak indicated that these two subunits are bound together.

Figure 2. Representative denaturation profiles constructed for RPA monitored by CD spectroscopy (filled triangles, solid line, right axis) and intrinsic protein fluorescence (open circles, dashed line, left axis). (Inset) The renaturation of RPA from 0 M to 2 M urea. The open circles and dashed line have the same meaning as just described. The black diamonds represent fluorescence intensities of samples incubated in buffer containing the indicated urea concentration and then diluted to 0.2 M urea (see Materials and Methods). Experiments were performed at 20°C using a protein concentration equal to 0.11 mg/mL.

Table 1. RPA unfolding parameters

Preparation	Method	50% unfolded [denaturant] (M)	ΔG (kcal/mole)	m/M values
RPA ^a	CD ^e	3.29 ± (0.09) ^l		−0.72 ± (0.01) ^{o,l}
RPA-dA15 ^b	CD ^e	3.81 ± (0.13)		−0.86 ± (0.03) ^o
RPA 14 ^c	Methylene ^f	3.55 ± (0.17)		−0.70 ± (0.05) ^o
RPA 32 central ^c	Methylene ^f	3.40 ± (0.26)		−0.94 ± (0.11) ^o
RPA 70 D4 ^c	Methylene ^f	4.04 ± (0.24)		−0.70 ± (0.05) ^o
RPA (GndHCl) ^d	CD ^e	2.39 ± (0.11)		−1.31 ± (0.03) ^o
RPA ^a	Fluorescence ^g	1.46 ± (0.08)	6.33 ± (0.98) ^l	−4.28 ⁿ ± (0.72)
RPA ^a	Fluorescence ^h	2.68 ± (0.10)		−1.54 ± (0.17) ^o
RPA ^a	Fluorescence ⁱ	4.18 ± (0.39)		−5.65 ± (2.98) ^o
RPA ^a	Fluorescence ^j	5.30 ± (0.17)		−2.46 ± (0.42) ^o
RPA ^k	dA 15 binding		8.85 ± (1.31) ^m	−2.29 p ± (0.27) ^m
RPA ^k	dT 8 binding		7.95 ± (2.48) ^m	−2.39 p ± (0.70) ^m

Denaturations performed as described in Materials and Methods using urea at pH 7.8, 20°C, with the specific modifications indicated under preparations.

^a [RPA] ≈ 1 μM.

^b [RPA] ≈ 1 μM preincubated in the presence of 10 μM dA 15.

^c Denaturation of specific folds.

^d Guanidine hydrochloride-induced denaturation, [RPA] ≈ 1 μM.

^e Denaturation reported by circular dichroism at 215 nm.

^f Denaturation monitored by methylene incorporation.

^g Denaturation reported by the ratio of fluorescence emission intensities at 340 nm to 304 nm, first urea transition.

^h Denaturation reported by the ratio of fluorescence emission intensities at 340 nm to 304 nm, second urea transition.

ⁱ Denaturation reported by ratio of fluorescence emission intensities at 340 nm to 304 nm, third urea transition.

^j Denaturation reported by the ratio of fluorescence emission intensities at 340 nm to 304 nm, fourth urea transition.

^k Equilibrium ssDNA binding affinity, [RPA] = 300 nM.

^l Standard deviation of average (at least three determinations).

^m Standard error of estimate.

ⁿ *m* value.

^o *M* value (see Materials and Methods).

Gel exclusion chromatography of RPA at 3.5 M urea (the end of the second fluorescence transition) revealed two peaks. The more prominent peak, which eluted as the larger species, was composed of the 70- and 32-kD subunits of RPA (as indicated by SDS-PAGE). The less prominent peak corresponded to the 14-kD subunit of RPA. This indicated that at 3.5 M urea, RPA 14 dissociated from the heterotrimeric complex. Inspection of the SDS gel revealed a small amount of free RPA 32 at an elution volume between that of the complex and RPA 14. This was probably a result of an equilibrium between the free and bound states of the subunits. Gel exclusion chromatography likely prevented complete reassociation of the complex after thermodynamic dissociation.

At 5 M urea (after the third transition in the fluorescence profile), all three subunits of RPA chromatographed independently, signaling complete dissociation of the protein complex. Interestingly, at 6 M urea the chromatographic positions of subunits shifted slightly to greater retention volumes. This probably reflects changes in hydrodynamic volumes of individual RPA subunits as a result of the unfolding of peptide chains.

To fully describe the denaturation of RPA, we examined the reversibility of the denaturation processes. By decreasing urea concentrations in samples previously equilibrated with the denaturant in unfolding experiments, and again incubating, we have shown that the process is reversible through the first denaturation transition (Fig. 2). We did not find conditions under which the subsequent transitions were reversible. Interestingly, through the first transition, the trimeric RPA complex remained intact (Fig. 3).

