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Function of Conserved Tryptophans in the *Aspergillus niger* Glucoamylase 1 Starch Binding Domain†

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ABSTRACT: Nuclear magnetic resonance (NMR) and ultraviolet (UV) difference spectroscopy were used to assess the role of a number of tryptophan residues in the granular starch binding domain (SBD) of glucoamylase 1 from *Aspergillus niger*. Wild-type SBD and three variant (W563K, W590K, and W615K) proteins were produced using an *A. niger* expression system. Titration studies were conducted with β-cyclodextrin (βCD), a cyclic analogue of starch, as the ligand. The NMR studies show that the W563K and W590K variants only bind 1 equiv while the wild-type protein forms a 2:1 (ligand:protein) complex. It also clearly demonstrates the abolition of binding at site 1 and site 2 in W590K and W563K, respectively. UV difference spectroscopy was used to calculate dissociation constants with addition of βCD: 14.4 μM (apparent) for the wild type, 28.0 μM for W563K, and 6.4 μM for W590K. The implication of this is that the two binding sites have unequal contributions to the overall binding of the SBD which may be related to functional differences between the two binding sites. The low stability of the third variant, W615K, suggests that this tryptophan is not involved in binding but has an essential structural role.

Glucoamylase 1 (G1,[1] 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) hydrolyzes primarily α-1,4 glucosidic linkages (but also α-1,6 linkages) to release β-D-glucose from nonreducing ends of starch and related polysaccharides and oligosaccharides. *Aspergillus niger* G1 consists of a catalytic domain (residues 1−470) and a granular starch binding domain (SBD, residues 509−616) which are connected by a heavily glycosylated serine/threonine-rich linker.

The protein sequences of various starch-degrading enzymes have been compared, and it has been shown that tryptophans at positions (relative to G1 SBD) 543, 563, 590, and 615 of the binding domains are well conserved (Coutinho & Reilly, 1994a; Henrissat et al., 1994). Using this information, deletion analysis of parts of the SBD of *Aspergillus awamori* glucoamylase has been investigated by Chen et al. (1995). Their studies showed that the C-terminal 103 residues of G1 are important for fully functional starch binding. Large deletions of SBD residues result in alteration of the folding and stability properties of the protein and eliminate ligand binding.

Recently, the solution structure of the G1 SBD has been solved by nuclear magnetic resonance (NMR) spectroscopy, and two substrate binding sites were proposed (Sorimachi et al., 1996). Binding site 1 contains residues W543, K578, W590, E591, and N595, and binding site 2 contains T526, Y527, G528, E529, N530, D554, and W563. Two substrate binding sites have previously been proposed in biochemical studies (Belshaw & Williamson, 1993; Sigurskjold et al., 1994). W615 and W590 have also been suggested by chemical modification to be involved in the binding of granular starch (Svensson et al., 1986), although other tryptophan residues were not discussed in their work. Knowledge of the structure of the SBD and the ability to produce large quantities of mutated SBDs using the *A. niger* expression system enable us to examine the role of individual tryptophan residues in substrate binding. We have specifically mutated two tryptophans, W590 and W563, which belong to binding sites 1 and 2, respectively. A third conserved tryptophan (W615), which was proposed as a binding site residue (Svensson et al., 1986) located near the C terminus of the SBD, has also been mutated.

Much remains unanswered about the precise role of the SBD, although clearly its main function is to attach to granular starch and increase the local concentration of the substrate at the active site. Previously, a report suggested that the cellulose binding domain from *Cellulomonas fimi* endoglucanase A played a direct role in the disruption of cellulose fibers, causing roughening of the surface or exfoliation of the fiber structure (Din et al., 1991). However, there is no evidence that the starch binding domain plays such a role with regard to starch molecules.

MATERIALS AND METHODS

Site-Directed Mutagenesis of the SBD DNA. Mutations at tryptophan residues 563, 590, and 615 were introduced by PCR. These residues were replaced by lysine using

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[1] Abbreviations: βCD, β-cyclodextrin; G1, glucoamylase 1 (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3); NMR, nuclear magnetic resonance; SBD, granular starch binding domain of G1 (residues 509−616); UV, ultraviolet; W563K, variant of SBD where W563 is replaced by a lysine; W590K, variant of SBD where W590 is replaced by a lysine; W615K, variant of SBD where W615 is replaced by a lysine; PCR, polymerase chain reaction.

