Bioorganic & Medicinal Chemistry Letters 18 (2008) 5503–5506

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Using proton nuclear magnetic resonance to study the mode of ribonuclease A inhibition by competitive and noncompetitive inhibitors

Kalyan Sundar Ghosh, Joy Debnath, Tanmaya Pathak, Swagata Dasgupta *

Department of Chemistry, Indian Institute of Technology, Kharagpur, West Bengal 721302, India

ARTICLE INFO

Article history:
Received 17 June 2008
Revised 2 September 2008
Accepted 4 September 2008
Available online 7 September 2008

Keywords:
Ribonuclease A
^{1}H NMR
Histidine pK_a
Competitive and noncompetitive inhibition

ABSTRACT

The C(2) proton resonances of the active site histidines (His 12 and His 119) of ribonuclease A have been exploited to study the inhibition pattern of both noncompetitive (four green tea polyphenols and their copper complexes) and competitive (3′-*O*-carboxy esters of thymidine and 3′-amino derivatives of uridine) inhibitors. Competitive inhibitors devoid of any phosphate group have the ability to change the pK_a of the histidine residues at the active site. Their mode of inhibition, albeit competitive, is found to be different compared to known phosphate inhibitors 2′-CMP and 3′-CMP as revealed by changes in the pK_a values. We find a correlation between the changes in the chemical shift of His 12 and the corresponding inhibition constants (K_i).

The proton nuclear magnetic resonance (NMR) spectra of proteins contain numerous overlapping resonances, which are not always appropriately resolved. However, the C(2) proton resonances of histidine (His) residues can clearly be resolved for a few proteins.[1] In case of bovine pancreatic ribonuclease A (RNase A), the C(2)–H resonances of all four His residues are observed separately.[1–3] Of these, His 12 and His 119 comprise the P_1 subsite of the active site of the enzyme and are mainly involved in the phosphodiester hydrolysis mechanism.[4,5] In the most widely accepted chemical mechanism,[6–8] His 12 acts as a base to deprotonate the 2′-oxygen atom of the substrate to form the cyclic 2′, 3′-phosphate intermediate. His 119 behaves as an acid to protonate the oxygen atom of the leaving group. This mechanism was confirmed by site directed mutagenesis studies.[8–10]

Changes in proton resonances of the two active site His residues due to changes in their protonation or deprotonation environment can be correlated to the mode of RNase A inhibition. When a competitive inhibitor binds to the enzyme, the chemical shifts (δ) of C(2)–H of the active site His residues are expected to change. However, for an inhibitor not going to the active site, little or no change in the δ value of C(2)–H is expected. This can easily be used for classification of the type of inhibitor of RNase A. In this report we have described the effect of binding of both competitive and noncompetitive types of inhibitors on the chemical shifts of the histidine residues of RNase A, particularly on the two histidines situated at the active site. To the best of our knowledge this is the first time a report on the effect of any noncompetitive inhibitor on RNase A histidines has been reported by NMR. The changes in pK_a of His 12 and His 119 on binding of the competitive inhibitors were calculated from NMR titrations. Among competitive inhibitors both phosphate and nonphosphate based inhibitors were employed. From the pK_a change, different modes of inhibition by phosphate and nonphosphate inhibitors were concluded. Finally a correlation between the change in δ of C(2)–H of His 12 on complexation with competitive inhibitors and their reported inhibition constants (K_i) was performed. This correlation suggests that a measure in the change in δ could be utilized to predict the K_i of an inhibitor. This study could also be extended to other proteins like angiogenin,[11] eosinophil-derived neurotoxin (EDN),[12] etc., belonging to the ribonuclease superfamily because the arrangement of ribonucleolytic residues (two histidines and one lysine) is conserved in all of them.[13] A rapid screening of inhibitors of such proteins would allow the further development of potent inhibitors of these proteins.

