A stable tetramer is not the only oligomeric state that mitochondrial single-stranded DNA binding proteins can adopt

Mitochondrial single-stranded DNA (ssDNA)–binding proteins (mtSSBs) are required for mitochondrial DNA replication and stability and are generally assumed to form homotetramers, and this species is proposed to be the one active for ssDNA binding. However, we recently reported that the mtSSB from Saccharomyces cerevisiae (ScRim1) forms homotetramers at high protein concentrations, whereas at low protein concentrations, it dissociates into dimers that bind ssDNA with high affinity. In this work, we are using a combination of analytical ultracentrifugation techniques and DNA binding experiments with fluorescently labeled DNA oligonucleotides, we tested whether the ability of ScRim1 to form dimers is unique among mtSSBs. Although human mtSSBs and those from Schizosaccharomyces pombe, Xenopus laevis, and Xenopus tropicalis formed stable homotetramers, the mtSSBs from Candida albicans and Candida parapsilosis formed stable homodimers. Moreover, the mtSSBs from Candida nivariensis and Candida castellii formed tetramers at high protein concentrations, whereas at low protein concentrations, they formed dimers, as did ScRim1. Mutational studies revealed that the ability to form either stable tetramers or dimers depend on a complex interplay of more than one amino acid at the dimer–dimer interface and the C-terminal unstructured tail. In conclusion, our findings indicate that mtSSBs can adopt different oligomeric states, ranging from stable tetramers to stable dimers, and suggest that a dimer of mtSSB may be a physiologically relevant species that binds to ssDNA in some yeast species.

In mitochondria, ssDNA-binding activity is provided by SSBs that bear similarity to the SSB from Escherichia coli (EcSSB). Human mitochondrial SSB (HsmtSSB) forms homotetramers that have DNA-binding activities similar to EcSSB (1–7). The mtSSB from Saccharomyces cerevisiae (ScRim1) forms tetramers in solution (8, 9). However, we recently showed that, at low protein concentrations, ScRim1 tetrads dissociate into dimers (10). ScRim1 dimers are the dominant species that binds with high affinity to ssDNA, and formation of tetramers is favored by a DNA-induced tetramerization that depends on the length of the ssDNA (10). These findings raise the question of whether this is a behavior exclusive to ScRim1 or a more general property shared among mitochondrial SS Bs.

In the known crystal structures of homotetrameric SSBs, one tetramer forms from two dimers (AB and CD) arranged in a head-to-head orientation. The interface between the AB and CD dimers is stabilized by four symmetric pairs of potential salt bridges, which is characteristic of different SSBs (3, 10–12). In addition, this interface is stabilized by hydrophobic interactions, of which the ones formed by a highly conserved tyrosine appear to be important for tetramer stability. Mutations Y78R and Y78W in EcSSB destabilize the tetramer, with Y78R leading to formation of dimers (13). Sequence analysis of ~200 known or predicted mtSSBs from different yeast species indicates that although tyrosine is the preferred amino acid at this position, leucine, isoleucine, phenylalanine, or histidine can also be found. Interestingly, we recently showed that a tyrosine to histidine mutation in ScRim1 is sufficient to destabilize the tetramer and generate stable dimers (10), suggesting that natural changes at this position may contribute to changes in the oligomeric state of mtSSBs.

In this work, we set out to answer two basic questions. First, is the ability of ScRim1 to bind DNA as a dimer a property shared with other mitochondrial SS Bs? Second, is the nature of the amino acid at the highly conserved position at the AB/CD interface generally occupied by a tyrosine, a major contributor to stability of a tetramer, and an indicator of oligomeric states other than a tetramer? To this end, we overexpressed, purified, and characterized mtSSBs from five yeast species and two Xenopus species. The mtSSB from Schizosaccharomyces pombe (SpRim1) was chosen because this protein has been proposed to form stable tetramers (14). The mtSSB from Candida parapsilosis (Mtp1/mtTBP) was chosen because, although it can form tetramers in the presence of cross-linking agents, it also forms dimers (15–17). Moreover, at the conserved position at the AB/CD interface, Mtp1/mtTBP harbors a natural isoleucine rather than a tyrosine. Our recent studies showed that mutation of this conserved tyrosine at the AB/CD interface in ScRim1 (Y85H) leads to formation of stable dimers (10), suggesting that this residue plays a crucial role in stability of a tetramer. Thus,
mtSSBs adopt different oligomeric states

![Diagram](image)

Figure 1. mtSSBs adopt different oligomeric states in solution. A and B, protein concentration dependence (monomers) of the s$_{20,w}$ (A) and molecular weight (B), determined in buffer HK$_{150}$. The color coding in A and B is the same. For CnRim1, the molecular weight (M.W.) was also determined in buffer HK$_{150}$, (open, red circles). Lines are for visual help only.

we chose to test Candida albicans mtSSB (CaRim1), which, by sequence analysis, harbors a natural histidine at this position. If the nature of the amino acid at this position has major contributions to the stability of tetramers, we would predict CaRim1 to form dimers as well. We also generated mtSSBs from Candida castelli (CcRim1) and Candida nivariensis (CnRim1) to test whether, in solution, they behave similarly as ScRim1. Finally, we generated mtSSBs from Xenopus laevis (XlmtSSB) and Xenopus tropicalis (XtmtSSB) as examples of higher eukaryote mtSSBs that, at the conserved position at the AB/CD interface rather than a tyrosine, harbor a natural isoleucine or phenylalanine, respectively.