The unfolding data just described measure an average of conformational changes sensed by the protein backbone, or by all monitored fluorophores. To directly monitor the unfolding of individual domains of multidomain RPA, we have used a novel methylene modification/peptolysis-based method. This method relies on identifying peptides from individual domains in limited proteolytic digests and characterizing the modification of these peptides/domains at different stages of the unfolding profile.

Limited digestion of RPA

Individual fold-containing fragments were evident in limited trypsin digests of RPA (Fig. 4). Except for the C-ter-

Figure 3. SDS-PAGE analysis (*right panels*) of RPA gel exclusion chromatography (*left panels*) experiments conducted at the indicated urea concentrations. Pure unmanipulated RPA was used as a molecular weight marker for SDS-PAGE experiments (*left lane*), and all gels were stained with silver. HPLC experiments were performed at 20°C using a protein concentration equal to 0.20 mg/mL.

minimal fold of RPA 32, all are OB folds of roughly comparable mass. The smallest subunit, RPA 14, and the family of cleavage products that encompass the RPA 32 central fold (43–171, 41–171, 43–180, 41–180, and 39–180) were the most prominent peaks in the spectrum (Fig. 4). Two other fragments from RPA are also visible, although somewhat obscured by the cleavage products of the RPA 32 central fragment. One spans RPA 70 D2 (168–311) and the other spans RPA 70 D1 (1–157). RPA 70 D4 (432–616) was easily resolved from the other peaks; it contains a zinc-binding fold that makes it substantially larger than the other fragments of RPA (Fig. 4). The identities of tryptic peptides just discussed were deduced by peptide mass and were confirmed by tryptic footprinting experiments in which peptides were first isolated by SDS-PAGE and digested exhaustively with trypsin (Supplemental Material; Bantscheff et al. 1999). It should be noted that peak intensities collected by MALDI-TOF MS are not only dependent on the concentration of a species, but also on the ability of the species to be desorbed from a solid support. Therefore, the relative amplitudes of different peaks do not directly correlate with the relative concentration of the individual species.

A CD spectrum of the limited digest was recorded and compared with CD spectra of native RPA and RPA denatured with 7 M urea (available in Supplemental Material). These results indicated that RPA in the limited digest re-

tained most of its secondary structure. The denatured sample, however, had nearly no ellipticity between 205 and 240 nm. These results indicate that fragments behaved similarly to native RPA in that they were still folded after trypsin digestion, but unfold in the presence of 7 M urea.

To characterize the association state of RPA folds after limited digestion with trypsin, the digest just mentioned was chromatographed on an HPLC gel exclusion column. Two multifragment species were identified (Fig. 5). MS analysis of peak fractions showed that the higher-molecular-weight complex consisted of the “minimum trimerization core” (Bochkareva et al. 2002) of RPA: the 14-kD subunit, the central region of RPA 32 (cut at 39 or 41 and 180), and the RPA 70 C terminus (D4; cut at position 432). The lower-molecular-weight species was comprised of a fragment spanning RPA 70 D1 (cleaved at position 157) and a fragment (168–311) that encompasses RPA 70 D2 (Fig. 1).

Monitoring denaturation by measuring methylene incorporation

To monitor the unraveling of proteolytically severed folds, RPA digests were labeled with methylene carbene. In these experiments, limited proteolytic digests (which have cleaved interfold linking regions; see above) were divided into aliquots, incubated in buffer with varying urea concentrations (as described in Materials and Methods), and then labeled with methylene carbene. Methylene carbene reacts with the protein (and solvent) to form stable products by inserting into X–H bonds (where X = O, N, S, or C) as well as adding into C=C bonds (Frey 1966; Turro et al. 1987). This indiscriminate reactivity dictates that the extent

Figure 4. MALDI-TOF mass spectrum of trypsin-resistant RPA fragments. Individual peaks are labeled by the peptide fragment to which they correspond and the position of the fragment in RPA's primary structure. Multiple peaks for the central fold of the 32-kD subunit arise from alternative N-terminal and C-terminal cleavage sites (see Materials and Methods).

Figure 5. Mass spectral analysis (*lower panels*) of the indicated gel filtration fraction obtained by HPLC chromatography of limited RPA tryptic digests (*upper panel*). RPA solutions (0.25 mg/mL in Zinc Buffer) were digested at 37°C and chromatographed in Running Buffer at 20°C (see Materials and Methods).

of methylation is determined primarily by the solvent accessibility of the protein (Richards et al. 2000).

MALDI-TOF MS was used to measure the extent of modification of the individual fragments. Figure 6 shows overlaid mass spectra of unmodified RPA 14, methylated native RPA 14, and RPA 14 methylated after incubation in 4 M and 7 M urea. Methylation of the protein leads to an increase in mass as demonstrated by a shift of the peak position (centroid) for the modified protein to higher molecular weights. Craig and coworkers have shown that carbene is more readily incorporated into the unfolded state of the protein than the native state, as judged by ^3H -labeled methylene and scintillation counting (Craig et al. 2002). Therefore, carbene modification is well suited to measure exposure through unfolding of various portions of a protein. Using MALDI-TOF MS, we found the greatest shift when the protein denatured with 7 M urea was labeled. In addition to shifting the centroid, carbene modification also increased the MS peak width. This indicates greater degrees of methylation and greater variation in extent of modification in the denatured protein.

