Identification of Binding Sites for Bepridil and Trifluoperazine on Cardiac Troponin C

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The solution structure of cardiac troponin C (cTnC) (Sia, S., Li, M. X., Spyraecopoulos, L., Gagne, S. M., Liu, W., Putkey, J. A. & Sykes, B. D. (1997) J. Biol. Chem. 272, 18216–18221) challenges existing structure/function models for this critical regulatory protein. For example, it is clear that the closed conformation of the regulatory N-terminal domain in Ca\(^{2+}\)-bound cardiac troponin C (cTnC) presents a much different binding surface for Ca\(^{2+}\)-sensitizing compounds than previously thought. We report here the use of Met methyl groups as site-specific structural markers to identify drug binding sites for trifluoperazine and bepridil on cTnC. Drug-dependent changes in the NMR heteronuclear single-quantum coherence spectra of [methyl\(^{-13}C\)]Met-labeled cTnC indicate that bepridil and trifluoperazine bind to similar sites but only in the presence of Ca\(^{2+}\). There are 3–4 drug binding sites in the N- and C-terminal domains of intact cTnC that exhibit fast exchange on the NMR time scale. Use of a novel spin-labeled phenothiazine and detection of isotope-filtered nuclear Overhauser effects allowed identification of drug binding sites in the shallow hydrophobic cup in the C-terminal domain and on two hydrophobic surfaces on the N-terminal regulatory domain. The data presented here, coupled with our previous study using covalent blocking groups, support a model in which the Ca\(^{2+}\)-sensitizing binding site includes Met-45 in helix B of site I, and Met-60 and -80 in helices B and C of the regulatory site II. This subregion in cTnC makes a likely target against which to design new and selective Ca\(^{2+}\)-sensitizing compounds.

The ability to sensitize cardiac muscle to Ca\(^{2+}\) would have promising therapeutic potential for the treatment for Ca\(^{2+}\) desensitization that is associated with congestive heart failure due to acute myocardial infarction and associated ischemia (1). Ideally, the mechanism of sensitization would not involve altering Ca\(^{2+}\) transients in myocardial cells that are already metabolically challenged. Regulatory proteins located on the thin filament of cardiac muscle are logical targets for such therapeutic compounds since they respond to cellular Ca\(^{2+}\) levels but are not involved in modulation of Ca\(^{2+}\) transients.

Cardiac troponin C (cTnC)\(^{1}\) is the EF-hand Ca\(^{2+}\) binding receptor on the thin filament of slow skeletal and cardiac striated muscle. Cardiac muscle contraction is initiated when Ca\(^{2+}\) binds to the N-terminal regulatory metal binding site II in cTnC. Muscle relaxation occurs upon release of Ca\(^{2+}\) from this regulatory site. This central role for cTnC makes it an attractive target for putative Ca\(^{2+}\)-sensitizing compounds designed to modify the Ca\(^{2+}\) dependence of cardiac muscle contraction. Indeed, precedents have been established for both desensitization and sensitization of cardiac muscle to Ca\(^{2+}\) via mechanisms that involve cTnC. Phosphorylation of Ser-22 and -23 on cardiac troponin I (cTnI), which is constitutively associated with cTnC in the troponin complex, leads to a decrease in the Ca\(^{2+}\) sensitivity of cardiac muscle fibers and myofibrils (2) and to a decrease in the affinity of site II in cTnC (3). In contrast, a variety of small hydrophobic compounds including the calmodulin antagonists bepridil (4–6), trifluoperazine (TFP) (7), and calmidazolium (7–9) have the opposite effect of increasing the Ca\(^{2+}\) sensitivity of cardiac muscle preparations. Bepridil has been shown to increase the affinity of cTnC for Ca\(^{2+}\) by decreasing the Ca\(^{2+}\) off-rate (5, 6). Such reports have led to a search for new generations of Ca\(^{2+}\)-sensitizing compounds with greater specificity for cTnC (1).