Results

Mitochondrial SS Bs from different yeasts have different propensities to form homotetramers in solution

The oligomeric state of purified mtSSBs from different yeast species (Fig. S1A) was examined in buffer HK$_{150}$ (20 mM HEPES pH 7.4, 1% (v/v) glycerol, 150 mM KCl) at different protein concentrations by both analytical sedimentation velocity (Fig. 1A, s$_{20,w}$) and equilbrium experiments (Fig. 1B, M.W.).

Consistent with a previous publication reporting that SpRim1 forms tetramers by criteria of analytical gel filtration (14), our analyses of both the s$_{20,w}$ and the molecular weight indicate that SpRim1 forms a stable tetramer in solution, with no evidence of smaller oligomeric species even at the lowest protein concentration tested (Fig. 1, A and B, black). On the other hand, the mtSSB from C. parapsilosis (Mtp1/mtTBP) has been shown to form tetramers in the presence of cross-linking agents, albeit a large fraction still formed dimers (15). Our data indicate that, in the absence of cross-linking agents, even at the highest protein concentration tested, Mtp1/mtTBP has an s$_{20,w}$ much lower than any of the mtSSBs examined, and analysis of the molecular weight indicates that Mtp1/mtTBP forms a species in solution that is no larger than a dimer (Fig. 1, A and B, gray). The ability to form stable dimers in solution is not limited to Mtp1/mtTBP. Analyses of the protein concentration dependence of both the s$_{20,w}$ and the molecular weight indicate that CaRim1 forms stable dimers in solution as well (Fig. 1, A and B, blue). The observation that both Mtp1/mtTBP and CaRim1 harbor natural substitutions at the conserved tyrosine at the AB/CD dimer–dimer, together with the finding that mutation of this tyrosine in ScRim1 (10) and EcSSB (13) generates stable dimers, points to this amino acid position as a major determinant for dimer/tetramer stability.

The situation is different for CnRim1 and CcRim1. Sedimentation velocity data are consistent with CnRim1 forming a tetramer at the highest protein concentration, whereas a smaller oligomeric species forms at lower protein concentrations, as indicated by the decreasing s$_{20,w}$ (Fig. 1A, green). This behavior is similar to what we recently reported for ScRim1 (10). Direct determination by equilibrium analytical sedimentation of the molecular weight of CnRim1 was hampered by instability of the samples over the long times needed to reach equilibrium. On the other hand, CcRim1 is well-behaved both in sedimentation velocity and equilibrium experiments, and the determined s$_{20,w}$ and molecular weight indicate that CcRim1 forms stable tetramers over the protein concentration range examined (Fig. 1, A and B, red).

Except for CnRim1, the yeast mtSSBs examined showed that, in solution, they form either tetramers or dimers. Next, we tested whether these apparently stable oligomeric species are the dominant ones that interact with ssDNA at the much lower protein concentrations expected for high-affinity binding to ssDNA.

S. pombe Rim1 binds DNA as a tetramer, whereas C. albicans Rim1 binds DNA as a dimer

To begin testing which oligomeric species of SpRim1 and CaRim1 is able to bind ssDNA, we first determined their occluded site size on ssDNA, a parameter needed to understand the stoichiometry of the protein–DNA complexes formed on different lengths of ssDNA. We note that, at the position corresponding to the conserved Trp-54 in EcSSB or Trp-60 in ScRim1, SpRim1 harbors a natural phenylalanine, resulting in a smaller quenching of protein fluorescence upon DNA binding (Fig. S3A) (10, 18, 19). The calculated occluded site size increases as the salt concentration increases (Fig. S3B), indicating that, similar to other known SSBs (1, 5, 20, 21), SpRim1 can access at least two DNA binding modes. DNA binding experiments monitoring the change in spectroscopic signals from the
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DNA (Cy3 fluorescence in Fig. 2A and Fig. S3C and fluorescence anisotropy in Fig. 2B) indicate that a tetramer of Sprim1 binds to ssDNA less than 59 nt long. However, for a 68-nt ssDNA, the change in FRET signal indicates that a second Sprim1 tetramer can bind to the ssDNA (Fig. S3D). This behavior is similar to what has been observed for both EcsSB (22, 23) and HsmtSSB (1), and it is consistent with the ability of Sprim1 to access alternative binding modes on DNA. From these data, we conclude that Sprim1 behaves as a stable tetramer and that this is the species that binds ssDNA.

