Kellermann, O., Brevet, A., Tonetti, H., & Waller, J. P. (1978) *Eur. J. Biochem. 88*, 205-210.
Kellermann, O., Brevet, A., Tonetti, H., & Waller, J. P. (1979) *Eur. J. Biochem. 99*, 541-550.
Kellermann, O., Tonetti, H., Brevet, A., Mirande, M., Pailliez, J. P., & Waller, J. P. (1982) *J. Biol. Chem. 257*, 11041-11048.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mayaux, J. F., & Blanquet, S. (1981) *Biochemistry 20*, 4647-4654.
Mayaux, J. F., Kalogerakos, T., Brito, K. K., & Blanquet, S. (1982) *Eur. J. Biochem. 128*, 41-46.
Mirande, M., Gache, Y., Le Corre, D., & Waller, J. P. (1982a) *EMBO J. 1*, 733-736.
Mirande, M., Cirakoglu, B., & Waller, J. P. (1982b) *J. Biol. Chem. 257*, 11056-11063.
Mirande, M., Kellermann, O., & Waller, J. P. (1982c) *J. Biol. Chem. 257*, 11049-11055.
Mirande, M., Cirakoglu, B., & Waller, J. P. (1983) *Eur. J. Biochem. 131*, 163-170.
Mirande, M., Le Corre, D., & Waller, J. P. (1985) *Eur. J. Biochem. 147*, 281-289.
Posorke, L. H., Cohn, M., Yanagisawa, N., & Auld, D. S. (1979) *Biochim. Biophys. Acta 576*, 128-133.
Saxholm, H. J. K., & Pitot, H. C. (1979) *Biochim. Biophys. Acta 562*, 386-399.
Sihag, R. K., & Deutscher, M. P. (1983) *J. Biol. Chem. 258*, 11846-11850.

Probing Histidine–Substrate Interactions in Tyrosyl-tRNA Synthetase Using Asparagine and Glutamine Replacements†

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ABSTRACT: We have analyzed the interactions of a histidine residue with a substrate using site-directed mutagenesis. Previous studies on tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* have shown that a histidine residue (His-48) makes an interaction with ATP, which is improved on mutating Thr-51 → Pro-51. We find on replacing His-48 in wild-type enzyme with either asparagine or glutamine that Asn-48 is equally as good as His-48 but His-48 → Gln-48 leads to a far lower activity. The side chain of an asparagine residue may be superimposed on that of a histidine so that the amide $-NH_2$ group of asparagine occupies the same position as the π-N of histidine, whereas the equivalent $-NH_2$ group of glutamine may be superimposed upon the τ-N. This suggests that it is the π-N of histidine that hydrogen bonds with ATP and that there is no significant electrostatic interaction between the histidine and ATP. Incorporating the Pro-51 mutation into each of the Asn-48 and Gln-48 mutants gives an improvement in the affinity of the enzyme for ATP, but this improvement is less than that seen with the wild-type enzyme.

Residues in the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* that are known from protein crystallographic studies to interact with the substrates are being systematically altered by site-directed mutagenesis of the gene (Winter et al., 1982; Fersht et al., 1984). The effects of these mutations on substrate binding and the enzyme kinetics allow a detailed analysis of the roles of such contacts in catalysis. This enzyme catalyzes the aminoacylation of $tRNA^{Tyr}$ in a two-step reaction (Fersht & Jakes, 1975) in which the tyrosine is activated to give enzyme-bound tyrosyl adenylate (eq 1) before being transferred to $tRNA^{Tyr}$ (eq 2).

$$E + Tyr + ATP = E\cdot Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\cdot Tyr\text{-}AMP + tRNA^{Tyr} = Tyr\text{-}tRNA^{Tyr} + AMP + E \quad (2)$$

