# Interaction of bepridil with the cardiac troponin C/troponin I complex

Ekram Abusamhadneh, M. Bret Abbott, Alex Dvoretsky, Natosha Finley, Soumya Sasi, Paul R. Rosevear*

*Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, College of Medicine, 231 Albert B. Sabin Way, Cincinnati, OH 45267, USA*

Received 5 June 2001; revised 31 July 2001; accepted 1 August 2001

First published online 14 September 2001

Edited by Thomas L. James

**Abstract We have investigated the binding of bepridil to calcium-saturated cardiac troponin C in a cardiac troponin C/troponin I complex. Nuclear magnetic resonance spectroscopy and [$^{15}$N,$^{2}$H]cardiac troponin C permitted the mapping of bepridil-induced amide proton chemical shifts. A single bepridil-binding site in the regulatory domain was found with an affinity constant of $\sim$140 $\mu M^{-1}$. In the presence of cardiac troponin I, bepridil binding to the C domain of cardiac troponin C was not detected. The pattern of bepridil-induced chemical shifts is consistent with stabilization of more open regulatory domain conformational states. A similar pattern of chemical shift perturbations was observed for interaction of the troponin I cardiac-specific amino-terminus with the cardiac troponin C regulatory domain. These results suggest that both bepridil and the cardiac-specific amino-terminus may mediate an increase in calcium affinity by interacting with and stabilizing open regulatory domain conformations. Chemical shift mapping suggests a possible role for inactive calcium-binding site I in the modulation of calcium affinity.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

*Key words:* Cardiac troponin C; Cardiac troponin I; Bepridil; Nuclear magnetic resonance; Drug binding

## 1. Introduction

Cardiac muscle contraction is regulated by calcium-dependent interactions between members of the troponin (Tn) complex and other thin filament proteins including actin and tropomyosin. This complex consists of TnC, I, and T. TnC is the $Ca^{2+}$-binding subunit of the troponin complex and a potential drug target in the therapy of congestive heart failure. TnT makes primary contact with tropomyosin while troponin I participates in the major protein–protein interaction with recombinant cTnC (desMet1-Ala2, Cys35Ser) (cTnC).

Cardiac TnC and TnI interact in head to tail fashion such that the C domain of cTnC interacts with the N domain of cTnI [1]. The C domain of cTnC contains two high affinity $Ca^{2+}/Mg^{2+}$-binding sites whereas the N domain, or regulatory domain, contains a single active low affinity $Ca^{2+}$-specific binding site, site II. Site I in the cardiac isoform is naturally inactive [2]. This region has been suggested to modulate conformational states within the regulatory domain. Structures for the N domain of cTnC bound to cTnI(147–163) [3] and the C domain of cTnC bound to cTnI(33–80) [4] have been determined. Structural information has not been obtained on the flexible linker connecting globular domains. Cardiac TnI contains a 32 amino acid N-terminal extension that can be phosphorylated at Ser residues 23 and 24 by cAMP-dependent protein kinase A [5]. In the absence of phosphorylation, this region is proposed to interact with the cTnC regulatory domain [6].

Many EF-hand proteins undergo a conformational change upon $Ca^{2+}$ binding leading to exposure of a hydrophobic pocket or cleft, referred to as the open/active form, which interacts with appropriate target proteins. The increased affinity of $Ca^{2+}$ for the open form is thought to provide the driving force for exposure of the hydrophobic cleft. In the cardiac system, activation of cTnC appears to require interactions with both $Ca^{2+}$ and the cTnI regulatory region [3,7,8]. Exchange between open and closed N domain conformational substates has been observed in the presence of $Ca^{2+}$ and cTnI [6,9,10]. Phosphorylation of the cardiac-specific amino-terminus, as a consequence of β-adrenergic stimulation, disrupts this regulatory domain interaction resulting in decreased $Ca^{2+}$ affinity and decreased $Ca^{2+}$ sensitivity of contraction [6,11,12].