Exchangeable hydrogen atoms of RNase A were replaced with deuterium by dissolving the protein in D_2O with incubation for 20 min at 50 °C and then lyophilized following the procedure of Quirk and Raines.[14] This procedure was performed thrice. The lyophilized protein was dissolved in D_2O containing 0.2 M NaCl. The pH of the samples was measured with a microelectrode assembled with a pH meter. Small amounts of DCl and NaOD were used to adjust the pH* for the range 3–8, where pH* is the direct measure of the pH which was not corrected for a deuterium isotope effect. The [ligand] to [enzyme] molar ratio was maintained at 5:1 for all the compounds. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer at 22.5 °C. The acquisition time was 2 s

* Corresponding author. Tel.: +91 3222 283306; fax: +91 3222 255303.
E-mail addresses: swagata@chem.iitkgp.ernet.in (S. Dasgupta), swagata_dasgupta@hotmail.com (S. Dasgupta).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.09.014

with 100 scans. The δ value was recorded with respect to 3-trimethylsilylpropane sulfonic acid (DSS). The values of δ_{obs} for histidine C(2) protons at different pH were fitted using least-squares iterative regression fit[15,16] by applying the following formulae, which are normally used for the calculation of the pK_a of various ribonucleases[17–19]:

(a) For simple proton association–dissociation equilibria

$$\delta_{obs} = \delta_0 + \frac{\Delta \times 10^{(pK-pH)}}{1 + 10^{(pK-pH)}} \quad (1)$$

where δ_{obs} is the observed chemical shift, δ_0 is the chemical shift of the unprotonated form and Δ is the chemical shift change upon protonation.

(b) In cases where an acid inflection is present the equation is

$$\delta_{obs} = \delta_0 + \frac{\Delta_1 \times 10^{(pK_1-pH)}}{1 + 10^{(pK_1-pH)}} + \frac{\Delta_2 \times 10^{(pK_2-pH)}}{1 + 10^{(pK_2-pH)}} \quad (2)$$

where Δ_1 and Δ_2 are the chemical shift changes for the acidic pH inflection (pK_1) and the basic pH inflection (pK_2), respectively.

The individual histidine resonances and their pK_a values for native RNase A and those of RNase A complexed with 2′-CMP, 3′-CMP, 3′-UMP and several other phosphate esters have been assigned and determined previously.[2,3,14,20,21] The pK_a of His 105 was calculated considering Eq. 1 and a better fit of the calculated curve to the experimental points was obtained for His 12 and His 119 considering the existence of an acid inflection as given in Eq. 2. The other histidine residue, His 48, is inaccessible to solvent and its titration curve shows anomalous behavior with pH, preventing determination of its pK_a value as was reported earlier.[22] By using Eq. 2 pK_1 and pK_2 values have been obtained. The microscopic environment surrounding the imidazole rings of histidine residues can be analyzed in detail by considering the microscopic pK values of the two tautomeric structures of the imidazole ring. This is because the pK values of the histidines are to a large extent dependent on the neighboring charged groups. The large error in estimated pK_1 values is generally encountered in all cases due to the uncertainty in the experimental points which is close to the width of the acid inflection.[17] Hence in our discussion of pK_a only pK_2 has been considered. The observed pK_a values for His 12, His 105 and His 119 are 5.88, 6.59 and 6.22, respectively, for free RNase A. The values correlate well with reported values.[17]

In this study, we have selected inhibitors that have reported inhibitory activity on RNase A for elucidation of their effects on the local environment of active site histidine residues. A previous study from our group identified the four green tea polyphenols namely (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) (compounds **1**–**4** in Fig. 1) as noncompetitive inhibitors of RNase A.[23,24] Noncompetitive inhibition of RNase A by the copper complexes of ECG and EGCG (compounds **9** and **10**) have also been reported.[25] In panel A of Figure 2, little or no change in the chemical shift of C(2) protons of the active site histidines was observed on binding of compounds **1**–**4**, **9** and **10**, which further confirmed that these compounds were indeed noncompetitive inhibitors.

In the competitive series (panel B) we have considered two well known inhibitors 2′-CMP and 3′-CMP along with the synthesized compounds. Replacement of the phosphate group by the amino group at the 3′-position in compounds **5** and **6** leads to competitive inhibition of RNase A.[26] The proximity of **5** to the active site of RNase A has been established by structural studies of the complex of RNase A with the compound.[27] 3′-*O*-Carboxy esters of thymidine (compounds **7** and **8**) also exhibited competitive inhibition of RNase A.[28] The observed competitive inhibition indicates that these compounds bind to the P_1 subsite despite the absence of a phosphate group, in turn inhibiting the enzymatic activity. In panel B of Figure 2, substantial changes in the chemical shift of C(2)–H ascertain that these compounds are in close proximity of His 12 and His 119. For all of these compounds, we observe that the change in δ is more in case of His 12 than His 119 due to the major interactions of the compounds with His 12 as evidenced from the crystal structure or from docked conformations (discussed later). The His 105 signal remains almost unaltered for 3′-CMP and compounds **6**, **7** and **8**. However, for 2′-CMP and compound **5**, the His 105 peak also shifts with His 12 and 119. This indicates some additional effect of these compounds on the environment of His 105 along with His 12 and His 119.

