Cohesin–dockerin interaction in cellulosome assembly: a single Asp-to-Asn mutation disrupts high-affinity cohesin–dockerin binding

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Abstract The cohesive cellulosome complex is sustained by the high-affinity cohesin–dockerin interaction. In previous work [J. Biol. Chem. 276 (2001) 9883], we demonstrated that a single Thr-to-Leu replacement in the *Clostridium thermocellum* dockerin component differentiates between non-recognition and high-affinity recognition by the interspecies rival cohesin from *C. cellulolyticum*. In this report, we show that a single Asp-to-Asn substitution on the cohesin counterpart also disrupts normal recognition of the dockerin. The Asp34 carboxyl group of the cohesin appears to play a central role in the resultant hydrogen-bonding network as an acceptor of two crucial hydrogen bonds from Ser45 of the dockerin domain. The results underscore the fragile nature of the intermolecular contact interactions that maintain this very high-affinity protein–protein interaction.

Keywords: Cellulosome; Protein–protein interaction; Cohesin–dockerin binding specificity; Multi-enzyme complex; *Clostridium thermocellum*; *Clostridium cellulolyticum*

1. Introduction

Cellulosomes are multi-enzyme extracellular complexes, produced by various anaerobic microorganisms for the efficient degradation of plant cell wall polysaccharides [1–4]. The various cellulosome components are assembled by virtue of a high-affinity protein–protein interaction between reciprocal modules on the interacting subunits – the cohesin and the dockerin. In early studies on the cellulosomes from two clostridial species, *Clostridium thermocellum* and *C. cellulolyticum*, the interaction between cohesins and dockerins was found to be generally species specific: experiments carried out with isolated modules from the two species revealed that cohesins from the scaffoldin of one species bind to the dockerins of its own enzymatic subunits with high affinity, but fail to recognize those of the other species despite the relatively high sequence homology among the analogous components [5,6].

Crystal structures of cohesins from the scaffoldin of *C. thermocellum* [7,8] and *C. cellulolyticum* [9] have been published. The cohesins form a nine-stranded β-sandwich with a jelly-roll topology. The β-sandwich results from the association of a four-stranded antiparallel β-sheet and a five-stranded mixed β-sheet, stabilized by a hydrophobic core. The two β-sheets are composed of strands 8, 3, 6, 5 and strands 9, 1, 2, 7, 4, respectively. In addition, a solution structure of a dockerin from *C. thermocellum* cellulosomal cellobiohydrolase CelS has been solved by NMR analysis [10]. The structure consists of two Ca^{2+}-binding loop-helix motifs that bear sequence homology to the EF-hand motif of eukaryotic calcium-binding proteins, such as calmodulin and troponin C. Very recently, a crystal structure of a cohesin–dockerin complex from *C. thermocellum* has also been solved [11]. The complex shows that, while the cohesin module remains essentially unchanged, the dockerin undergoes conformational adjustments upon binding. The protein–protein contact between one face of the cohesin and α-helices 1 and 3 of the dockerin is mediated mainly by hydrophobic interactions and relatively few intermolecular hydrogen bonds. Although the structure of the heterodimer sheds additional light on the structural basis of the cohesin–dockerin interface, the function and importance of specific amino acids involved in recognition and binding are not entirely apparent from the structural data.

To determine the exact role of the contact residues in binding and affinity, various residues on the *C. thermocellum* cohesin surface were replaced with matching residues of *C. cellulolyticum*. The binding specificity and affinity of the resultant mutated proteins were tested using an enzyme-linked assay. Although the mutated cohesins maintained their original specificity, a dramatic reduction in the affinity was observed for several of the mutants, the common denominator being the D34N mutation. This single conservative amino acid replacement reduces the affinity of the interaction by more than 3 orders of magnitude.