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mutagenic primers. The template DNA that was used corresponded to a cDNA clone of the *A. niger* glucoamylase gene. Sequences of the primers were as follows: 5′-CGAATATCTAGACTACCGCTTGGTGTCGGT-3′ for W615K, 5′-TCCGTGGAGAAGGAGAGTG-3′ and its reverse complement for W590K, and 5′-GACCCGCTCAAGTATGTCACTG-3′ and its reverse complement for W563K. The last two mutants were constructed using the overlap extension method described by Ho et al. (1989). The DNA fragments were purified, *Xba*I treated, and cloned into the pIGF expression vector (Archer et al., 1994) which has previously been used for production of recombinant wild-type SBD in *A. niger* (Le Gal-Coëffet et al., 1995). The orientation of the insert was checked by a restriction digest with *Pvu*II. Correctly oriented clones were sequenced on an automated ABI sequencer to verify that the desired mutation was present and that no unwanted mutations were introduced during the PCR. *A. niger* strain AB4.1 was cotransformed with pAB4.1 which contains the selectable *pyr*G gene (van Hartingsveldt et al., 1987). Two culture media were used: *Aspergillus* complete medium with starch and additional nitrogen buffered with phosphate (ACMS/N-P) (Archer et al., 1990) and soya milk medium (SMM) (MacKenzie et al., 1994). *A. niger* transformants were grown in 100 mL of medium in shake flasks at 25 °C.

Purification of the Variants. The *A. niger* mycelium was separated from the culture supernatant by filtering through two layers of Miracloth. The supernatant was then filtered through a 5 μm cellulose nitrate filter. The three variants were partly purified using an affinity column of β-cyclodextrin (βCD) coupled to activated Sepharose 6B. The equilibrating and elution buffers and the next steps of purification have been described previously (Le Gal-Coëffet et al., 1995). An alternative purification procedure was used for W563K. After the affinity column, the Taka-amylase digestion was omitted and the eluate was lyophilized. Proteins in the dried eluate were then dissolved in 50% sucrose/50% H_2O and separated by gel filtration on a Sephacryl S200 HR column (2.5 cm × 90 cm) in 50 mM borate buffer/400 mM NaCl at pH 8.2. The purified variant proteins were finally dialyzed against H_2O.

Estimation of the Protein Concentration. The amount of SBD protein was determined on the eluate after gel filtration purification by measuring the absorbance at 280 nm using a calculated value for ϵ of 3.07×10^4 M^{-1} cm^{-1}, based on the amino acid composition (Gill & von Hippel, 1989).

Protein Characterization. The proteins were first characterized by SDS−PAGE. Gels (12%) were run according to Laemmli (1970), and immunoblot procedures were performed as previously described (Archer et al., 1990).

Titrations by NMR Spectroscopy. Protein samples used for NMR titration experiments were prepared in 90% H_2O/10% D_2O. The initial protein concentrations varied between 0.58 and 1.03 mM. A 14.1 mM stock solution of βCD was prepared in 100% H_2O, and small aliquots were added directly to the protein sample in the NMR tube by using a 10 μL Hamilton syringe. The addition of ligand was initially in steps of 0.1−0.2 mol/(mol of protein), increasing to 0.3−0.5 mol/(mol of protein) steps until a ligand:protein molar ratio of 4.8, 2.5, or 2.1 was achieved for wild-type SBD, W590K, and W563K, respectively.

One-dimensional ^{1}H NMR spectra were acquired on a Bruker AMX 500 spectrometer at 300 or 310 K. The solvent signal was suppressed by presaturation. Typically, 256 or 512 scans and 8192 complex points were recorded over a spectral width of 12 500 Hz. For each protein sample, all titration spectra were recorded successively, immediately after each aliquot of ligand was added.

Titrations by UV Difference Spectroscopy. Titrations of the SBD and the tryptophan variants with βCD were performed by UV difference spectroscopy as described previously (Belshaw & Williamson, 1991), except that two cuvettes with a 10 mm path length were used.

