Binding of a Calcium Sensitizer, Bepridil, to Cardiac Troponin C

A FLUORESCENCE STOPPED-FLOW KINETIC, CIRCULAR DICHROISM, AND PROTON NUCLEAR MAGNETIC RESONANCE STUDY*

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Stopped-flow fluorescence kinetic measurements, circular dichroism (CD), and 1H nuclear magnetic resonance (NMR) spectroscopy at 360 MHz have been used to study the interaction of the calcium-channel blocker and calmodulin antagonist bepridil with cardiac troponin C (cTnC) in the presence of calcium. The kinetic data show that bepridil reduces the rate of calcium release only from the low affinity, calcium-specific site and not from the two high affinity calcium/magnesium sites.

CD measurements indicate that drug binding leads to a small increase in the a-helical content of the complex. 1H NMR shows that the protein binds one equivalent of bepridil, with a dissociation constant of ~20 μM, only when the low affinity calcium site is occupied. Exchange is fast or intermediate on the chemical shift time scale. Drug binding is shown to be largely localized in the N-terminal domain, containing the low affinity calcium site, by observing the shifting and broadening of several resonances associated with that domain. These include assigned aromatic signals together with methionyl and other methyl signals. Observation of intermolecular nuclear Overhauser effects was precluded by extensive spectral overlap. Consideration of the data from the three techniques permitted a model of the bepridil-cTnC complex to be constructed, using the model of cTnC derived from the x-ray structure of calmodulin (MacLachlan L. K., Reid D. G., and Carter, N. (1990) J. Biol. Chem. 265, 9754–9763).

Binding of bepridil to a prominent hydrophobic depression in the N-terminal domain can be invoked to explain many of the induced changes in the spectral and kinetic properties of the protein. The implications of the model for the calcium sensitizing action of bepridil are discussed.

In congestive heart failure, cardiac function is impaired, and long term survival of patients is poor. New drugs are needed which improve the pumping of the failing myocardium (Wetzel and Haul, 1988). One approach to this end is to identify compounds which increase the affinity of troponin C, the calcium receptor component of troponin. Such compounds, which have been designated "calcium sensitizers," ought to produce an increase in the force of myocardial contraction at a given intracellular calcium concentration,

and hence help to restore the hemodynamic performance of the heart (Ruegg, 1987).

Bepridil (I) is a drug which affects calcium handling outside and inside cardiac cells, in that it shows the properties of a calcium-channel blocker and calmodulin antagonist (Vogel et al., 1979; Hoh et al., 1984). Bepridil also acts on the contractile proteins themselves; it increases the isometric contractile force of skinned cardiac fibers at submaximal calcium concentrations and modifies the calcium dependence of the ATPase activity of cardiac myofibrils (Salaru et al., 1986). These effects may be due to an increased affinity of cardiac troponin C (cTnC)

1 for calcium. We have constructed a model of cTnC, based on the crystal structures of the homologous proteins skeletal muscle troponin C (sTnC) and calmodulin (CaM), to deduce a number of resonance assignments in its 1H NMR spectrum (MacLachlan et al., 1990).

This paper reports the use of two other techniques as well as NMR to investigate the effects of bepridil binding to cTnC. The drug may be shown by fluorescence kinetic methods to slow the release of calcium from the regulatory site of the protein. Circular dichroism spectroscopy, which is sensitive to protein secondary structure, has been employed to study conformational changes in cTnC induced when bepridil binds.

Knowledge of the three-dimensional structure of receptors and drug-receptor complexes derived by NMR (Jardetzky and Roberts, 1981) has been shown to be of tangible assistance to the drug discovery process (Birdsall et al., 1984). 1H NMR experiments suggest that bepridil binding is localized in the N-terminal domain of cTnC and permit a dissociation constant for the interaction to be measured; global conformational changes do not occur when the drug binds. This specificity for the N-terminal domain, and a number of other changes in the NMR spectrum of the protein, can be qualitatively explained by the model of cTnC (MacLachlan et al.,

The abbreviations used are: cTnC, cardiac troponin C; sTnC, skeletal muscle troponin C; CaM, calmodulin; EGTA, (ethylenebis(oxyethylenenitrilo)tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid.