2. Materials and methods

2.1. Protein constructs and cloning

The protein construct containing the cohesin from *C. thermocellum* consists of cohesin-2 and a cellulose-binding domain from CipA. The construct containing the cohesin from *C. cellulolyticum* comprises a cellulose-binding domain, a hydrophilic domain, and cohesin-1 from CipC. Details of the cloning of cohesin constructs (termed Coh2CBD-t

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and miniCipC-c, where t and c denote domains derived from *C. thermocellum* and *C. cellulolyticum*, respectively) were described elsewhere [5,12].

The dockerin constructs comprise the dockerin domain of CelS from *C. thermocellum* [13] or the dockerin domain of CelA from *C. cellulolyticum* [14], fused downstream of the non-cellulosomal family-10 xylanase T-6 from *Geobacillus stearothermophilus* [15,16]. These constructs, termed XynDocS-t and XynDocA-c, respectively, were cloned using a specially designed cassette produced for this purpose. The cassette consisted of the gene for the *G. stearothermophilus* xylanase T-6 with a His-tag and a *Bsp*HI site at the 5′-terminus and a *Kpn*I site at the 3′-terminus. This construct was ligated at the *Kpn*I site with the PCR product of a *C. thermocellum* CelS (Cel48A) dockerin (containing a 5′-terminal *Kpn*I site and a 3′-terminal *Bam*HI site) and inserted into the pET9d vector at the *Nco*I and *Bam*HI sites. This plasmid allows replacement of the CelS dockerin with any other desired dockerin by digesting with *Kpn*I and *Bam*HI, and the resultant expressed product constitutes a His-tagged xylanase T-6 fusion-protein bearing a dockerin at the C-terminus.

2.2. Site-directed mutagenesis

The mutagenesis of the cohesin domain was carried out as previously described [17]. Generally, mutated cohesins containing combined mutations were produced in a sequential manner, in which one mutant served as a template for the subsequent one.

2.3. Expression and purification of proteins

All proteins were expressed in *Escherichia coli* BL21(DE3) grown overnight in Terrific Broth medium [18]. For the production of miniCipC-c cloned in pET22b, the medium was supplemented with 0.1 mg/ml ampicillin, and protein expression was induced with 0.4 mM isopropyl β-D-thiogalactoside. For all other proteins cloned in pET9d, the medium was supplemented with 25 μg/ml kanamycin, and growth was carried out without induction. Following growth, cells were harvested, resuspended in TrisNC buffer (50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, and 0.02% sodium azide, pH 7.5), disrupted by two passages through a French® press (Spectronic Instruments, Inc., Rochester, NY, USA), and centrifuged for the production of clear crude protein extracts, that were further purified as described below.

Xylanase-containing constructs (XynDocS-t and XynDocA-c) were purified by gel filtration using a Superdex 200 26/60 column, AKTA explorer (Pharmacia), running at 2.5 ml/min with TrisNC buffer. CBD-containing constructs (Coh2CBD-t and Coh1-c) were purified by affinity chromatography on cellulose. Microcrystalline cellulose (Avicel Type PH-101 FMC) was added to the crude protein extract, originating from a 1-l cell culture. The ratio of cellulose to cells was 0.7 g per 1 unit OD600. The resultant suspension was stirred for 1 h. After centrifugation, the pellet was washed twice with TrisNC buffer, containing 0.1 M NaCl and twice with TrisNC buffer, containing 1 M NaCl. The CBD-containing proteins were eluted from the cellulosic matrix with 11 ml of 1% (v/v) triethylamine. The eluent fractions were neutralized with TrisNC buffer. Purity of all proteins was estimated by SDS–PAGE and protein concentration was estimated by Bradford [19].

2.4. Non-competitive enzyme-linked interaction assay

Microtiter plates (MaxiSorp-immunoplates, NUNC A/S, Roskilde, Denmark) were coated overnight at 23 °C with the cohesin test samples (200 μl/well, 270 nM of miniCipC-c, wild-type or mutated Coh2CBD-t). The plates were blocked for 2.5 h with blocking solution (300 μl/well, 3% (w/v) of bovine serum albumin in TrisNC buffer) and washed three times with TrisNC buffer (300 μl/well). The cohesin–dockerin interaction was initiated upon addition of dockerin samples (200 μl/well, 94 nM of XynDocA-c or XynDocS-t), and the plates were incubated for 2.5 h. After five washes, the bound dockerins were detected by means of the fused-xylanase activity: substrate solution (240 μl/well, 2.9 mM of *p*-nitrophenyl β-D-cellobioside) was added followed by incubation at 60 °C. Optical density was determined at 420 nm on a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale CA).

