Identification of residues in the TPR domain of Ssn6 responsible for interaction with the Tup1 protein

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Abstract Ssn6, a yeast protein that comprises 10 tandem tetratricopeptide repeat (TPR) motifs, associates with Tup1 repressor protein and acts as a transcriptional corepressor. In this report we identify point mutations in the TPR1 of Ssn6 that disrupt Tup1 interaction. Furthermore, we construct a 3D model of the TPR domain of Ssn6, which is responsible for Tup1 binding, based on the known structure of protein phosphatase 5. According to this model all selected mutations reduce the ability of Ssn6 to interact with Tup1 by affecting the structural integrity of TPR1 and/or the correct spatial arrangement of TPR1 relative to TPR2 and TPR3.

Key words: Tetratricopeptide repeat motif; Transcription; Protein–protein interaction; Ssn6; Tup1

1. Introduction

Ssn6 and Tup1, two physically associated proteins, repress the transcription of a diverse set of genes in *Saccharomyces cerevisiae* cells [1]. The Ssn6–Tup1 complex, which does not bind DNA directly, is recruited to different promoters via interactions with pathway-specific DNA binding repressor proteins. These protein–protein interactions are predominantly mediated by Ssn6, while the transcriptional repression function is performed by a specific domain of Tup1 [2]. Ssn6 does not possess repression activity, in contrast it can activate transcription in the absence of Tup1 [3].

The Ssn6 protein contains 10 copies of a 34 amino acid sequence motif which is known as tetratricopeptide repeat (TPR). TPR motifs have been found in a wide variety of proteins of all organisms – including humans – and are known to mediate protein–protein interactions ([4,5] and references therein). TPR1 to TPR3 of Ssn6 are known to be responsible for Tup1 binding, whereas combinations of TPR4 to TPR10 mediate interactions with different repressor proteins specific for each gene family regulated by the Ssn6–Tup1 complex [2]. Recently, members of the Groucho/transducin-like enhancer of split (TLE) family – which is functionally related to Tup1 – have been shown to interact with Ssn6 and the mammalian TPR containing protein, UTY/X [6]. These findings suggest that repression mechanisms mediated via TPR–non-TPR protein complexes may be evolutionarily conserved.

Apart from transcription, TPR containing proteins play an important role in a wide variety of cellular functions ([5] and references therein) including viral infection [7–10] and genetic diseases such as Down [11] and Williams syndromes [12]. The majority of TPR containing proteins have been found as members of protein complexes mediated by their TPR domains. The involvement of the TPR motif in protein–protein interactions and the functional diversity and importance of TPR containing proteins suggest that TPR motifs may represent an ancient general protein–protein interaction module adopted by functionally different proteins and adapted for specific functions [5]. However, questions such as: are there any particular residues which determine the specificity of TPRs to different target proteins? are still open.

TPR motifs are characterized by a consensus sequence pattern of small and large hydrophobic residues. The protein sequence similarity and biochemical properties of TPR containing proteins suggest that TPR repeats may fold into a similar tertiary structure [13]. The crystal structure of the human protein phosphatase 5 (Pp5), the only TPR containing protein with known structure so far, has revealed that each TPR motif folds in a pair of antiparallel α-helices [14]. Helices of adjacent TPR repeats are arranged in such a way as to form a right-handed super-helical conformation. This conformation yields the formation of a channel which has been proposed to be responsible for the accommodation of the non-TPR counterparts in TPR-mediated protein complexes. The number of simultaneous interactions of TPR proteins with different target proteins depends, according to this model, on the number of TPR motifs and, hence, the size of the created groove [14]. However, the role of individual TPR motifs and specific residues therein remains to be elucidated.

In this report we identify, by random mutagenesis work, specific residues within the TPRs of Ssn6, which are responsible for its interaction with the Tup1 repressor. In order to analyze the role of each selected mutation we also produced a model of the three-dimensional structure of the TPR domain of Ssn6 which is involved in Tup1 binding and comprises TPR1 to TPR3. The crystal structure of Pp5 (PDB entry: 1A17) was used as a template for this purpose.

