Articles

Duplicated Dockerin Subdomains of *Clostridium thermocellum* Endoglucanase CelD Bind to a Cohesin Domain of the Scaffolding Protein CipA with Distinct Thermodynamic Parameters and a Negative Cooperativity†

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ABSTRACT: Mutagenized dockerin domains of endoglucanase CelD (type I) and of the cellulosome-integrating protein CipA (type II) were constructed by swapping residues 10 and 11 of the first or the second duplicated segment between the two polypeptides. These residues have been proposed to determine the specificity of cohesin−dockerin interactions. The dockerin domain of CelD still bound to the seventh cohesin domain of CipA (CohCip7), provided that mutagenesis occurred in one segment only. Binding was no longer detected by nondenaturing gel electrophoresis when both segments were mutagenized. The dockerin domain of CipA bound to the cohesin domain of SdbA as long as the second segment was intact. None of the mutated dockerins displayed detectable binding to the noncognate cohesin domain. Isothermal titration calorimetry showed that binding of the CelD dockerin to CohCip7 occurred with a high affinity [$K_a = (2.6 \pm 0.5) \times 10^9$ M^{-1}] and a 1:1 stoichiometry. The reaction was weakly exothermic ($\Delta H° = -2.22 \pm 0.2$ kcal mol^{-1}) and largely entropy driven ($T\Delta S° = 10.70 \pm 0.5$ kcal mol^{-1}). The heat capacity change on complexation was negative ($\Delta C_p = -305 \pm 15$ cal mol^{-1} K^{-1}). These values show that cohesin−dockerin binding is mainly hydrophobic. Mutations in the first or the second dockerin segment reduced or enhanced, respectively, the hydrophobic character of the interaction. Due to partial enthalpy−entropy compensation, these mutations induced only small changes in binding affinity. However, the binding affinity was strongly decreased when both segments were mutated, indicating strong negative cooperativity between the two mutated sites.

Clostridium thermocellum, a thermophilic, anaerobic bacterium, produces a very active exocellular cellulolytic complex termed cellulosome (*1*). The cellulosome consists of about two dozen different cellulases and hemicellulases (*2*−*4*), which are organized around a large, multifunctional scaffolding subunit termed CipA (*5*, *6*). It is thought that the clustering of enzymes with complementary specificities (endoglucanases, cellobiohydrolases) maximizes their synergism and brings about the high specific activity of the cellulosome, particularly toward crystalline cellulose (*1*, *7*, *8*). The basic principle of the quaternary organization of the cellulosome has been established. Each of the catalytic subunits carries a conserved, noncatalytic domain, termed dockerin domain (*9*). This domain mediates the interaction of each subunit with CipA (*10*, *11*). CipA is a multimodular polypeptide, which consists of a cellulose-binding domain, of nine highly conserved modules, termed cohesin domains, and of a C-terminal dockerin domain whose sequence diverges clearly from the consensus established for the dockerin domains of the catalytic subunits. The cohesin domains have been shown to act as binding partners for the dockerin domains of the catalytic subunits (*12*, *13*). There seems to be little or no binding preferences between the dockerin domains of the various catalytic subunits and the cohesin domains of CipA (*14*, *15*). By contrast, the C-terminal dockerin domain of CipA binds specifically to a set of polypeptides located in the cell envelope (*13*, *16*, *17*), which are thought to mediate attachment of the cellulosome to the surface of *C. thermocellum* cells. These polypeptides contain one or more cohesin domains, which differ strongly in their sequence and binding specificity from those borne by CipA (*16*). Hence, the cohesin domains of CipA and the dockerin domains of the catalytic subunits have been designated as domains of type I, whereas the dockerin domain of CipA and the complementary cohesin domains borne by the cell envelope proteins are termed domains of

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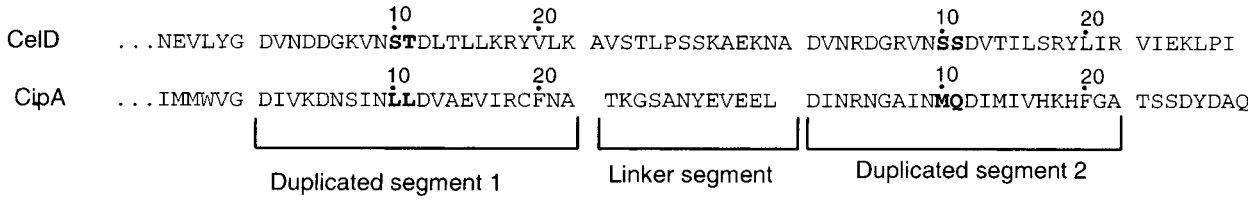

FIGURE 1: Sequences of the dockerin domains of CelD and CipA. Residues 10 and 11 of each segment, which were proposed to act as selectivity determinants, are shown in boldface type.

type II (*16*). A further type of binding specificity has been demonstrated for the cohesin and dockerin domains of *Clostridium cellulolyticum*, which are not compatible with those of *C. thermocellum* and are termed domains of class III (*18*). In the three types of cohesin−dockerin complexes, the interaction is strongly dependent on the presence of Ca^{2+} (*14*, *17*, *18*).

The sequence of dockerin domains comprises two highly similar segments of 22 residues each joined by a variable linker region of 10−18 residues (Figure 1). In all cases, residues 10 and 11 of both segments are conserved within the same type of dockerin domain but different between different types, suggesting that they are involved in determining binding specificity. This was demonstrated by swapping these residues between the dockerin domains of *C. thermocellum* CelS and *C. cellulolyticum* CelA: each of the mutated dockerins acquired the ability to bind to its noncognate cohesin domain (*19*).

In this study, we constructed various mutants of the dockerin domains of endoglucanase CelD (type I) and of CipA (type II) by exchanging residues 10 and 11 in each segment (Figure 1). The binding specificity of each of the wild type and mutated dockerin domain was tested by nondenaturing electrophoresis, looking for the formation of complexes with the seventh cohesin domain of CipA (type I) or with the cohesin domain of the cell envelope protein SdbA (type II). The association between the seventh cohesin domain of CipA and wild type or mutated forms of the dockerin domain of CelD was further analyzed by isothermal titration calorimetry.