RESULTS AND DISCUSSION

In this paper, we have followed the binding site numbering convention of Lawson et al. (1994) in domain E of cyclodextrin glycosyltransferase. This is opposite to the numbering adopted by Klein and Schulz (1991) and Coutinho and Reilly (1994b).

Production of the Variants. The three variants, W615K, W590K, and W563K, were all secreted by *A. niger*. The yields of each SBD following purification from 1 L of SMM culture were greatly reduced when compared with that of the wild-type SBD: less than 1 mg for W615K and approximately 5 mg for W590K compared to more than 200 mg for the wild-type SBD. We obtained a better yield for W563K, 30−50 mg. The W615K and W590K proteins and recombinant wild-type SBD were purified according to the same method. The W563K protein, however, was degraded during the Taka-amylase digestion step which was used to remove traces of βCD following the βCD affinity column. The W563K protein was unstable during this step irrespective of the temperature of the digestion. The purification procedure was therefore modified as described in Materials and Methods. In order to improve the amount of W615K, we also attempted to purify it according to the alternative method. Unfortunately, this did not improve the recovered amount of W615K. During the gel filtration step, elution of the proteins is followed by UV absorbance at 280 nm. The typical pattern of elution for the G1/wild-type SBD protein mixture presents two major peaks, the first one corresponding to G1 and the second one to SBD. In the case of the variants, we have noticed that the pattern is different as there is a third peak, quite large and misshapen, corresponding to small molecular weight molecules, which is probably due to degraded proteins. This probably explains why the amount of purified variant SBDs is low.

The three variants were characterized by gel electrophoresis and immunoblotting. On SDS−PAGE gels, both recombinant and proteolytically derived SBDs run anomalously, owing to glycosylation and a low isoelectric point; the same is also true for the tryptophan variants (data not shown). The proteins appear to have a higher molecular weight than expected, and the band is diffuse and difficult to stain with Coomassie. Antibodies raised against the wild-type SBD (Le Gal-Coëffet et al., 1995) also recognized the variant proteins. From the SDS−PAGE, immunoblotting, and NMR, we estimate the purity of the wild type, W590K, and W563K to be >95% and of W615K to be at least 90%.

Titration Studies by NMR Spectroscopy. We have previously shown that titration of βCD into wild-type SBD causes chemical shift changes to a number of ^{1}H resonances in or around the binding sites and that 2 equiv of the ligand is required for the formation of a complex (Le Gal-Coëffet et

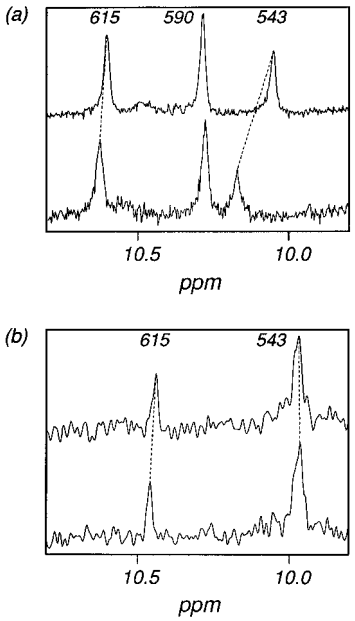

FIGURE 1: Expansion of one-dimensional ^{1}H NMR spectra for (a) wild-type SBD and (b) W590K. The downfield region is shown where $H^{\epsilon 1}$ peaks for three tryptophan residues (as numbered) are observed. For both proteins, the upper spectrum is that of the free protein while the lower spectrum shows the bound state.

al., 1995; Sorimachi et al., 1996). Here, we analyze binding curves for different resonances to understand the nature of ligand binding in the variant proteins compared to that in wild-type SBD.