2. Materials and methods

2.1. Yeast strains and cultures

The yeast strain FT5, a derivative of FY105 [15], was used for all the in vivo experiments. SC containing the required amino acids and YPD standard synthetic media were used for yeast cultures. For in vivo selection of mutated LexA TPR clones that derepressed *HIS3* gene transcription minimal media were supplemented with 3-amino-

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triazole (3-AT), a competitive inhibitor of the His3 enzymatic activity, at a concentration of 10 mM.

2.2. Plasmids, random mutagenesis and two-hybrid assays

Plasmids expressing LexA-fused TPR1, TPR2 and TPR3 (LexA–TPR1–3) or the entire TPR domain (LexA–TPR), and reporter plasmids expressing *LacZ* (JK103) or *HIS3* (lop-HIS3) have been described previously [15]. LexA–TPR1–3 hybrids carrying point mutations in TPR1 were constructed by inserting the *Sma*I–*Bst*XI fragments (TPR1 to TPR3) from the respective LexA–TPR mutant clones into the *Sma*I–*Nco*I sites of the YCp91-LexA vector [15].

The DNA sequence encoding the TPR domain was mutagenized as previously described [16] and cloned into the *Sma*I and *Nco*I sites of the YCp91-LexA vector. A mutant collection with a complexity of ~10 000 clones and mutation frequency ~2% transformed into the FT5 yeast strain containing the *lop-HIS3* reporter gene in place of the wild-type *HIS3* locus. Yeast colonies grown on Min-3AT plates were purified and the LexA–TPR encoding plasmids were rescued and sequenced.

Two-hybrid interaction assays were performed as described previously [15] using plasmids expressing various LexA–TPR derivatives along with either Tup1–VP16 (a hybrid containing the Ssn6 interaction domain of Tup1, residues 1–72, fused to the potent activation domain of VP16) or the Tup1 control protein (residues 1–72).

2.3. Molecular modeling

The 3D model of the three N-terminal TPRs of Ssn6 (TPR1–3) is based on the 2.5 Å resolution crystal structure of the TPR domain of human protein Ser/Thr phosphatase Pp5 [14]. Pp5 is the only TPR containing protein with known structure, so far. The sequences of TPR1–3 of Ssn6 and of the TPR domain of Pp5 were aligned against each other with the BESTFIT program of the GCG package [17]. A graphical output of the alignment is shown in Fig. 1.

The above alignment and the coordinates of Pp5 (PDB entry: 1A17) as a template were used within the program MODELLER [18] in order to obtain an initial model of the Tup1 binding domain of Ssn6. The structure was subsequently energy-minimized using X-PLOR 3.1 [19] and the parameters of Engh and Huber [20] as restraints. Charges of all net charged groups were removed and a dielectric constant $\varepsilon = 20$ was used. The stereochemical quality of the final model was assessed using the PROCHECK suite of programs [21].

2.4. Electrostatic calculations

As one method to find possible protein–protein interaction sites of Ssn6 with Tup1 we performed an analysis of the electrostatic potential of the Ssn6 model. The isopotential surfaces were calculated using the program GRASP [22] with the following parameters: a probe parameter of 1.4 Å, an ion exclusion layer of 2 Å, a salt concentration of 0 M, standard van der Waals radii, a solvent dielectric of 80 and a solute dielectric of 2.

3. Results and discussion

3.1. Specific point mutations within TPR1 prevent interaction with Tup1

When bound to a promoter via the DNA binding domain of the bacterial LexA protein, a LexA–TPR hybrid associates with Tup1 and represses transcription. However, in the absence of Tup1, e.g. in a *tup1*Δ yeast strain, the Ssn6 TPR domain does not repress, but in fact activates transcription [3,15]. In order to identify specific TPR amino acid residues that are important for Tup1 interaction, we looked for mutants that were unable to repress transcription even in the presence of the Tup1 protein.