Results similar to those observed for RPA 14 (Fig. 6) were found for other fragments. The centroid for each peak

in the mass spectrum was recorded and plotted versus urea concentration as shown in Figure 7. Denaturation curves for RPA 14 and peptides spanning central 32 and RPA 70 D4 regions are shown. Unfolding profiles of all the cleavage products of the RPA 32 central fragment were similar. We focused on the denaturation of the most prominent peptide (RPA 32 43–171) for this manuscript. Peak broadening occurred upon methylation of all fragments obscuring the smaller peaks corresponding to RPA 70 D1 and RPA 70 D2 peptide fragments. Hence, we were unable to monitor the unfolding of these domains.

Analysis of denaturation of RPA's component domains

Denaturation profiles for fragments of RPA measured by methylene modification, and for the entire protein, measured by CD, are shown in Figure 7. Parameters obtained from these profiles, m or M values and transition midpoints, are summarized in Table 1. RPA 14 and the peptide containing D4 have M values similar to the average M value obtained for the entire protein as monitored by CD. The M value of the central 32 fragment is greater than that of RPA

Figure 6. MALDI-TOF mass spectra of methylation adducts of native RPA 14 (solid line), RPA 14 denatured in 4 M urea (dashed line), and RPA 14 denatured in 7 M urea (dashed/dotted line). Unmodified RPA 14 (dotted line) is also shown for comparison purposes. Each peptide is labeled with its centroid molecular weight.

14, the RPA 70 D4 fragment, and the average denaturation transition observed in CD experiments. The other parameter calculated, denaturant concentration leading to 50% unfolded, describes the stability of a protein. RPA 14 and the central 32 fragment were half-denatured at urea concentrations close to the average RPA value observed in CD experiments. The D4 fragment unfolded at higher urea concentrations than RPA 14, central 32, or the overall protein as monitored by CD spectroscopy.

Energetic linkage between ssDNA binding and RPA stability

Binding energies describing the association of two substrates to RPA at various urea concentrations are shown in Figure 8. In these experiments, RPA was incubated in buffers with different urea concentrations (as described in Materials and Methods) and then allowed to bind to either dT8 or dA15. Binding isotherms were hyperbolic and showed no deviation from a simple noninteracting binding sites mechanism (data not shown). For both substrates, a similar urea dependence of binding affinity was observed. Only small changes in binding energy were found until urea concentration exceeded 2 M.

As anticipated, nucleotide binding stabilized RPA with respect to denaturation. Figure 9 compares denaturation profiles of the free protein and the protein bound to dA15 (both monitored by CD). Like the free protein, the profile of the DNA-bound protein was characterized by a single transition. However, the midpoint of denaturation increased to 3.8 M urea. In addition, the urea dependence of the unfolding of the RPA–dA15 complex was apparently more cooperative than that of the free protein (data summarized in Table 1).

Discussion

RPA is an essential protein for nucleotide excision repair, replication, and recombination in eukaryotes. It is composed of multiple OB folds (Fig. 1). Unfolding profiles measured using fluorescence spectroscopy show that RPA unfolds in a complex process that involves multiple transitions (Fig.

Figure 7. Denaturation profiles for three RPA folds (as reported by methylene modifications) and for the entire protein (as reported by CD; diamonds, solid line). Individual profiles for RPA 14 (crosses, dotted line), RPA 70 D4 (circles, dashed line), and the central fold from RPA 32 (triangles, large/small dashed line) are shown. Experiments were performed at 20°C, as described in Materials and Methods.

Figure 8. Urea dependence of dT8 (filled circles, dashed line) and dA15 (filled triangles, solid line) binding to RPA. The standard state free energies plotted here were calculated from binding isotherms measured as described in Materials and Methods at each urea concentration indicated. Standard deviations involving at least three determinations are represented by error bars. The lines in this figure were calculated using parameter values determined by nonlinear least square fitting of experimental data to equation 1 as described in Materials and Methods. The constants derived from this procedure are presented in Table 1. Experiments were performed at 20°C using a protein concentration equal to 300 nM (~ 0.03 mg/mL).

2). CD analysis of the unfolding process (Fig. 2) and chromatographic analysis of RPA at various stages in the unfolding profile (Fig. 3) form the basis for interpreting these transitions.