2.5. Competitive enzyme-linked interaction assay

Microtiter plates were coated overnight with wild-type *C. thermocellum* cohesin samples (200 μl/well, 270 nM of Coh2CBD-t). Plates were blocked for 2.5 h with the above-described blocking solution and washed three times with TrisNC buffer. The cohesin–dockerin interaction was carried out by the addition of 100 μl of the desired competitor cohesin sample (i.e., wild-type or mutant Coh2CBD-t at various concentrations, up to a maximum of 1.3 μM), immediately followed by the addition of dockerin solution (100 μl of XynDocS-t to a final concentration of 47 nM). Dilutions of the competitor cohesins were carried out in TrisNC buffer containing BSA, to maintain a constant protein concentration. After incubation for 2.5 h, the wells were washed five times, and the amount of dockerin bound to the coating cohesin was detected by means of the fused-xylanase activity, as described above.

Results were expressed as percentage of binding, derived from the mean optical density values of five repetitions for each competitor concentration (percentage of binding = 100 × optical density of the test competitor concentration/optical density without competitor). Data were analyzed using a 4-parameter fit in Grafit 5 software [20,21].

3. Results and discussion

The cohesin–dockerin interaction is the molecular adhesive that defines and secures the cellulosome complex. To elucidate the structural basis behind the tenacious interaction, a combined bioinformatics-mutagenesis approach was exploited. Based on amino acid sequence alignment of dockerins with divergent specificities, we previously predicted a group of dockerin residues that would serve as cohesin-recognition codes. Site-directed mutagenesis was used to validate the prediction, and several amino acids located on the duplicated segments of the dockerin domain were indeed proved to be important for the binding specificity [22,23].

In this work, a similar approach was applied to the complementary module – the cohesin, to reveal the role of its interacting residues and their contribution to binding and specificity. The residues chosen for this study were based on cohesin sequence alignment (Fig. 1) and the superposition of related cohesin structures from two species – cohesins from *C. thermocellum* and *C. cellulolyticum*. The amino acids important for the binding process were assumed to be surface residues, conserved within one species but dissimilar between the divergent species. On the basis of these criteria, various positions on the surface of the test cohesin from *C. thermocellum* were subjected to site-directed mutagenesis, in which the designated residues were replaced with their counterparts from the cohesin of *C. cellulolyticum*. The mutated positions were mainly located on the conserved 8,3,6,5-face of the cohesin and included N32, D34, V36, D65, V76, A80, and D114, as well as replacement of the small loop connecting strands 5 and 6 (mutant 28). Several mutations (mutant 20) were also designed to test the previously proposed involvement of residues at the "crown" of the cohesin [24]. The mutated genes contained single or combined mutations, and the gene products were overexpressed, purified, and tested for their binding specificity and affinity.

To test the interactions of the mutated proteins, a simple direct enzyme-linked interaction assay (ELIA) was developed. In the non-competitive form of this assay, a cohesin solution is used to coat microtiter plates and is allowed to interact with an enzyme-linked dockerin solution. The enzymatic activity obtained after the appropriate washings provides a direct indication of cohesin–dockerin interaction. The dockerin-fused enzyme was xylanase T6 from *Geobacillus stearothermophilus*, which is known for its exceptionally high propensity towards expression in *E. coli* host cell systems [15]. Additional desirable