A LexA–TPR encoding plasmid was randomly mutagenized and was expressed in a wild-type (TUP1) yeast strain in which the *HIS3* gene is transcribed under the control of a LexA operator (see Section 2). In contrast to the wild-type LexA–TPR, which interacts with Tup1 and inhibits *HIS3* gene transcription, five independent LexA–TPR mutant clones permitted expression of the *HIS3* gene and supported cell growth in histidine-depleted media (Fig. 2A,B). Interestingly, sequencing analysis revealed that all five mutations map within the first N-terminal TPR motif of Ssn6, known to be essential for Tup1 interaction [2]. The residues that correspond to disruptive mutations are indicated with asterisks in Fig. 1.

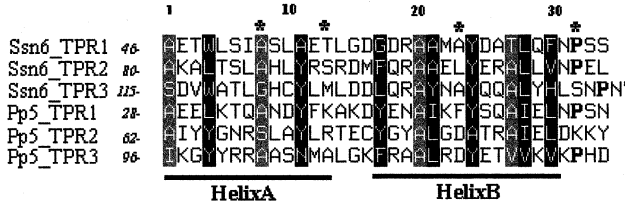

Fig. 1. Sequence alignment of the three N-terminal TPRs of Ssn6 with the TPR domain of Pp5. Consensus TPR motif residues are shown in black and gray background for large and small hydrophobic residues, respectively. The consensus proline is shown in bold. Residues that correspond to disruptive mutations are indicated with asterisks.

Of the five selected mutations only two (A53V and P77L) correspond to consensus TPR motif residues: mutation A53V changes the TPR consensus alanine 53 (TPR position 8) to a valine, while P77L mutation replaces the consensus proline 77 by a leucine. Mutations at TPR consensus position 8 in other TPR containing proteins also result in a disruption of their function [23–25]. Two mutations (A68T and A68V) change the non-consensus TPR motif alanine 68 to a threonine and

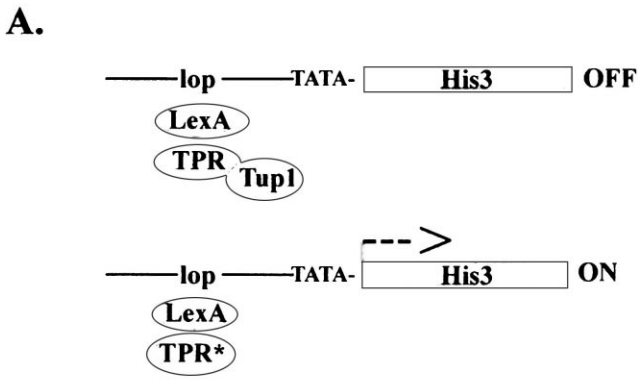

Fig. 2. A: Schematic representation of the selection scheme that was used (see text for details). TPR*: mutated TPR molecules impaired for Tup1 interaction. B: Growth of yeast strains expressing the *HIS3* gene under the control of various LexA–TPR hybrids. Wild-type (WT) LexA–TPR and its derivatives carrying the indicated point mutation were transformed into a TUP1 yeast strain that expresses the *HIS3* gene under the control of a LexA operator and incubated in media that either lack (−His) or contain (+His) histidine for 2 and 4 days, respectively. In contrast to wild-type LexA–TPR, mutant derivatives defective for Tup1 interaction and repression of *HIS3* transcription support cell growth in histidine-depleted media.