MATERIALS AND METHODS

Construction of Recombinant Clones. Owing to difficulties experienced in maintaining isolated dockerin domains in solution, we fused the dockerin domains to be studied to the catalytic core of CelD. A His_6 tag was added to the COOH end of the constructs to facilitate purification. All clones encoding polypeptides containing mutants of the dockerin domains of CipA and CelD were derived from pCT671 and pCT672, respectively (Figure 2). Both plasmids were constructed using the pQE-17/pQE-51 system developed to express polypeptides carrying a COOH-terminal His_6 tag (Qiagen). To facilitate cloning of isogenic cassettes encoding mutagenized dockerin domains, a *Xho*I site was introduced immediately upstream of the sequence encoding the dockerin domain of CelD. Thus, an appropriate linker fragment was cloned immediately downstream of the *Hinc*II site bordering the 3′ end of the sequence encoding the catalytic domain of CelD. The *Hinc*II site was converted into a *Sal*I site in the process. The *Xho*I site originally present in the pQE-51 vector (Qiagen) was destroyed by filling in with the Klenow fragment of DNA polymerase followed by

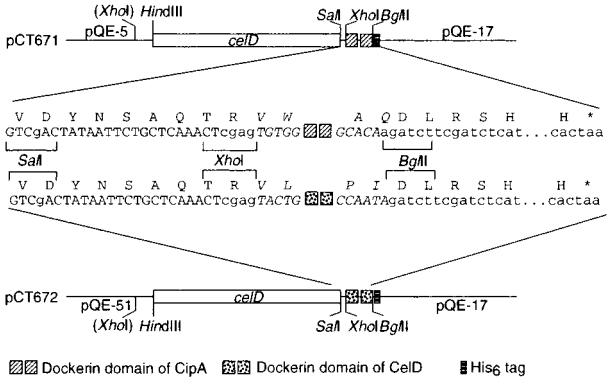

FIGURE 2: Structure of the pCT671 and pCT672 plasmids encoding fusions of the catalytic domain of endoglucanase CelD with the dockerin domains of CipA and CelD, respectively. Nucleotides belonging to the dockerin domains are in italics; nucleotides that were mutagenized to introduce restriction sites or belong to the pQE-17 vector are in lower case type. The position of the deleted *Xho*I site originally present in the vector is shown in parentheses.

religation. PCR[1] using appropriate primers was used to generate fragments encoding the dockerin domains of CelD and CipA, flanked at the 5′ end by a *Xho*I site and at the 3′ end by a *Bgl*II site. This altered the original stop codon and allowed in-frame fusion with the His_6 tag-encoding sequence borne by the vector. Fragments encoding mutagenized dockerin domains were obtained by overlap extension of PCR fragments synthesized using pCT671 or pCT672 as templates (*20*) and recloned between the *Xho*I and *Bgl*II sites of the same plasmids. All sequences subjected to PCR amplification were verified by DNA sequencing (Génome Express, Paris). The CelD polypeptides encoded by plasmids pCT671, pCT6711 to pCT6713, pCT672, pCT6721 to pCT6723, and pCT678 to pCT687 are designated as $CelD_{671}$, $CelD_{6711}$ to $CelD_{6713}$, $CelD_{672}$, $CelD_{6721}$ to $CelD_{6723}$, and $CelD_{678}$ to $CelD_{687}$, respectively. Their sequence at positions 10 and 11 of each segment is indicated in Table 1.

The pCT1836 plasmid, encoding the cohesin domain of SdbA, was obtained by recloning the *Bgl*II-*Sac*I fragment of pCT1834 (*21*) into pQE-31 (Qiagen) cut by *Bam*HI and *Sac*I.

Purification of Recombinant Polypeptides. Wild-type CelD ($CelD_{wt}$) was purified from *Escherichia coli* JM101(pCT603) as described (*11*). For expression of genes encoding recombinant polypeptides fused to His_6 tags, plasmids were introduced into *E. coli* BL21(pREP4) (*22*) or M15(pREP4) (Qiagen). The His_6-tagged cohesin domains corresponding to the seventh domain of CipA (CohCip7) and to the cohesin

[1] Abbreviations: CohCip7, seventh cohesin domain of *Clostridium thermocellum* CipA; CohSdbA, cohesin domain of C. *thermocellum* SdbA; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside.

Table 1: Binding Properties Conferred on the Dockerin Domains of CelD and CipA by Mutations Affecting Residues 10 and 11 of the First or the Second Duplicated Segment

		residues 10−11		binding to CohCip7		binding to CohSdbA	
polypeptide	dockerin domain	first segment	second segment	monomer	dimer	monomer	dimer
$CelD_{672}$	CelD wt	...ST...	...SS...	+	NA[a]	−	NA[a]
$CelD_{6721}$	CelD	...LL...	...SS...	+	NA[a]	−	NA[a]
$CelD_{6722}$	CelD	...ST...	...MQ...	+	NA[a]	−	NA[a]
$CelD_{6723}$	CelD	...LL...	...MQ...	−	NA[a]	−	NA[a]
$CelD_{671}$	CipA wt	...LL...	...MQ...	−	−[b]	+	+[b]
$CelD_{6711}$	CipA	...ST...	...MQ...	−	−[b]	+	+[b]
$CelD_{6712}$	CipA	...LL...	...SS...	−	−[b]	−	−[b]
$CelD_{6713}$	CipA	...ST...	...SS...	−	−[b]	−	−[b]
$CelD_{678}$	CelD	...CT...	...SS...	+	+[c]	NT[d]	NT[d]
$CelD_{684}$	CelD	...SC...	...SS...	+	+[c]	NT[d]	NT[d]
$CelD_{679}$	CelD	...ST...	...CS...	+	+[c]	NT[d]	NT[d]
$CelD_{685}$	CelD	...ST...	...SC...	+	+[c]	NT[d]	NT[d]
$CelD_{680}$	CelD	...CT...	...MQ...	−	−	NT[d]	NT[d]
$CelD_{681}$	CelD	...LL...	...CS...	+	−	NT[d]	NT[d]
$CelD_{682}$	CipA	...CL...	...MQ...	NT[d]	NT[d]	+	+[c]
$CelD_{686}$	CipA	...LC...	...MQ...	NT[d]	NT[d]	+	+[c]
$CelD_{683}$	CipA	...LL...	...CQ...	NT[d]	NT[d]	±[e]	−[c]
$CelD_{687}$	CipA	...LL...	...MC...	NT[d]	NT[d]	±[e]	−[c]

[a] NA, not applicable (no dimer formed). [b] Dimer formed between Cys_{19} residues. [c] Dimer formed between introduced Cys residues; Cys_{19} mutagenized to Ser. [d] NT, not tested. [e] ±, partial conversion of the dockerin polypeptide into a complexed form in the presence of an excess of cohesin polypeptide.