It is known from the crystal structure of the enzyme-bound tyrosyl adenylate (Rubin & Blow 1981) that a histidine at position 48 is in close proximity to the ribose ring oxygen of ATP (Figure 1). Mutagenesis experiments are consistent with there being a hydrogen bond from the histidine (Carter et al., 1984). It was not possible, however, to identify which of the two nitrogens of the imidazole ring is likely to be making a hydrogen-bond contact with the ribose ring oxygen or whether the imidazole ring is positively charged and contributes an electrostatic interaction to the binding of the negatively charged phosphate groups of ATP. In an attempt to answer these questions, we have made mutations at position 48 to asparagine and glutamine. These amino acids have a nitrogen atom placed, respectively, three and four atoms distant from the α-carbon atom, in equivalent positions to the nitrogens in a histidine imidazole (see Figure 2). By studying the energetics of the mutated enzymes, we have deduced the most likely orientation of the histidine ring and have ruled out any large electrostatic contributions in its binding to ATP in the wild-type enzyme.

In a previous study it was shown that mutation of residue 51 from a threonine to a proline greatly improves the strength of the interaction of His-48 with ATP in the transition state

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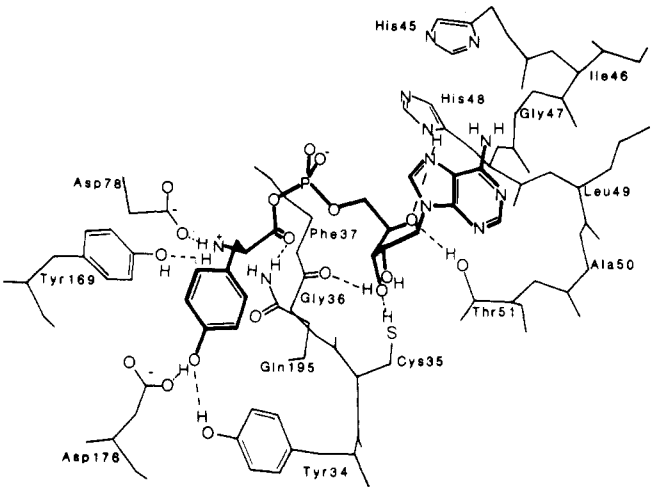

FIGURE 1: Perspective sketch of the active site of tyrosyl-tRNA synthetase with the bound tyrosyl adenylate [modified from Fersht et al. (1984)].

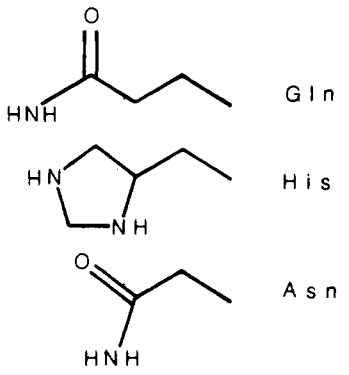

FIGURE 2: Comparison of side chains of histidine, asparagine, and glutamine, illustrating the approximate equivalence of the nitrogen atoms.

(Carter et al., 1984). We have incorporated this mutation into the Asn-48 and Gln-48 mutants to investigate whether the effect of Pro-51 upon His-48 is caused just by an improved hydrogen bond or, if the histidine is protonated, whether it now makes an improved electrostatic interaction with ATP.

Experimental Procedures

Mutant Constructions. Mutants were constructed from the TyrTS[1] gene cloned in the phage M13mp93 as described previously (Winter et al., 1982; Wilkinson et al., 1983, 1984; Carter et al., 1984). The following synthetic primers were used to direct the mutations: His-48 → Asn-48, GCCAAGTT*GCCGATAT; His-48 → Gln-48, GCCAAC*TGGCCGATAT; His-48 → Lys-48, GGCCAAC*TT*GCCGATAT (where an asterisk follows a mismatched base). The Asn-48, Pro-51 and Gln-48, Pro-51 double mutants were constructed stepwise by using the gene of TyrTS(Pro-51) as template and the above mutagenic primers (Wilkinson et al., 1984). The genes of all mutants were fully sequenced.