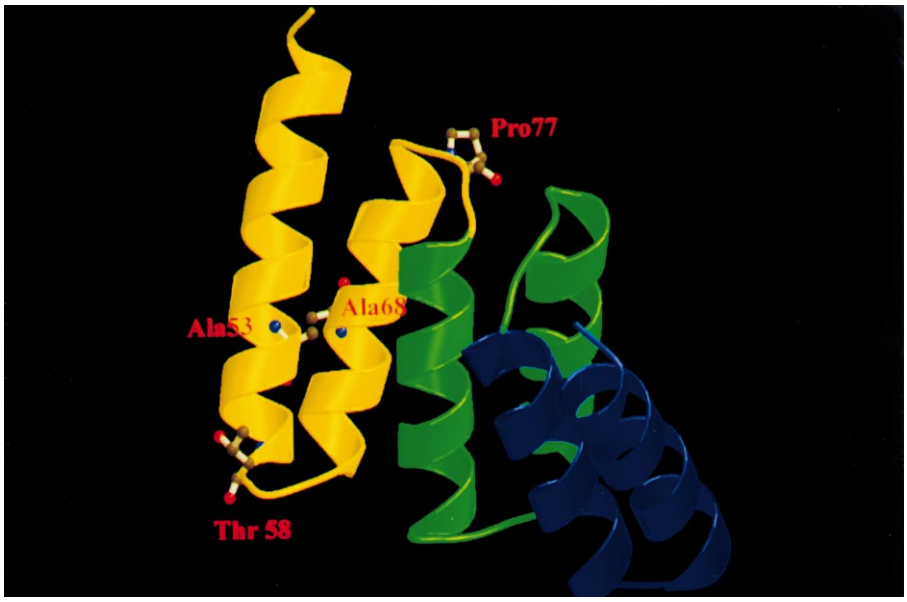

Fig. 3. Ribbon model of the three N-terminal TPRs of Ssn6 which are responsible for Tup1 binding. TPR1, TPR2 and TPR3 are colored in yellow, green and blue, respectively. Residues corresponding to disruptive mutations are labelled and depicted as balls-and-sticks. The figure was drawn using the MOLSCRIPT [32] and RASTER3D [33] programs.

a valine, respectively. Finally, the T58P mutation changes a non-conserved threonine to a proline residue.

We employed an in vivo two-hybrid assay in order to confirm that these mutations disrupt the interaction between TPRs and Tup1. As has been previously shown [15], LexA–TPR1–3, a molecule that contains the first three N-terminal TPRs of Ssn6, activated transcription 65-fold when expressed in vivo along with a Tup1–VP16 hybrid molecule (see Section 2), indicating that TPR1–3 interact with Tup1. However, LexA–TPR1–3 hybrids carrying either one of the above listed point mutations in TPR1 activated transcription at a much lower level when combined with the Tup1–VP16 hybrid (Table 1). P77L and A68V had the most severe effect, activating transcription only 1.5-and 0.8-fold, respectively. A68T and A53V activated transcription 8- and 5-fold, respectively, while T58P had the least severe effect activating transcription 18-fold. Thus, all five TPR1 mutations either weaken or completely prevent interaction with the Tup1 protein.

3.2. The 3D model

The 3D model of the TPR1–3 domain of Ssn6 is shown in Fig. 3. The Ramachandran plot for the final model is within the acceptable range for structures at 2.5 Å with no outliers: 91.2% of the residues are located in the most energetically