domain of SdbA (CohSdbA) were purified from the cytoplasmic supernatant of isopropyl β-D-thiogalactoside- (IPTG-) induced cells harboring pCip7 (*22*) and pCT1836, respectively, as described previously (*23*). CelD polypeptides containing His_6-tagged wild type or mutated dockerin domains derived from CipA were obtained in the same manner. CelD polypeptides containing His_6-tagged wild type or mutated dockerin domains derived from CelD were obtained from inclusion bodies. Washed inclusion bodies were prepared from IPTG-induced cells and dissolved in 0.1 M Tris-HCl, pH 8.5, containing 5 M urea, as described (*24*). The material from a 0.5 L culture was loaded onto a 2 mL Ni^{2+}-nitrilotriacetic column, and unbound material was washed with 50 mM Tris-HCl, pH 7.5, containing 5 mM imidazole and 5 M urea followed by 50 mM Tris-HCl, pH 7.5. Bound material was eluted with 50 mM Tris-HCl, pH 7.5, containing 250 mM imidazole and dialyzed against 50 mM Tris-HCl, pH 7.5. Preparations subjected to microcalorimetric analysis were further purified by ion-exchange chromatography. Affinity-purified material from a 1 L culture was made 5 M in urea by adding 1.66 volumes of Tris-HCl, 50 mM, pH 7.5, containing 8 M urea and loaded onto a 12 mL column of Q-Sepharose Fast Flow (Amersham Pharmacia Biotech) equilibrated in 50 mM Tris-HCl, pH 7.5, containing 5 M urea. The protein was eluted with a 100 mL gradient containing 0−200 mM NaCl in the same buffer, concentrated by ultrafiltration, and dialyzed against 50 mM Tris-HCl, pH 7.5.

Formation and Analysis of Cohesin−Dockerin Interactions by Native Gel Electrophoresis. CelD polypeptides containing wild-type or mutagenized dockerin domains were mixed with an excess of CohCip7 or CohSdbA in the presence of 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$ and incubated overnight at 4 °C. Complexes formed by each dockerin mutant were analyzed as described by nondenaturing gel electrophoresis (*21*).

Microcalorimetric Analysis of Cohesin−Dockerin Complex Formation. Isothermal titration calorimetry (ITC) experiments were carried out using a MCS ultrasensitive titration calorimetry system (MicroCal Inc., Northampton, MA) as previously described (*25*, *26*), with the following modifications. Protein samples were dialyzed against buffer of the same batch to minimize artifacts due to any differences in buffer composition and were thoroughly degassed under vacuum before use. In a typical calorimetric experiment, the solution of endoglucanase CelD protein (4−10 μM) in the calorimetric cell was titrated with a solution of CohCip7 protein (70−150 μM) in the injection syringe. Raw calorimetric data, i.e., heats evolved after each ligand injection (5−10 μL) into the protein solution (Q_i), were obtained from the integral of the calorimetric signal and corrected for the heat of dilution of the ligand alone. Analysis of the data was performed using the software package ORIGIN (*27*, *28*) provided by the manufacturer. The calorimetric binding isotherm was fitted by an iterative nonlinear least-squares algorithm (the Marquardt method).

The association process was biphasic and could be fitted assuming two independent reactions (Figure 3). An association reaction corresponding to the major fraction (85%) of the CelD preparation occurred with a strong binding affinity and was observed from the first injection of CohCip7 until a molar ratio of cohesin to dockerin of 0.8 was reached in the titration cell. A second reaction, corresponding to a minor fraction (15%) with a reduced binding affinity, occurred at higher molar ratio values until all binding sites were titrated. Indirect evidence suggests that the latter fraction corresponds to a truncated form of CelD in which the second segment of the dockerin is missing (see Supporting Information). Thus, analysis of the data was focused on the properties of the major, high-affinity species, which probably represents the genuine form of the dockerin.

The association constants (K_a), molar binding stoichiometry (N), and molar binding enthalpy ($\Delta H°$) were determined directly from the fitted curve. The Gibbs free energy and molar entropy of binding were calculated using equations $\Delta G° = -RT \ln K_a$ and $\Delta S° = (\Delta H° - \Delta G°)/T$, respectively, where R is the gas constant and T the absolute temperature in kelvin. The molar change in heat capacity (ΔC_p) accompanying binding was determined using a linear regression analysis of plots of binding enthalpies versus temperature. Prior to performing the ITC experiments, the thermal stability of CelD and CohCip7 was checked by differential scanning calorimetry to ensure that the two proteins remained folded in the temperature range studied (not shown).

All reported experiments were performed in 50 mM Tris-HCl buffer containing 2 mM $CaCl_2$, pH 7.5 at 25 °C. However, to determine if binding was pH-dependent and involved protonation/deprotonation events, the effect of pH on binding was also tested at 25 °C at pH 6.5 and 7.5 in MOPS buffer and at pH 8.5 in Tris-HCl buffer, all buffers of the same composition. This variation of pH is larger than the temperature-dependent variation of pH of the Tris-HCl buffer used in the binding experiments performed at temperatures between 20 °C (pH 7.6) and 50 °C (pH 6.8). The ionization enthalpies, $\Delta H°_{ioniz}$, of Tris-HCl and MOPS buffers are 11.34 and 4.90 kcal mol^{-1} at 25 °C, respectively (*29*).