Knowledge of the structure of cTnC and identification of potential drug binding sites on this protein would help facilitate the design or selection of Ca\(^{2+}\)-sensitizing compounds with desired pharmacological effects. Until recently, high resolution structural information was only available for the fast skeletal isoform of TnC (sTnC) (10, 11). Structural models for Ca\(^{2+}\)-bound cTnC have been proposed based on the structures of sTnC and calmodulin. Not surprisingly, these models predict an open N-terminal regulatory domain of Ca\(^{2+}\)-bound cTnC, with an exposed hydrophobic surface similar to the structure of sTnC and calmodulin. Existing models for drug binding to cTnC propose that these compounds bind to this exposed N-terminal hydrophobic pocket (5, 12, 13). Recently, the NMR solution structures of Ca\(^{2+}\)-bound intact cTnC (14), and the apo and Ca\(^{2+}\)-saturated N-terminal regulatory fragment (15) were reported. The most striking feature of these structures is that the Ca\(^{2+}\)-bound N-terminal regulatory domain is partially closed, resulting in significantly less exposed hydrophobic surface than found in Ca\(^{2+}\)-bound sTnC. These structures have significant implications with respect to drug binding sites in cTnC.

The goal of the current study was to generate structural information on the binding of bepridil and TFP to full-length cTnC and to correlate these data with the solution structure of this critical Ca\(^{2+}\)-regulatory protein. We chose both drugs based on their known Ca\(^{2+}\)-sensitizing affects on cTnC. The data presented here, coupled with our previous study using covalent blocking groups (16), support a model in which bepridil and trifluoperazine; TFP, trifluoperazine; TEMPO-TFP, N-(3-(4-Amino-TEMPO)propyl)-2-(trifluoromethyl)phenothiazine.
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RESULTS

Drug and Protein Structures—In previous studies, we assigned the 10 methionyl methyl 1H-13C correlations (18) in recombinant cTnC and used them as general markers to monitor Ca2+ and cTnI binding to cTnC (23–25). The solution structure of cTnC allows these assignments to be used as positional markers for drug binding. Fig. 1 shows the location methionyl methyl carbons in a ribbon rendering of intact cTnC (Fig. 1A), and solvent accessible surfaces in the N-terminal (Fig. 1, B and C) and C-terminal (Fig. 1D) domains. Hydrophobic residues are shown in blue, all other residues are in white, and the methionyl methyl groups are shown in red. Fig. 1E shows the structures of the compounds used in this study. Both bepridil and TFP have a positively charged nitrogen at pH 7.0. The spin-labeled phenothiazine, which we will call TEMPO-TFP, was designed to mimic the primary structural features of TFP including the intact phenothiazine rings and a positively charged amine. Synthesis of this compound is described under “Experimental Procedures.”

Effect of Bepridil and TFP on cTnC—Initial experiments involved titration of [methyl-13C]Met-labeled cTnC with bepridil or TFP to assign chemical shifts in the presence of the drugs, and to identify similarities or differences in patterns of chemical shift change. Unless otherwise indicated, all experiments were performed using recombinant cTnC(A-Cys) (17). This protein has both Cys-35 and -84 converted to Ser to prevent formation of inter- and intramolecular disulfide bonds during NMR analysis that can affect the hydrophobic surfaces and functional characteristics of cTnC. cTnC(A-Cys) is active and was used in determining the solution structure of Ca2+-bound full-length cTnC (14). The methionyl methyl 1H-13C correlations observed for free cTnC(A-Cys) in the presence Ca2+ were essentially identical to those previously reported for cTnC(C35S) (18).

The HSQC spectra in Fig. 2, panel A, shows the effect of bepridil on the methionyl methyl 1H-13C correlations in cTnC-(A-Cys) in the absence of Ca2+. The addition of drug in molar excess over protein resulted in small chemical shift changes for all the Met residues. The apparent binding constant for association of bepridil with 3Ca2+-cTnC is approximately 10–20 μM (4, 5). We do not attribute the small chemical shift changes seen in Fig. 2 to high affinity binding of bepridil to specific sites since 1) equilibrium dialysis at lower drug and protein concentrations (200 μM of cTnC(A-Cys)) showed no evidence of bepridil binding in the absence of Ca2+. Equilibrium dialysis in the presence of Ca2+ yielded an apparent binding constant of 20 μM (data not shown); 2) we were unable to detect NOEs between the N-terminal Met methyl groups of cTnC(A-Cys) and protons on the aromatic rings of bepridil in the absence of Ca2+ (see below); and 3) the magnitude of drug-induced chemical shift changes seen in the absence of Ca2+ were minor relative to those observed in the presence of Ca2+ (see below). These data suggest the minor chemical shift changes and line broadening seen for the Mets in the absence of Ca2+ are due to nonspecific weak binding of the drug at mM concentrations used in the NMR experiments.