Bepridil is one member of a class of pharmacological compounds capable of binding cTnC and modulating regulatory domain $Ca^{2+}$ affinity [13,14]. Early studies found an apparent bepridil $K_a$ of 10 $\mu M^{-1}$ for binding to $Ca^{2+}$-saturated cTnC [14]. In the presence of bepridil, $Ca^{2+}$ affinity at site II, the regulatory site, increased [15,16]. This increase was observed in both slow and fast skeletal muscle fibers, with the effect being greater in slow fibers [17]. Nuclear magnetic resonance (NMR) studies, using Met residues as structural markers, identified three or four bepridil-binding sites in cTnC [18]. Recently, an X-ray crystal structure of $Ca^{2+}$-saturated cTnC with three bound bepridil molecules has been obtained [19]. The overall cTnC structure was compact with the two globular domains close together [19]. Two bepridil molecules are located in the hydrophobic cavity between domains and make contact with both domains. A single bepridil appears to stabilize an open regulatory domain conformation by inserting between helical pairs A-D and B-C, sterically preventing domain closing [19].

These studies prompted us to explore bepridil binding to the cTnC/cTnI complex. Amide proton chemical shift pertur-

*Corresponding author. Fax: (1)-513-558 847.
*E-mail address:* rosevear@proto.med.uc.edu (P.R. Rosevear).

*Abbreviations:* cTnC, recombinant cardiac troponin C (desMet1-Ala2, Cys35Ser); cTnI, recombinant mouse cardiac troponin I; cTnI(1–80), cardiac troponin I corresponding to residues 1–80

0014-5793/01/$20.00 © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0014-5793(01)02790-9

bation mapping was used to monitor effects of bepridil on cTnC in [$^{15}$N,$^{2}$H]cTnC/cTnI(1–211). Bepridil binding induces $^{1}$H–$^{15}$N amide chemical shift changes within the cTnC regulatory domain consistent with a shift in conformational equilibria toward more open states. Stabilization of a more open conformation is the expected activity of a $Ca^{2+}$-sensitizing agent. In the presence of cardiac TnI, no binding of bepridil to the C domain of cardiac TnC was detected. Our studies demonstrate the usefulness of this system for screening compounds capable of stabilizing open/active regulatory conformations.

## 2. Materials and methods

### 2.1. Proteins

[$^{15}$N,$^{2}$H]cTnC and cTnI(1–211) were expressed and purified as previously described [9,20]. $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI complexes were prepared at a known concentration, usually 0.4–0.6 mM, in 10% $^{2}H_2O$, 20 mM Tris-$d_{11}$ (pH 6.8), 500 mM KCl, 10 mM $CaCl_2$, 5 mM dithiothreitol, 0.1 mM pefabloc and 0.1 mM leupeptin.

### 2.2. Bepridil titration

Stock solutions of bepridil (Sigma) were prepared fresh in 10% $d_4$-methanol, and added in aliquots to $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI. Sample pH was corrected to 6.8 as necessary. Binding of bepridil to $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI was monitored by following bepridil-induced $^{1}H_N$ chemical shift changes. Chemical shift changes were judged significant when they exceeded the sum of the average plus one S.D. of all bepridil-induced $^{1}H_N$ shifts. Normalized average $^{1}H_N$ chemical shift data as a function of total bepridil were utilized to estimate $K_a$ for drug binding.

### 2.3. NMR spectroscopy

Experiments were carried out on a Varian Inova 800 MHz spectrometer. $^{1}$H–$^{15}$N correlation experiments utilized sensitivity-enhanced $^{1}$H–$^{15}$N HSQC-based pulse sequences [21]. Data collection and processing parameters have been previously described [8].

## 3. Results

Our laboratory has developed an intact cTnC/cTnI model useful for understanding structural mechanisms involved in activation and modulation of contraction. One of the many advantages in utilizing intact cTnC/cTnI for structural studies is the ability to avoid or reduce non-specific peptide and drug interactions [22]. To further our structural understanding of activation and modulation of the $Ca^{2+}$ signal, we have studied the binding of a known $Ca^{2+}$-sensitizing agent, bepridil, to the $Ca^{2+}$-saturated cTnC/cTnI complex having a molecular mass of $\sim$40 kDa.