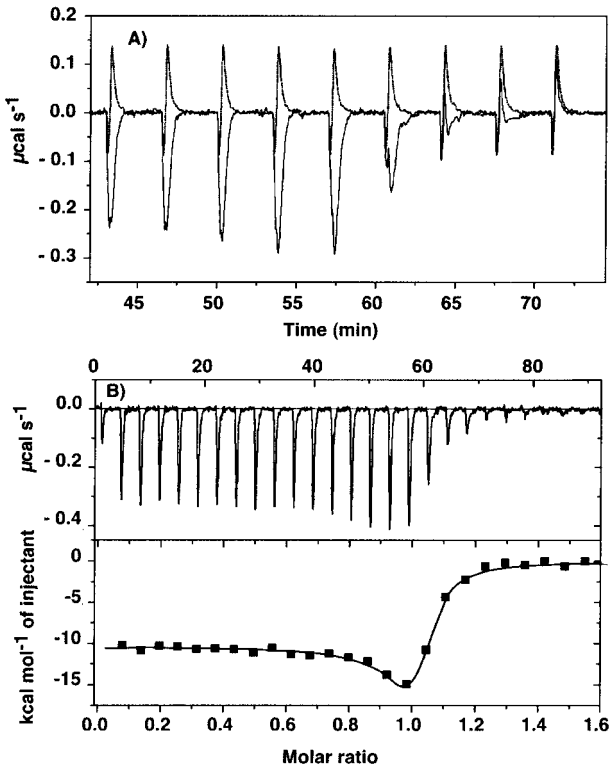

FIGURE 3: Isothermal calorimetric titration of endoglucanase CelD with CohCip7 at 50 °C. Panel A shows the raw heat signal for injections of 5 μL of 150 μM CohCip7 into a reaction cell containing 1.34 mL of buffer with (full line) and without (dashed line) 10.3 μM CelD, all at 50 °C. Panel B shows (top) the heat signal after subtraction of the heat of dilution of CohCip7 and (bottom) the integrated heat of each injection normalized to the amount of CohCipA injected (filled squares). In (A), injections of 5 s duration spaced with 3.5 min intervals are shown only in the transition zone from partial to full saturation. In (B) (bottom), the complete transition curve is shown. The curve through the points represents the best fit to a model with a heterogeneous population of CelD proteins composed of intact and modified CelD, each bearing a single binding site. The parameters defining the fitted curve are $K_a = 4.8 \times 10^8$ M^{-1} and $\Delta H = -10.15$ kcal mol^{-1} for the intact, major CelD fraction and $K_a = 1.9 \times 10^7$ M^{-1} and $\Delta H = -15.50$ kcal mol^{-1} for the modified minor fraction, with the intact and modified fractions representing 85% and 15% of the total amount of CelD protein in the titration cell, respectively.

The effect on binding of the COOH-terminal His_6 tag added to dockerin mutants for the purpose of protein purification was tested using a sample of $CelD_{672}$ purified from BL21(pREP4)(pCT672), which encodes wild-type CelD fused to a His_6 tag. Parameter values obtained with the two CelD proteins were equal within experimental error (data not shown). Similar data were reported for the dockerins of *C. cellulolyticum* (*30*). Possible artifacts due to the p*K* variation of Tris with temperature were ruled out, since the thermodynamic parameters of the cohesin−dockerin association remained constant between pH and pH (data not shown).

RESULTS

Exchange of Residues 10 and 11 of Either Duplicated Segment between the Dockerin Domains of CelD and CipA. To assess the influence of residues 10 and 11 on the binding preference of dockerin domains of type I and type II, these amino acids were exchanged in the first, in the second, or in both duplicated segments between the dockerin domains of CelD and CipA. The wild-type and mutated dockerin domains were then compared for their ability to form stable complexes with CohCip7 (type I cohesin domain) or with CohSdbA (type II cohesin domain). Figure 4 shows the analysis by nondenaturing polyacrylamide gel electrophoresis of samples containing each dockerin polypeptide alone or in combination with either cohesin domain. All CelD-derived dockerin domains failed to form detectable complexes with CohSdbA (panel B), as did all CipA-derived dockerin domains combined with CohCip7 (panel C). Thus, no extended binding specificity could be detected, even when residues 10 and 11 were exchanged in both segments of either domain. When tested against CohCip7, CelD-derived dockerin domains were still able to form visible complexes, provided that exchange of residues 10 and 11 took place in one segment only, as in $CelD_{6721}$ and $CelD_{6722}$ (panel A, LL...SS and ST...MQ). In the case of CipA-derived dockerin domains, exchange was tolerated in the amino-terminal segment only, as in $CelD_{6711}$ (panel D, ST...MQ). Preparations of CelD bearing CipA-derived dockerins contained two species. The slower migrating band corresponded to a disulfide dimer, whose appearance could be prevented by mutagenizing into Ser the Cys_{19} residue present in the amino-terminal segment of the dockerin of CipA (data not shown). Interestingly, formation of the disulfide dimer did not prevent association with CohSdbA.

Substitution by Cys Residues of Residues 10 and 11 in the Dockerin Domain of CelD and CipA. A possible explanation for the fact that mutations in both dockerin segments are required to abolish binding to CohCip7 may be that the mutations introduced in either single segment are too weak by themselves to interfere with complex formation as detected by native gel electrophoresis. To rule out this possibility, we sought to modify residues 10 and 11 in such a way as to reasonably preclude any intra- or intermolecular contact in which the side chain of the original amino acid may participate. The observation that Cys residues promoted the formation of disulfide dimers provided the opportunity to introduce very drastic changes in the side chains of mutagenized residues. Indeed, introducing a Cys residue that forms a disulfide dimer amounts to substituting the original side chain by a side chain of some 73 kDa. Thus, if complex formation could be observed with disulfide dimers involving Cys residues introduced at positions 10 and 11 of either segment, it would strongly suggest that the corresponding residues do not need to make contact with the cohesin domain and that they are not required for the folding of the dockerin domain.

Consequently, dockerin polypeptides were constructed in which Cys residues were introduced at positions 10 and 11 of either duplicated segment in the dockerin domains CelD and CipA. In order not to confuse the results, Cys_{19} was exchanged for Ser in the polypeptides derived from the dockerin domain of CipA. The polypeptides were mixed with their cognate cohesin domain and analyzed by electrophoresis under nondenaturing, nonreducing conditions. As an example, Figure 5 shows the results obtained with mutants of the CelD dockerin domain carrying Cys residues at position 10 of the first or the second duplicated segment. Both polypeptides are present as monomers and disulfide dimers, yielding two bands when electrophoresis is run under nonreducing conditions (panel A), whereas only one band is observed under reducing conditions (panel B). Both the