Drug Solutions—Stock solutions of each drug were prepared initially before use. Bepridil was prepared in a 80% D2O, 20% methanol-d4 solution and a stock solution of TFP was prepared in D2O. TEMPO-TFP was prepared in methanol-d4. The stock solutions of TFP that are light sensitive were stored in the dark. For each drug titration, the sample pH was adjusted when necessary. To reduce the nitroxide radical on the spin-label, TEMPO-TFP, a 2-fold molar excess of ascorbic acid was added to the NMR sample.
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Fig. 1. Solution structure of cTnC and chemical structures of bepridil, TFP, and TEMPO-TFP. Panels A-D show different structural aspects of the solution structure of Ca\textsuperscript{2+}-bound cTnC. Hydrophobic residues are shown in purple, all other polar and charged residues are in white, whereas the Met methyl groups are in red. Panel A shows the positions of all 10 Met methyl groups in a ribbon structure of cTnC. Panels B and C show two different perspectives of solvent accessible surfaces in the N-terminal domain, and panel D shows the solvent accessible surface of the shallow hydrophobic cup in the C-terminal domain. Panels A-D were generated using Insight 95. Accessible surface was calculated using the Connolly algorithm and a 1.4 Å probe. Panel E shows the chemical structures of the compounds used in this study.

Fig. 2. Titration of apo (panel A) and 2Ca\textsuperscript{2+}-cTnC (A-Cys) (panel B) with bepridil. HSQC spectra of \([\text{methyl-}^{13}\text{C}]\text{Met cTnC (A-Cys)}\) showing the Met methyl \(^{1}H-^{13}C\) correlations at bepridil:protein ratios of 0, 1, and 2 equivalents. The initial peaks without drug are shown in green. The \(^{1}H-^{13}C\) Met methyl correlation for Met-45 shown in the box is at a 4-fold lower contour level.
and B) or TFP (panels C and D). Each panel shows the superimposition of spectra obtained at different ratios of drug:protein. The 1H-13C correlations attributed to Met groups located in the N-terminal domain (panels A and C) are plotted separately from those in the C-terminal domain (panels B and D). Bepridil was added in 0.5 equivalents to a drug/protein ratio of 4 (panels A and B). TFP was added in 1.0 equivalents to a drug:protein ratio of 6 (panels C and D). All drug additions (blue peaks) are shown only for Met-45 and -157, respectively. The methyl 1H-13C correlation for Met-45 was observed only at a greater than 10-fold lower contour after addition of one equivalent of either drug.

The data show that 1H chemical shift changes are observed for all groups except Met-137, which suggests binding sites in both the N- and C-terminal domains. The drug binding sites for both bepridil and TFP exhibit fast exchange characteristics relative to the chemical shift time scale as evidenced by single cross peaks in the HSQC spectra at a given drug:protein ratio, and the fact that these changes are seen for all affected Met's at drug to protein ratios below 1:1. Met-45 shows the greatest drug-induced change at increasing bepridil or TFP concentrations. The resonance for Met-45 broadens significantly at intermediate levels of drug, requiring a greater than 10-fold lower contour level to observe this peak, but then sharpens somewhat at saturating drug levels. This is indicative of intermediate exchange taking place for the methyl group of Met-45 between the free and drug-bound states of cTnC(A-Cys), possibly due to a localized slow conformational transition.