A 1:1 complex of $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC and cTnI was prepared and characterized as previously described [8]. We have previously assigned both amide proton, $^{1}H_N$, and nitrogen,$^{15}N_H$, resonances in $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC bound to full-length cTnI. $^{1}$H–$^{15}$N HSQC spectra were used to map bepridil binding to cTnC during titration of $Ca^{2+}$-saturated cTnC/cTnI. Fig. 1 compares $^{1}H_N$ chemical shift differences for $Ca^{2+}$-saturated cTnC bound to cTnI in the absence and presence of saturating drug. Chemical shift differences map primarily to defunct site I and $Ca^{2+}$-binding site II (Fig. 1). No significant bepridil-induced amide chemical shift changes were observed in the cTnC C domain demonstrating that any potential drug-binding sites in the C domain are blocked in the presence of cTnI (Fig. 1). This is in contrast to crystallographic studies and solution studies on free $Ca^{2+}$-saturated cTnC where at least two C domain bepridil-binding sites were detected [18,19].

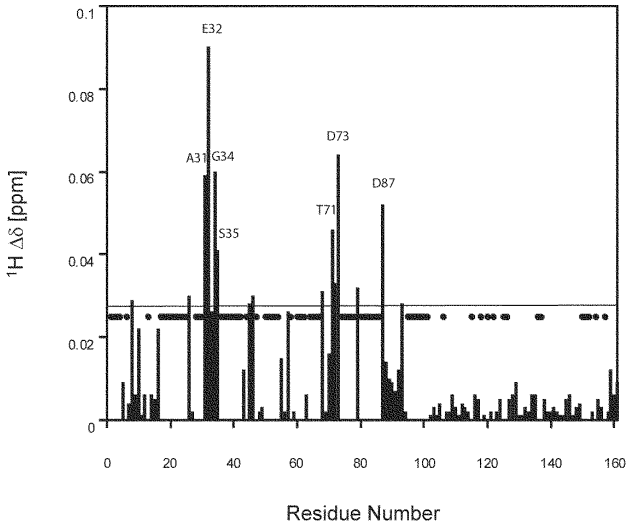


Fig. 1. Comparison of absolute value amide chemical shift differences between $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI and $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI in the presence of saturating bepridil. Chemical shift differences were measured for $^{1}H_N$ resonances of cTnC in a 0.5 mM $Ca^{2+}$-saturated [$^{15}$N,$^{2}$H]cTnC/cTnI complex in the absence and presence of 2 mM bepridil. The horizontal line represents the average chemical shift difference plus one S.D. Filled circles mark residues for which resonance assignments in one of the $^{1}$H–$^{15}$N correlation spectra were broadened as a consequence of chemical exchange or could not be confirmed.

Binding site chemical shift perturbations indicate where the magnetic environment is changing upon addition of bepridil. While chemical shift changes often map to the binding interface, changes resulting from perturbation of regulatory domain equilibria may also be detected. Single $^{1}$H–$^{15}$N correlations were observed for amide protons whose chemical shift could be followed with increasing bepridil concentration, consistent with fast exchange between conformational substates relative to chemical shift differences between free and drug-bound forms. In addition to residues in and around sites I and II, chemical shift perturbations were also observed for selected residues in the A-, B-, and D-helices (Fig. 1). Broadening of $^{1}H_N$ resonances for cTnC residues 36–41, 50–54, 60–62, 64–67, 74–78, and 80–86 in either the presence or absence of saturating bepridil precluded mapping drug-induced chemical shift perturbations within these regions (Fig. 1). Many of these residues have been previously demonstrated to undergo chemical exchange in several different cTnC/cTnI complexes [8]. The observed broadening is consistent with conformational exchange that persists even in the presence of bepridil. Taken together, our data suggest that bepridil-induced $^{1}H_N$ chemical shifts not only localize bepridil binding to the cTnC N domain, but also reveal changes in regulatory domain conformational substates. Identification of the bepridil-binding interface in the regulatory domain requires measurement of distance restraints, NOEs, in appropriately isotope-labeled cTnC/cTnI complexes. In this study, the presence of protonated cTnI precluded the possibility of detecting NOE interactions between bepridil and regulatory domain $^{1}H_N$ resonances.