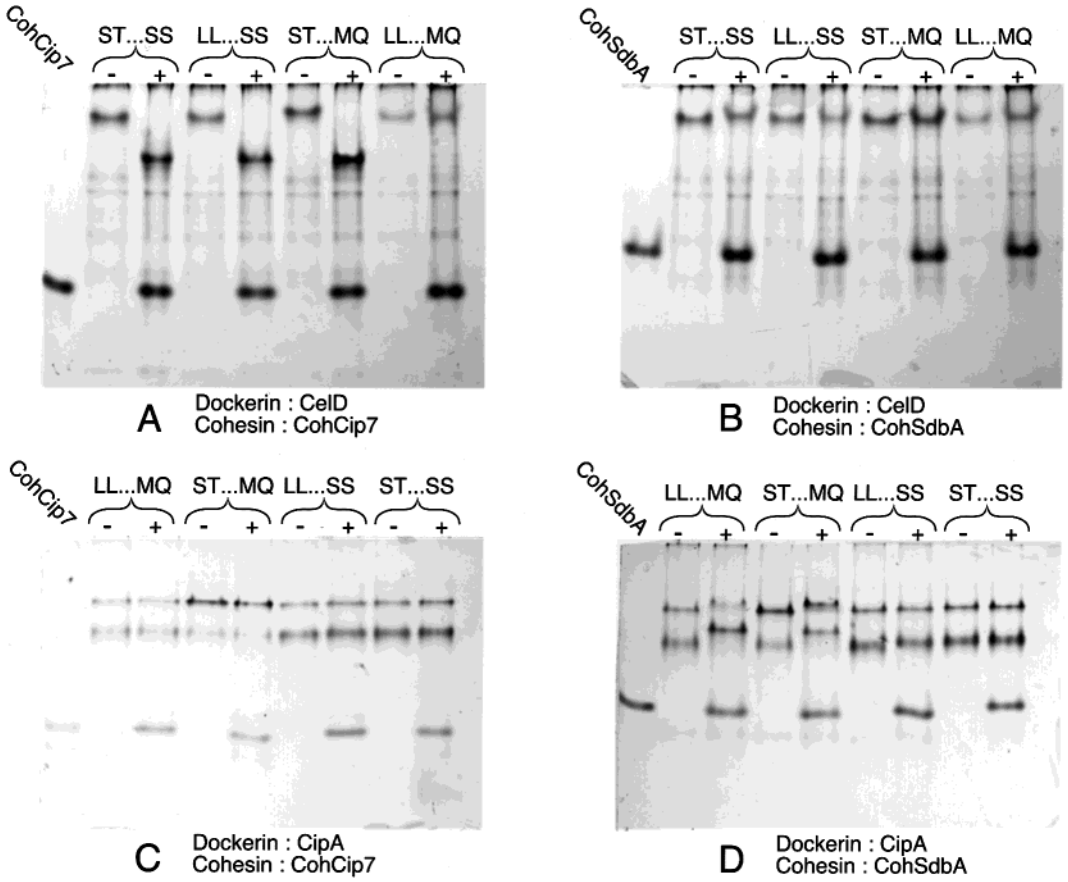

FIGURE 4: Analysis of complexes formed by CohCip7 and CohSdbA with CelD harboring dockerin domains in which residues 10 and 11 of each duplicated segment were swapped between the dockerin domains of CelD and CipA. (A) $CelD_{672}$ (ST...SS), $CelD_{6721}$ (LL...SS), $CelD_{6722}$ (ST...MQ), and $CelD_{6723}$ (LL...MQ) were incubated overnight at 4 °C in the absence (−) or in the presence (+) of CohCip7 in 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$. Each mixture was separated on a 7.5% polyacrylamide gel containing 2 mM $CaCl_2$ in the absence of SDS and mercaptoethanol (*21*). CohCip7 was run alone in lane 1. (B) Same as (A) except that CohCip7 was replaced by CohSdbA. (C) $CelD_{671}$ (LL...MQ), $CelD_{6711}$ (ST...MQ), $CelD_{6712}$ (LL...SS), and $CelD_{6713}$ (ST...SS) were incubated in the absence (−) or in the presence (+) of CohCip7 and subjected to nondenaturing gel electrophoresis as in panels A and B. CohCip7 was run alone in lane 1. (D) Same as (C) except that CohCip7 was replaced by CohSdbA.

monomer and the disulfide dimer are shifted upon mixing with CohCip7 (panel A), indicating that both are capable to form complexes. This was confirmed by two-dimensional gel electrophoresis (panel C). A mixture of $CelD_{678}$ and CohCip7 was run on a nonreducing, nondenaturing gel. The lane was cut out, boiled in sample buffer containing SDS and mercaptoethanol, and laid across a second, denaturing gel. Next to the material precipitating at the top of the gel, it is seen that the two slowly migrating bands in the first dimension are indeed complexes containing both the cohesin and the dockerin polypeptides. The results obtained with the whole set of mutants are summarized in Table 1.

In the case of the CelD dockerin domain, both the monomeric forms and the disulfide dimers induced by the introduction of Cys at each of the four sites tested retained the ability to bind CohCip7 provided that at least one duplicated segment was intact. No binding was observed with either the monomeric or the dimeric form of mutants $CelD_{680}$, which contained a Cys residue on the NH_2-terminal segment and residues derived from the CipA dockerin on the COOH-terminal segment. With $CelD_{681}$, carrying a Cys residue in the COOH-terminal segment and residues derived from the CipA dockerin in the first segment, partial complex formation was observed with the monomeric form, but no complex was seen with the Cys dimer. In the case of the CipA dockerin domain, the introduction of Cys at either position of the amino-terminal segment was tolerated, both for the monomer and for the disulfide dimer. When Cys residues were inserted at positions 10 and 11 of the carboxy-terminal segment, binding could only be observed for the monomeric form.

Binding of CohCip7 to Wild-Type CelD. A complete thermodynamic characterization of the association of CohCip7 with CelD was obtained by performing titration experiments at temperatures between 20 and 50 °C. Results of these experiments are shown in Figure 6 and Table 2. The binding parameters in Table 2 were not changed by replacing Tris-HCl buffer by MOPS buffer, indicating that no change in protonation occurred upon binding at pH 7.5, and binding was pH-independent between pH 6.5 and pH 8.5 (data not shown). The cohesin−dockerin interaction was characterized by a strong affinity, with a binding constant of $(2.6 \pm 0.5) \times 10^9\ M^{-1}$. Figure 6 shows that the association reaction was both enthalpy- and entropy-favored throughout the temperature range studied. The binding enthalpy was negative, with a linear and sharp temperature dependence corresponding to a negative heat capacity change during complexation ($\Delta C_{p,wt} = -305$ cal mol^{-1} K^{-1}; Table 2). With increasing temperature, the binding entropy decreased almost in parallel with the binding enthalpy, so that enthalpy and entropy changes nearly canceled out in the binding free

Table 2: Thermodynamic Parameters at 25 °C of the Association of Cohesin Domain 7 of the Scaffolding Protein CipA (CohCip7) with Endoglucanase CelD Carrying either Wild-Type (WT) or Mutated Dockerin Domains (6721, 6722, and 6723)[a]

CelD	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	$(2.6 \pm 0.5) \times 10^9$	-12.92 ± 0.17	-2.22 ± 0.20	10.70 ± 0.35	-305 ± 15
6721	$(1.2 \pm 0.3) \times 10^9$	-12.48 ± 0.12	-3.99 ± 0.13	8.48 ± 0.25	-113 ± 15
6722	$(5.7 \pm 0.8) \times 10^8$	-12.02 ± 0.08	1.74 ± 0.10	13.77 ± 0.18	-498 ± 23
6723	$(4.5 \pm 2.4) \times 10^6$	-9.14 ± 0.3	3.12 ± 0.15	12.27 ± 0.45	-165 ± 35

[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.