Fig. 4 plots 1H chemical shift changes as a function of added

### Table I

Drug-induced changes in chemical shifts of methyl 1H and 13C resonances in Ca2+-saturated cTnC(A-Cys)

| Met-45 | Met-47 | Met-60 | Met-80 | Met-81 | Met-85 | Met-103 | Met-120 | Met-137 | Met-157 |
|-------|-------|-------|-------|-------|-------|--------|--------|--------|--------|
| Bepridil:cTnC ratio |
| 0     | 1.83/16.1 | 2.21/15.4 | 1.95/15.3 | 1.84/15.8 | 1.38/15.3 | 2.10/15.1 | 1.91/14.9 | 1.89/15.7 | 1.92/15.6 | 1.82/14.3 |
| 0.5   | 1.72/16.0 | 2.14/15.4 | 1.97/15.4 | 1.85/15.7 | 1.42/15.3 | 2.10/15.1 | 1.91/15.0 | 1.82/15.6 | 1.92/15.5 | 1.76/14.4 |
| 1.0   | 1.59/15.8 | 2.09/15.3 | 1.98/15.4 | 1.85/15.7 | 1.45/15.3 | 2.09/15.1 | 1.90/15.1 | 1.75/15.4 | 1.92/15.5 | 1.71/14.5 |
| 1.5   | 1.45/15.6 | 2.04/15.3 | 1.99/15.5 | 1.84/15.6 | 1.45/15.3 | 2.08/15.2 | 1.88/15.2 | 1.70/15.3 | 1.92/15.5 | 1.66/14.6 |
| 2.0   | 1.35/15.5 | 2.02/15.3 | 1.99/15.5 | 1.83/15.5 | 1.45/15.3 | 2.07/15.2 | 1.86/15.2 | 1.83/15.3 | 1.92/15.4 | 1.63/14.7 |
| 2.5   | 1.26/15.4 | 2.00/15.2 | 1.99/15.6 | 1.80/15.4 | 1.44/15.2 | 2.05/15.2 | 1.83/15.4 | 1.65/15.1 | 1.92/15.4 | 1.61/14.8 |
| 3.0   | 1.19/15.3 | 1.99/15.2 | 1.98/15.6 | 1.77/15.3 | 1.41/15.2 | 2.03/15.2 | 1.78/15.4 | 1.63/15.1 | 1.92/15.4 | 1.59/15.0 |
| 3.5   | 1.15/15.3 | 1.98/15.2 | 1.97/15.6 | 1.74/15.3 | 1.40/15.2 | 2.02/15.2 | 1.76/15.5 | 1.63/15.1 | 1.92/15.4 | 1.58/15.0 |
| 4.0   | 1.13/15.2 | 1.98/15.2 | 1.96/15.6 | 1.73/15.3 | 1.40/15.2 | 2.01/15.2 | 1.75/15.5 | 1.63/15.1 | 1.92/15.3 | 1.57/15.0 |
| TFP:cTnC ratio |
| 0     | 1.82/16.2 | 2.22/15.4 | 1.94/15.3 | 1.84/15.8 | 1.38/15.3 | 2.11/15.1 | 1.92/14.9 | 1.89/15.7 | 1.92/15.6 | 1.82/14.3 |
| 1.0   | 1.73/16.0 | 2.14/15.4 | 1.94/15.4 | 1.80/15.8 | 1.39/15.5 | 2.09/15.1 | 1.93/15.1 | 1.63/15.1 | 1.92/15.4 | 1.63/14.4 |
| 2.0   | 1.57/15.6 | 2.06/15.3 | 1.92/15.5 | 1.76/15.7 | 1.34/15.5 | 2.06/15.1 | 1.91/15.2 | 1.56/14.9 | 1.92/15.3 | 1.49/14.5 |
| 3.0   | 1.46/15.4 | 2.03/15.2 | 1.89/15.5 | 1.71/15.7 | 1.27/15.4 | 2.03/15.2 | 1.88/15.3 | 1.54/14.8 | 1.92/15.3 | 1.40/14.7 |
| 4.0   | 1.39/15.3 | 2.00/15.2 | 1.85/15.5 | 1.68/15.7 | 1.25/15.4 | 2.01/15.2 | 1.86/15.4 | 1.53/14.8 | 1.92/15.2 | 1.38/14.7 |
| 6.0   | 1.32/15.2 | 1.97/15.1 | 1.80/15.5 | 1.60/15.7 | 1.27/15.5 | 1.98/15.1 | 1.82/15.3 | 1.52/14.8 | 1.92/15.2 | 1.40/14.7 |
bepridil for Met-45, -47, -120, and -157, which experience the greatest overall change. Drug-induced changes reach 90–100% of maximal at a drug to protein ratio of 3:1. Table I shows that \(^{1}\text{H}\) chemical shifts for the other Met residues reach a plateau after addition of 4 mol of bepridil per mol of protein. Additional drug molecules may bind but without altering the Met methyl groups. Binding of TFP to cTnC appears more complex. Although the major changes are seen upon the addition of 3 equivalents of TFP, additional but smaller changes in the \(^{1}\text{H}\) chemical shifts are induced upon the addition of 4 and 6 equivalents of TFP. Nevertheless, it is clear that both bepridil and TFP induce similar patterns in chemical shift changes and that the majority of these changes are achieved at a drug to protein ratio of 3.