A dimer of Caram1 binds ssDNA with an occluded site size of ~28 nt that is little dependent on the salt concentration (Fig. S3B), suggesting that, like Scrim1 (10), Caram1 does not efficiently access alternative binding modes on DNA. Fig. 2, A and B, shows DNA binding experiments for Caram1 compared with Sprim1. The data indicate that a dimer of Caram1 binds to ssDNA independent of the signal monitored. Binding of a dimer of Caram1 to ssDNA further confirmed by analytical sedimentation equilibrium experiments monitoring the signal of short, Cy3-labeled DNAs in the presence of a large excess of protein (Fig. S4A). The calculated molecular weight of the Caram1-DNA complexes indicates that more than a dimer of Caram1 binds (Fig. S4B), even at high protein and DNA concentrations. This behavior is different from what we recently reported for Scrim1 (10), where binding of DNA to a dimer of Scrim1 induces tetramerization, even for lengths of ssDNA that are sufficient to accommodate only one dimer of the protein. Binding of a second dimer of Carm1 occurs on a 68-nt ssDNA (Fig. S3D), as expected from a site size of ~28 nt. However, unlike Sprim1, only two Caram1 dimers bind to this length of ssDNA.

Finally, Fig. 2, C and D, shows DNA binding experiments for Mtp1/mtTBP compared with Carm1. Although Mtp1/mtTBP appears to have acquired ssDNA binding specificity for the ends of the linear mtDNA of C. parapsilosis (15, 17), it binds homo-oligomeric ssDNA with sufficient affinity. Consistent with being a dimer in solution, a dimer of Mtp1/mtTBP is the dominant species that binds to these lengths of ssDNA.

C. nivariensis and C. castellii Rim1s bind DNA as dimers of dimers

Next we tested how Cnr1 and Ccr1 bind to ssDNA and which is the oligomeric species responsible for ssDNA binding at low protein concentrations. Fig. 3A shows DNA binding experiments for Cnr1 and Ccr1, monitoring FRET...
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between the ends of the ssDNA. Binding of CnRim1 is accompanied by biphasic behavior of the monitored signal, providing direct evidence that more than one molecule of CnRim1 binds to this ssDNA. The initial increase in FRET peaks at two CnRim1 molecules, e.g. one dimer, followed by a decrease in FRET that we associate with lower-affinity binding of a second CnRim1 dimer. This is the same behavior we recently reported for ScRim1 (10). Consistent with the sedimentation velocity data (Fig. 1A) suggesting that, at low protein concentration, CnRim1 forms an oligomeric species smaller than a tetramer, these DNA binding data indicate that, at low protein concentrations, CnRim1 exists predominately as a dimer and that this is the species that dominates the high-affinity binding to ssDNA. Surprisingly, CcRim1 behaves in a similar manner as CnRim1 (Fig. 3A), and the biphasic behavior of the signal provides strong evidence that CcRim1 does not bind as a single, stable tetramer but rather as a dimer. This is in stark contrast to the expectation from the analytical sedimentation analysis indicating that, at the higher protein concentrations used in these experiments (>3 μM monomer), CcRim1 forms tetramers and provides the first evidence that CcRim1 tetramers are unstable and dissociate into dimers at low protein concentrations.

We further corroborated the data in Fig. 3A by performing DNA binding experiments monitoring both the change in fluorescence intensity and anisotropy of short, carboxyfluorescein–labeled DNA as a function of protein concentration. Fig. 3B shows DNA binding data for CnRim1 and CcRim1 to a FAM-labeled dT20. The change in fluorescence intensity of the labeled DNA is biphasic for both proteins, and for CcRim1, the change in anisotropy is also biphasic. The behavior of the monitored signals is consistent with binding of more than one dimer, even for this short ssDNA. The solid lines in Fig. 3A are fits of the data to a 2:1 binding model with K and L constants characterizing the binding of the first and second dimer, respectively (Supporting information). Estimates of the K and L constant are as follows: K20 = 1.3 x 10^9 M^-1 and L20 = 1.4 x 10^7 M^-1 for CnRim1 and K20 = 2 x 10^9 M^-1 and L20 = 8 x 10^7 M^-1 for CcRim1. The same values we reported for ScRim1 are K20 = 1.6 x 10^8 M^-1 and L20 = (1.5 - 3) x 10^6 M^-1 (10). The L20 constant changes in the order ScRim1 < CnRim1 < CcRim1, indicating that a major difference among these three mtSSBs is their propensity to tetramerize on DNA.

For ScRim1, we showed that the L constant increases as the ssDNA length increases (10). As more DNA becomes available, interaction of the second dimer with DNA stabilizes formation of a tetramer. Next we tested whether this was the case for CnRim1 and CcRim1 as well. Fig. 3, C and D, shows DNA binding data monitoring both the change in fluorescence anisotropy and intensity of a FL-labeled dT38. Again, the biphasic behavior of the fluorescence intensity change is a clear indication of binding of multiple proteins. Analysis of the data with a 2:1 binding model (Fig. 3, C and D, solid lines) provides estimates of the K and L constant as follows: K38 = 3 x 10^9 M^-1 and L38 = 3 x 10^8 M^-1 for CnRim1 and K38 = 6 x 10^9 M^-1 and L38 = 6 x 10^8 M^-1 for CcRim1. The same values we reported for ScRim1 are K38 = 3 x 10^9 M^-1 and L38 = 7 x 10^7 M^-1 (10). Similar to what we reported for ScRim1 (10), increasing the length of the ssDNA (from 20 to 38 nt) increases the L38 constant for both CnRim1 and CcRim1, consistent with interaction of the second dimer with DNA stabilizing the tetramer. Also, similar to what is observed with the shorter ssDNA, the L38 constant changes in the order ScRim1 < CnRim1 < CcRim1, further supporting the conclusion that the propensity to tetramerize on DNA is a major property distinguishing these mtSSBs.