Analysis of the $^{1}H_N$ chemical shift dependence with increasing concentrations of drug was used to estimate an apparent

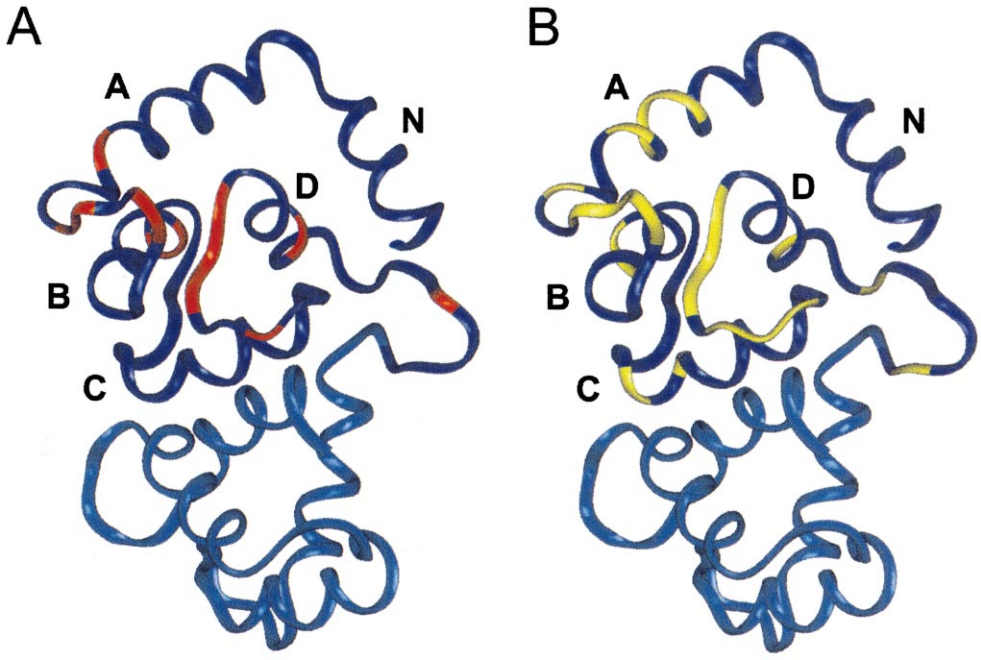


Fig. 2. The conformation of $Ca^{2+}$-saturated cTnC when bound to cTnI is shown with regions colored to indicate $^1H_N$ chemical shift changes upon binding bepridil (A) and the cTnI cardiac-specific amino-terminus (B). The conformation of $Ca^{2+}$-saturated cTnC bound to cTnI was determined using long-range distance and orientation restraints (Abusamhadneh, unpublished). A: Calcium-saturated cTnC residues whose $^1H_N$ chemical shift changes in the presence of bepridil (Fig. 1) are shown in red. B: Cardiac TnC residues whose $^1H_N$ chemical shifts are perturbed by binding the cTnI cardiac-specific amino-terminus are shown in yellow. Residues whose $^1H_N$ chemical shifts exhibit significant chemical shift perturbations were obtained by comparing the absolute values of the $^1H_N$ chemical shift differences between cTnC/cTnI(1–80) and cTnC/phosphorylated cTnI(1–80) [22]. Residues experiencing significant perturbations in the presence of the cTnI cardiac-specific amino-terminus include 21, 22, 23, 25, 29, 32, 33, 34, 35, 42, 43, 55, 57, 67, 68, 69, 71, 72, 73, 81, 84, and 90.

$K_a$ of $\sim 140\ \mu M^{-1}$ for bepridil binding to the cTnC regulatory domain in $Ca^{2+}$-saturated cTnC/cTnI. This value is 10-fold greater than the published value for bepridil binding to free cTnC [14]. At the high protein concentration used in the NMR titrations, care must be taken to look for and minimize non-specific, weak associations. Absence of bepridil-induced cTnC C domain chemical shifts, even in the presence of excess drug, suggests $^1H_N$ chemical shifts in the regulatory domain reflect specific association. The apparent weak binding of bepridil to the cTnC regulatory domain in the complex may in part explain its poor pharmacological effects [14,15].