Effect of TEMPO-TFP on Met Methyl Chemical Shifts—A number of the Met residues reside in close proximity to the Phe residues in the protein and could be affected by local ring current fields. Thus, the chemical shifts changes seen in Fig. 3 and Table I could be attributed to either direct ring current effects from TFP and bepridil or from a secondary effect resulting from drug-induced protein conformational changes that alter the positions of the Met methyl groups relative to Phe side chains. The latter mechanism would be more likely for Met residues such as Met-81 and -157, which are within ~3.4 Å of the Phe ring, rather than Met-45 and -60 which are no closer than ~6.5 Å from the nearest Phe ring.

To distinguish between direct and indirect effects of the drugs, we chose two lines of experiments. The first involved the use of a spin-labeled phenothiazine (TEMPO-TFP in Fig. 1E), the second uses \(^{13}\text{C}\)-labeled NOESY NMR experiment to identify NOEs between drug and cTnC. The paramagnetic effect of spin labels on chemical shift line widths is a low resolution technique that can measure distances from between 10 and 15 Å (26). We have used spin labels successfully in measuring solvent exposure of Met methyl groups as well as determining central helix flexibility in cTnC when free or bound to cTnI (23, 24). If the Met methyl groups participate in the formation of the drug binding sites, then TEMPO-TFP will result in line broadening of the \(^{1}\text{H}-^{13}\text{C}\) correlations for those Met. Thus, TEMPO-TFP is used here simply to identify which Met side chains are in the drug binding sites.

The HSQC spectra in Fig. 5, panel A, compares the Met methyl chemical shifts of Ca\(^{2+}\)-bound cTnC in the presence and absence of reduced diamagnetic TEMPO-TFP at a probe-to-protein ratio of 0.8. The reduced compound should not affect the chemical shift line width but should alter chemical shift positions if it binds in a manner similar to TFP. Indeed, TEMPO-TFP-induced chemical shift changes comparable in magnitude and nature to those induced by TFP at a similar probe to protein ratio. Fig. 5, panel B, shows the paramagnetic effect of oxidized TEMPO-TFP on Met methyl groups. All \(^{1}\text{H}-^{13}\text{C}\) correlations are broadened beyond detection at this contour level except that for Met-137. These data demonstrate that: 1) the paramagnetic effect of TEMPO-TFP is due to specific binding since a nonspecific effect of the soluble compound would likely result in broadening of all resonances including Met-137; 2) Met-137, which is on the opposite side of the C-terminal domain relative to the C-terminal hydrophobic surface, is not included in a drug binding site; and 3) all Met methyl groups except Met-137 are within about 10 Å from a bound drug. TEMPO-TFP will prove very useful for subsequent studies of drug binding sites on cTnC when associated with the cTnI or the intact troponin complex.

NOEs between Bepridil and Met Methyl Groups—Bepridil was chosen for additional experiments designed to identify NOEs between drug and Met residues in 3Ca\(^{2+}\)-cTnC. cTnC(A-Cys) was labeled with both [methyl-\(^{13}\text{C}\)]Met and L-Phe[\(^{13}\text{C}\)] to eliminate intraprotein NOEs between the Met methyl groups and Phe side chains. Isotope editing allowed the select observation of NOEs between the Met methyl protons attached to \(^{13}\text{C}\) and aromatic protons on bepridil. Control 1D spectra confirmed the efficiency of labeling with L-Phe[\(^{13}\text{C}\)] (data not shown).