**mtSSBs tetramer stability depends on more than one residue at the AB/CD interface**

We showed that, in ScRim1, mutation to histidine of a conserved tyrosine at the AB/CD tetramer interface leads to destabilization of a tetramer into dimers (10). The data in the previous section showed that CaRim1, which harbors a natural histidine to tyrosine change at this conserved position, forms stable dimers in solution. Thus, we tested whether the nature of this amino acid at the AB/CD interface, generally occupied by a tyrosine, makes major contributions to the stability of a tetramer and whether it is an indicator of oligomeric states other than a tetramer.

To this end, we determined the oligomeric state of two mtSSBs from the *Xenopus* genus. On the hand, we chose XlmtSSB because, instead of a tyrosine, it harbors a natural leucine, and it forms tetramers (24); on the other hand, XlmtSSB harbors a natural phenylalanine, and its oligomeric state is unknown. Analytical ultracentrifugation experiments performed as a function of protein concentration (Fig. 4A) indicate that both XlmtSSB and XlmtSSB form stable tetramers. Importantly, both *Xenopus* mtSSBs bind ssDNA as stable tetramers (Fig. 4B).

The ability of the *Xenopus* mtSSB to form stable tetramers provides a direct indication that the presence of a tyrosine at the AB/CD interface is not a prerequisite for formation of stable tetramers. This conclusion is further supported by mutational studies at the AB/CD interface of SpRim1, which, in solution, forms stable dimers. SpRim1 harboring a Y91H mutation (SpRim1<sup>Y91H</sup>) still forms tetramers at the lowest protein concentration tested (Fig. 4C, gray), indicating that the change of tyrosine to histidine is not enough to destabilize the AB/CD interface. However, mutation Y91R (SpRim1<sup>Y91R</sup>), which introduces a large positively charged amino acid, leads to formation of a dimer of SpRim1 (Fig. 4C, red), like EcSSB (13). Surprisingly, and unlike EcSSB, SpRim1<sup>Y91R</sup> can still form tetramers at high protein concentration, suggesting that SpRim1 forms a stronger dimer–dimer interface. To further test this point, we made the same mutation in human mtSSB and found that HsmtSSB<sup>Y109R</sup> forms a stable dimer even at high protein concentration (Fig. 4D), consistent with HsmtSSB having a weaker dimer–dimer interface.

Next we tested the opposite scenario and asked which changes within a stable dimer would be required to form stable tetramers. For this, we used CaRim1, which forms stable and well-behaved dimers in solution. First, we made the single point mutation H87Y, which reintroduces the conserved tyrosine at the AB/CD dimer–dimer interface. Sedimentation velocity analysis indicates that the distribution of sedimentation coefficients of CaRim1<sup>H87Y</sup> is shifted to larger values (Fig. 5A, blue). However, although an oligomer larger than a dimer may be
forming, this mutation does not appear to be enough to form stable tetramers, even at the highest protein concentration tested. It is only with the introduction of a second mutation within the putative dimer–dimer interface (CaRim1H87Y/F18I) that a second oligomeric species appears, consistent with formation of significant amounts of tetramer (Fig. 5A, red). Again, these data indicate that multiple amino acids at the dimer–dimer interface are needed to form a stable tetramer.

Finally, we found that the C-terminal tail of CaRim1, predicted to be unstructured, has a role in modulating the oligomeric state of the protein. We generated a CaRim1 construct that removes the last 25 amino acids, leaving what, by sequence analysis, is predicted to be the core of the OB-fold (oligonucleotide/oligosaccharide-binding fold). Fig. 5B shows the distribution of sedimentation coefficients of CaRim1H87Y/Δ25 determined at three protein concentrations. Although at low protein concentration CaRim1H87Y/Δ25 forms dimers, at higher protein concentration, the $s_{20,w}$ shifts to larger values, suggesting that a larger oligomeric species may form, similar to what is observed in Fig. 5A for the H87Y mutation. We also generated the same deletion in the context of the H87Y mutation (CaRim1H87Y/Δ25). Surprisingly, CaRim1H87Y/Δ25 forms a large fraction of tetramers even at the lowest concentration tested (Fig. 5B, red). Formation of tetrers was further confirmed by sedimentation equilibrium experiments (Fig. S4C). Interestingly, the last 25 residues of the C terminus tail (Fig. S1B) contain a peculiar distribution of amino acids, with three positively charged amino acids in the first seven residues and eight glutamates in the next 18 residues, six of which are arranged in a Glu-Gly repeat. Thus, we tested whether removal of the negatively charged region was enough to lead to stabilization of tetramers. To this end, we generated the constructs CaRim1Δ18 and CaRim1H87Y/Δ18. The sedimentation velocity analysis in Fig. 5C shows that CaRim1Δ18 forms dimers even at high protein concentration. Also, different from CaRim1H87Y/Δ25, CaRim1H87Y/Δ18 does not appear to form...
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stable tetramers (Fig. 5C, blue). These data strongly suggest that tetramer stabilization upon removal of the last 25 amino acids originates mainly from removal of the first seven amino acids rather than from removal of the negatively charged region.