Purification of Tyrosyl-tRNA Synthetase. Preparations of enzymes were purified as described previously (Wilkinson et al., 1983), except that the final material was applied to a fast protein liquid chromatography (FPLC) Mono Q column (Pharmacia) equilibrated in 20 mM Tris-HCl, pH 7.8, and eluted with a gradient of 0–400 mM NaCl dissolved in the equilibration buffer. The enzyme eluted at about 200 mM NaCl and was judged to be homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Kinetic Procedures. The concentrations of all enzymes were determined by active site titration with nitrocellulose disks (Wilkinson et al., 1983). Activation of tyrosine was measured by pyrophosphate exchange (Calendar & Berg, 1966). tRNA charging and equilibrium dialysis experiments were performed as described previously (Jakes & Fersht, 1975).

[1] Abbreviations: TyrTS, tyrosyl-tRNA synthetase; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate. Mutants of tyrosyl-tRNA synthetase are denoted by the amino acid change: His-48 → Asn-48 = histidine residue 48 in wild-type enzyme changed to asparagine.

Table I: Effect of Mutation of His-48 and Thr-51 on Activation of Tyrosine by Tyrosyl-tRNA Synthetase[a]

enzyme	k_{cat} (s^{-1})	K_M for ATP (mM)	K_M for Tyr (μM)	k_{cat}/K_M for ATP (s^{-1} M^{-1})	k_{cat}/K_M for Tyr (s^{-1} M^{-1})
His-48, Thr-51 (wild type)	8.4	1.1	2.2	7640	3.82×10^6
His-48, Pro-51[b]	12.4	0.058	1.7	213790	7.29×10^6
Asn-48, Thr-51	7.9	1.4	3.8	5640	2.08×10^6
Asn-48, Pro-51	11.2	0.4	1.0	27320	11.2×10^6
Gln-48, Thr-51	0.34	2.0	15.5	170	0.02×10^6
Gln-48, Pro-51	1.6	1.5	5.0	1070	0.32×10^6
Gly-48, Thr-51[b]	2.0	1.3	3.2	1540	0.63×10^6
Gly-48, Pro-51[b]	2.8	2.1	2.9	1330	0.97×10^6

[a] All kinetic experiments were performed at 25 °C in 144 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 2 mM PP_i. Kinetic constants for variation of ATP were determined in the presence of 0.05 mM tyrosine and for variation of tyrosine in the presence of 2 mM ATP. The standard deviations for these results were typically about 5%. Values of k_{cat} are extrapolated to infinite concentration of tyrosine and ATP, assuming Michaelis–Menten kinetics. [b] From Carter et al. (1984).

Table II: Effect of Mutation of His-48 and Thr-51 on ATP Dependence of $tRNA^{Tyr}$ Charging[a]

enzyme	k_{cat} (s^{-1})	K_M for ATP (mM)	k_{cat}/K_M for ATP (s^{-1} M^{-1})
His-48, Thr-51	4.5	2.0	2250
His-48, Pro-51[b]	1.8	0.019	95800
Asn-48, Thr-51	4.9	2.1	2330
Asn-48, Pro-51	5.4	0.4	13500
Gln-48, Thr-51	0.3	25.7	12
Gln-48, Pro-51	1.8	6.7	270
Gly-48, Thr-51[b]	2.4	8.7	280
Gly-48, Pro-51[b]	3.4	6.7	510

[a] Assay conditions as in Table I. [Tyrosine] was 0.1 mM. [b] From Carter et al. (1984).

Table III: Effect of Mutation of His-48 on Binding of Tyrosine to Tyrosyl-tRNA Synthetase[a]

enzyme	dissociation constant (μM)	enzyme	dissociation constant (μM)
His-48[b]	11.6	Gly-48	23[c]
Asn-48	22[c]	Lys-48	~80

[a] Equilibrium dialysis was performed as described previously (Jakes & Fersht, 1975) in 144 mM Tris-HCl, pH 7.8, and 10 mM $MgCl_2$ at 25 °C. [b] From Fersht et al. (1975). [c] T. N. C. Wells and A. R. Fersht, unpublished kinetic data.