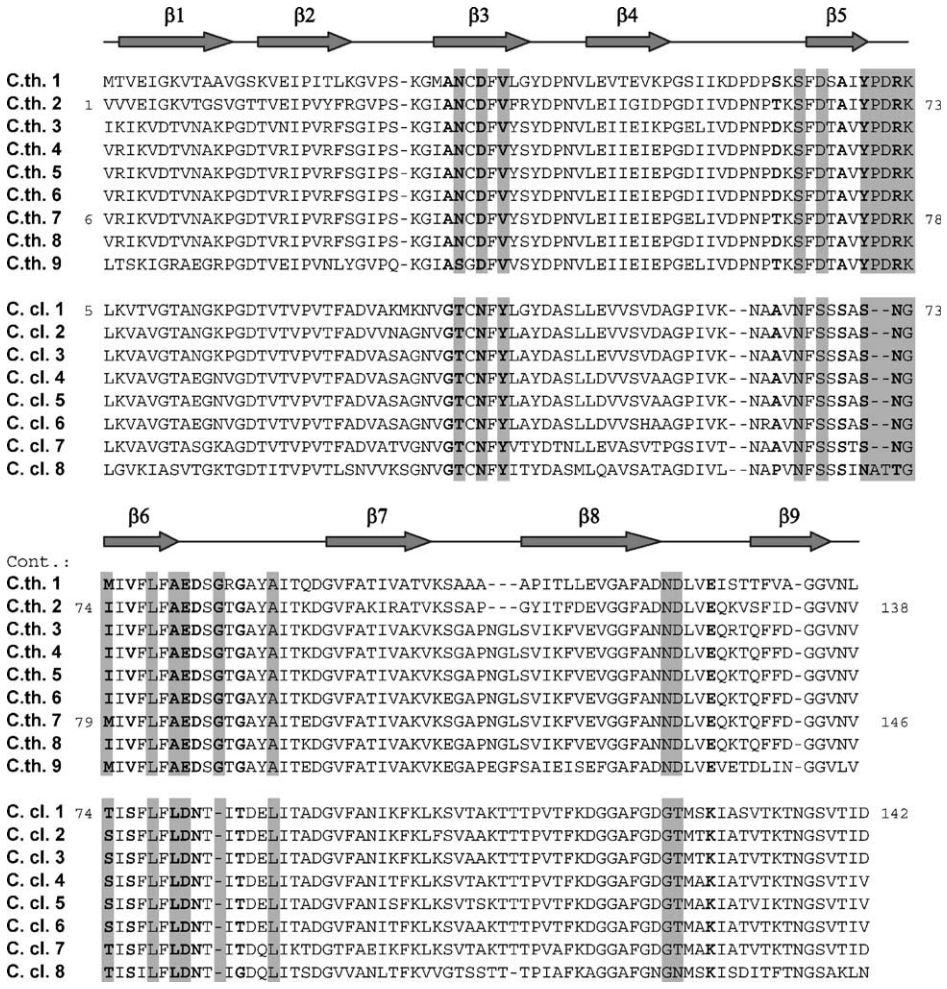

Fig. 1. Amino acid alignment of cohesin modules from *C. thermocellum* CipA and *C. cellulolyticum* CipC. Contact residues, as reported by Carvalho et al. [11] for the *C. thermocellum* cohesin-2, are shown in bold as are the homologous residues of *C. cellulolyticum*. Residues that were replaced in this work are highlighted in gray. The position of secondary structures (β strands) is indicated by arrows; residues are numbered according to the available crystal structures [7–9].

features of this enzyme include its high thermostability, which facilitates its isolation and resistance to denaturation, and its ability to hydrolyze a series of commercially available chromogenic substrates, which allows rapid measurement of enzyme activity. The His-tagged xylanase T-6 fused to the N-terminus of the dockerin domain also serves to stabilize the relatively small dockerin domain, which is otherwise unstable in solution. The assay described here enables us to examine the binding ability and specificity of a large number of cohesin–dockerin pairs in a relatively rapid and economical manner.