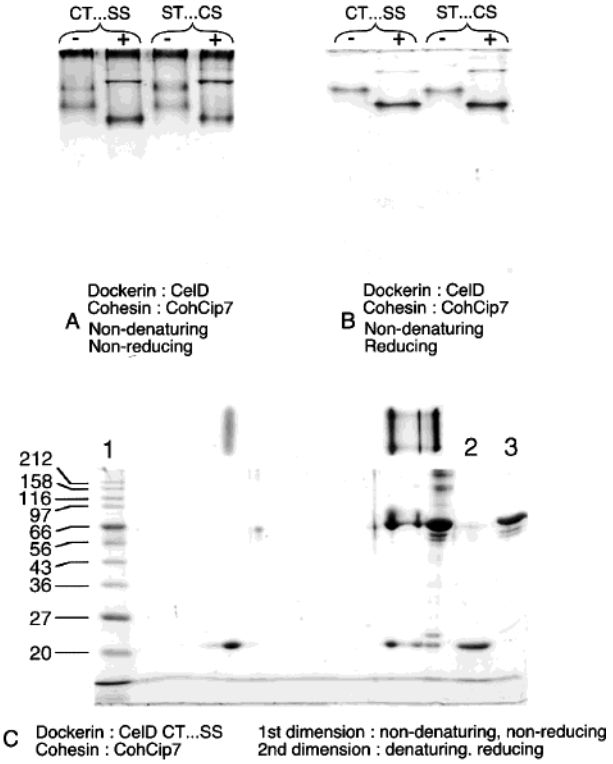

FIGURE 5: Analysis of complexes formed by CohCip7 with $CelD_{678}$ and $CelD_{679}$. (A) $CelD_{678}$ (CT...SS) or $CelD_{679}$ (ST...CS) was incubated overnight at 4 °C in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of CohCip7 in 20 μL of 50 mM Tris-HCl, pH 7.5, containing 2 mM $CaCl_2$. Each mixture was separated on a nondenaturing 15% polyacrylamide gel containing 2 mM $CaCl_2$. Electrophoresis was run for three times the time required for bromophenol blue to reach the bottom of the gel, so that excess CohCip7 was no longer present in the gel. (B) Same as (A) except that the samples were heated for 15 min at 60 °C in the presence of 10 mM dithiothreitol prior to electrophoresis. (C) Two-dimensional electrophoresis of the complexes formed by CohCip7 and $CelD_{678}$ (CelD CT...CS) under nondenaturing, nonreducing conditions. A mixture of $CelD_{678}$ and CohCip7 was run on a nondenaturing, nonreducing gel as in panel A. The lane was cut off and incubated for 45 min at room temperature in 25 volumes of denaturing sample buffer containing 2% SDS and 5% mercaptoethanol (*45*), followed by heating for 5 min at 100 °C. It was then loaded on top of a 12.5% polyacrylamide gel and electrophoresed in the presence of 0.1% SDS. Molecular weight markers (Biolabs), CohCip7, and $CelD_{678}$ were added to reference troughs 1, 2, and 3, respectively.

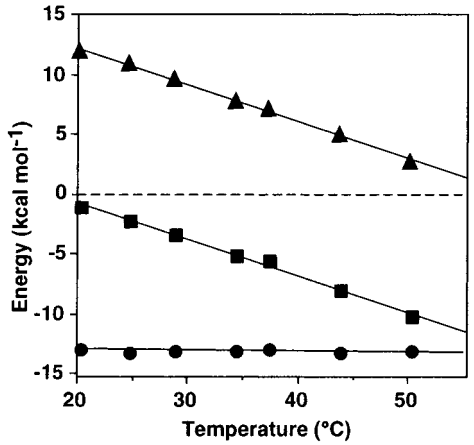

FIGURE 6: Free energy, enthalpy, and entropy changes for binding of CohCipA to $CelD_{wt}$ versus temperature. ΔG (circles), ΔH (squares), and $T\Delta S$ (triangles) were fitted to equations of the form $\Delta H(T) = \Delta H(T_R) + \Delta C_p(T - T_R)$, $\Delta S(T) = \Delta S(T_R) + \Delta C_p \ln(T/T_R)$, and $\Delta G(T) = \Delta H(T) - T\Delta S(T)$, with T_R, a reference temperature, taken as 25 °C. The solid lines were calculated from best-fit parameters obtained from regression analysis. Best-fit parameters are given in Table 2. Temperatures at which the binding enthalpy and binding entropy equal zero are $T_H = 17.7$ °C and $T_S = 64.6$ °C.

energy, which varied little in the temperature range tested. Thus, the association reaction was entropy-driven at 20 °C ($-\Delta H_{wt} \ll T\Delta S_{wt}$) and enthalpy-driven ($-\Delta H_{wt} \gg T\Delta S_{wt}$) at 50 °C (Figure 6). At 25 °C, the binding enthalpy $\Delta H°_{wt}$ amounted to 17.2% of the binding free energy $\Delta G°_{wt}$ (Table 2). Taken together, a negative heat capacity change on binding and an association reaction that is entropy-driven at low temperatures and enthalpy-driven at high temperatures are typical of an association reaction dominated by hydrophobic interactions.

Binding of CohCip7 to CelD Mutants. Concerning the binding of CohCip7 to the major CelD fraction, examination of thermodynamic parameters indicates that mutations in the first and the second segment of the dockerin domain had clearly detectable but quite different consequences (Table 2). The $CelD_{6721}$ mutation made the reaction at 25 °C more exothermic, whereas the entropy change was less favorable than with wild-type CelD; the two phenomena nearly compensated each other, and the binding constant at 25 °C was reduced only by a factor of 2. The heat capacity change decreased to about 37% of that observed with the wild type. Temperature changes were accompanied by nearly exact entropy−enthalpy compensation, so that the affinity constant remained nearly the same between 25 and 45 °C. As compared to the wild type, the larger binding enthalpy at 25 °C, the smaller binding in entropy, and the lower heat capacity change on binding observed with $CelD_{6721}$ indicate that the association reaction is less dependent on hydrophobic interactions.