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Isolation and purification of bovine cTnC is described in MacLachlan et al. (1990). Protein samples were dialyzed at 4 °C against 100 mM potassium phosphate, 100 mM KCl, 0.5 mM dithiothreitol, pH 7.4, in 0.4 ml of 99.8% isotopic purity H2O solution, until the pH NMR spectrum resembled that previously obtained in the Ca2+-saturated form by addition of aliquots of a 0.2 M CaCl2 solution. The protein was obtained in the range 7.0–7.8.

RESULTS AND DISCUSSION

Stopped-flow Kinetic Measurements

Fig. 1 shows stopped-flow traces illustrating the increase in fluorescence on mixing solutions of cTnC (Fig. 1A), or cTnC and bepridil, (Fig. 1B) with excess Quin-2. The fluorescence transients collected over 50 ms are biphasic and may be fitted to a double exponential equation. In the absence of bepridil (Fig. 1A), the fitted curve corresponds to two calcium release rates of 164 and 0.9 s⁻¹. The faster process contributes approximately one-third of the total signal amplitude when corrected for the dead time of the instrument. The transient acquired in the presence of bepridil (50 μM final concentration) (Fig. 1B) gives two rates of 71 and 4.25 s⁻¹. Data collected over a longer time period (not shown) gave rates for the slower process of 1.8 and 2.2 s⁻¹ in the absence and presence of bepridil.

These fast and slow rates correspond, respectively, to calcium release from the single, low affinity, calcium-specific site and the pair of high affinity calcium/magnesium-binding sites in cTnC (Potter and Gergely, 1975; Crouch and Klee, 1980; Drabikowski et al., 1980). Thus, in the presence of bepridil, the rate of calcium release from the low affinity, N-terminal-binding site is reduced, whereas there is minimal effect at the high affinity C-terminal sites.

An alternative method of monitoring calcium release is by the use of fluorescence probes covalently attached to residues on the protein. The fluorescence of cTnC labeled with IAANS at Cys-35 and Cys-84 is sensitive to the occupancy of the low affinity calcium-specific site (Johnson et al., 1980). Solaru et al. (1986), using this method, have found that bepridil modifies the calcium dependence of the fluorescence of this derivative of cTnC and thus presumably its affinity for calcium.

The results shown here have confirmed this observation di-
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Circular Dichroism Experiments

The CD spectra recorded during the titration of cTnC (1.14 mg/ml) with bepridil, in the presence of a saturating concentration of CaCl₂ (0.5 mM), are shown in Fig. 2, a and b. The increase in Δε at 220 nm (a) shows that a small increase in α-helicity of cTnC occurs as the bepridil concentration is increased. Simultaneously, in the near UV region (b) increases in AC at 220 nm (a) shows that a small increase in LY-9766 Bepridil Binding to cTnC.

In AA are observed below 275 nm, overlapping the sharp

Four bands at 262 and 268 nm due to the aromatic side chains of the protein, and an extra band appears at 298 nm. Throughout the titration a fine suspension formed in the solution, probably due to some precipitation of bepridil as it was added to the buffered cTnC solution. However, the scintillation counting results indicate that any loss of bepridil due to precipitation was minimal. Light scattering from the suspension may, though, have resulted in some loss of intensity in the CD spectra, particularly in the near UV region where a 1.0-cm pathlength was used.

By comparison with the UV spectrum of bepridil (Fig. 2c), the changes observed in the near UV CD spectra are attributed to the appearance of a CD spectrum of bepridil, arising either from preferential binding of one enantiomer of the compound to cTnC, or from additional asymmetry induced in the compound when it complexes with the protein.

The changes in the far UV spectra indicate a small increase in α-helical content of the protein on binding bepridil. Klevit et al. (1985) have shown that the binding of the 27 residue peptide M13 from skeletal muscle myosin light chain kinase to the homologous calmodulin results in an increase of about 20% in the α-helical content of the complex in the presence of calcium; it is expected that this includes contributions from both the peptide and the protein. Similar increases in α-helical content have been observed for complexes of calmodulin with other peptides (see, for example, Maulet and Cox, 1983; McDowell et al., 1985).

