Mapping by Site-Directed Mutagenesis of the Region Responsible for Cohesin−Dockerin Interaction on the Surface of the Seventh Cohesin Domain of *Clostridium thermocellum* CipA†

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ABSTRACT: To locate the region involved in binding dockerin domains, 15 mutations were introduced across the surface of the seventh cohesin domain of the scaffolding protein CipA, which holds together the cellulosome of *Clostridium thermocellum*. Mutated residues were located on both faces of the nine-stranded β-sandwich forming the cohesin domain and on the loops connecting β-strands 4 and 5, 6 and 7, and 8 and 9. The loop region was previously proposed, on the basis of sequence comparisons, to form a contiguous "recognition strip". Individual mutants of four residues, D39, Y74, E86, and G89, formed no complexes detectable by nondenaturing gel electrophoresis after incubation with $CelD_{664}$, a shortened form of endoglucanase CelD lacking the residues linking the catalytic domain with the dockerin domain. The four sensitive residues encompass a hydrophobic region on the 5-6-3-8 face of the molecule, which overlaps partially with the recognition strip and with a hydrophobic zone involved in the formation of cohesin−cohesin dimers. Isothermal titration calorimetry showed that single cohesin mutations affecting the binding of $CelD_{664}$ had significant effects on the enthalpy or entropy of binding of wild-type CelD but much lesser effects on the association constant, owing to enthalpy−entropy compensation. However, the affinity for wild-type CelD of the triple mutant affecting D39, Y74, and E86 was reduced by 2 orders of magnitude, due to negative cooperativity between mutations affecting D39 + Y74 on one hand and E86 on the other hand.

The cellulase system of *Clostridium thermocellum* consists of a large multienzyme complex termed the cellulosome (*1*). The various cellulases and hemicellulases forming the complex are tethered to a large scaffolding component termed CipA. Each of the enzymes bears a conserved, noncatalytic domain, termed dockerin domain, which consists of two highly similar segments of 22 residues each. Dockerin domains bind to a set of modules, termed cohesin domains, which are reiterated 9-fold within the sequence of CipA (*2*). The three-dimensional structures of the second and the seventh cohesin domains have been elucidated (*3*, *4*). Both of them are highly similar and consist of nine-stranded β-sandwiches forming a flattened β-barrel with an overall "jelly roll" topology. The two principal β-sheets comprise β-strands 5, 6, 3, and 8 on one face of the barrel and β-strands 4, 7, 2, and 9 on the other face. The surface of cohesin domains features no obvious binding cleft or pocket. Crystallization of both cohesin domains leads to the formation of dimers owing to extensive intermolecular contacts between two adjacent molecules. The interaction buries a contact surface of 520 Å^2, located for the most part around the centers of the 5-6-3-8 faces of both polypeptides and characterized by extended hydrophobic contacts. This region is suspected to overlap with the dockerin binding site, since binding of dockerin domains interferes with dimer formation (*4*, *5*).

In the accompanying article (*24*), we show that the association between cohesin and dockerin domains is largely driven by hydrophobic interactions. Hydrophobic interactions are also compatible with the observation that dissociation of the cellulosome requires the presence of detergents such as sodium dodecyl sulfate (*6*, *7*). Binding experiments indicate that each of the dockerin domains borne by the catalytic subunits can interact with any of the cohesin domains borne by CipA or by the surface protein OlpA (*8*−*10*), suggesting that the residues involved in the interaction are conserved. Conversely, although the cohesin and dockerin domains present in the *Clostridium cellulolyticum* cellulosome are quite similar to their *C. thermocellum* homologues, they show no cross-species recognition (*11*). Consequently, the residues involved in cohesin−dockerin contacts would be expected to diverge between cohesin domains of the two species. On the basis of these considerations, Bayer et al. (*12*) proposed that the contact region involves a set of residues located in the loops connecting strands 4 and 5, 6 and 7, and 8 and 9 of the cohesin domain. These residues are contiguous and were suggested to form a "recognition strip" running approximately along the edge between the two faces of the molecule. In this study, we attempted to map