To further understand the effect of the inhibitors on the local environment of the active site histidines for probing the inhibition mechanism, changes in pK_a of the histidines were monitored. When one representative from the noncompetitive inhibitors, compound **4** (the most effective of the green tea polyphenols) binds with the enzyme, the pK_a values for His 12, His 105 and His 119 are 5.82, 6.59 and 6.19, respectively (Fig. 3B). These are almost identical to those for uncomplexed RNase A (Fig. 3A), which

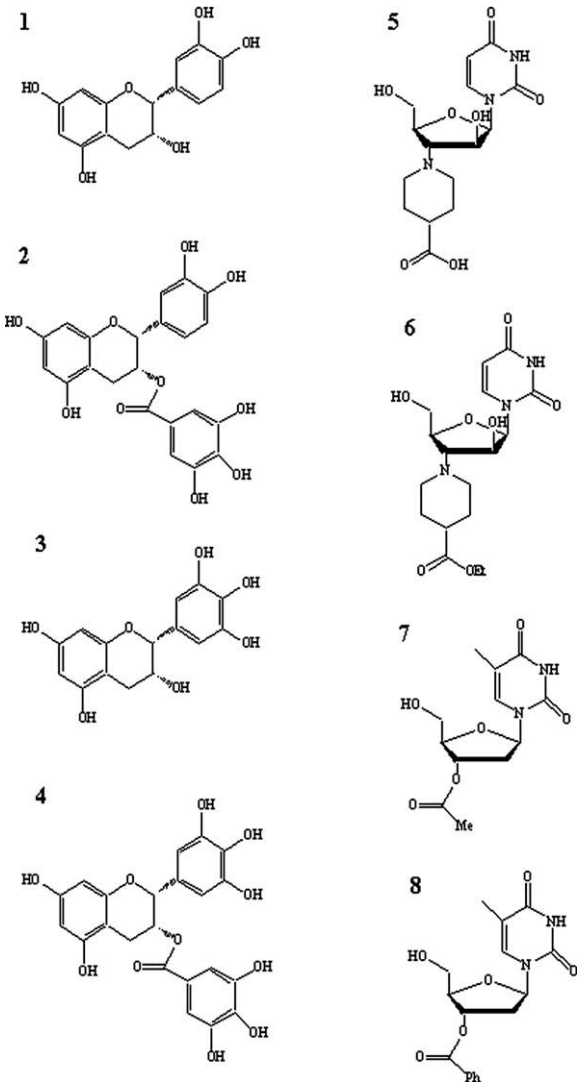

Figure 1. Structures of (−)-epicatechin (EC) (**1**), (−)-epicatechin gallate (ECG) (**2**), (−)-epigallocatechin (EGC) (**3**), (−)-epigallocatechin gallate (EGCG) (**4**), 3′-[1-(4-carboxylpiperdinyl)]-3′-deoxy-*ara*-uridine (**5**), 3′-[1-(4-ethoxycarbonylpiperdinyl)]-3′-deoxy-*ara*-uridine (**6**), 3′-*O*-acetyl thymidine (**7**) and 3′-*O*-benzoyl thymidine (**8**).

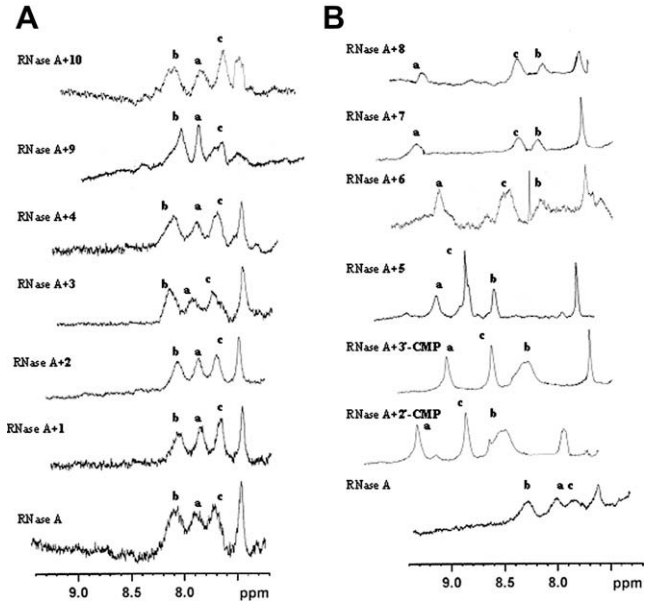

Figure 2. NMR spectra of RNase A and its complexes with noncompetitive inhibitors (A) and competitive inhibitors (B). The signal for C(2) protons of His 12, His 105 and His 119 are indicated by a, b and c, respectively.