To quantitatively assess the binding affinity of mutated cohesins, a competitive form of the assay (cELIA) was used, in which high binding constants can be determined in a comparative manner. In this approach, microtiter plates are coated with the native cohesin and allowed to interact subsequently with an enzyme-linked dockerin solution in the presence of various dilutions of a competitor cohesin (native or mutated). The measured activity reflects the amount of dockerin bound to the coating cohesin, and the value of IC_{50}, i.e., the concentration of competitor that results in 50% inhibition of binding, is proportional to the dissociation constant (K_d) of the competitor. By comparing the interaction of the mutated cohesin with that of the native molecule, the relative binding affinity can thus be assessed. Representative results of cELIA are presented in Fig. 2.

The mutated *C. thermocellum* cohesins were first examined qualitatively by direct ELIA, in which mutant proteins were allowed to interact with dockerins from both *C. thermocellum* and *C. cellulolyticum*. All of the tested mutants retained their original binding preference (data not shown), i.e., all were capable of binding to the dockerin from *C. thermocellum* but failed to bind the dockerin from *C. cellulolyticum*.

To determine the possible role and relative importance of these residues to cohesin–dockerin binding, quantitative cELIA was employed, in which the mutated cohesins served as competitors for the wild-type cohesin (Table 1). Using this approach, many of the mutations failed to reduce significantly the binding affinity of the cohesin for the dockerin, despite their location in the contact area between the two modules. For example, the single mutations – N32T, V36Y, V76S and A80L (Table 1 and Fig. 3B) – all showed relatively low alterations in IC_{50}, despite the fact that they all appear to play a direct role in binding to the dockerin [11] and that some of the latter mutations introduced larger amino acids. This could be explained by the reported structural flexibility of the dockerin component and its purported ability to adopt a new

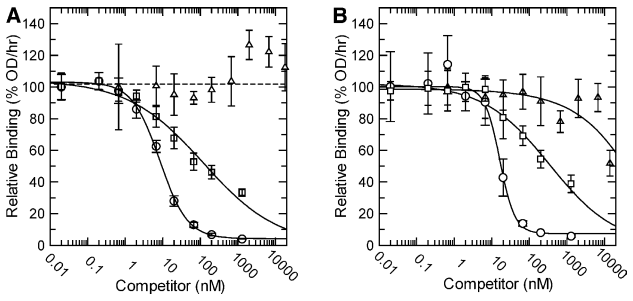

Fig. 2. Determination of relative binding affinity by competitive enzyme-linked interaction assay (cELIA). (A) Native cohesin (◯) and combined mutations: YPDRKI(69–74)SNGT, mut 28 (□); D65S + D34N + V76S + YPDRKI(69–74)SNGT, mut 33 (△). (B) Single mutations: A80L (◯), V76S (□), and D34N (△). Microtiter plates were coated with a solution of native cohesin (270 nM), and the immobilized sample was allowed to interact with a solution of enzyme-linked dockerin (94 nM) in the presence of competitor cohesin (native or mutated). The measured activity reflects the relative amount of dockerin bound to the coating cohesin.

conformation upon binding as revealed by the comparison of the free dockerin structure with that of the crystal complex [10,11]. The latter replacements were rather conservative, except, perhaps for V76S (hydrophobic to polar), which exhibited a relatively moderate 30-fold increase in IC_{50}. A similar increase was observed for mutant 20, which included six mutations, two of which were contact residues. In the latter mutant, the E81D mutation is admittedly conservative, as the functional group of the side-chain remains and can presumably serve the same role in forming a hydrogen bond with Arg53 of the dockerin. The deletion of G84, whose main-chain nitrogen forms a water-mediated H bond with the latter arginine, can also be compensated by flanking residues. Even replacement of the entire loop between β-strands 5 and 6 (mutant 28) failed to radically perturb the binding (10-fold change in IC_{50}), despite the fact that three crucial contact residues are inherent in this loop. Moreover, deletion of two of these residues (mutant 27) had only a modest effect on the affinity for the dockerin. Thus, according to the nature of the mutations made, the results may suggest that these positions and the substituted residues have a similar role in *C. thermocellum* and *C. cellulolyticum*. In general, these findings are in accord with those of Miras et al. [25], who tested the effect of several mutations in the cohesin domain and found only a small reduction in the association constants, which emphasizes the tenacious nature of the cohesin–dockerin interaction.