Unfolding transitions of RPA

Urea dependencies that were monitored by fluorescence suggest there are at least four unfolding transitions. The first

transition (midpoint 1.5 M urea; Fig. 2) is associated with little if any domain unfolding as judged by CD spectroscopy, and no changes in quaternary structure (Fig. 3). In contrast, several observations indicate the second fluorescence transition (midpoint 2.7 M urea) reports the substantial loss of the protein's secondary structure. Transition midpoint urea concentrations and steepness parameters for this transition are very similar to those for the CD-monitored unfolding (Table 1). The latter method reports secondary

Figure 9. Unfolding profiles of RPA (filled circles, solid line) and RPA bound to dA15 (filled triangles, dashed line) as monitored by CD spectroscopy at 215 nm. Experiments were performed at 20°C using a protein concentration equal to 0.11 mg/mL (~ 1 μ M) and an oligonucleotide concentration equal to 10 μ M.

structure directly. Interestingly, although RPA 14 was dissociated in the 2.7 M urea transition, the 70- and 32-kD subunits were still associated (Fig. 3). This is very consistent with significant secondary structure remaining at 3.5 M urea.

Two additional fluorescence transitions (midpoint urea concentrations of 4.2 and 5.3 M urea) occur at denaturant concentrations higher than the midpoint for loss of most secondary structure. At the end of the 4.2 M urea transition, RPA 70 and RPA 32 subunits are dissociated (Fig. 3). It is reasonable that fluorescence changes detected between 4 M and 5 M urea are linked to this dissociation. The last (5.3 M midpoint) fluorescence transition must reflect conformational rearrangements of the most stable structural elements of the protein. This assertion is supported by evidence from gel exclusion chromatography experiments. The chromatogram from the experiment conducted at 6 M urea exhibits subtly changed elution volumes and chromatographic profiles compared with the elution profile at 5 M urea. This is consistent with conformational properties of the subunits changing between 5 and 6 M urea (Fig. 3).

Carbene modification of RPA

Frequently, protein unfolding is monitored using reporter groups distributed throughout the protein structure, for example, as with the peptide backbone when using CD, or aromatic amino acid side chains when using fluorescence. It is often difficult to deconvolute the composite responses observed to obtain information specific to a particular portion of the protein. In multiple domain proteins such specific information is particularly important because conformational properties of domains, as well as interactions among domains, may be critical determinants of the proteins' functions. Therefore, we have applied the method of carbene modification to RPA unfolding. It has the advantage of reporting conformational changes at varying degrees of resolution, determined by the analysis of the reacted protein. Here we are particularly interested in accessing the stability of individual folds in this multifold protein.

We and others (Gomes et al. 1996; Bochkareva et al. 2000) have found RPA is readily cleaved into large fragments that span folds (Fig. 4). We have identified several of these (Fig. 4; Supplemental Material) using MS analyses of limited RPA digests. During urea denaturation of these digests, the methylene incorporation into the individual fragments increased in a single transition (Fig. 7), which is very reminiscent of the CD transition that characterizes the whole protein (Fig. 2). We conclude CD experiments conducted on the intact protein and methylene incorporation experiments conducted on proteolytic fragments monitor the same processes: the unraveling of folds. This conclusion is very consistent with observations of Craig et al. (2002), who reported a striking similarity between CD-monitored

and carbene modification-monitored unfolding experiments in a single-domain protein.

In the analysis used here, we produced domains by proteolytic cleavage prior to methylene modification. This focuses on the stability of cleaved portions of the RPA structure. An alternative approach would be to perform the methylene carbene modification at various urea concentrations and then produce peptides. Although this directly monitors the stability of the uncleaved protein, the generation of peptides is complicated by the denaturant.

Several observations do suggest that the carbene modifications used here monitor, but do not drive the unfolding process. First, the modification is sparse. Figure 6 shows a representative result. The total shift of the centroid, which reflects the extent of modification, corresponds to the addition of slightly less than two methyl groups in this 121-amino-acid peptide. Second, because the reaction is non-specific (Richards et al. 2000), it is likely that in doubly labeled peptides, the labeling occurs at many different sites throughout the fragment. Lastly, the unfolding transitions monitored by carbene modification in RPA (after limited proteolysis) are characterized by urea midpoints, and steepness parameters that are similar to those characterizing CD-monitored unfolding of native RPA (Table 1). We conclude that it is very unlikely that modification affects unfolding. This is supported by binding studies that indicate the native protein, after being subjected to carbene modification, binds ssDNA with equal avidity as native RPA (data not shown).

Independence of OB folds

Although unfolding transitions of the individual fragments of RPA that we have examined are similar, they are not identical. In addition, they are also not identical with the CD-monitored transition of native RPA. However, the fragment transitions (Fig. 7) occur within the urea concentration range for the CD-monitored unfolding. This is quite consistent with the CD transition being a weighted sum of transitions like those in Figure 7 for each fold fragment. Unfortunately, we were not able to analyze the unfolding of all fragments of RPA, thus a quantitative summation is not feasible.