'H NMR Experiments

Fig. 3 shows the effects of titrating bepridil into a phosphate-buffered solution of calcium-saturated cTnC (2.5 mM); the final bepridil concentration was 3.75 mM. Addition of bepridil up to a drug/protein ratio of 1:1 causes well-defined and specific changes of chemical shift and line width in the 'H NMR spectrum of the protein. These are clearly evident in resolution-enhanced spectra and in comparisons of spectra obtained at compound to protein ratios intermediate between those shown in Fig. 3. Thereafter, the changes are much less pronounced, being limited to generalized broadening of the protein spectrum.

In the aromatic spectral region, the prominently downfield-shifted signals R1 and R2 move upfield and broaden; signal R1 was previously assigned (MacLachlan et al., 1990) to Phe-77, and R2 to Phe-153. Changes are also noted for other components of these spin systems, namely R3, R5 (Phe-77) and R7, R5 (Phe-153); however, these are partly obscured by drug signals. The signals R8, R9, previously assigned to Phe-24 I12,6 and Phe-27 I12,6 broaden and shift downfield, and R11 (Phe-74 H2,6) shifts downfield from 6.59 ppm to ~6.8 ppm. In addition, intensity due to bepridil appears in the spectral envelope corresponding to signals R4 and R5.

Some increase in dispersion of the downfield-shifted α-proton resonances A1-A4 is observed. Two of these signals have been assigned to Glu-76 Ha and Tyr-111 Ha, which are in the limited regions of β-sheet structure which are present in both domains of the protein. These regions of β-sheet structure, between adjacent calcium-binding loops, are a common feature of the calcium-binding proteins such as S1TnC, CaM, intestinal calcium-binding protein, and parvalbumin. They appear to be an essential feature for the stability of the calcium-binding domains (Babu et al., 1987). This could imply subtle drug-induced changes in the polypeptide backbone conformation, which may be significant in view of the proximity of one of the perturbed regions to the low affinity calcium site.

Some of the most striking bepridil-induced changes occur
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FIG. 3. 360 MHz 'H NMR spectra at 25 °C of 2.5 mM calcium-saturated cTnC in 'H2O, 100 and 50 mM with respect to KCl and potassium phosphate at a pH meter reading of 7.4. The resolution of each spectrum has been enhanced by a convolution difference procedure using the manufacturer's software. Successive traces correspond to the following bepridil/cTnC ratios: 0.01:1; 0.10:1; 0.20:1; 0.35:1; 0.50:1; 0.65:1; 0.80:1; 1.00:1; 1.25:1; 1.50:1. Starred signals are due to some resonances of bepridil. Selected signals which shift during the titration are marked with dashed lines.

in the spectral region corresponding to the terminal methyl groups of methionine residues. Signals M1, M4 and M5, and M7 broaden dramatically and shift in the very earliest stages of the titration, corresponding to bepridil/cTnC ratios of about 0.2:1, although M2, M3, and M6 are little affected. M1 and M3 have been assigned to either Met-81 CH₃ or Met-157 CH₃. The marked broadening observed for several of these resonances implies immobilization of the methionine side chains as a result of contact with the drug.

The most evident changes in the aliphatic methyl region are associated with the envelope of signals T2 and T3. These have been associated with the N-terminal domain and tentatively assigned to Leu-41 δ-CH₃ and Ile-36 δ-CH₃. Signal T2 moves considerably upfield and only slightly broadens, while signal T3 moves downfield into the aliphatic methyl envelope.

All the spectral changes induced by bepridil in the protein spectrum are characteristic of fast or intermediate exchange processes on the 'H chemical shift time scale corresponding to rates of about 10⁻¹⁰ s⁻¹; observed chemical shifts appear to be weighted averages of their values in the free and bound states. The chemical shifts of selected resonances (R1, R2, and T2) are plotted against drug/protein ratio in Fig. 4. A smooth change in chemical shift as a function of added bepridil is observed, reaching a limiting value near the 1:1 drug/protein ratio. Concentrations of bepridil-bound protein, P_b, may be calculated from the known total protein concentration, P_t, using the relationship

\[ \Delta_{\text{obs}} = P_b \Delta_{\text{obs}} / P_t \]