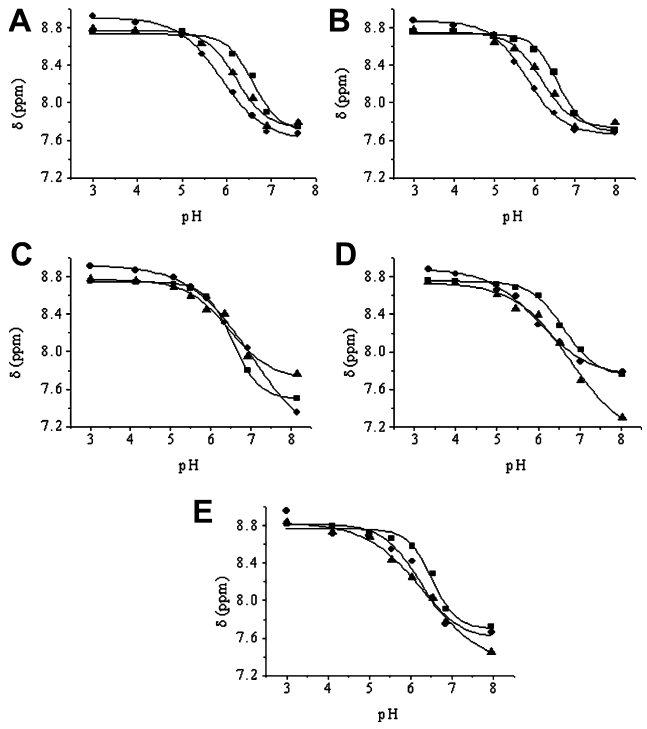

Figure 3. pH dependence of the histidine C(2) proton signals of RNase A (A) and its complexes with compound **4** (B), compound **5** (C), compound **7** (D), and compound **8** (E). Chemical shifts (δ) of histidine residues: His 12 (●), His 105 (■), His 119 (▲).

confirms that there is no effect on the active site histidine residues suggestive of a noncompetitive mode of inhibition. The unchanged chemical shifts and pK_a values of His 12 and His 119 on complexation with polyphenols corroborate our previous studies on the noncompetitive mode of inhibition of the polyphenols.[24] In the docking results reported, we find that a nonregular region of RNase A encompassing residues 34–39 is primarily involved. Interaction with Arg 39 may alter the orientation of Lys 41, which is very close to Arg 39 and thereby causing noncompetitive inhibition. In addition interactions with Lys 1, Glu 2, Lys 7 and Arg 10 were also found in some cases, which are not the part of P_1 subsite but affect the ribonucleolytic mechanism. Thus, apart from enzyme kinetic studies that reveal a noncompetitive mode of inhibition, structural insights from docking studies also corroborate the unchanged pK_a values of the two active site histidines for noncompetitive inhibition. In addition the unchanged accessible surface area of His 12 and His 119 on binding with four polyphenols explains the unaltered pK_a of these two active site histidines.[24]

We choose compound **5** from the competitive inhibitors because of its available crystal structure with RNase A.[27] For the RNase A–compound **5** complex, pK_a values of His 12, His 105 and His 119 residues become 7.01, 6.55 and 6.47, respectively (Fig. 3C). Structural studies indicated that the carboxyl group of the 4-carboxypiperidine moiety of the inhibitor molecule is in close proximity with His 12, whereas the uracyl group is close to His 119.[27] At physiological pH, the interaction of the carboxylate anion causes an increase in pK_a for His 12 by ~20%, as is found in the case of phosphate esters.[2,3]