Discussion

mtSSBs bear similarity to the homotetrameric E. coli SSB and the ones that have been characterized form homotetramers that bind ssDNA with high affinity. Of these, the best studied is HsmtSSB, which forms stable homotetramers in solution (1, 2), as do SpRim1 and XlmtSSB (14, 24). Consistent with these observations, in this work, we showed that XlmtSSB, XtmtSSB, and SpRim1 form stable homotetramers at all protein concentrations tested and that this oligomeric species is the one responsible for high-affinity DNA binding.

However, our recent observation that ScRim1 does not form stable homotetramers in solution and binds to DNA as a dimer of dimers (10) raises the question of whether this behavior is exclusive to ScRim1 or a more general property shared among yeast mitochondrial SSBS. Phylogenetic analysis (Fig. S2A) indicated that the mtSSB from Candida glabrata, CrRim1, and CcRim1 cluster with ScRim1 and that they have larger sequence homology to ScRim1 than to CaRim1. Thus, we tested the possibility that, like ScRim1, these mtSSBs may not form stable tetramers in solution. Indeed, we showed that CnRim1 and CcRim1 behave similarly as ScRim1. At the protein concentrations achievable in analytical ultracentrifugation experiments, CcRim1 appears to form stable tetramers, whereas CnRim1 dissociates into a lower oligomeric species. However, at low protein concentration, both mtSSBs bind ssDNA as dimers, and interaction with ssDNA favors tetramerization, as we showed for ScRim1 (10). Although the dimers of all three mtSSBs can bind with similar affinity as ssDNA, their tetramerization constant changes in the order ScRim1 < CnRim1 < CcRim1, indicating that the propensity to tetramerize on DNA is a distinguishing property of these mtSSBs. This observation suggests that the ability to form dimers or tetramers via changes in protein concentration, changes of the length of ssDNA available for interaction, or modulation by still unidentified interacting partners may be a means to regulate the function of these mtSSBs. For example, the ability of mtSSBs to form dimers or tetramers may be a means to modulate their ability to engage in cooperative interactions on DNA and/or long-range interactions.

In E. coli, mutation Y78R leads to dissociation of the tetramer into dimers (13); however, this mutation does not yield viable strains, indicating that dimers are not functional in E. coli. Whether a dimer of mtSSB is sufficient to sustain mitochondrial functions in some species remains to be determined. The ability of ScRim1, CnRim1, and CcRim1 to be intrinsically able to form dimers that are competent for high-affinity DNA binding suggests the possibility that a dimer may be physiologically functional in these yeast species. This proposal is further supported by our findings that two of the mtSSBs studied in this work behave as stable dimers in solution and bind ssDNA as dimers. Work by Nosek et al. (15) showed that the mtSSB from C. parapsilosis (Mtp1/TBP) can form tetramers in solution in the presence of cross-linking agents (15). Analytical ultracentrifugation experiments in this work indicate that, in the absence of cross-linking agents, Mtp1/TBP forms an oligomeric species that is no larger than a dimer and that this species is active for binding to the short ssDNAs tested, with no evidence of tetramerization on DNA. However, we note that Mtp1/TBP appears to have gained specificity for sequence repeats at the end of mtDNA (15, 17). Whether ssDNA sequence composition can modulate the oligomeric state of Mtp1/TBP on DNA remains to be determined.

We also found that the CaRim1 forms stable dimers in solution. Different from ScRim1, CnRim1, and CcRim1, binding of CaRim1 to short ssDNAs does not induce formation of tetramers, strongly suggesting that the tetramerization constant for this mtSSB is very weak. However, two dimers of CaRim1 can bind to ssDNA that is at least twice its occluded site size, and the increase in FRET between the ends of the DNA suggests that a tetramer of CaRim1 can form on longer ssDNA.

Sequence analysis of Mtp1/TBP and CaRim1 (Fig. S1B) indicates that, in the region that forms the AB/CD dimer–dimer interface in known tetrameric SSBS, these two mtSSBs harbor either an isoleucine or a histidine rather than a highly conserved tyrosine. We showed that tyrosine to histidine mutation at this position in ScRim1 leads to formation of stable dimers (10). Thus, it would be tempting to reach the simple conclusion that the natural changes at this position in CaRim1 and Mtp1/TBP are the reason why these two mtSSBs form stable dimers. However, the data in this work show that the ability of mtSSBs to form either tetramers or dimers is the result of changes of at least two or more amino acids and not necessarily at the AB/CD dimer interface.