## 4. Discussion

We have utilized our $^1H_N$ assignments for [$^{15}N$,$^2H$]cTnC bound to cTnI to study bepridil binding and its effects on regulatory domain conformational substates. Observed regulatory domain chemical shift changes map primarily to defunct site I and $Ca^{2+}$-binding site II (Fig. 1). Both the direction and magnitude of Ala-31 and Asp-73 $^1H_N$ chemical shift changes are consistent with stabilization of open regulatory domain conformations. A similar pattern of chemical shift perturbations has been attributed to interaction of the cTnI cardiac-specific amino-terminus with the cTnC regulatory domain [8,20]. Observed chemical shift changes are similar to those detected upon opening of the N domain in response to cTnI(129–166) binding [23].

The apparent bepridil-binding constant is weaker than that determined for free cTnC [14,15,18]. Several possible explanations could account for the apparent difference in binding affinities. Analysis of the crystal structure of bepridil bound to cTnC shows an overall collapsed structure. Collapse of cTnC results in all three bound bepridil molecules having interactions with both cTnC domains [19]. In the presence of cTnI, cTnC is more elongated and therefore it is less likely that both cTnC domains contribute to the binding affinity of bepridil [8,24,25]. Our studies show that in the presence of cTnI, two apparent bepridil-binding sites within the C domain are blocked (Fig. 1). This observation is consistent with available structures suggesting that C domain drug-binding sites overlap with the cTnI N domain-binding site [4,19]. Recently, it has been demonstrated that both bepridil and a cTnI regulatory peptide can bind to the isolated N domain of cTnC [26]. This observation would suggest that decreased bepridil affinity for cTnC in the cTnC/cTnI complex does not result from competitive binding interactions. Our recent comparison of regulatory conformational substates in cTnC/cTnI(1–80)/cTnI(129–166) and cTnC/cTnI(1–167) suggests that covalent attachment of the cTnI N domain to the cTnI regulatory region, via residues 81–128, modulates interactions between the cTnI regulatory region and the cTnC N domain [23], leaving open the possibility that cTnI may alter bepridil binding in the intact system.

We have previously demonstrated that interaction of the cTnI cardiac-specific amino-terminus with cTnC alters regulatory domain conformational substates, presumably toward more open/active conformations [9,20,23]. Phosphorylation of Ser-23 and Ser-24 in the cardiac-specific amino-terminus is thought to result in the loss of these cTnC regulatory domain interactions. Regulatory domain chemical shift changes observed upon drug binding and upon interaction with the cTnI cardiac-specific amino-terminus [20] suggest that both

induce similar cTnC regulatory domain conformational changes (Fig. 2). Bepridil binding to the regulatory domain has been shown to extend the N-terminus of helix B with formation of a side chain interaction, between Glu-40 and Ser-37 [19]. These structural changes have been suggested to stabilize defunct site I and facilitate regulatory domain opening [19]. It is interesting to speculate that the cTnI cardiac-specific amino-terminus also stabilizes site I and thereby increases the population of open/active conformations. Thus, the inability of site I to bind $Ca^{2+}$ produces a conformationally active region capable of modulating the overall conformational entropy and therefore populations of apo/closed and $Ca^{2+}$-cTnI/open states. Consistent with this proposal, binding of the cardiac-specific amino-terminus and bepridil are both known to increase the apparent $Ca^{2+}$ affinity for $Ca^{2+}$-binding site II. Thus, our studies provide evidence for a conformationally active region within the cTnC regulatory domain that may be targeted for development of $Ca^{2+}$-sensitizing compounds capable of increasing $Ca^{2+}$ affinity in cardiac troponin.

*Acknowledgements:* This work supported by Grant AR 44324 (P.R.R.) from the National Institutes of Health.