In contrast to the latter mutants, however, major increases in IC_{50} were observed for multiple mutations in mutants 30 and 33. The common residues in all of these mutations were D65S, D34N and V76S. In fact, the most striking mutation was the single substitution of the Asp34 to Asn, which resulted in more than a thousandfold reduction in the affinity (Fig. 2). Such an increase in the K_d corresponds to a $\Delta\Delta G$ of about 4 kcal/mol, and Asp34 may thus be considered a hot-spot residue for the interaction [26,27]. Indeed, Asp34 of the cohesin is located at the protein–protein interface of the heterodimer (Fig. 3B) and is in direct contact with the dockerin domain [11]. Asp34 forms direct hydrogen bonds with Ser45 of the dockerin, and water-mediated hydrogen bonds with Val21 and Ile43 (Fig. 3C and D). As seen in many other systems [28,29], water molecules may participate in stabilizing the complex by providing polar interactions between the two proteins as well as contributing to the close-packing that ensures complementarity between the two protein surfaces.

The approach employed in this work involved replacement of *C. thermocellum* cohesin residues with their counterparts from the *C. cellulolyticum* cohesin. Hence, considering the high homology and structural similarity between the two cohesins, it is unlikely that destabilization of the complex would result from unfolding of the mutated protein (which is often the case in alanine scanning, carried out in similar studies). Furthermore, shape complementarity is known to be one of the primary criteria for protein interfaces [26,30]; thus, a mutation that changes the shape of the protein surface may interfere with the interaction and might be misinterpreted. These concerns are diminished by using the *C. cellulolyticum* cohesin as a conceptual template for mutagenesis of the *C. thermocellum* cohesin, owing to the similar structures and conserved aromatic/hydrophobic core residues. Sequence alignment shows that in *C. thermocellum*, position 34 of all 9 scaffoldin-borne cohesins is exquisitely conserved as Asp, whereas the 8 cohesins of *C. cellulolyticum* all exhibit Asn in the same position. Since aspartate and aspargine share similar volume and shape, there is no obvious reason to suspect that the Asp-to-Asn mutation would cause any shape changes in the cohesin molecule.

Table 1
IC_{50} values of mutated cohesin-2 from *C. thermocellum*

Cohesin	Position and mutations[a]	IC_{50} (nM)[d]
Wild-type		1×10^1
mut 20	A89L + N122G + D123T + S63N + **E81D** + **ΔG84**[b]	2×10^2
mut 22	**D34N**	$>1 \times 10^4$[c]
mut 23	D65S	1×10^1
mut 27	**ΔY69** + **ΔI74**	7×10^1
mut 28	**YPDRKI**(69–74)SNGT	1×10^2
mut 30	D65S + **D34N** + **V76S**	$\gg 2 \times 10^3$[c]
mut 33	D65S + **D34N** + **V76S** + **YPDRKI**(69–74)SNGT	$\gg 2 \times 10^4$[c]
mut 35	**V76S**	3×10^2
mut 36	**V36Y**	4×10^1
mut 41	**N32T**	2×10^1
mut 43	**A80L**	1×10^1

[a] Numbering refers to the positions of the indicated residues in the cohesin crystal structure [7].
[b] Residues in bold denote contact residues, as reported by Carvalho et al. [11].
[c] Maximal concentration tested in this experiment.
[d] IC_{50} values were calculated using Grafit software [21].