The change of the conserved tyrosine at the AB/CD dimer–dimer interface with amino acids that are naturally found in other mtSSBs (Fig. S1C) is not sufficient to destabilize strong tetramers. For example, in place of the conserved tyrosine, XlmtSSB and XtmtSSB harbor either a natural leucine or phenylalanine, respectively, but both proteins form stable tetramers that bind with high-affinity ssDNA. Thus, for XlmtSSB and XtmtSSB, other amino acids must be contributing to the formation of stable tetramers. Moreover, in SpRim1, mutation of the conserved tyrosine to a naturally occurring histidine does not destabilize the tetramer. Only introduction of an arginine, not naturally occurring (Fig. S1C), offers sufficient destabilization of the AB/CD interface to favor formation of dimers. Interestingly, even introduction of arginine in place of the conserved tyrosine has a different effect on the propensity of HsmtSSB and SpRim1 to form tetramers, suggesting that, even though both proteins form tetramers, multiple and different amino acids contribute to their stability. Taken together, these observations suggest the presence of a complex network of interactions that contributes to the stability of different homotetrameric mtSSBs, possibly originating from compensatory amino acid changes. This latter point is further reinforced by mutational studies in E. coli SSB. Mutation of histidine 55, at the AB (or CD) dimer interface, to tyrosine or lysine leads to unstable tetramers of EcSSB that, at low protein concentrations, cooperatively dissociate into monomers (25). However, second-site compensatory mutations outside of the AB interface (Q76L and
The contribution to tetramer stability of at least two or more amino acids, and not necessarily at the AB/CD dimer–dimer interface, is also evident from our study, where we asked which amino acid changes would be needed to generate a tetramer from a stable dimer. In place of the conserved tyrosine at the putative AB/CD interface, CaRim1 harbors a natural histidine. If this naturally occurring change in CaRim1 were the sole contributor to formation of dimers, then we would expect that reverting this amino acid back to a tyrosine would lead to formation of CaRim1 tetramers. However, reverting the histidine back to a tyrosine (CaRim1<sup>H87Y</sup>) does not lead to a significant population of tetramers. Multiple sequence alignment shows that Phe-18 in CaRim1 is conserved in ScRim1, CrRim1, and CcRim1, which can form dimers at low protein concentrations. However, in SSBSs that form stable tetramers, this is a leucine in HsmtSSB, XtmtSSB, and Plasmodium falciparum SSB, an isoleucine in SpRim1, a methionine in XtmtSSB, and a valine in <i>E. coli</i> SSB. In known crystal structures, this residue is at the dimer–dimer interface and is involved in hydrophobic interactions (3, 10–12). Although all of these amino acids are hydrophobic in nature, we reasoned that the aromatic ring of phenylalanine is bulkier and may hinder the packing of the dimer–dimer interface. Thus, we mutated Phe-18 in CaRim1 to isoleucine based on SpRim1 being the nearest homolog that forms stable tetramers. Consistent with our expectation, mutation F18I in the background of H87Y (<i>i.e. CaRim1<sup>H87Y/F18I</sup></i>) leads to a significant fraction of tetramers. Again, this observation points to multiple residues at the dimer–dimer interface being involved in formation of stable tetramers.

Moreover, we found that, in CaRim1, amino acids within its unstructured C-terminal tail contribute to maintaining a dimer form of the protein. Upon deletion of the last 25 amino acids, CaRim1 begins to form an oligomeric species larger than a dimer, similar to what is observed with the H87Y mutation. However, the same deletion coupled to the H87Y mutation leads to a large fraction of tetramers even at the lowest concentration tested. The data strongly suggest that the first seven amino acids within the 25 amino acid tail, and not its highly negatively charged region, play a role in maintaining a dimer of CaRim1.

In conclusion, the data presented in this work indicate that mtSSBs can adopt different oligomeric states ranging from stable tetramers to stable dimers. The repertoire of oligomeric states yeast mtSSBs can access is richer than expected based on the best-characterized homo-oligomeric SSBSs. The observation that more than one yeast mtSSB can form dimers, intrinsic or at low protein concentrations, suggests that, in these species, a dimer of mtSSB is a physiologically relevant species that binds to ssDNA.

**Experimental procedures**

**Protein expression and purification**

The sequences for the mtSSBs, codon-optimized for overexpression in <i>E. coli</i>, were either synthesized (GenScript) or ordered as G-blocks (Integrated DNA Technologies) and cloned in pET28a as described previously (10). The sequences of mtSSBs from <i>X. laevis</i> (NP_001095241.1) and <i>X. tropicalis</i> (NP_001072658.1) were retrieved from the NCBI database, and the mature forms that were cloned (excluding the mitochondrial targeting sequence) comprise residues 17–146 and 17–148, respectively. The sequence of the mtSSB from <i>S. pombe</i> (SPAC2F3.04c) was retrieved from the Pombase database, and the mature form cloned comprises residues 24–150. The sequences of mtSSBs from <i>C. albicans</i> (C1_05680C_A) and <i>C. parapsilosis</i> (CPAR2_107440) were retrieved from the PhylomeDB database, and the mature forms cloned comprise residues 17–143 and 17–133, respectively. The sequences of different mtSSBs studied in this work were generated by site-directed mutagenesis. Proteins were overexpressed and purified following protocols we described recently for ScRim1 and HsmtSSB (10), with modifications detailed in the Supporting information. Before use, the proteins were dialyzed in buffer HK<sub>150</sub>M<sub>v</sub>, where H is 20 mM HEPES (pH 7.4) and 1% (v/v) glycerol, K<sub>150</sub> is 150 mM KCl, and M<sub>v</sub> is 5 mM MgCl<sub>2</sub> when present. Protein concentrations were determined spectrophotometrically using the extinction coefficients in Table S1.