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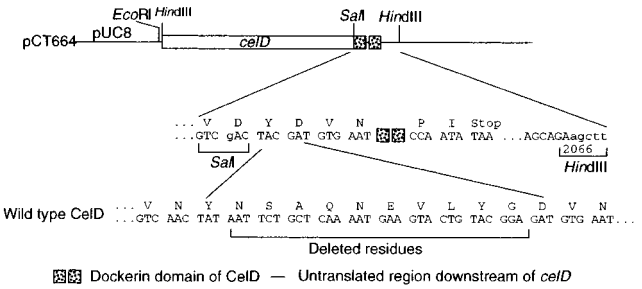

FIGURE 1: Structure of pCT664 encoding $CelD_{664}$. Mutagenized nucleotides and nucleotides that belong to the vector are in lower case type. Numbering starts at the initiation codon of *celD*.

by site-directed mutagenesis cohesin residues making contacts with dockerin domains. Mutations were targeted on amino acids whose side chains point toward the solvent and included the residues proposed to belong to the recognition strip.

MATERIALS AND METHODS

Construction and Purification of Cohesin Mutants. DNA fragments encoding mutants of the seventh cohesin domain of CipA (CohCip7)[1] were obtained by overlap extension of PCR fragments synthesized using pCip1 (*13*) as a template (*14*) and recloned between the *Bam*HI and *Sac*I sites of pQE-31 (Qiagen). All sequences subjected to PCR amplification were verified by DNA sequencing (Génome Express, Paris). Wild-type and mutated polypeptides were purified by Ni^{2+}-nitrilotriacetic acid affinity chromatography as described (*13*).

Construction and Purification of CelD Containing a Shortened Linker Peptide. To construct pCT664, a DNA fragment encoding the dockerin domain of CelD, but missing the residues connecting the catalytic domain and the dockerin domain, was synthesized by PCR using pCT603 (*15*) as a template. Primers were designed to flank the 5′ end with a *Sal*I site to be fused in-frame with the catalytic domain of CelD and the 3′ end with *Hin*dIII and *Eco*RI sites. The fragment was cloned between the *Sal*I and *Eco*RI sites of pCT607, which contains the *Hin*dIII−*Hin*cII fragment encoding the catalytic domain of CelD inserted between the *Hin*dIII and *Sal*I sites of pUC19 (*16*). The 2 kb *Hin*dIII fragment was recloned in pUC8 (*17*) to yield pCT664 (Figure 1). Wild-type CelD and $CelD_{664}$ were purified from the cytoplasmic inclusion body fraction of *Escherichia coli* JM101 (*18*) harboring pCT603 and pCT664, respectively, as described previously (*19*), except that inclusion bodies were washed with STET buffer instead of 2.5% *n*-octyl glucoside.

Assay of Cohesin−Dockerin Complex Formation by Native Gel Electrophoresis. Wild-type CelD or $CelD_{664}$ was mixed with wild-type or mutated CohCip7 in 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$. Complexes formed after overnight incubation at 4 °C were analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions, as described previously (*20*).

Assay of Cohesin−Dockerin Complex Formation by Isothermal Titration Calorimetry. The thermodynamics of

[1] Abbreviations: CohCip7, seventh cohesin domain of *Clostridium thermocellum* CipA; $CelD_{664}$, mutated form of endoglucanase CelD with no connecting residues between the catalytic domain and the dockerin domain; PCR, polymerase chain reaction.