Limited proteolysis does change the structure of RPA substantially. Chromatographic and SDS-PAGE analyses show the proteolyzed protein is reduced to two complexes; one contains three peptides including RPA 14, the RPA 32 central fold, and the RPA 70 D4 fold. This is called the trimerization core (Bochkareva et al. 2002). The second is comprised of the first and second folds of the 70-kD subunit (not linked covalently; Figs. 1, 5). Yet similarities in the unfolding of native RPA and mildly proteolyzed RPA suggest a substantial degree of folding autonomy of domains. This is an essential characteristic of folding domains. Our

observations, then, argue in favor of the relevance to the several structural (X-ray crystallographic and NMR; Bochkarev et al. 1997, 1999; Jacobs et al. 1999; Mer et al. 2000; Bochkareva et al. 2001, 2002) and functional (Bochkareva et al. 1998) studies on RPA fragments reported by other researchers.

Linkage between DNA binding and RPA unfolding

DNA binding influences RPA unfolding by stabilizing the protein, as monitored by CD spectroscopy (Fig. 9; Table 1). As just mentioned, we have assigned the transition observed by CD spectroscopy to the unraveling of folds. We conclude that DNA binding protects these structures. The shift in midpoint of the transition that is caused by dA15 association reflects an increase in fold stability (resistance to unfolding; Table 1; Fig. 9). This is consistent with individual domains providing binding sites for short (2–4-nt portions of ssDNA) substrates (Bochkarev et al. 1997; Bastin-Shanower and Brill 2001).

Whether dT8 or dA15 binding is energetically linked to the first fluorescence transition is less straightforward (Figs. 2, 8). Because DNA binding was measured by decreases in intrinsic protein fluorescence, which is our unique monitor of the 1.5 M urea transition, we were unable to saturate RPA with either of the oligonucleotides and then monitor unfolding through the first fluorescence transition. However, we did monitor dA15 and dT8 binding affinity at various urea concentrations as large as 3 M. This covers urea concentrations necessary to induce the first and part of the second fluorescence transition. The data show no decrease in oligonucleotide affinity at 1.5 M urea. We also fit these urea dependencies to a model in which binding changes were attributed to a conformational change between a binding-competent and a binding-incompetent conformation. The data in Figure 8 are well described by this two-state model (lines in Fig. 8), but only when the loss of activity is a consequence of domain unfolding (second fluorescence transition) rather than domain unpacking (first fluorescence transition). If differences in binding energy associated with the low urea transition are sufficiently small, we would not be able to detect them in this analysis. We estimate this difference would have to be much less than 1 kcal.

A model for RPA unfolding

Figure 10 summarizes the results of experiments reported here. Our results are consistent with a two-step process. The second step corresponds to loss of organized domain structures, and is characterized by changes in CD spectra as well as carbene chemical modification. We conceptualize that the first step (the first fluorescence transition of Fig. 2) corresponds to a conformational change, perhaps “unpack-

ssDNA Binding and Conformational Interactions

Figure 10. RPA unfolding scheme. RPA_c, RPA_b, and RPA_{coil} correspond to the native, an alternate conformation with changed domain juxtaposition, and an unfolded coil conformation of RPA. The same nomenclature is used to describe the various conformations in the presence of a dA15 substrate. When possible, the equilibrium between conformations is labeled with experimentally determined free energy.

ing” of the domains of RPA, transitioning from a compact structure (RPA_c in Fig. 10) to a “beads-on-a-string” structure (RPA_b in Fig. 10) where domain juxtaposition is altered. This view of the protein changing its domain juxtaposition is consistent with electron microscopy studies suggesting that free RPA exists in a compact structure that elongates substantially upon DNA binding (Blackwell et al. 1996).

Analysis of the first fluorescence transition indicates that in the absence of urea, 6.3 kcal separates RPA_b and RPA_c energetically (Table 1; Fig. 10). As summarized in Figure 10, this reversible conformational intermediate unfolds in multiple transitions with midpoint urea concentrations of 2.5 to 5.5 M. Domains and secondary structures are lost and subunits dissociate, as discussed above.

Other investigators have provided evidence that oligonucleotide binding occurs through interaction with multiple OB fold domains that comprise the RPA structure (Bastin-Shanower and Brill 2001). Furthermore, they have suggested that an oligonucleotide-binding site in an OB fold can accommodate only 3–4 nt (Bochkarev et al. 1997). Because only one 15-nt oligonucleotide binds to RPA at a time (Lavrik et al. 1999; Bastin-Shanower and Brill 2001), a 15-nt oligonucleotide may simultaneously interact with and link several domains. Our results suggest that, at least for substrates as long as 15 nt, binding affinity to a beads-on-a-string or compact conformation is the same (Bochkarev et al. 1997). It may be that equally avid binding occurs at alternative domains before and after the first fluorescence transition, or that the first fluorescence transition does not change the relative orientation of the domains used in the binding reactions we observed. Either of these possibilities seems consistent with proposed properties other investigators have attributed to the 13–14-nt or the 8-nt binding modes. Dramatic structural changes that accompany binding are linked to longer polynucleotides than used here (Gomes et al. 1996).