**DNA substrates**

Fluorescently modified oligo(dT)<sub>n</sub> of lengths up to 38 nt were purchased from Integrated DNA Technology. Fluorescently modified oligo(dT)<sub>n</sub> longer than 38 nt and the ones modified with a donor–acceptor couple were a kind gift from Dr. Lohman (Washington University School of Medicine, St. Louis, MO). DNA concentrations were determined spectrophotometrically using the extinction coefficient ε<sub>260</sub> = 8,100 M<sup>-1</sup> cm<sup>-1</sup> for dT, corrected for the contribution at 260 nm of the fluorophores.

**Analytical ultracentrifugation and DNA-binding experiments**

All analytical sedimentation experiments were collected on an Optima XL-A analytical ultracentrifuge using an An60Ti rotor (Beckman Coulter) as described previously (10). Sedimentation velocity experiments were performed using Epon charcoal-filled double-sector centripetals at 50,000 rpm, monitoring absorbance at either 280 nm or 230 nm. Sedimentation equilibrium experiments were performed using Epon charcoal-filled six-sector centripetals at 12,000–27,000 rpm, monitoring absorbance at either 280 nm or 230 nm. Sedimentation equilibrium and velocity data were processed and analyzed with SedFit/SedPhat (National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health). All fluorescence titrations were performed at 20 °C with an L-format PC1 spectrofluorimeter (ISS, Champaign, IL) equipped with Glan-Thompson polarizers, as described previously (10). Excitation and emission wavelengths were as follows: λ<sub>excitation</sub> = 520 nm and λ<sub>emission</sub> = 565 nm for Cy3-labeled DNA, λ<sub>excitation</sub> = 520 nm and λ<sub>emission</sub> = 662 nm for Cy5-Cy3-labeled DNA, λ<sub>excitation</sub> = 520 nm and λ<sub>emission</sub> = 706 nm for Cy5.5-Cy3-labeled DNA, and λ<sub>excitation</sub> = 490 nm and λ<sub>emission</sub> = 578 nm for Cy5-Cy3-labeled DNA.
mtSSBs adopt different oligomeric states

\[ \lambda_{\text{emission}} = 530 \text{ nm for FAM- and FL-labeled DNA. Details of the 2:1 model used to analyze the binding isotherms are provided in the Supporting information.} \]

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**References**

1. Qian, Y., and Johnson, K. A. (2017) The human mitochondrial single-stranded DNA-binding protein displays distinct kinetics and thermodynamics of DNA binding and exchange. *J. Biol. Chem.* 292, 13068–13084 [CrossRef] [Medline]

2. Wong, T. S., Rajagopalan, S., Townsley, F. M., Freund, S. M., Petrovich, M., Loakes, D., and Fersht, A. R. (2009) Physical and functional interactions between human mitochondrial single-stranded DNA-binding protein and tumour suppressor p53. *Nucleic Acids Res.* 37, 568–581 [Medline]

3. Yang, C., Curth, U., Urbanke, C., and Kang, C. (1997) Crystal structure of human mitochondrial single-stranded DNA-binding protein at 2.4 A resolution. *Nat. Struct. Biol.* 4, 153–157 [CrossRef] [Medline]

4. Webster, G., Genschel, J., Curth, U., Urbanke, C., Kang, C., and Hilgenfeld, R. (1997) A common core for binding single-stranded DNA: structural comparison of the single-stranded DNA-binding proteins (SSB) from *E. coli* and human mitochondria. *FEBS Lett.* 411, 313–316 [CrossRef] [Medline]

5. Lohman, T. M., and Overman, L. B. (1985) Two binding modes in *Escherichia coli* single strand binding protein-single stranded DNA complexes. Modulation by NaCl concentration. *J. Biol. Chem.* 260, 3594–3603 [Medline]

6. Lohman, T. M., and Ferrari, M. E. (1994) *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. *Annu. Rev. Biochem.* 63, 527–570 [CrossRef] [Medline]

7. Lohman, T. M., Bujalowski, W., Overman, L. B., and Wei, T. F. (1988) Interactions of the *E. coli* single strand binding (SSB) protein with ss nucleic acids: binding mode transitions and equilibrium binding studies. *Biochim. Biophys. Acta.* 978, 178–192 [CrossRef] [Medline]