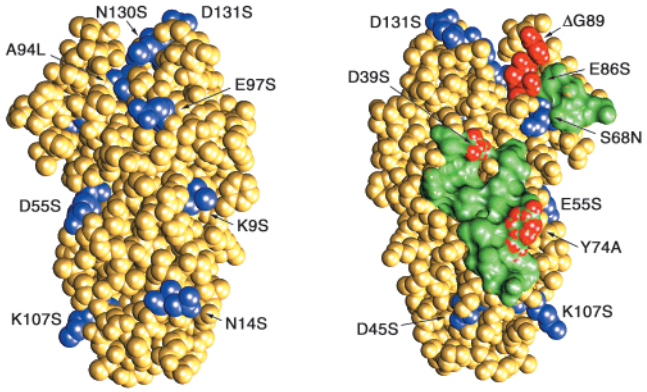

FIGURE 2: Map of the mutagenized residues on the three-dimensional structure of CohCip7. Residues whose mutagenesis impaired the formation of complexes with $CelD_{664}$ are shown in red. Residues whose mutagenesis did not prevent the formation of complexes with $CelD_{664}$ are shown in blue. The region participating in cohesin−cohesin contacts in the crystallographic dimer is shown in green.

complex formation between wild-type CelD and CohCip7 mutants was analyzed as described in the accompanying paper (*24*). Like the wild-type cohesin domain, mutated cohesin domains displayed biphasic titration curves. Curves were fitted assuming two independent reactions, with about 80−90% of the dockerin binding with a high affinity and the remainder with a lower affinity (*24*). Analysis of the data was focused on the high-affinity reaction, which most likely represents titration of the genuine dockerin domain.

RESULTS

Choice of Residues To Be Mutagenized. The position of mutated residues on the surface of the CohCip7 polypeptide is shown in Figure 2. Two criteria dictated the targeting of site-directed mutations. First, the surface of CohCip7 was probed without preconceived idea by mutagenizing residues more or less evenly distributed across the whole surface of the protein. The mutations selected were K9S, N14S, D39S, D45S, E55S, D87S, E97S, and K107S. Second, the recognition strip hypothesis was tested by systematically probing all residues proposed by Bayer et al. (*12*) to belong to the contact zone. These mutations included S68N, E86S, ΔG89, A94L, N130S, and D131S. G89 was deleted rather than substituted, since sequence alignments show that it is absent in the cohesin domains of *C. cellulolyticum* CipC, which do not bind dockerin domains from *C. thermocellum* (*11*). In all selected residues, the side chains point toward the solvent, and substitutions were designed to retain the hydrophobic or hydrophilic character of the side chain, so that mutations were unlikely to impair the folding of the protein significantly.

Complex Formation by Single Mutants of CohCip7. None of the CohCip7 single mutants displayed any obvious difference in complex formation when tested against wild-type CelD in the nondenaturing gel assay (data not shown). However, the test might fail to detect the modest loss of affinity caused by a single mutation if the interaction between wild-type partners is too strong. Consequently, we tried to reduce the stability of the complexes in order to bring them closer to the threshold at which the complex formed with wild-type CohCip7 begins to fall apart. For this, cohesin polypeptides were incubated with $CelD_{664}$, which is deleted

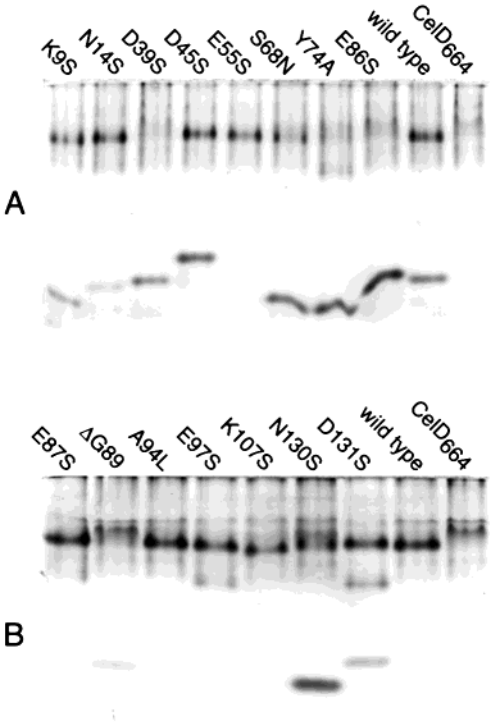

FIGURE 3: Band-shift analysis of complex formation between $CelD_{664}$ and various single mutants of CohCip7. Panels A and B show the formation of a complex migrating as a sharp band when $CelD_{664}$ is mixed with wild-type CohCip7 or with most of the mutated cohesin polypeptides, except those harboring the mutations D39S, Y74A, E86S,and ΔG89. A control lane containing $CelD_{664}$ alone is shown for both gels.