## References

[1] Krudy, G.A., Kleerekoper, Q., Guo, X., Howarth, J.W., Solaro, R.J. and Rosevear, P.R. (1994) J. Biol. Chem. 269, 23731–23735.
[2] Van Eerd, J.P. and Takahashi, K. (1976) in: Calcium Transport in Contraction and Secretion (Carafoli, E., Clement, E., Drabikowski, W. and Margreth, A., Eds.), pp. 427–430, Elsevier Science Publishing Co., New York.
[3] Li, M.X., Spyracopoulos, L. and Sykes, B.D. (1999) Biochemistry 38, 8289–8298.
[4] Gasmi-Seabrook, G.M.C., Howarth, J.W., Finley, N., Abusamhadneh, E., Gaponenko, V., Brito, R.M.M., Solaro, R.J. and Rosevear, P.R. (1999) Biochemistry 38, 8313–8322.
[5] Moir, A.J., Solaro, R.J. and Perry, S.V. (1980) Biochem. J. 185, 505–513.
[6] Abbott, M.B., Gaponenko, V., Abusamhadneh, E., Finley, N., Li, G., Dvoretsky, A., Rance, M., Solaro, R.J. and Rosevear, P.R. (2000) J. Biol. Chem. 275, 20610–20617.
[7] Sia, K.S., Li, M.X., Spyracopoulos, L., Gagne, M., Liu, W., Putkey, J.A. and Sykes, B.D. (1997) J. Biol. Chem. 272, 18216–18221.
[8] Dong, W.J., Xing, J., Chandra, M., Solaro, R.J. and Cheung, H.C. (2000) Protein Sci. 41, 438–447.
[9] Gaponenko, V., Abusamhadneh, E., Abbott, M.B., Finley, N., Gasmi-Seabrook, G., Solaro, R.J., Rance, M. and Rosevear, P.R. (1999) J. Biol. Chem. 274, 16681–16684.
[10] Pääkkönen, K., Annila, A., Sorsa, T., Pollesello, P., Tilgmann, C., Kilpeläinen, I., Karisola, P., Ulmanen, I. and Drakenberg, T. (1998) J. Biol. Chem. 273 (25), 15633–15638.
[11] Robertson, S.P., Johnson, J.D., Holroyde, M.J., Kranias, E.G., Potter, J.D. and Solaro, R.J. (1982) J. Biol. Chem. 257, 260–263.
[12] Chandra, M., Kim, J.J. and Solaro, R.J. (1999) Biochem. Biophys. Res. Commun. 263, 219–223.
[13] Kischel, P., Bastide, B., Potter, J.D. and Mounier, Y. (2000) Br. J. Pharmacol. 131, 1496–1502.
[14] Solaro, R.J., Bousquet, P. and Johnson, J.D. (1986) J. Pharmacol. Exp. Ther. 238, 502–507.
[15] MacLachlan, L.K., Reid, D.G., Mitchell, R.C., Salter, C.J. and Smith, S.J. (1990) J. Biol. Chem. 265, 9764–9770.
[16] Hatakenaka, M. and Ohtsuki, I. (1992) Eur. J. Biochem. 205, 985–993.
[17] Kischel, P., Stevens, L. and Mounier, Y. (1999) Br. J. Pharmacol. 128, 767–773.
[18] Kleerekoper, Q., Liu, W., Choi, D. and Putkey, J.A. (1998) J. Biol. Chem. 273, 8153–8160.
[19] Li, Y., Love, M.L., Putkey, J.A. and Cohen, C. (2000) Proc. Natl. Acad. Sci. USA 97, 5140–5145.
[20] Finley, N., Abbott, M.B., Abusamhadneh, E., Gaponenko, V., Dong, W.-J., Gasmi-Seabrook, G.M.C., Howarth, J.W., Rance, M., Solaro, R.J., Cheung, H.C. and Rosevear, P.R. (1999) FEBS Lett. 453, 107–112.
[21] Kay, L.E., Xu, G.Y. and Yamazaki, T. (1994) J. Magn. Reson. 109, 129–133.
[22] Abbott, M.B., Dvoretsky, A., Gaponenko, V. and Rosevear, P.R. (2000) FEBS Lett. 469, 168–172.
[23] Abbott, M.B., Dong, W.J., Dvoretsky, A., DaGue, B., Caprioli, R.M., Cheung, H.C. and Rosevear, P.R. (2001) Biochemistry (in press).
[24] Kleerekoper, Q., Howarth, J.W., Guo, X., Solaro, R.J. and Rosevear, P.R. (1995) Biochemistry 34, 13343–13352.
[25] Olah, G.A., Rokop, S.E., Wang, L.-L.A., Blechner, S.L. and Trewhella, J. (1994) Biochemistry 33, 8233–8239.
[26] Li, M.X., Wang, X., Spyracopoulos, L. and Sykes, B.D. (2001) Biophys. J. 393.