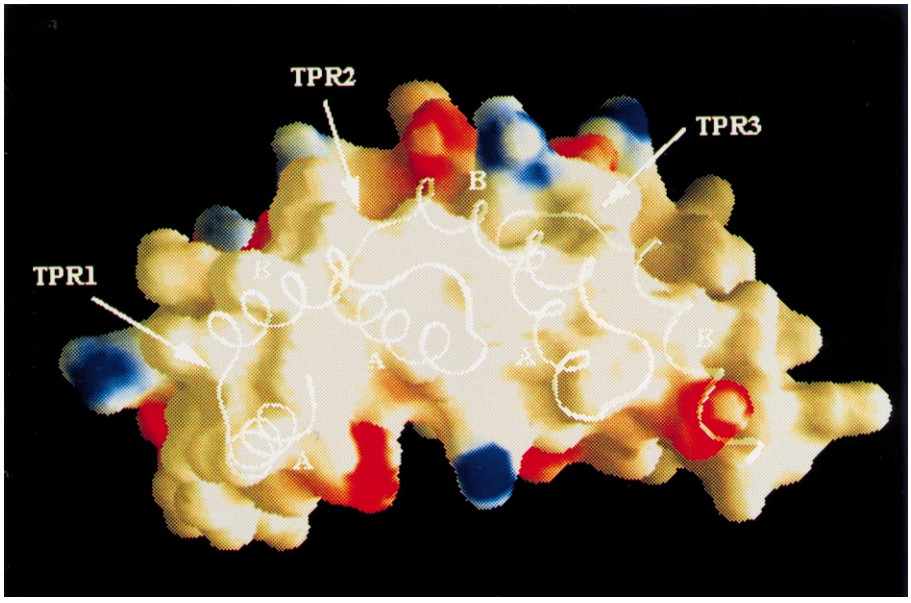

Fig. 4. Electrostatic isopotential surfaces calculated upon the 3D model of the TPR1–3 domain of Ssn6 showing that the created groove is not amphipathic and the highly hydrophobic character of helix A of TPR1. The figure was drawn using the GRASP program [22], color-coded on the surface from blue (12 kT/e) to red (−11 kT/e).

favored regions and the rest, 8.8%, in additionally allowed regions. All other PROCHECK stereochemical criteria are acceptable and correspond to a very high quality model.

As in Pp5, each TPR motif is composed of a pair of antiparallel α-helices (helix A and helix B) with a crossing angle of ~24°. The TPRs pack against each other in a similar arrangement to that of the helices within each TPR domain (Fig. 3). This regular repeat of α-helices yields to a right-handed super-helical conformation which, as in Pp5 [14], creates a channel. However, and in contrast to the groove created by the TPRs of Pp5, this channel in Ssn6 is not amphipathic (shown by the electrostatic isopotential surfaces calculated upon the 3D model (Fig. 4)). Furthermore, in all three TPRs the A helices, which form the surface of the channel, are more hydrophobic than the equivalent helices in the case of Pp5 [14] with helix A of TPR1 being the most hydrophobic one.

3.3. Disruptive mutations affect the structural integrity of the TPR1 to TPR3 domain of Ssn6

We used the 3D model of the TPR domain of Ssn6 responsible for Tup1 binding in order to elucidate the role of each particular residue in the formation of the Ssn6–Tup1 complex. Residues which correspond to disruptive mutations are labeled and depicted as balls-and-sticks on the 3D model shown in Fig. 3.

The correct packing of the helices within TPRs was suggested to be very important for the function of other TPR containing proteins [14]. In the 3D model, Ala-53 is located at the position of closest contact between helices A and B of TPR1 and corresponds to TPR consensus position 8. Mutation of this particular residue would result in an incorrect packing of the helices within TPR1.

Proline 77 is a consensus TPR motif residue and in the 3D model it is located at the interface between TPR1 and TPR2 (Fig. 3). It is known that proline causes a ~20° change in the direction of the polypeptide chain due to breakage of intrachain hydrogen bonds. Mutation of proline 77 most probably changes the relative positioning of TPR1 and TPR2 and disrupts the overall structure of this domain required for Tup1 binding.

Residue Ala-68 is located on helix B of TPR1 with its C^{β} atom in a position very close to the neighboring helix A. Substitution of Ala-68 by valine, a residue whose side chain is only allowed in the *trans* conformation on α-helices [26], would introduce steric hindrance effects. This, as in the case of the A53V mutation, would also result in a severe disruption of the correct packing of helices A and B within TPR1. On the other hand, replacement of Ala-68 by threonine (A68T mutation) may have less destabilizing effects than valine due to the ability of the hydroxyl group of threonine to form a shared hydrogen bond with the helical backbone, as was also proposed in other studies [27].