for all amino acids connecting the last residue visible in the crystal structure of the catalytic domain of CelD (Tyr_{574}) and the first residue of the first duplicated segment of the dockerin domain (Asp_{585}). As shown in Figure 3, wild-type CohCip7 and most of the mutated cohesin polypeptides were still able to shift the band corresponding to uncomplexed $CelD_{664}$, with the exception of the polypeptides carrying the D39S, Y74A, E86S, and ΔG89 mutations. These mutations are clustered in a region extending from the putative recognition strip to the center of the 5-6-3-8 face of the cohesin domain and overlapping with the contact zone seen in the crystallographic dimer (Figure 2). For polypeptides carrying these mutations, as well as the A94L mutation, the association reaction with wild-type CelD was studied by isothermal titration calorimetry. As shown in Table 1, single mutations affecting residues A39, Y74, E86, G89, and A94

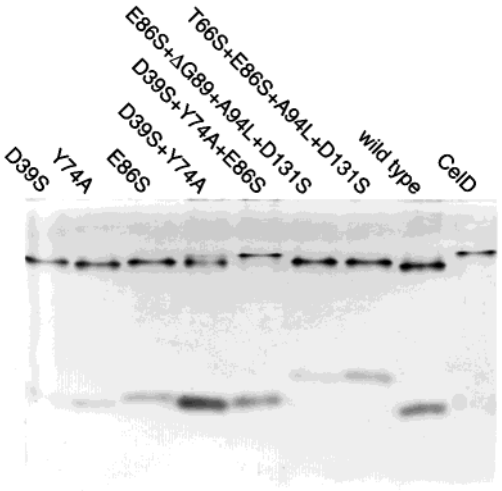

FIGURE 4: Band-shift analysis of complex formation between wild-type CelD and mutants of CohCip7 harboring two or more mutations. A complex migrating faster than CelD is formed when CelD is mixed with wild-type CohCip7 or with most mutated cohesin polypeptides except that harboring the triple mutation D39S + Y74L + Y74A.

had very significant effects on the enthalpy and entropy of the association, indicating that these residues participate in binding. However, since the changes in binding enthalpy and entropy largely compensated one another, the effect of the mutations on the binding free energy and the association constant was much less dramatic. Mutations affecting the β-sheet, such as D39S and Y74A, resulted in a less favorable enthalpy change and a more favorable entropy change, which suggests that the association was driven by enhanced hydrophobic interactions as compared to the wild type. By contrast, mutations affecting the loop connecting strands 6 and 7, such as E86S, ΔG89, or A94L, resulted in a more favorable enthalpy change and a less favorable entropy change, suggesting that hydrophilic interactions played a more important role than in the wild type. The mutation E97S, which is located on the other side of the β-sandwich, failed to alter the thermodynamic parameters of the association significantly.

Complex Formation by Double and Triple Mutants of CohCip7. Since the effect of single mutations on the binding affinity of CohCip7 with wild-type CelD was rather modest, the mapping of the contact region was further confirmed by analyzing several combinations of mutations. As shown in Figure 4, the combination of the D39S, Y74A, and E86S mutations led to loss of detectable complex formation with

Table 1: Thermodynamic Parameters at 28.7 °C of the Association between Wild-Type Endoglucanase CelD and the Seventh Cohesin Domain of CipA (CohCip7) Harboring Various Point Mutations[a]