Results

Effect of Mutation of His-48 on Catalytic Activity. It is seen in Tables I and II that mutation of His-48 to Asn-48 results in very little change in the kinetic parameters for ATP in the pyrophosphate exchange or the $tRNA^{Tyr}$ charging reactions. This compares with a 15–25-fold decrease in the value of k_{cat} and a raising of the K_M when position 48 is mutated

to a glutamine residue. Smaller reductions in k_{cat} and increases in K_M were reported for mutation to Gly-48 (Carter et al., 1984).

All three of these mutations at position 48 were found to raise the K_M for tyrosine in the pyrophosphate exchange reaction (Table I). An increase of 7-fold was found for TyrTS(Gln-48). The mutations to Asn-48 and Gly-48 caused a 1.5–1.7-fold increase in the K_M for tyrosine. The dissociation constant for tyrosine was raised from 11.6 μM, found for the native enzyme (Fersht et al., 1975), to about 23 μM for the Gly-48 mutant and 22 μM for the Asn-48 mutant (Table III). K_M for tyrosine (and ATP) in the pyrophosphate exchange reaction is a complex quantity composed of the true dissociation constant and an attenuation factor comprised of the rate constants for the formation of Tyr-AMP and its pyrophosphorolysis. Changes in K_M can result from changes in these rate constants.

Inserting a lysine residue at position 48 abolished all catalytic activity. However, the resulting enzyme still binds tyrosine with a dissociation constant of about 80 μM (Table III).

Effect of Double Mutations of His-48 and Thr-51 on Catalytic Activity. Converting Thr-51 to Pro-51 in the wild-type TyrTS causes a massive increase in the value of k_{cat}/K_M, mainly due to a decrease in K_M for ATP (Tables I and II). This has been shown to be mediated via His-48, which, in the His-48, Pro-51 mutant, has improved its interaction with the transition state by 2.0 kcal/mol (Carter et al., 1984). Converting Thr-51 to Pro-51 in the Asn-48 and Gln-48 mutants in both cases causes an increase in the value of k_{cat}/K_M for ATP and tyrosine, involving both a decrease in the K_M's and an increase in k_{cat} (Tables I and II). By comparison, the mutant Gly-48, Pro-51 has approximately the same activity as the TyrTS(Gly-48, Thr-51) mutant (Table I). Thus, the presence of a hydrogen-bond donor at position 48 seems to be essential for the structural change introduced by the Pro-51 mutation to bring about an improvement in the transition-state stabilization.

Discussion

Mutation of His-48 to a glycine residue was found to lower enzyme activity, and it was calculated that the imidazole side chain contributes 1.2 kcal/mol of binding energy with the substrate in the transition state for activation (Carter et al., 1984). It was not clear, however, which of the two nitrogens of the imidazole ring is making a hydrogen bond with the ribose ring oxygen or whether the positive charge on the imidazole ring makes any contribution toward substrate binding. By substituting an asparagine at position 48, we have shown that the resulting enzyme possesses very similar enzymic activity to the wild-type TyrTS. This was not the case when glutamine was substituted at position 48, which resulted in a large loss in enzyme activity and a large effect on the K_M for tyrosine. This is not caused simply by the steric bulk of the glutamine side chain since it is smaller than that of histidine. Possibly, the greater flexibility of a glutamine side chain enables it to form a hydrogen bond elsewhere in the protein. Lysine is larger and more flexible than glutamine, and the substitution His-48 → Lys-48 abolishes all measurable activity and weakens the binding of tyrosine. Irrespective of the detailed reasoning of why there is low activity on His-48 → Gln-48, the ability of asparagine to substitute for His-48 and the failure of glutamine to do so is strong evidence that the π-N of His-48 is the one that forms the hydrogen bond with the substrate. Further, the similarity of kinetics on His-48 → Asn-48 suggests that there is little electrostatic interaction between His-48 and ATP.