Finally, mutation T58P changes a non-consensus threonine to a proline. In the 3D model residue 58 is located at the C-terminus of helix A (Fig. 3) and more specifically two positions before its C-cap. Proline, in general, causes a helix break one or two residues before its actual occurrence induced by steric hindrance effects from its ring. This results in a disruption of hydrogen bonds in the last turn of helices. Among 1131 helices found in 205 non-homologous proteins determined at high resolution, this position was never occupied by a proline suggesting that hydrogen bonds in the last turn of a helix are essential for its stability [28]. In the case of Ssn6 shortening of helix A by one turn, induced by the introduction of a proline at position 58, could result in a reduction of the interface surface between helices A and B of TPR1. However, the rest of helix A is expected to remain intact explaining why the effect of this mutation is not so dramatic.

Interestingly all five mutations of Ssn6 that disrupt its association with Tup1 are located in TPR1 although it is known that TPR2 and TPR3 are also necessary and essential for Tup1 binding. It is also known that only one molecule of Ssn6 [29,30] is necessary for the formation of the Ssn6–Tup1 complex. These findings in conjunction with the hydrophobic character of the groove contradict the model for the association of TPR proteins with their non-TPR counterparts, proposed by Das et al. [14]. According to this model five to six TPR motifs and the inside surface of the created super-helix would be needed for this interaction. However, this does not seem to be the case for the Ssn6–Tup1 complex.

Our results can be summarized as follows:

1. The structural integrity of TPR1 of Ssn6 and its correct spatial arrangement relative to TPR2 and TPR3 are essential for Tup1 binding. These latter two TPRs probably provide the structural framework to present TPR1 effectively to Tup1.
2. No specific residue is responsible for direct binding to Tup1 via electrostatic interaction. The interaction of Ssn6 and Tup1 has a rather hydrophobic character. This observation is in agreement with mutagenesis work on Tup1, which showed that mutation of a hydrophobic residue (Leu-62) yielded a disruption of Ssn6 binding [31].
3. The interaction of Ssn6 with Tup1 does not seem likely to be performed via the groove created by the three tandemly arranged TPRs of Ssn6. The way Tup1 interacts in space with Ssn6 remains to be elucidated. Only the three-dimensional structure of the Ssn6–Tup1 complex will help in understanding the mechanism of this particular interaction.

Table 1
Two-hybrid assays for Ssn6–Tup1 interaction

LexA hybrid	β-Galactosidase activity		Fold activation
	Tup1	Tup1–VP16	
WT	2.0	130.0	65.0
A53V	1.6	8.0	5.0
T58P	1.2	22.0	18.0
A68V	1.2	1.0	0.8
A68T	2.2	18.0	8.0
P77L	1.0	1.5	1.5

β-Galactosidase activity from yeast cells expressing the indicated LexA–TPR hybrids along with either Tup1 or with the Tup1–VP16 hybrid protein. The *LacZ* reporter gene (JK103) is transcribed by a promoter that contains four LexA binding sites upstream of the *GAL1* TATA element. For each LexA–TPR hybrid, 'fold activation' represents the strength of interaction with Tup1 and is expressed as the ratio of β-galactosidase activities obtained from cells expressing Tup1–VP16 versus those expressing a control Tup1 protein.