CohCip7	K_a (M^{-1})	$\Delta G°$ (kcal·mol^{-1})	$\Delta H°$ (kcal·mol^{-1})	$T\Delta S°$ (kcal·mol^{-1})	ΔC_p (cal·mol^{-1}·K^{-1})
WT	$(2.0 \pm 0.5) \times 10^9$	-12.93 ± 0.17	-3.33 ± 0.20	9.60 ± 0.35	-305 ± 23
D39S	$(8.3 \pm 1.5) \times 10^8$	-12.39 ± 0.10	-0.44 ± 0.15	11.96 ± 0.40	-289 ± 36
Y74A	$(2.3 \pm 0.5) \times 10^9$	-13.02 ± 0.08	-1.72 ± 0.20	11.29 ± 0.32	-343 ± 22
D39S + Y74A	$(3.4 \pm 1.8) \times 10^8$	-11.87 ± 0.3	1.52 ± 0.17	13.39 ± 0.47	-252 ± 21
E86S	$(1.1 \pm 0.2) \times 10^9$	-12.55 ± 0.09	-3.91 ± 0.32	8.64 ± 0.41	-294 ± 30
D39S + Y74A + E86S	$(2.0 \pm 0.5) \times 10^7$	-10.16 ± 0.15	0.38 ± 0.30	10.54 ± 0.45	-289 ± 36
A94L	$(8.0 \pm 1.5) \times 10^8$	-12.38 ± 0.11	-5.72 ± 0.15	6.66 ± 0.26	-300 ± 25
ΔG89	$(8.1 \pm 2.1) \times 10^8$	-12.38 ± 0.16	-6.14 ± 0.10	6.24 ± 0.26	ND[b]
G97S	$(1.7 \pm 0.4) \times 10^9$	-12.83 ± 0.15	-3.19 ± 0.15	9.64 ± 0.30	-305 ± 15

[a] Values of K_a, $\Delta G°$, $\Delta H°$, and $T\Delta S°$ were obtained from the average of at least two titration experiments and are given ± the standard error of the mean. ΔC_p was calculated using at least four titration experiments performed at different temperatures. [b] ND, not determined.

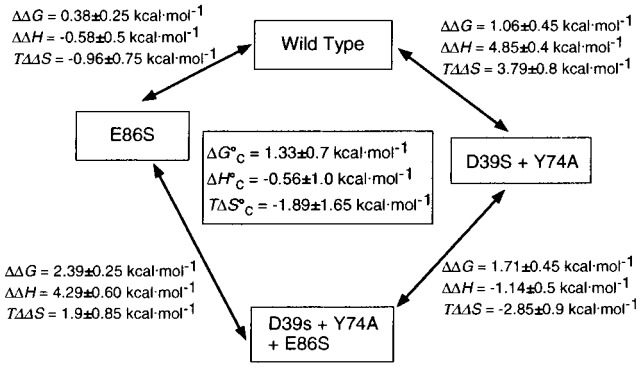

FIGURE 5: Coupling energetics between the double mutation D39S + Y74A in the 8-3-6-5 β-sheet and the E86S mutation in the $\beta6-\beta7$ connecting loop. The wild type is shown at the top and the triple mutant at the bottom. $\Delta\Delta G°$, $\Delta\Delta H°$, and $T\Delta\Delta S°$ represent the differences in the thermodynamic parameters of binding between the indicated cohesin domains. The values for the coupling parameters $\Delta G°_c$, $\Delta H°_c$, and $T\Delta S°_c$ are shown in the center of the cycle. For each indicated value, the uncertainty was calculated by propagation of the errors indicated in Table 1.

wild-type CelD, as detected by nondenaturing polyacrylamide gel electrophoresis. However, wild-type CelD still formed complexes with CohCip7-derived polypeptides harboring other combinations of mutations, such as T66A + E86S + A94L + D131S or E86S + ΔG89 + A94L + D131S, both of which bore four different mutations and up to two mutations preventing association with $CelD_{664}$ (E86S and ΔG89).