Figure 1 shows a downfield expansion of one-dimensional ^{1}H NMR spectra of wild-type SBD and W590K. In the wild-type spectrum, the three labeled tryptophan $H^{\epsilon 1}$ resonances are observed, clearly resolved with good signal-to-noise. Of these three resonances, the largest chemical shift change observed upon formation of a wild-type SBD−βCD complex is due to residue W543 (0.121 ppm). The observed change for W615 is much smaller at 0.022 ppm. From the spectrum for W590K, it is readily apparent that the $H^{\epsilon 1}$ resonance for W590 is missing, thus confirming the result of the mutation. The peaks for W543 $H^{\epsilon 1}$ and W615 $H^{\epsilon 1}$ are present, and their chemical shifts, which are similar to that of wild-type SBD, suggest that the protein is correctly folded. The chemical shift difference observed for W615 $H^{\epsilon 1}$ between the free and bound states of W590K is 0.022 ppm (the same as for wild-type SBD) and therefore confirms that W615 is not in the binding site and any large structural change in this region associated with addition of ligand is unlikely. The titration results for the W543 $H^{\epsilon 1}$ peak, on the other hand, are quite different. This resonance shows no change in chemical shift at any ligand concentration. This is consistent with the W590K mutation resulting in the abolition of binding at site 1 since W543 and W590 are both key binding residues at this site. By contrast, signals associated with binding site 2 shift by similar amounts in wild-type SBD and W590K (results not shown). Comparable results were obtained from W563K, showing the absence of W563, and no chemical shift changes for residues associated with site 2 on addition of βCD.

In addition to the low yield obtained for W615K, its one dimensional ^{1}H NMR spectrum was very different compared to that of the other variants. It appears that the protein may be unable to adopt the correctly folded conformation,

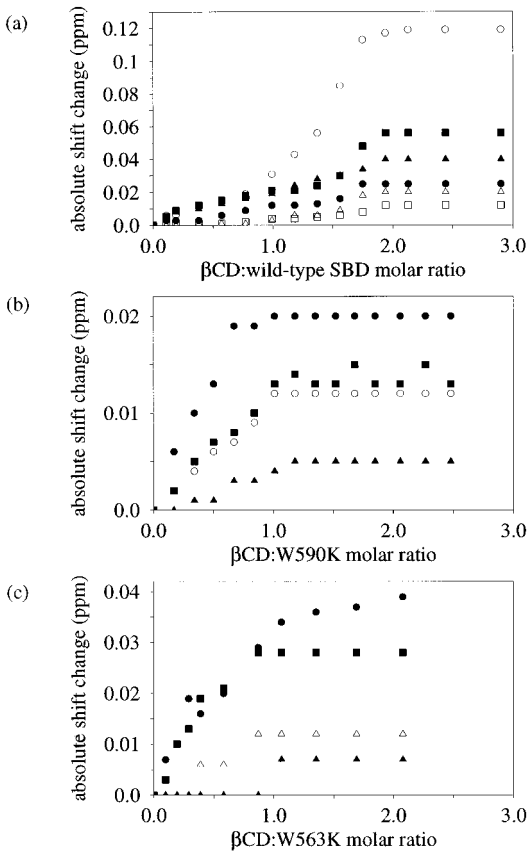

FIGURE 2: Absolute ^{1}H chemical shift changes observed for selected resolved resonances in the NMR titration experiments of βCD and (a) wild-type SBD [(○) W543 $H^{\epsilon 1}$, (■) E576 H^{α}, (▲) L521 H^{β}, (●) W615 $H^{\epsilon 1}$, (△) L521 H^{δ}, and (○) E591 H^{α}], (b) W590K [(●) W615 $H^{\epsilon 1}$, (■) L521 H^{δ}, (○) I531 H^{δ}, and (▲) E591 H^{α}], and (c) W563K [(●) W543 $H^{\epsilon 1}$, (■) W615 $H^{\epsilon 1}$, (△) I580 H^{δ}, and (▲) I537 $H^{\gamma 1}$]. The scales for the vertical axes are different.

suggesting that this tryptophan is required for structural stability of the SBD. Consequently, titration studies were not carried out for this variant.

The NMR titration binding curves obtained for each protein with the addition of βCD are shown in Figure 2. Overall, the observed changes in ^{1}H chemical shifts are larger for the wild-type protein. The most notable finding is that, unlike the 2:1 (ligand:protein) molar ratio required for wild-type SBD, both variants are fully bound after addition of 1 equiv of ligand; *i.e.* one binding site is abolished in each variant. The titration curves level off very close to 1 equiv of βCD, indicating a dissociation constant significantly below the concentrations used in the titration, *i.e.* a value of K_d of less than 200 μM. However, all the SBD resonances are in fast exchange on the chemical shift time scale (*i.e.* they move smoothly from free to bound positions with no obvious exchange broadening), indicating a K_d of greater than 0.1 μM. Thus, the NMR titrations imply a dissociation constant between 0.1 and 200 μM.