Fig. 6 shows the spectra from the \(^{13}\text{C}\)-edited 2D NOESY experiment at drug:protein ratios of 1.5:1 (panels A, B, and C) and 3.5:1 (panels D, E, and F). Panels A and D show the \(^{1}\text{H}\) dimension of the 2D \(^{1}\text{H}-^{13}\text{C}\) HSQC, whereas panels B and E show the \(^{1}\text{H}-^{13}\text{C}\) HSQC that were used to assign and determine the intensity of each resonance at the given drug concentration. NOEs between the aromatic protons in bepridil and protons attached to [methyl-\(^{13}\text{C}\)]Met are shown in panels C and F. At the lower drug to protein ratio, strong NOEs are observed between the 3.5 aromatic protons on the drug and the methionyl protons of Met-60 and -80. Weaker NOEs are detected between the drug and the other Met residues. Due to resonance overlap with Met-81, the NOE to Met-45 cannot be observed, however at a drug:protein ratio below 1 a NOE to Met-45 was detected. At high drug:protein ratios, NOEs between bepridil and Met-60 and -80 persist, and NOEs between bepridil and other Met residues are strengthened in intensity. The NOE at 1.89 ppm in panel C cannot be clearly assigned to Met-103 or -137 due to resonance overlap. However, we have tentatively assigned this NOE to Met-103 since: 1) panel F shows no apparent NOE to Met-137; 2) TEMPO-TFP has no effect on the chemical shift of Met-137; and 3) neither TFP nor bepridil have a significant effect on the \(^{1}\text{H}\) chemical shift of Met-137 (Table I). These filtered NOEs show that all methyl groups except 137 are likely within 5 Å of aromatic rings in bound bepridil molecules and that the hydrophobic surface that includes Met-60 and -80 appears to constitute a preferred binding site at lower concentrations of drug.
DISCUSSION

The overall goal of the current study was to identify sites on cTnC that can bind cardiotonic compounds and potentially alter the Ca\(^{2+}\) binding affinity of site II. We chose bepridil and TFP as test compounds since previous reports show that they bind to sTnC (4, 5, 27) or sTnC peptides (28–30) and have clear Ca\(^{2+}\)-sensitizing effects on muscle preparations (4, 7, 9, 31, 32).

It was reasoned that identification of binding sites for these compounds and the unique opportunity to correlate structural drug binding data with the recent high resolution solution structure of cTnC (14, 15) would implicate drug binding sites that contribute to Ca\(^{2+}\) sensitization and provide a foundation for the development of compounds that are specific for cTnC.

In a previous study, MacLachlan et al. (5) reported binding of one bepridil molecule to the N-terminal domain of cTnC. The drug was proposed to make contact with Met-81, and a model for drug binding was presented based on tentative chemical shift assignments and a structure for Ca\(^{2+}\)-bound sTnC that was based on the open conformation of sTnC. In contrast to MacLachlan's study, our data differ markedly in both the number and location of bepridil binding sites. Differences in the number of drug binding sites can be attributed to the experi-
mental conditions used in each study. At pH 7.0 used here, bepridil is fairly soluble, whereas at a higher pH used in the previous study, bepridil precipitates at drug:protein ratio of greater than 1:1. Differences in locations of bound bepridil can be attributed to incorrect chemical shift assignments in the previous study. Met-81 was assigned a chemical shift of 2.15 ppm, whereas its correct assignment is 1.38 ppm. Confidence in the location of the binding sites reported here is based on the unambiguous assignments of methionyl Met chemical shifts, and the use of heteronuclear NMR to clearly monitor chemical shift changes and for the collection NOE distance constraints.

Bepridil and TFP bind to cTnC only in the presence of Ca$^{2+}$ with rapid exchange of drug between multiple binding sites in the N- and C-terminal domains. The stoichiometry of binding either drug was roughly the same, and the observed patterns of Met methyl chemical shift changes were remarkably similar for both drugs. This suggests that bepridil and TFP bind similar sites on cTnC. The C-terminal drug binding sites appear to be restricted to the hydrophobic inside surface of a shallow cup formed by the C-domain upon Ca$^{2+}$ binding to sites III and IV (see Fig. 1D). This surface includes side chains for Met-103, -120, and -157, but not -137. This conclusion is based on: 1) the large chemical shift changes seen for the methyl groups of Met-120 and -157; 2) the paramagnetic effect of the oxidized TEMPO-TFP on the methyl $^1$H-$^1$C correlations for Met-103, -120, and -157, but not -137; and 3) observed NOEs between bepridil and Met-103, -120, and -157.