The cumulative effect of the D39S, Y74A, and E86S mutations was studied quantitatively by performing the thermodynamic analysis of the association of wild-type CelD with the double mutant D39S + Y74A and with the triple mutant D39S + Y74A + E86S. As shown in Table 1, the double mutant D39S + Y74A displayed changes in the enthalpy and entropy of association that were nearly equal to the sum of the changes induced by either of the individual mutations. Consequently, these changes largely compensated one another in the free energy of association of the double mutant, as they did for the individual mutations. With the triple mutant, however, enthalpy−entropy compensation was much reduced, and the association constant was decreased by 2 orders of magnitude. As shown in Figure 5, the double mutation D39S + Y74A on one hand, and the E86S mutation on the other hand, acted on binding with negative cooperativity: the decrease in the free energy of binding caused by the E86S mutation was larger when the mutation was introduced in the D39S + Y74A mutant instead of the wild-type background. The difference, which defines the coupling free energy $\Delta G°_c$, amounted to 1.33 ± 0.71 kcal·mol^{-1} (Figure 5). The coupling enthalpy $\Delta H°_c$ was not significantly different from zero, while $T\Delta S°_c$ was significant and negative, indicating that the coupling free energy was of entropic origin.

DISCUSSION

Out of the 15 loci tested, none of the single mutations was sufficient to abolish complex formation with wild-type CelD as detected by the nondenaturing gel assay. Thus, complex formation was assayed with $CelD_{664}$, which carries a deletion covering the linker segment between the catalytic domain and the dockerin domain and the first conserved Gly residue of the dockerin domain. Mutations at four loci were found to affect the association of CohCip7 with $CelD_{664}$. These are located on the face of the cohesin domain comprising the 5-6-3-8 β-sheet. D39 and Y74 are located close to the center of the 5-6-3-8 β-sheet. E86 and G89 belong to the loop connecting strands $\beta6$ and $\beta7$, which is part of the recognition strip proposed by Bayer et al. on the basis of sequence comparisons between cohesin domains from *C. thermocellum* and *C. cellulolyticum* (*12*). All residues whose mutation affected binding of $CelD_{664}$ also had significant effects on the enthalpy and entropy of the association with wild-type CelD. One additional locus, A94, also located on the $\beta6-\beta7$ loop, was also found to influence the enthalpy and entropy of binding of wild-type CelD. The effects of the mutations are likely to reflect direct interference with cohesin−dockerin contacts, since the side chains of mutated residues were exposed to the solvent. Furthermore, the mutations are unlikely to destabilize the structure of the cohesin domain, since the latter is known to possess a compact structure that is highly resistant to proteases and to denaturing agents (*7*, *13*, *21*). The proposed binding region overlaps the hydrophobic contact region between the two cohesin molecules forming the dimer observed by X-ray crystallography. Sedimentation equilibrium analysis revealed no dimers in solutions of isolated CohCip7 (*4*). However, such dimers were observed by gel filtration for the first cohesin module of *C. cellulolyticum* CipC (*5*). The same cohesin−cohesin contacts probably occur within the scaffolding protein, in which cohesin domains are covalently linked together by short Pro/Thr-rich linkers. Thus, dockerin binding and cohesin dimer formation are expected to compete with each other. Indeed, binding of dockerin domains to a pair of covalently linked cohesin domains is highly cooperative (*22*), as expected if cohesin−cohesin interactions between adjacent domains mask the respective dockerin-binding sites. Furthermore, molecular weight estimates of the complexes formed between the cohesin domain of *C. cellulolyticum* CipC and cellulases CelA and CelF suggest that the cohesin dimer dissociates upon association with the dockerin domain borne by CelA and CelF (*5*).