The $CelD_{6722}$ mutation modified the thermodynamic parameters for binding in a direction opposite to that observed with $CelD_{6721}$. At 25 °C, the reaction was strongly entropy-driven: the change in enthalpy was unfavorable ($\Delta H°_{6722} > 0$) and the change of entropy strongly favorable ($\Delta H°_{6722}/\Delta G°_{6722} = -14.5\%$ and $-T\Delta S°_{6722}/\Delta G°_{6722} = 114.6\%$). Again, the two phenomena nearly compensated

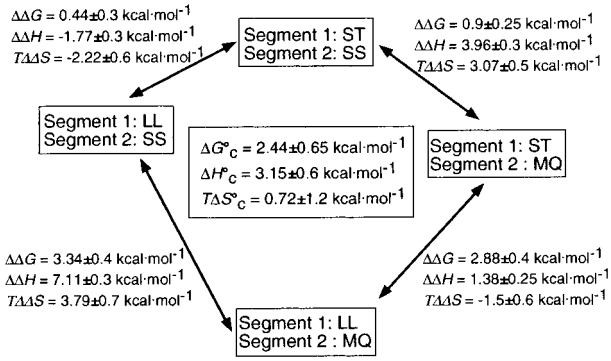

FIGURE 7: Coupling energetics between residues of the two duplicated segments of a type I dockerin domain. The double mutant cycle for the interaction between the pairs of residues 10 and 11 of the NH_2-terminal and COOH-terminal segments of the CelD dockerin domain is shown. The wild type carrying Ser-Ser in the first segment and Ser-Thr in the second segment is shown at the top. $\Delta\Delta G°$, $\Delta\Delta H°$, and $T\Delta\Delta S°$ represent the differences in the thermodynamic parameters of binding between the indicated dockerin 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 2.

each other, and the association constant at 25 °C decreased only 5-fold (Table 2). As compared to the wild type, the positive change in enthalpy at 25 °C, the larger change in entropy, and the strong negative variation in heat capacity change observed with $CelD_{6722}$ indicate that the association reaction has a more hydrophobic character.

Mutagenizing the dockerin domain in both segments induced drastic changes in the binding reaction. At 25 °C, binding of CohCip7 to $CelD_{6723}$ was endothermic and overwhelmingly entropy-driven ($\Delta H°_{6723}/\Delta G°_{6723} = -34.1\%$ and $-T\Delta S°_{6723}/\Delta G°_{6723} = 134.2\%$; Table 2). Changes of the enthalpy and entropy of binding due to the double mutation were the largest observed. These changes did not compensate in the binding free energy, giving the smallest association constant $K_{a,6723} = 4.5 \times 10^6\ M^{-1}$; Table 2). The heat capacity change on binding was intermediate between the values obtained for $CelD_{6721}$ and wild-type CelD ($\Delta C_{p,6723} = -165$ cal mol^{-1} K^{-1}; Table 2).

The changes induced in the thermodynamic parameters by exchanging residues 10 and 11 in either segment were highly dependent on the context provided by the other segment (Figure 7). Mutating one segment clearly had stronger effects on the binding enthalpy and binding free energy when the other segment was already mutated. The negative cooperativity between the two mutated sites can be assessed from the coupling free energy $\Delta G°_c$. $\Delta G°_c$ is equal to the difference between the change in binding free energy induced by either mutation when the other segment is mutated and the change induced by the same mutation when the other segment is intact: $\Delta G°_c = (\Delta G°_{6723} - \Delta G°_{6722}) - (\Delta G°_{6721} - \Delta G°_{wt}) = 2.44 \pm 0.7$ kcal mol^{-1}. Similar calculations lead to $\Delta H°_c = 3.15 \pm 0.6$ kcal mol^{-1} and $T\Delta S°_c = 0.72 \pm 1.2$ kcal mol^{-1}. $\Delta G°_c$ was large and positive, indicating that binding at the mutagenized sites in both segments showed negative cooperativity. $\Delta H°_c$ was also large and positive, whereas $T\Delta S°_c$ was not significantly different from zero, showing that the coupling free energy was of enthalpic origin. Applying the same difference calculation to the heat capacity change on binding gives a large positive value (141 ± 88 cal mol^{-1} K^{-1}), indicating that the negative cooperativity between the two mutated sites increases with temperature.

DISCUSSION

The recently published structure of the CelS dockerin domain shows that the side chains of residues 10 and 11 are exposed on the surface of the folded polypeptide (*31*). Therefore, it is likely that mutations of these amino acids act by altering the contact surface directly.

Residues 10 and 11 of dockerin domains were proposed by Pagès et al. (*18*) to be involved in determining the specificity of cohesin−dockerin interactions. Even when performed in both duplicated segments, exchange of residues 10 and 11 between the dockerin domains of CelD and CipA was not sufficient to confer cross-specific binding capacity on the dockerin domains tested. This result runs counter to the data of Mechaly et al. (*19*, *32*, *33*), who performed similar exchanges between the type I dockerin of *C. thermocellum* CelS and the type III dockerin of *C. cellulolyticum* CelA. In this case, exchange of residue 10 in the first segment was sufficient to promote binding of the CelS dockerin domain to the *C. cellulolyticum* cohesin. A possible explanation for our failure to detect cross-specific binding is that the nondenaturing gel analysis used in the present study may be less sensitive than the blotting technique and the plasmon resonance measurements used by Mechaly et al. Indeed, nondenaturing gel electrophoresis showed no detectable affinity between CohCip7 and the dockerin of $CelD_{6723}$, which was mutated in both segments (Figure 4A). However, thermodynamic analysis indicated that the binding constant of $CelD_{6723}$, although over 500 times weaker than that of the wild type, was still $4.5 \times 10^6\ M^{-1}$ (Table 2). Furthermore, *C. thermocellum* type I domains and *C. cellulolyticum* type III domains are more similar to each other than *C. thermocellum* type I and type II domains. Thus, the conversion between type I and type III may require fewer changes than the conversion between type I and type II.