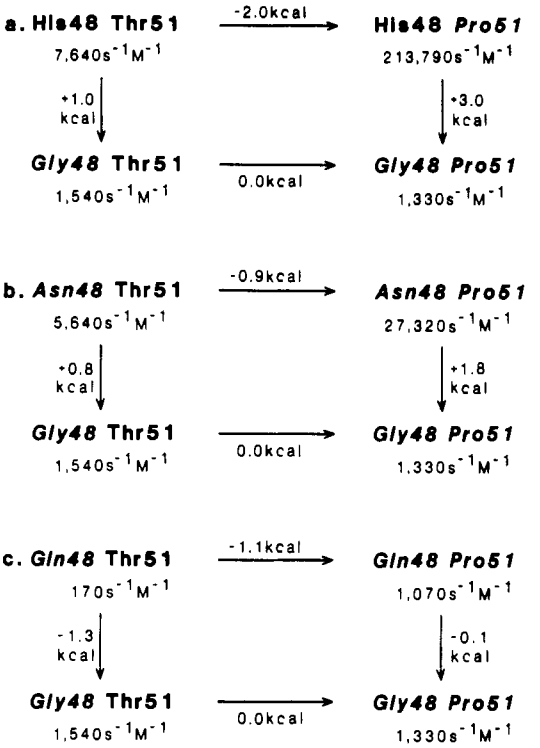

FIGURE 3: Energies of interaction of side chains with the transition state for activation of tyrosine. Calculations were performed as described by Wilkinson et al. (1983) and Carter et al. (1984) from the ratios of k_{cat}/K_M (Table I) with

$$\Delta G = -RT \ln [(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wild\,type}]$$

An asparagine residue has been found at position 48 in the TyrTS from another thermophile, *Bacillus caldotenax* (M. D. Jones, D. M. Lowe, T. Borgford, and A. R. Fersht, unpublished work), and it is also present in yeast methionyl-tRNA synthetase, whereas histidine is conserved at this position in other types of tRNA synthetases (Barker & Winter, 1982). Asn-48 is thus a natural residue that can make an equivalent interaction with the ATP substrate as His-48.

Mutation of His-48 to either of the uncharged residues glycine or asparagine lowers the affinity of the enzyme for its other substrate, tyrosine. In these mutants, the K_M for tyrosine is raised by about 1.6-fold (Table I), and the dissociation constant is raised by about 2-fold (Table III). If His-48 is positively charged, this is consistent with an electrostatic interaction with the negatively charged carboxyl of the tyrosine, which strengthens its binding. However, because the substitution of His-48 → Gln-48 considerably raises the K_M for tyrosine, the data may simply result from small structural changes in the ATP binding site that are transmitted to the tyrosine site.

Changing Thr-51 to Pro-51 in the wild-type TyrTS improves the interaction of His-48 with the transition state by 2.0 kcal/mol (Figure 3), whereas the double mutant TyrTS-(Gly-48, Pro-51) has very similar kinetics to the single mutant TyrTS(Gly-48) (Carter et al., 1984). Changing Thr-51 to Pro-51 in the Asn-48 mutant improves the interaction with ATP by 0.9 kcal/mol and in the Gln-48 mutant by 1.1 kcal/mol (Figure 3). Thus, the presence of a hydrogen-bond donor at position 48 seems to be necessary for the Thr-51 → Pro-51 mutation to result in an improved interaction with ATP, but the extent of this improvement is greater by about 1 kcal/mol when position 48 is a histidine. Again, if His-48 is charged, then part of the effect of mutating Thr-51 → Pro-51 in the wild-type TyrTS could result from an improved charge

interaction between His-48 and ATP, whereas in the Asn and Gln mutants the increase is due only to an improved hydrogen bond. Alternatively, the increased affinity of ATP with TyrTS(Pro-51) could result simply from improved van der Waals' contacts. The total energy of interaction of His-48 with the transition state of the reaction in TyrTS(Pro-51), some 3 kcal/mol, is far larger than we have found in any of our experiments in which a simple hydrogen bond has been removed from the enzyme–substrate complex. These are generally 0.5–1.5 kcal/mol (Fersht et al., 1984, 1985). The high value implies that there are additional interactions superimposed on the energy of the hydrogen bond, either electrostatic or van der Waals'.