Pan and Johnson (33) recently showed that binding of EMD 57033 to cTnC, required the high affinity sites III and IV to be occupied with Ca$^{2+}$. Thus, the hydrophobic surface in the C-terminal domain of free cTnC appears to present binding sites for calmodulin antagonists TFP and bepridil as well as EMD 57033. A pertinent question is whether binding of these compounds to the C-terminal domain can affect the characteristics of Ca$^{2+}$-binding to the N-terminal domain. Intradomain communication in cTnC has been reported previously (34). In addition, the Ca$^{2+}$ binding affinity of site II in cTnC is increased by inactivation of sites III and IV (35), whereas the affinity of sites III and IV in C-terminal fragments of both cTnC (35) and $\alpha$TnC (36) are increased relative to the intact protein. However, these observations should be interpreted cautiously with respect to an interdomain effect of drugs in the troponin complex. The cTnI inhibitory peptide was shown to shield Met-120 and -157 from solvent (23), and association of cTnI with cTnC causes significant change in the Met chemical shifts of Met-120 and -157 (24, 25). This suggests that association of cTnI may displace compounds bound to the C-terminal domain of cTnC.

Drug binding to the N-terminal domain must be considered with respect to the pattern of N-terminal hydrophobic surfaces, distances between Met methyl groups and NOEs between protons in bepridil and the methionyl methyl groups. Fig. 1 shows the N-terminal domain of cTnC to contain discrete hydrophobic surfaces. One surface includes the side chains of Met-47, -81, and -85 (Fig. 1B), whereas the other includes Met-45, -60, and -80 (Fig. 1C). The methyl groups of Met-47 and -60 are 16.7 Å apart and on different surfaces of the N-terminal domain. Thus, it seems unlikely that they could participate in the same drug binding site. Even if bepridil were to induce a separation of helices A and B, to more fully open the N-terminal domain and generate a contiguous hydrophobic surface, the methyl groups of Met-47 and -60 would be separated by about 17 Å. Given these structural constraints and the fact that at least 3 mol of drug bind per mol of protein, it is more likely that the N-terminal domain has multiple drug binding sites. Modeling of bepridil binding to the hydrophobic surface seen in Fig. 1B shows that NOEs could be generated between one aromatic ring in bepridil and Met-47 and between the other aromatic ring and Met-81 and -85. In addition, stabilizing electrostatic interactions could form between bepridil and Glu-19. The other binding site would include the hydrophobic patch seen in Fig. 1C. The methyl group of Met-60 is 5.5 and 7.5 Å from those of Met-45 and -80, respectively, and the methyl groups of Met-45 and -80 are separated by only a 3.5 Å distance. Thus a single drug molecule bound to this site could generate NOEs with the methyl groups of all three Mets.

In a previous study we tested the functional consequence of covalently coupling a 9 amino acid peptide to single Cys residues in cTnC (16). These experiments were designed to mimic the steric blocking effects of noncovalently bound drugs. Our general conclusion was that the N-terminal regulatory domain had discrete hydrophobic surfaces with different functions. One surface, which includes Met-81, is important for activity since blocking groups attached to Cys at position 81 greatly inhibited the activity of cTnC. This surface appears to interact with cTnI since position 81 can be readily cross-linked to cTnI (16) and since the methyl $^1$H-$^1$C correlation of Met-81 is significantly affected by association of cTnC with cTnI or cTnI peptides (25). In contrast, no significant functional effect was seen when blocking groups were attached to Cys at position 45, and the methyl $^1$H-$^1$C correlation of Met-45 is not affected by cTnI. Together these data suggest that drugs that are noncovalently bound to the region of Met-81 would either be displaced by cTnI or that the nature of binding would be altered such that cTnC activity is not inhibited. Drug binding to a region that includes Met-45 as well as Met-60 and -80 could persist in the presence of cTnI and not inhibit activity. Drug binding to this site may sensitize cTnC to Ca$^{2+}$ since Met-60 and -80 are in the helices C and D of the regulatory Ca$^{2+}$ binding site II. This provides potential structural basis for the bepridil-induced decrease in Ca$^{2+}$ off rate at site II (5, 6) and an attractive target against which to direct Ca$^{2+}$-sensitizing compounds.

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