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References

[1] Keheler, C.A., Reed, J.J., Schultz, J., Carlson, M. and Johnson, A.D. (1992) Cell 68, 709–719.
[2] Tzamarias, D. and Struhl, K. (1995) Genes Dev. 9, 821–831.
[3] Conlan, R.S., Gounalaki, N., Hatzis, P. and Tzamarias, D. (1999) J. Biol. Chem. 274, 205–210.
[4] Lamb, J.R., Tugendreich, S. and Heiter, P. (1995) Trends Biochem. Sci. 20, 257–259.
[5] Blatch, G.L. and Lassle, M. (1999) BioEssays 21, 932–939.
[6] Grbavec, D., Lo, R., Liu, Y., Greenfiels, A. and Stifani, S. (1999) Biochem. J. 337, 13–17.
[7] Callahan, M.A., Handley, M.A., Lee, Y.H., Talbolt, K.J., Harper, J.W. and Panganiban, A.T. (1998) J. Virol. 72, 5189–5197.
[8] Meyer, B.K., Pray-Grant, M.G., vanden Heuvel, J.P. and Perdew, G.H. (1998) Mol. Cell. Biol. 18, 978–988.
[9] Cziepluch, C., Kordes, E., Poirey, R., Grewenig, A., Rommelaere, J. and Jauniaux, J.C. (1998) J. Virol. 72, 4149–4156.
[10] Morimoto, R.I. and Katze, M.G. (1999) J. Biol. Chem. 274, 3797–3803.
[11] Tsukahara, F., Hattori, M., Muraki, T. and Sakaki, Y. (1996) J. Biochem. 120, 820–827.
[12] Meng, X., Lu, X., Morris, C.A. and Keating, M.T. (1998) Genomics 52, 130–137.
[13] Hirano, T., Kinoshita, N., Morikawa, K. and Yanagida, M. (1990) Cell 60, 319–328.
[14] Das, A.M., Cohen, P.T.W. and Barford, D. (1998) EMBO J. 17, 1192–1199.
[15] Tzamarias, D. and Strul, K. (1994) Nature 369, 758–761.
[16] Ling, M.M. and Robinson, B.H. (1997) Anal. Biochem. 254, 157–178.
[17] Wisconsin Package, SeqLab®, SeqWeb®, Wisconsin Package Version 10.0, Genetics Computer Group (GCG), Madison, WI.
[18] Sali, A. and Blundell, T.L. (1993) J. Mol. Biol. 234, 779–815.
[19] Brunger, A.T. (1992) X-PLOR: version 3.1, Yale University Press, New Haven, CT.
[20] Engh, R.A. and Huber, R. (1991) Acta Crystallogr. A47, 392–400.
[21] Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) J. Appl. Crystallogr. 26, 283–291.
[22] Nicholls, A., Sharp, K.A. and Honig, B.H. (1991) Proteins 11, 281–296.
[23] de Boer, M., Hilarius-Stokman, P.M., Hossle, J.-P., Verhoeven, A.J., Graf, N., Kenney, R.T., Seger, R. and Roos, D. (1994) Blood 83, 531–536.
[24] Sikorski, R.S., Michaud, W.A. and Hieter, P. (1993) Mol. Cell. Biol. 13, 1212–1221.
[25] Koga, H., Tarasawa, H., Nunoi, H., Takeshige, K., Inagaki, F. and Sumimoto, H. (1999) J. Biol. Chem. 274, 25051–25060.
[26] Janin, J., Wodak, S., Levitt, M. and Maigret, B. (1978) J. Mol. Biol. 125, 357–386.
[27] Horovitz, A., Matthews, J.M. and Fersht, A.R. (1992) J. Mol. Biol. 227, 560–568.
[28] Kumar, S. and Bansal, M. (1998) Proteins 31, 460–476.
[29] Redd, M., Arnaud, M. and Johnson, A.D. (1997) J. Biol. Chem. 272, 11193–11197.
[30] Varanasi, U., Klis, M., Mikesell, P.B. and Trumbley, R.J. (1996) Mol. Cell. Biol. 16, 6707–6714.
[31] Carrico, P.M. and Zitomer, R.S. (1998) Genetics 148, 637–644.
[32] Kraulis, P. (1991) J. Appl. Crystallogr. 24, 946–950.
[33] Merritt, E.A. and Murphy, M.E.P. (1994) Acta Crystallogr. D50, 869–873.