Nevertheless, our data show that substituting residues 10 and 11 in both segments strongly affected binding of the type I dockerin of CelD to the cognate cohesin domain CohCip7. This suggests that, in dockerin domains of type I, residues 10 and 11 of both segments can interact with the cognate cohesin domains. However, it is particularly striking that both segments of the type I dockerin domain had to be mutated in order to prevent binding. Even when drastic modifications were introduced in one segment, as was the case with the Cys dimers, binding still occurred if the other segment was intact. This suggests that either segment is able to make contact and provide a strong binding with the cohesin domain. In the case of the type II dockerin, the second segment appears to be critical, but auxiliary contacts made by the first segment cannot be ruled out at this stage.

Quantitative analysis by isothermal titration calorimetry provided a more refined picture of the interaction between CohCip7 and wild-type or mutated dockerin domains. Binding of cohesin domain 7 of the scaffolding protein CipA (CohCip7) to the dockerin domain of endoglucanase CelD occurred in 1:1 stoichiometry with a high association constant ($K_a = 2.6 \times 10^9\ M^{-1}$ at 25 °C).

The association between CohCip7 and CelD was both exothermic and entropy-driven at 25 °C. The change in heat

capacity ΔC_p was constant between 20 and 50 °C, indicating that folding of the cohesin−dockerin complex did not change within this range of temperature. The negative heat capacity change and the large positive entropy change of the binding reaction are the hallmark of a process governed by hydrophobic interactions involving the removal of nonpolar surface from water with a reorganization of the water molecules (*34*−*36*). Enthalpy−entropy compensation with varying temperature was observed, leading to only small variations of the Gibbs free energy of binding in the temperature range studied ($K_a = 4.8 \times 10^8$ M^{-1} at 50 °C; Figure 6).

Mutations at sequence positions 10 and 11 of the first or the second duplicated segment of the dockerin domain clearly induced significant changes in the enthalpy and entropy of binding, which confirms that both segments of the dockerin domain contribute to the association with the cohesin domain. However, these changes largely compensated each other, and the mutations had only small effects on the binding affinity. Enthalpy−entropy compensation has been described for many binding events in aqueous solution involving changes in hydrogen bonding (*37*−*39*). It is successfully accounted for with general thermodynamic models involving the coupling between some type of transition state of the protein (or the ligand) and the binding process (*40*).

Our data suggest that the two segments are responsible for different types of interactions with the cohesin domain. Mutating residues 10 and 11 in segment 1 from ST to LL ($CelD_{6721}$) rendered the association less hydrophobic. The heat capacity change on binding decreased by half, the reaction being more exothermic with a smaller positive binding entropy at 25 °C (Table 2). Mutating SS to MQ in segment 2 ($CelD_{6722}$) acted in the opposite way, increasing the hydrophobic character of the association. The heat capacity change was 1.6 times larger, the association reaction being less exothermic at low temperatures with a large positive increase in binding entropy (Table 2). Thus, it may be that the first segment makes hydrophobic contacts, which are perturbed in $CelD_{6721}$, while the second segment makes more hydrophilic contacts, which are impaired in $CelD_{6722}$. The differences between the heat capacity changes on binding of the $CelD_{6721}$ and $CelD_{6722}$ mutants were much larger than expected on the basis of the nature of the substituted amino acids [calculations not shown (*36*)]. This suggests that the different thermodynamic parameters observed for wild-type and mutant dockerin domains are due to differences in the structure (including both protein folding and binding of solvent molecules) of the complexes formed by each polypeptide.

When combined, mutations in the first and in the second segment of the dockerin domain were strongly cooperative in impairing the binding process, as demonstrated by the properties of $CelD_{6723}$. This was reflected in a large and positive coupling free energy ($\Delta G°_c = 2.44$ kcal mol^{-1}), which was of enthalpic origin ($\Delta H°_c = 3.15$ kcal mol^{-1}). Hence, for either of the dockerins mutated in only one segment, the detrimental effect of the mutation was largely compensated by a conformational rearrangement and/or a different solvation of the dockerin−cohesin complex leading to an energetically more favorable structure. Compensation was no longer possible when both segments were mutated. In other words, simultaneous binding of residues 10 and 11 in both dockerin segments to the cohesin domain must be accompanied by a structural change involving the buildup of strain, which nearly cancels the gain in free energy provided by interactions at both sites instead of one. In addition to solvation phenomena, such a strain may be due to an alteration of the structure of the dockerin domain, the cohesin domain, or both. However, a modification of the dockerin structure appears more likely. Cohesin domains are highly stable structures, as evidenced by their resistance to proteases and denaturing agents (*7*, *22*, *41*, *42*). By contrast, several observations argue that the structure of dockerin domains is flexible. NMR spectroscopy has shown that the folding of the noncomplexed dockerin domain of CelS is induced by Ca^{2+} (*43*). Furthermore, the complex formed between CelD and CohCip7 migrates faster than noncomplexed CelD in nondenaturing gels (Figure 4A), and this is also the case in polyacrylamide gradient gels (*44*). This suggests that complexation results in a conformational change leading to a more compact structure of the dockerin domain.

The association constants reported in this paper are generally lower that those reported by Mechaly et al. (*32*) for the interaction between the dockerin domain of CelS and the third cohesin domain of CipA ($>10^{11}$ M^{-1} for the wild-type domains). However, the latter were determined by a different technique (surface plasmon resonance), and the values cannot be compared in absolute terms (*25*). Nevertheless, the effect of the mutations studied by Mechaly et al. is quite compatible with that reported in this paper: all the mutated dockerin domains bearing substitutions in one segment only had association constants too high to be measured ($>10^{11}$ M^{-1}), whereas mutant mS5, which carries substitutions in both segments, had an affinity of 10^{10} M^{-1}.

If either of the dockerin segments is able to make specific contacts with the cohesin domain, the zone of interaction between cohesin and dockerin domains should extend over a significant surface area. Furthermore, effects symmetric to those observed with mutations of the dockerin segments might be obtained by mutagenizing different areas of the contact zone on CohCip7. The accompanying study investigates these points by mapping amino acid residues of the cohesin domain whose side chains are exposed to the solvent and whose mutagenesis affects binding to the dockerin domain (*47*).

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

One figure showing calorimetric binding isotherms measured for binding of CohCip7 to wild-type CelD, $CelD_{6721}$, $CelD_{6722}$, and $CelD_{6723}$ at 28.7 and 37.2 °C and evidence that the second transition observed in the thermodynamic titration of CelD by CohCip7 is due to the presence of a molecular species of CelD in which the second segment is absent or damaged. This material is available free of charge via the Internet at http://pubs.acs.org.

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