Determination of Dissociation Constants. Dissociation constants (K_d) for the wild-type recombinant SBD and the tryptophan variants binding to βCD were measured by UV difference spectroscopy. The absorbance change was quantified by measuring the height of the peaks at 290 and 281 nm relative to the trough at 285 nm [$(A_{281} - A_{285}) + (A_{290} - A_{285})$]. Figure 3 shows the offset maximum UV difference spectrum obtained for each of the variants and the wild type, normalized to equivalent protein concentrations. For W563K,

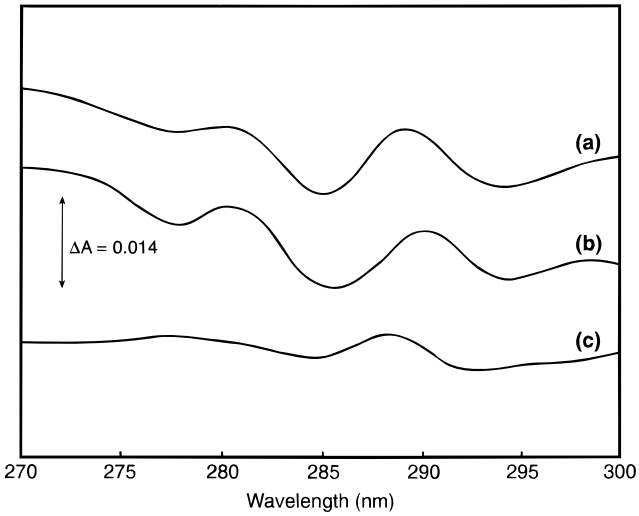

FIGURE 3: Offset maximum UV difference spectra of (a) wild-type recombinant SBD, (b) W590K, and (c) W563K in 5 mM sodium acetate (pH 4.5 and 25 °C) normalized to an equivalent protein concentration of 25.9 μM.

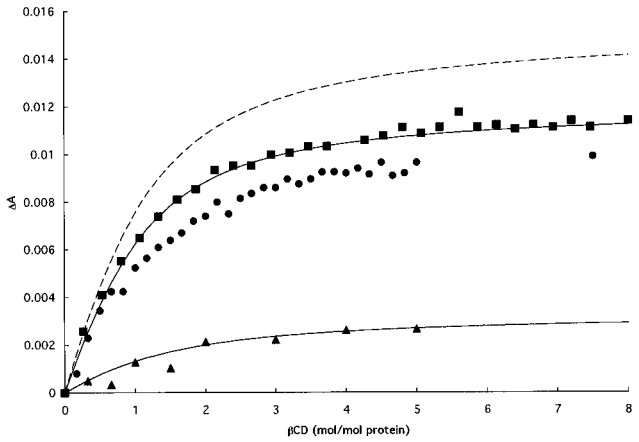

FIGURE 4: Binding curves for the interaction of wild-type recombinant SBD (●), W590K (■), and W563K (▲) with βCD in 5 mM sodium acetate (pH 4.5 and 25 °C). The solid lines represent the best-fit curves as calculated by the methods described in the text. The dotted line represents the curve of wild-type SBD simulated by summation of the curves for the two variants. All curves are normalized to an equivalent protein concentration of 25.9 μM.

there are comparatively smaller changes on addition of β-cyclodextrin compared to those for the wild-type SBD and W590K, with no peak at 281 nm and a shift of the other peak from 290 to 288.5 nm. Quantitatively, the W590K difference spectrum is very similar to that of the wild type.

The changes in absorbance were plotted against β-cyclodextrin concentration and the points fitted to a simulated curve using the appropriate equation for a protein interacting with a single ligand (Belshaw & Williamson, 1993):

$$\Delta A = \Delta A_{max}\,{}^{1}/_{2}\{[L]_0 + [P]_0 + K_d + [([L]_0 + [P]_0 + K_d)^2 - 4[P]_0[L]_0]^{1/2}\}/[P]_0$$

where $[P]_0$ and $[L]_0$ are the total concentration of protein and ligand, respectively.