Materials and methods

Chemicals

Immobilized trypsin and guanidine hydrochloride, “sequal grade,” were purchased from Pierce. A Plusone silver staining kit was purchased from Amersham Biosciences. Enzyme grade urea was purchased from USB. Formamide was obtained from EM Science. Sinapinic acid (SPA) and IGEPAL CA 630, were purchased from Sigma. HPLC grade acetonitrile and paraformaldehyde were obtained from Fisher. All other chemicals were purchased from Sigma and were at least reagent grade or better. Water was deionized using a Milipore Mili Q Plus water purification system.

Buffers

HI Buffer (30 mM HEPES at pH 7.8, 0.25% inositol, 1 mM DTT, and 0.25 mM EDTA) was used in experiments that were conducted on the heterotrimeric protein. A zinc-containing buffer (termed Zn Buffer: 20 mM TRIS with pH adjusted to 7.8, 10 mM DTT, 0.01% IGEPAL CA 630, and 10 μ M ZnCl₂) was used in experiments conducted on individual domains of RPA. Running buffer (10 mM MOPSO at pH 7.0 and 100 mM NaCl) was used in gel exclusion HPLC experiments.

RPA purification

Plasmid p11d-tRPA, which contains DNA corresponding to all RPA subunits, was obtained as a generous gift from Marc Wold (Department of Biochemistry, University of Iowa College of Medicine). It was used to transform the BL21 (DE3) strain of *Escherichia coli*. The transformed cells were then induced to over-express human recombinant RPA (rhRPA), and the protein was purified with a slight modification of a previously described method (Henricksen et al. 1994). To ensure zinc was maintained in the protein, EDTA was omitted from all buffers during purification of RPA that was used to generate fold-containing peptide fragments. All buffers were supplemented with 10 mM DTT and 10 μ M ZnCl₂ (Bochkareva et al. 2000).

RPA denaturation profiles

In typical denaturation experiments, RPA was incubated with varying urea (or guanidine hydrochloride) concentrations as described in the “Protein Denaturation” section below. At the end of that incubation, samples were analyzed by fluorescence or CD spectroscopy, as indicated in the respective sections below, or analyzed by methylene modification. Samples analyzed by the latter method were subjected to limited proteolysis, as described in the Limited Proteolysis of RPA section, and reacted with diazirine, as described in the Methylene Carbene section below; the fragments were analyzed as described in the MALDI-TOF section.

Limited proteolysis of RPA

To separate RPA into individual domains, limited proteolytic digestions of RPA were performed in Zn buffer at 37°C. Typically, 100 mg of immobilized trypsin gel was added to an RPA solution, 250–500 μ L (the RPA concentration for methylene labeling experiments equaled 2.38 mg/mL, whereas the concentration for gel exclusion experiments was 0.25 mg/mL prior to proteolysis). The

reaction was allowed to proceed for 2 h with occasional mixing and then stopped by separating the protein from the protease beads using a brief low-speed centrifugation. The protein solution was removed and saved for further analysis.

Gel exclusion

All HPLC gel exclusion experiments were conducted using a Dionex HPLC system and a Biorad Biosil SEC-125 column (300 $\times$ 7.8 mm). The elution profiles were constructed by continuously monitoring the effluent absorbance at 280 nm.

HPLC experiments conducted on the trimeric protein were performed by incubating 250 μ L of RPA (0.20 mg/mL) in a buffered solution containing 2.0 M, 3.5 M, 5.0 M, or 6.0 M urea for at least 18 h (see the Protein Denaturation section below for details). The samples were then injected onto the gel exclusion column, which was equilibrated with running buffer that contained the pertinent concentration of urea. To achieve acceptable separation, the urea-containing running buffer was continuously pumped through the column at a flow rate of 0.2 mL/min. Throughout the experiment, fractions were collected at 1-min intervals using an ISCO FOXY JR. fraction collector. Fractions were later analyzed by SDS-PAGE (see SDS-PAGE section below).

HPLC experiments conducted on the limited trypsin digests (0.25 mg/mL prior to digestion) were performed by injecting 250 μ L of the digest onto the column. Different molecular weight species were well resolved by pumping running buffer through the column at a flow rate of 0.2 mL/min. Fractions were collected continuously at 1-min intervals throughout the experiment. Fractions were later analyzed by SELDI-TOF mass spectrometry (see Mass Spectrometry section below).

SDS-PAGE

SDS-polyacrylamide gel electrophoresis was used to analyze fractions from gel filtration experiments. A 10- μ L aliquot of each chromatographic fraction was diluted into 30 μ L of a buffer containing 62.5 mM Tris at pH 6.8, 2% SDS, 10 mM DTT, and 10% glycerol (concentrations after dilution). The samples were boiled for 5 min and loaded onto a 10% acrylamide gel. RPA (0.1 μ g of total protein) was used as a molecular weight standard. All gels were electrophoresed at 35 mA of constant current. Gels were then stained with the Plusone silver staining kit (Amersham Biosciences) and photographed with a Fuji LAS-3000 digital imager.