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Registry No. His, 71-00-1; Asp, 70-47-3; Gln, 56-85-9; TyrTS, 9023-45-4; Tyr, 60-18-4; ATP, 56-65-5.

REFERENCES

Barker, D. G., & Winter, G. (1982) *FEBS Lett. 145*, 191–193.
Calendar, R., & Berg, P. (1966) *Biochemistry 5*, 1681–1690.
Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell (Cambridge, Mass.) 38*, 835–840.
Fersht, A. R., & Jakes, R. (1975) *Biochemistry 14*, 3350–3356.
Fersht, A. R., Mulvey, R. S., & Koch, G. L. E. (1975) *Biochemistry 14*, 13–18.
Fersht, A. R., Shi, J.-P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., & Winter, G. P. (1984) *Angew. Chem. 23*, 467–473.
Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238.
Jakes, R., & Fersht, A. R. (1975) *Biochemistry 14*, 3344–3350.
Rubin, J., & Blow, D. M. (1981) *J. Mol. Biol. 145*, 489–500.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581–3586.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, P., & Winter, G. (1984) *Nature (London) 307*, 187–188.
Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756–758.

Thermodynamics of the Binding of *Streptomyces* Subtilisin Inhibitor to α-Chymotrypsin†

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ABSTRACT: The binding of *Streptomyces* subtilisin inhibitor (SSI) to α-chymotrypsin (CT) (EC 3.4.21.1) was studied by isothermal and differential scanning calorimetry at pH 7.0. Thermodynamic quantities for the binding of SSI to the enzyme were derived as functions of temperature from binding constants (S. Matsumori, B. Tonomura, and K. Hiromi, private communication) and isothermal calorimetric experiments at 5–30 °C. At 25 °C, the values are $\Delta G°_b = -29.9$ kJ mol^{-1}, $\Delta H_b = +18.7$ (±1.3) kJ mol^{-1}, $\Delta S°_b = +0.16$ kJ K^{-1} mol^{-1}, and $\Delta C_{p,b} = -1.08$ (±0.11) kJ mol^{-1}. The binding of SSI to CT is weak compared with its binding to subtilisin [Uehara, Y., Tonomura, B., & Hiromi, K. (1978) *J. Biochem. (Tokyo) 84*, 1195–1202; Takahashi, K., & Fukada, H. (1985) *Biochemistry 24*, 297–300]. This difference is due primarily to a less favorable enthalpy change in the formation of the complex with CT. The hydrophobic effect is presumably the major source of the entropy and heat capacity changes which accompany the binding process. The unfolding temperature of the complex is about 7 °C higher than that of the free enzyme. The enthalpy and the heat capacity changes for the unfolding of CT were found to be 814 kJ mol^{-1} and 17.3 kJ K^{-1} mol^{-1} at 49 °C. The same quantities for the unfolding of the SSI–CT complex are 1183 kJ mol^{-1} and 39.2 kJ K^{-1} mol^{-1} at 57 °C.

Streptomyces subtilisin inhibitor (SSI) (Murao et al., 1972; Sato & Murao, 1973) having a molecular weight of 23 000 (dimer) specifically binds to alkaline proteases to inhibit their proteolytic action. It inhibits subtilisin BPN′ (SBPN′) most strongly to form a complex, E_2I_2, with a dissociation constant as small as 10^{-11} mol dm^{-3} for each of two identical, independent sites (Uehara et al., 1978). The formation of the subtilisin complex with SSI has been studied by various methods such as X-ray crystallography (Mitsui et al., 1979; Hirono et al., 1984), reaction kinetics (Inoue et al., 1977; Uehara et al., 1980), and NMR spectroscopy (Kainosho et al., 1985).

In previous papers, we reported studies of the binding of SSI to SBPN′ at pH 7.0 by the methods of isothermal and differential scanning calorimetry (DSC) and reported the thermodynamic properties for the interaction between the two proteins (Takahashi & Sturtevant, 1981; Takahashi & Fukada, 1985). It was found that the inhibitor binds to SBPN′ so tightly that it does not dissociate from the enzyme even after the complex unfolds at about 87 °C and that the strong binding

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