On the other hand when **7** and **8** are complexed with RNase A the pK_a values of His 12, His 105 and His 119 are shifted to 6.01, 6.62 and 6.77 for compound **7** and 6.23, 6.53 and 6.39 for compound **8**, respectively (Fig. 3D and E, respectively). For His 105, the pK_a values remain practically unchanged (<1%) which is similar to that reported for the pK_a for His 105 in case of 2′-CMP and 3′-CMP. The increase in the pK_a value (~9%) of His 119 indicates that the negative charge center on the carbonyl oxygen of the ester group has a definite influence on the environment of the imidazole moiety. A similar increase in pK_a is also observed when the negatively charged phosphate group is in close proximity to the His residues in complexes with 2′-CMP[2] and 3′-CMP[3]. However, the changes observed for phosphate esters are greater (~28%). This may be attributed to the presence of the dianion that is likely to perturb the environment to a higher degree than compounds **7** or **8**, where only a negatively polarized charge center is resident on the carbonyl oxygen. This is also evident from the fact that the pK_a of His remains unaltered in the cytidine–RNase A complex, where such an interaction with the phosphate dianion is absent.[2] In case of compound **8**, a ~6% change is observed for His 12 illustrating its preferable interaction with His 12. Due to the presence of the phenyl ring in compound **8**, a steric interaction with Phe 120 of RNase A probably prevents it to go near His 119 which shows a change of ~3%. In addition, for compound **8**, due to cross-resonance the population of the resonating structure in which the carbonyl oxygen bears the negative charge is lower compared to compound **7**, where no such cross-conjugation is present. The predicted preferred docking poses are consistent with this observation as reported earlier.[28]

Finally a correlation between the changes in the chemical shift ($\Delta\delta$) of C(2)–H of His 12 of RNase A on complexation with some phosphate based inhibitors [2′-CMP[2], 3′-CMP[3], 3′-UMP[14],

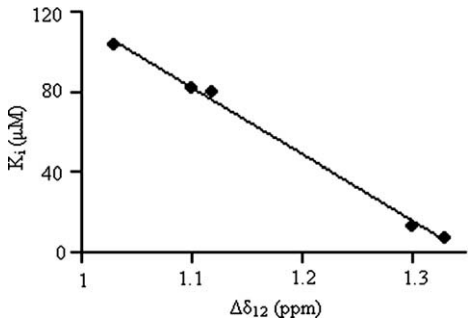

Figure 4. Correlation of $\Delta\delta$ of His 12 and K_i for competitive inhibitors.

Table 1
Predicted and reported inhibition constants of compounds **5**–**8**

Compound	Reported K_i (μM)	Observed $\Delta\delta$ of His 12	Predicted K_i (μM)
5	103[26]	1.08	89
6	120[26]	1.03	106
7	42[28]	1.24	36
8	95[28]	1.11	79

5′-AMP[17] and 3′-TMP (obtained from this study)] and their reported K_i values[5,29,30] was obtained which gave an R^2 value of 0.95 (Fig. 4). The inhibition constants of the synthesized competitive inhibitors have been predicted from this correlation, a comparison with the reported experimental values determined is presented in Table 1. Despite the lack of the phosphate group, the predicted K_i values are within 12% for the 3′-amino derivatives of uridine and within 24% for the 3′-*O*-carboxy esters of thymidine. The trends are similar to the reported values obtained from enzyme inhibition kinetics.

It is apparent that with a preliminary indication of the inhibitory nature of a compound towards RNase A, determination of the chemical shift ($\Delta\delta$) of C(2)–H of His 12 may act as a tool to decipher the mode of inhibition. This may be well utilized in the search for inhibitors of ribonuclease type proteins such as angiogenin where the ribonucleolytic activity of the protein is essential for its angiogenic activity.[31] This can also be extended to estimate details of the interaction for other competitive inhibitors in complex with RNase A where crystal structures are not available.

Acknowledgments

S.D.G. is grateful to the Department of Science and Technology (DST), Government of India for financial support (Grant No. SR/S5/OC-13/2002). The authors are thankful to Mr. Pranab K. Bhattacharya and Mrs. Mukulika Sarkar for recording NMR. K.S.G. and J.D. thank CSIR, New Delhi for providing fellowships.