Methylene carbene methylation of protein samples

Protein samples were modified by methylene carbene generated from the photolytic decomposition of diazirine (DZN) gas. DZN was synthesized from formamide and paraformaldehyde in a scheme producing the stable intermediate, methylene diammonium sulfate as described by Ohme and Schmitz (1964) and more recently by Richards et al. (2000). In a typical modification, 100 μ L of sample was placed in a gas-tight UV-transparent cuvette and purged with argon. DZN gas was introduced to the space above the sample in a slow stream. The sample was incubated in the dark for 30 min to allow the gas to equilibrate between gas and liquid phases. DZN was photolyzed using 320-nm light produced by a Bausch & Lomb SP200 Mercury light source powered by a SP 200 Mercury power supply and equipped with a Bausch & Lomb monochromator (2700 grooves/mm). The extent of DZN photolysis was quantitated spectrophotometrically by measuring absor-

bance at 320 nm ($E = 180 \text{ M}^{-1} \text{ cm}^{-1}$) in a Cary 300 Bio UV-Vis spectrophotometer (Craig et al. 2002). Conditions were arranged so that 389 nmole of DZN was consumed during the reaction. Reacted samples were diluted to a volume of 1 mL and dialyzed against 5 mM HEPES (pH 7.8) in preparation for MALDI-TOF analysis.

Protein denaturation

Protein denaturation profiles were constructed using a series of solutions containing varying (0.0–7.2 M) concentrations of urea. These solutions were incubated for 18 h at 20°C in a Lauda Brinkman Ecoline RE 120 temperature-controlled waterbath. Protein fluorescence, UV circular dichroism, or MALDI-TOF mass spectrometry was used to monitor RPA's conformation. When RPA was denatured in the presence of substrate, the protein (~1 μM) was incubated as described above but in the presence of 10 μM dA15. RPA's conformation was then monitored using CD spectroscopy. In the latter experiments, ssDNA concentrations were ~25-fold greater than the binding constant for the oligonucleotide, whereas RPA concentrations were within a factor of 3 of the binding constant. Under these conditions, native RPA is virtually saturated with the oligonucleotide (Kim et al. 1994).

Binding affinities of individual domains are not known, and were not measured here. Therefore, we do not know the fractional saturation of individual domains.

Protein renaturation

Samples were incubated as described in the previous section in solutions containing 0.2 M, 1.6 M, and 2.0 M urea. The samples were then diluted 10-fold with the appropriate buffer to yield a final concentration of 0.2 M urea after dilution. These samples were then incubated for at least 18 h at 20°C in a Lauda Brinkman ecoline RE 120 temperature-controlled waterbath. The intrinsic fluorescence of the samples was measured as described in the fluorescence section below. To directly compare fluorescence before and after renaturation (dilution), the fluorescence values of diluted samples were normalized to the 0.2 M sample before and after dilution. The protein concentration in all samples was equal to 0.11 mg/mL before dilution.

CD measurements

CD-monitored unfolding experiments were performed at 20°C using a JASCO J500 A spectropolarimeter. RPA samples monitored by CD were placed in a 1-mm pathlength cuvette. RPA denaturation profiles were constructed by monitoring ellipticity changes at 215 nm at different concentrations of urea. Three individual trials were used to construct an average denaturation profile and to calculate standard deviations (Fig. 2). Baseline ellipticities were subtracted from all spectra. The concentration of RPA for all CD experiments was 0.11 mg/mL (~1 μM).

Fluorescence measurements

Fluorescence-monitored denaturation experiments were performed at 20°C in a 1-cm pathlength cell using a Cary Eclipse spectrofluorimeter with the emission slit set at 5 nm and the excitation slit set at 2.5 nm. Denaturation was monitored by measuring the ratio of fluorescence emission at 340 nm to 304 nm, while using an excitation wavelength of 280 nm. RPA concentrations in fluores-

cence-monitored denaturation experiments were 0.11 mg/mL. Three separate experiments were conducted and average fluorescence amplitudes along with standard deviations are reported (Fig. 2).

Mass spectrometry

MALDI-TOF and SELDI-TOF MS experiments were performed using a Ciphergen Protein Chip System, model PBS II. To monitor methylene incorporation, MALDI-TOF mass spectrometry was used. Mass spectra were typically acquired by placing 1 μL of a protein sample (0.27 mg/mL) and 1 μL of the internal standard, horse heart cytochrome c, on a gold chip (Ciphergen). The sample was dried, and 0.5 μL of a saturated solution of SPA in 50% acetonitrile, 0.1% TFA was added to the spot. Samples were ionized using a spot protocol with a laser intensity of 273. Masses of each peak were determined with the centroid tool contained in the Protein Chip analysis package (Ciphergen User's Guide).