8. Ramanagoudr-Bhojappa, R., Blair, L. P., Tackett, A. J., and Raney, K. D. (2013) Physical and functional interaction between yeast Pif1 helicase and RIM1 single-stranded DNA binding protein. *Nucleic Acids Res.* 41, 1029–1046 [CrossRef] [Medline]

9. Van Dyck, E., Foury, F., Stillman, B., and Brill, S. J. (1992) A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB. *EMBO J.* 11, 3421–3430 [CrossRef] [Medline]

10. Singh, S. P., Kuksahl, V., De Bona, P., Antony, E., and Galletto, R. (2018) The mitochondrial single-stranded DNA binding protein from *S. cerevisiae*, RIM1, does not form stable homo-tetramers and binds DNA as a dimer of dimers. *Nucleic Acids Res.* 46, 7193–7205 [CrossRef] [Medline]

11. Antony, E., Welland, E. A., Korolev, S., and Lohman, T. M. (2012) *Plasmodium falciparum* SSB tetramer wraps single-stranded DNA with similar topology but opposite polarity to *E. coli* SSB. *J. Mol. Biol.* 420, 269–283 [CrossRef] [Medline]

12. Raghunathan, S., Ricard, C. S., Lohman, T. M., and Waksman, G. (1997) Crystal structure of the homo-tetrameric DNA binding domain of *Escherichia coli* single-stranded DNA-binding protein determined by multi-wavelength x-ray diffraction on the selenomethionyl protein at 2.9-A resolution. *Proc. Natl. Acad. Sci. U.S.A.* 94, 6652–6657 [CrossRef] [Medline]

13. Landwehr, M., Curth, U., and Urbanke, C. (2002) A dimeric mutant of the homotetrameric single-stranded DNA binding protein from *Escherichia coli*. *J. Biol. Chem.* 283, 1325–1333 [Medline]

14. Nosek, J., Tomáška, L., Pagácová, B., and Fukuhara, H. (1999) Mitochondrial telomere-binding protein from *Candida parapsilosis* suggests an evolutionary adaptation of a nonspecific single-stranded DNA-binding protein. *J. Biol. Chem.* 274, 8850–8857 [CrossRef] [Medline]

15. Tomaska, L., Makhov, A. M., Nosek, J., Kucejova, B., and Griffith, J. D. (2001) Electron microscopic analysis supports a dual role for the mitochondrial telomere-binding protein of *Candida parapsilosis*. *J. Mol. Biol.* 305, 61–69 [CrossRef] [Medline]

16. Tomáška, L., Nosek, J., and Fukuhara, H. (1997) Identification of a putative mitochondrial telomere-binding protein of the yeast *Candida parapsilosis*. *J. Biol. Chem.* 272, 3049–3056 [CrossRef] [Medline]

17. Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., and Chase, J. W. (1987) Investigation of the role of individual tryptophan residues in the binding of *Escherichia coli* single-stranded DNA binding protein to single-stranded polynucleotides: a study by optical detection of magnetic resonance and site-selected mutagenesis. *J. Biol. Chem.* 262, 10938–10945 [Medline]

18. Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., and Chase, J. W. (1987) Role of tryptophan 54 in the binding of *E. coli* single-stranded DNA-binding protein to single-stranded polynucleotides. *FEBS Lett.* 211, 155–159 [CrossRef] [Medline]

19. Bujalowski, W., and Lohman, T. M. (1986) *Escherichia coli* single-stranded binding protein forms multiple, distinct complexes with single-stranded DNA. *Biochemistry* 25, 7799–7802 [CrossRef] [Medline]

20. Bujalowski, W., Overman, L. B., and Lohman, T. M. (1988) Binding mode transitions of *Escherichia coli* single strand binding protein—single-stranded DNA complexes: cation, anion, pH, and binding density effects. *J. Biol. Chem.* 263, 4629–4640 [Medline]

21. Antony, E., Kozlov, A. G., Nguyen, B., and Lohman, T. M. (2012) *Plasmodium falciparum* SSB tetramer binds single-stranded DNA only in a fully wrapped mode. *J. Mol. Biol.* 420, 284–295 [CrossRef] [Medline]

22. Roy, R., Kozlov, A. G., Lohman, T. M., and Ha, T. (2007) Dynamic structural rearrangements between DNA binding modes of *E. coli* SSB protein. *J. Mol. Biol.* 369, 1244–1257 [CrossRef] [Medline]

23. Barat, M., and Mignotte, B. (1981) A DNA binding protein from *Xenopus laevis* oocyte mitochondria. *Chromosoma* 82, 583–593 [CrossRef] [Medline]

24. Bujalowski, W., and Lohman, T. M. (1991) Monomer–tetramer equilibrium of the *Escherichia coli* ssb-I mutant single strand binding protein. *J. Biol. Chem.* 266, 1616–1626 [Medline]

25. Carlini, L., Curth, U., Kindler, B., Urbanke, C., and Porter, R. D. (1998) Identification of amino acids stabilizing the tetramerization of the single stranded DNA binding protein from *Escherichia coli*. *FEBS Lett.* 430, 197–200 [CrossRef] [Medline]