References and notes

1. Meadows, D. H.; Markley, J. L.; Cohen, J. S.; Jardetzky, O. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *58*, 1307.
2. Meadows, D. H.; Roberts, G. C. K.; Jardetzky, O. *J. Mol. Biol.* **1969**, *45*, 491.
3. Meadows, D. H.; Jardetzky, O. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 406.
4. Findlay, D.; Herries, D. G.; Mathias, A. P.; Rabin, B. R.; Ross, C. A. *Nature* **1961**, *190*, 781.
5. Iwahashi, K.; Nakamura, K.; Mitsui, Y.; Ohgi, K.; Irie, M. *J. Biochem.* **1981**, *90*, 1685.
6. Nogués, M. V.; Vilanova, M.; Cuchillo, C. M. *Biochim. Biophys. Acta* **1995**, *1253*, 16.
7. Raines, R. T. *Chem. Rev.* **1998**, *98*, 1045.
8. Thompson, J. E.; Raines, R. T. *J. Am. Chem. Soc.* **1994**, *116*, 5467.
9. Trautwein, K. P.; Holliger, P.; Stackhouse, J.; Benner, S. A. *FEBS Lett.* **1991**, *281*, 275.
10. Messmore, J. M.; Fuchs, D. N.; Raines, R. T. *J. Am. Chem. Soc.* **1995**, *117*, 8057.
11. Fett, J. W.; Strydom, D. J.; Lobb, R. R.; Alderman, E. M.; Bethune, J. L.; Riordan, J. F.; Vallee, B. L. *Biochemistry* **1985**, *24*, 5480.
12. Sorrentino, S.; Glitz, D. G.; Hamann, K. J.; Loegering, D. A.; Checkel, J. L.; Gleich, G. J. *J. Biol. Chem.* **1992**, *267*, 14859.
13. Russo, N.; Acharya, K. R.; Vallee, B. L.; Shapiro, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 804.
14. Quirk, D. J.; Raines, R. T. *Biophys. J.* **1999**, *76*, 1571.
15. Shrager, R. I.; Cohen, J. S.; Heller, S. R.; Sachs, D. H.; Schechter, A. N. *Biochemistry* **1972**, *11*, 541.
16. Westmoreland, D. G.; Matthews, C. R.; Hayes, M. B.; Cohen, J. S. *J. Biol. Chem.* **1975**, *250*, 7456.
17. Arús, C.; Paolillo, L.; Llorens, R.; Napolitano, R.; Parés, X.; Cuchillo, C. M. *Biochim. Biophys. Acta* **1981**, *660*, 117.
18. Laurents, D. V.; Huyghues-Despointes, B. M. P.; Bruix, M.; Thurlkill1, R. L.; Schell, D.; Newsom, S.; Grimsley, G. R.; Shaw, K. L.; Treviño, S.; Rico, M.; Briggs, J. M.; Antosiewicz, J. M.; Scholtz, J. M.; Pace, C. N. *J. Mol. Biol.* **2003**, *325*, 1077.
19. Lou, Y.; Huang, Y.; Pan, Y.; Chen, C.; Liao, Y. *J. Mol. Biol.* **2006**, *355*, 409.
20. Meadows, D. H.; Jardetzky, O.; Epand, R. M.; Ruterjans, H. H.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *60*, 766.
21. Lenstra, J. A.; Bolscher, B. J. M.; Stob, S.; Beintema, J. J.; Kaptein, R. *Eur. J. Biochem.* **1979**, *98*, 385.
22. Markley, J. L. *Biochemistry* **1975**, *14*, 3554.
23. Ghosh, K. S.; Maiti, T. K.; Dasgupta, S. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 807.
24. Ghosh, K. S.; Maiti, T. K.; Debnath, J.; Dasgupta, S. *Proteins* **2007**, *69*, 566.
25. Ghosh, K. S.; Maiti, T. K.; Mandal, A.; Dasgupta, S. *FEBS Lett.* **2006**, *580*, 4703.
26. Maiti, T. K.; De, S.; Dasgupta, S.; Pathak, T. *Bioorg. Med. Chem.* **2006**, *14*, 1221.
27. Leonidas, D. D.; Maiti, T. K.; Samanta, A.; Dasgupta, S.; Pathak, T.; Zographos, S. E.; Oikonomakos, N. G. *Bioorg. Med. Chem.* **2006**, *14*, 6055.
28. Ghosh, K. S.; Debnath, J.; Dutta, P.; Sahoo, B. K.; Dasgupta, S. *Bioorg. Med. Chem.* **2008**, *16*, 2819.
29. Anderson, D. G.; Hammes, G. C.; Walz, F. G. *Biochemistry* **1968**, *7*, 1637.
30. Russo, N.; Shapiro, R.; Vallee, B. L. *Biochem. Biophys. Res. Commun.* **1997**, *231*, 671.
31. Shapiro, R.; Riordan, J. F.; Vallee, B. L. *Biochemistry* **1986**, *25*, 3527.