To identify the domains of RPA in gel filtration fractions, SELDI-TOF mass spectrometry was used. One μL of each fraction was placed on a spot of a reverse phase chip (H4 chip from Ciphergen) and placed in a humidity chamber for 15 min at room temperature. The spots were then washed several times with water, and the samples were then allowed to dry. Next, SPA matrix was added as described above. For these experiments, spectra were collected manually by adjusting the laser intensity until satisfactory signal to noise was achieved. At least 50 individual spectra were averaged to produce the spectra reported here. The use of SELDI-TOF technology enabled easy removal of components of the running buffer that were found to interfere with matrix crystallization.

ssDNA-binding activity measurements in the presence of urea

RPA (300 nM) was incubated overnight in solutions containing 0.0, 0.4 M, 0.7 M, 1.25 M, 2.0 M, and 3.0 M urea. Single-stranded DNA binding activity at each urea concentration was measured by successive additions of dA15 or dT8 to the reaction mixture. Quenching of intrinsic tryptophan fluorescence was measured after each addition (Kim et al. 1994). DNA-binding experiments were performed using a Cary Eclipse spectrofluorimeter. The excitation wavelength was set at 300 nm, and emission was monitored at 350 nm using excitation and emission slit widths of 5 nm. The fluorescence data were fit to the Langmuir binding equation, and equilibrium binding constants were extracted (Kim et al. 1994). The values of binding energies are in agreement with previously measured results (Kim et al. 1994). The urea dependence of binding free energies was fit to a two-state model in which full binding was assigned to the first state (equation 1)

$$\Delta G_{\text{Oligo}} = RT \ln \left[K_{\text{RPA}_b} + \frac{K_{\text{RPA}_c} - K_{\text{RPA}_b}}{1 + e^{(m[\text{urea}] - \Delta G^\circ)/RT}} \right] \quad (1)$$

where ΔG_{Oligo} is the dissociation free energy describing oligonucleotide binding at the urea concentration [urea], K_s are association constants for oligonucleotide binding to the subscripted RPA conformations, m is a cooperativity coefficient described below for equation 3, and ΔG° is the free energy for the transition form RPA_c to RPA_b at 0 M urea. Adjustable parameters in this model initially were the oligonucleotide binding affinity of the second state, the folding free energy difference between states, and

the value m . The value of oligonucleotide binding free energy for the second state was indeterminate, and was fixed at values varying from 1 kcal less than the native state to having no binding affinity at all. Results were not substantially changed over the range of binding affinity used for the second state. Therefore, data were routinely analyzed assuming the second state had no oligonucleotide binding affinity. The results for dA15 (dashed line) and dT8 (solid line) are shown in Figure 6, and best fit parameters are included in Table 1. Because urea concentrations cause affinity changes, and m values determined in this analysis are consistent with the second fluorescence transition, an irreversible process, this analysis is of little quantitative significance. It does, however, indicate little if any net energetic linkage between this first fluorescence transition and oligonucleotide binding.

Analysis of denaturation profiles

Denaturation profiles constructed from either methylene incorporation data or spectroscopic data were normalized to the maximum change in the measured property in each transition. When curves were interpreted in terms of multiple transitions, endpoints for each transition were taken to be the inflection point of a smooth curve in the plateau region between transitions.

The first fluorescence transition, the reversible transition, was further analyzed assuming a two-state denaturation model (equation 2)

$$\Delta G_u = -RT \ln K_u = -RT \ln \frac{[U]}{[N]} = -RT \ln \frac{f_u}{(1-f_u)} \quad (2)$$

where ΔG_u is the free energy of unfolding reaction, K_u is the equilibrium constant for the process, and U and N indicate the RPA conformations at 2 M and 0 M urea, respectively. Lastly, f_u is the fraction of RPA in the 2 M urea conformation. Using equation 2, f_u s for each urea concentration in the first fluorescence transition were used to calculate ΔG_u s, which were plotted in turn as indicated by equation 3

$$\Delta G_u^C = \Delta G_u^0 - (mC) \quad (3)$$

where superscripts C and 0 indicate ΔG at concentration C of urea and in the absence of urea, respectively. In this way, we obtained the free energy for the transition from the 0 to the 2 M urea conformation in the absence of urea (Goldenberg 1992).

When reversibility of unfolding transitions was not established, we used similar formalisms to describe the shape of unfolding profiles. Specifically, equation 3 was modified as follows:

$$\ln \frac{\text{fractchange}}{1 - \text{fractchange}} = \text{Const} - (MC) \quad (4)$$

where fractchange is the proportion of the maximum change in the measured parameter for a specific transition observed at urea concentration C, and Const is a constant.

Electronic supplemental material

Three figures are available as Supplemental Material. Supplemental Figure 1 shows the CD spectra of RPA conducted under native conditions, after limited proteolysis, and after denaturation with 7 M urea. Supplemental Figures

2 and 3 show data describing how limited tryptic fragments of RPA were identified. We also have a paragraph describing the conditions of the experiments and a brief statement regarding the results from each figure.

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