Figure 4 shows the β-cyclodextrin binding curves for the variant proteins and for the wild-type SBD. Fewer data points could be collected for W563K as it was unstable and turbidity started to increase after 45 min at 25 °C. There was a significant difference in the ΔA_{max} for the two variants.

Table 1: Comparison of Measured and Calculated Parameters of the Interaction of β-Cyclodextrin with Wild-Type and Variant SBD Proteins

	recombinant SBD	W590K	W563K
ϵ_{280} (mM^{-1} cm^{-1})[a]	30.7	25.0	25.0
ΔA_{max}	0.01068	0.0067	0.0040
K_d (μM)	14.4	6.4	28.0
ΔA_{max}/[protein]	0.41	0.49	0.14
$\Delta A_{max}/A_{280}$	0.0130	0.0200	0.0055
ΔG (kJ mol^{-1})	−27.62	−29.63	−25.97
λ_{max} (nm)	289.75	289.40	288.40
λ_{min} (nm)	285.50	286.00	284.50

[a] Calculated from the amino acid composition.

There were more changes in the difference spectrum on binding of β-cyclodextrin to W590K than to W563K, showing that the binding at site 2 (*i.e.* the site that remains in W590K) contributes more to the overall UV absorbance changes of the wild-type SBD than binding at site 1. A curve for the wild-type SBD was simulated by summation of the two curves for the mutants, assuming $\Delta A = \Delta A_{site1} + \Delta A_{site2}$. The resulting curve has a much higher ΔA_{max} for the wild-type SBD than that measured by the experimental data. This difference may arise because there is some interaction between the binding sites and/or from the contribution of the nonmutated tryptophans W615 and W543 that are common to both variants. This difference is reflected by the ΔA_{max}/[protein] and $\Delta A_{max}/\Delta A_{280}$ ratios (see Table 1).

K_d and ΔA_{max} values were determined by fitting these ideal curves to the experimentally obtained data. ΔG was calculated using the equation $\Delta G = -RT \ln K_d$. Table 1 compares the K_d, ΔA_{max}, and ΔG values for variants and the wild-type SBD. The ϵ_{280} is also shown and was calculated for the variants on the basis of the known values for tryptophan (Belshaw & Williamson, 1990). A K_d value of 14.4 μM was obtained for the wild-type SBD and fitted the experimental data well. This value is slightly higher than that obtained by Le Gal-Coëffet et al. (1995) (9 ± 0.55 μM), but the ΔG value of −27.62 kJ mol^{-1} is consistent with that found by Sigurskjold et al. (1994) using calorimetry (−27.08 ± 0.15 kJ mol^{-1}). However, the K_d for the wild type is an apparent value, as it represents a weighted average over two binding sites. The K_d values obtained for the variants are 28 and 6.4 μM for W563K and W590K, respectively. The data therefore suggest that the K_d is 28 μM for binding site 1 and 6.4 μM for binding site 2. These data are consistent with the differences observed in the values for the ΔA_{max} and the UV difference spectra for the variants, W563K showing smaller changes and a lower ΔA_{max} than W590K, and indicate an asymmetrical contribution of the binding sites to the overall UV absorbance changes of the protein. We have simulated UV changes in the wild type but, because the sum of the ΔA_{max} values of the variants does not equal ΔA_{max} of the wild type, a wide variety of simulated data can be produced depending on how the absorbance changes at each site are weighted. This highlights the difficulties associated with the use of UV data in obtaining dissociation constants when more than one site is present.

This study clearly shows that binding site 2 of SBD has a markedly higher affinity for the ligand than site 1. From structural studies which are in progress, we are able to say that site 1 is a compact and exposed binding site that shows virtually no structural change on binding, which is consistent

with the small UV difference measured. Site 2 on the other hand spans a much larger area and undergoes conformational rearrangement upon ligand binding. This supports an earlier hypothesis (Dijkhuizen et al., 1995) which held that site 1 acts as the initial recognition site while site 2 is more involved in preparing the substrate for catalysis. Thus, it appears that the SBD is actually involved in assisting the catalytic process in addition to its function in attaching the enzyme to starch granules. Finally, the conserved residue W615, although not involved in ligand binding, plays an important role in maintaining the structure of the protein.

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