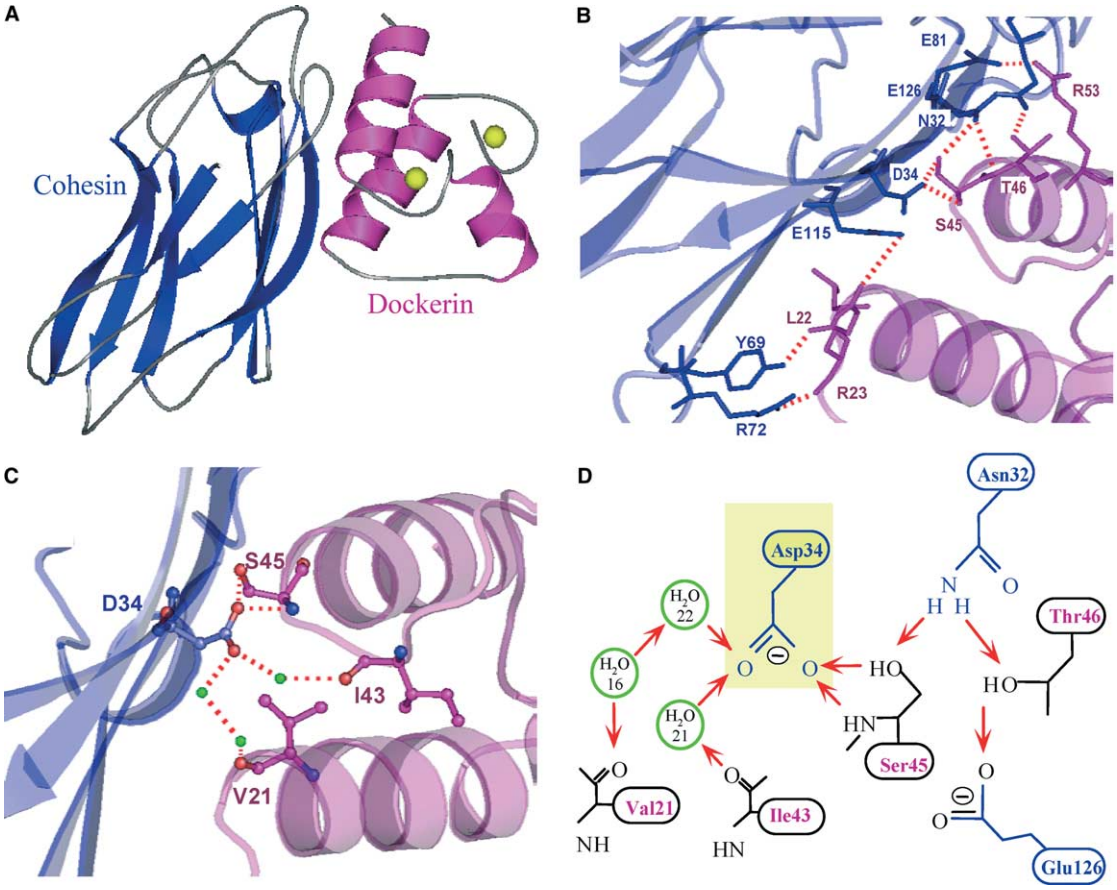

Fig. 3. Contribution of cohesin residue Asp34 in the binding of the dockerin domain. (A) Overview of the 3D structure of the cohesin–dockerin complex from *C. thermocellum* (PDB code 1OHZ). The cohesin module is rendered in blue and the dockerin in pink. Calcium ions are represented as yellow spheres. (B) Enlargement of the cohesin–dockerin contact area. Residues involved in direct contact between domains are displayed as sticks: N32, D34, Y69, R72, E81, E115 and E126 of the cohesin and L22, R23, R53, S45, and T46 of the dockerin. (C) Interaction of Asp34 with the dockerin residues. Water molecules are represented as green spheres. (D) Schematic representation of the proposed hydrogen-bonding network around Asp34. Cohesin residues are rendered in blue and the dockerin in black.

Taken together, it appears that the combination of negative charge and H-bond acceptor capacity of Asp34 plays a critical functional role in the cohesin–dockerin interaction in *C. thermocellum*, both by direct contact with the dockerin residues and via water-mediated interactions. Consequently, replacement of the carboxyl group with the amide of aspargine, which cannot fulfill the same role, results in a dramatic decrease in affinity and destabilization of the complex. The results underscore the fragile nature of the high-affinity intermolecular interactions that maintain the integrity of the cellulosome complex.

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