Analysis of the thermodynamic parameters indicates that the mutations had quite different effects depending on the position of the mutated residues. Mutations D39S and Y74A, which affected the center of the 8-3-6-5 β-sheet, resulted in higher values of enthalpy and entropy, consistent with an enhanced hydrophobic character of the interaction. Mutations E86S, ΔG89, and A94L in the $\beta6-\beta7$ connecting loop had the opposite effect, with lower values of enthalpy and entropy, suggesting enhanced hydrophilic contacts. It is tempting to correlate this behavior with the properties of mutations affecting the first or second reiterated segment of the dockerin domain (*24*). As shown in Figure 6, mutation of residues S10T11 in the first dockerin segment resulted in changes that were similar to those induced by mutants of the $\beta_6-\beta_7$ loop in the cohesin domain (decreased hydrophobicity). By contrast, mutation of residues S10S11 in the second segment induced changes that were similar to those induced by mutations targeted to the center of the 8-3-6-5 β-sheet. This correlation would be consistent with a mode of binding involving two subsites: one encompassing the $\beta_6-\beta_7$ loop, and interacting with the first dockerin segment, and the other one covering the center of the 8-3-6-5 β-sheet

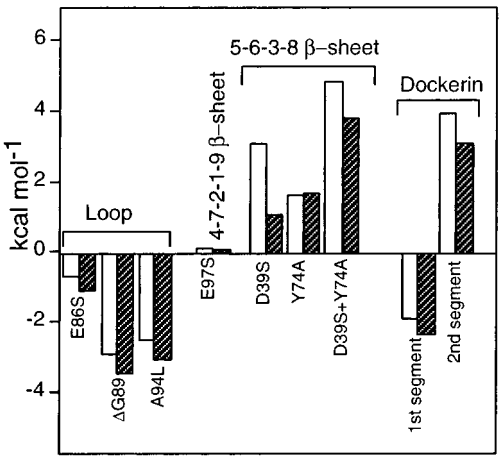

FIGURE 6: Variation of the cohesin−dockerin binding enthalpy and binding entropy as a function of the location of the mutations studied. Differences $\Delta\Delta H_{mutant} = \Delta H_{mutant} - \Delta H_{wt}$ (white bars) and $T\Delta\Delta S_{mutant} = T\Delta S_{mutant} - T\Delta S_{wt}$ (hatched bars) at 28.7 °C are shown for CohCip7 mutants of the loop connecting strands 6 and 7 (E86S, ΔG89, and A94L) and for mutants of the 8-3-6-5 β-sheet (D39S and Y74A). For purpose of comparison, the same parameters are shown for the mutant E97S, which belongs to the 4-7-2-1-9 β-sheet and for the dockerin mutants affected in residues S10T11 and S10S11 of the first and the second dockerin segment, respectively (*24*).

and interacting with the second dockerin segment. However, further data on the structure of the cohesin−dockerin complex are required to confirm or disprove this interpretation.

The combination of the three mutations D39S, Y74A, and E86S strongly reduced the affinity of wild-type CelD. ITC analysis indicated that, within experimental error, the two mutations D39S and Y74A had additive effects on the thermodynamic parameters of binding, suggesting that they had little effect on the structure and/or solvation of the complex. Introducing the third mutation E86S was accompanied by a significant negative cooperativity of entropic origin. This suggests that the formation of the wild-type complex generates some strain, which is relieved in the double mutant D39S + Y74A and in the E86S mutant, owing to a structural rearrangement, possibly including a change in solvation of the mutated complexes.

The properties of the cohesin mutants discussed in this study indicate that the formation of cohesin−dockerin complexes involves a relatively large surface area of both partners. Together with the results presented in the accompanying paper (*24*), the data show that binding is accompanied by induced fit phenomena, which account for the negative cooperativity between different loci. Indeed, it will be of interest to compare the three-dimensional structure of the two domains within the complex with those already known for the individual partners (*3*−*5*, *23*).

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

One figure showing calorimetric binding isotherms measured for the binding of CohCip7 mutants D39S, Y74A, D39S + Y74A, D39S + Y74A + E86S, E97S, A94L, E86S, and ΔG89 to wild-type CelD. This material is available free of charge via the Internet at http://pubs.acs.org.

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