\( \Delta_{\text{obs}} \) is the observed change in chemical shift of a specific
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Figure 4. Dependence of the chemical shifts (in ppm) of selected protein resonances on cTnC/bepridil molar ratio. The concentration of the calcium-saturated protein was 2.5 mM throughout the titration. A, signal T2 (Ile-36 or Leu-41). B, signal R1 (O) (Phe-77) and signal R2 (■) (Phe-153).

protein is induced by a given total bepridil concentration, $B_h$ and $\Delta \delta_h$ is the limiting chemical shift change for saturating amounts of bepridil. Both $\Delta \delta_{obs}$ and $\Delta \delta_h$ for signal T2 were measured from Fig. 4. $K_D$ may be calculated from the mass action equation, expressed as

$$K_D = \frac{[P_1 \cdot P_2 - P_1]}{[P_1 \cdot P_2 - P_2]}$$

which is based on the assumption that the concentration of bound bepridil equals that of bound protein. We estimate a $K_D$ of ~20 $\mu$M, with an associated error of about 50%.

Titration of bepridil into calcium-free cI'nC, or dicalcium-cTnC, produced no significant chemical shift changes, and only a slight generalized broadening (results not shown) indicating that the drug binding is considerably weaker in these cases.

Construction of a Model of the Bepridil-cTnC (Ca$^{2+}$) Complex

The model of cI'nC, based on CaM and sTnC x-ray structures and NMR data, (MacLachlan et al., 1990) was used to interpret the results of the bepridil-binding study. The construction of the drug-protein complex was guided by a consideration of several factors.

(i)—The results of the stopped-flow kinetics studies indicate that bepridil appears to have an effect only on the N-terminal, low affinity calcium site.

(ii)—The $^1$H NMR studies show that bepridil binds most strongly to calcium-saturated cTnC, and with lower affinity to the dicalcium or calcium-free protein, thus indicating that high affinity binding of bepridil requires the N-terminal calcium site to be occupied.

(iii)—Most of the resonances which are strongly perturbed by bepridil binding have been assigned to specific residues in the N-terminal domain of cTnC or have been generally associated with that domain.

(iv)—Several of the most striking changes in the $^1$H NMR spectrum observed when bepridil is added involve the immobilization of methionine-terminal methyl groups. The CD results suggest that there are no major conformational changes in the protein when the drug binds; thus the observed immobilization of these residues is most likely to be a result of direct contact of the side chains with the drug itself, with a consequent reduction in side chain mobility. In a similar study of drug binding to the homologous protein CaM, it was shown using paramagnetically labeled analogues, that methionine methyl signals broadened by the interaction are in fact at or near the binding site (Reid et al., 1990).

(v)—A number of studies of CaM (see for example, Prozi-aleck and Weiss, 1982; Mauet and Cox, 1983) and sTnC (Drabikowski et al., 1985; Gariepy and Hodges, 1983) support the notion that hydrophobic patches on the proteins' surfaces exposed by calcium-induced conformational changes provide binding sites for amphipathic drugs and peptides.

Taking these factors into consideration, the model of cTnC was examined for possible binding sites for a hydrophobic drug, such as bepridil. Gross structural differences are obvious between the hydrophobic structures which are exposed to solvent in each domain: the hydrophobic region of the N-terminal domain is extensive, and forms a distinct pocket or cleft, whereas the one in the C-terminal domain is more limited in extent, rather more exposed to solvent, and is more of a "patch" than a "pocket" in structure. This is clearly shown in Fig. 5A.

Because most bepridil signals are overlapped by protein resonances, there is no direct evidence to indicate the conformation of the bound drug. A stereochemically reasonable energy minimized conformation of the R enantiomer of the drug was therefore used.

Bepridil was then docked interactively with the hydrophobic surfaces of the protein with minimal clash of van der Waals' surfaces and with such an orientation that the aromatic rings protruded into the hydrophobic cavity. It is obvious that the N-terminal cleft provides the drug with much more opportunity for hydrophobic bonding than the C-terminal patch. Two stereo views of the drug-protein complex are shown in Fig. 5. In the absence of reliable drug-protein nuclear Overhauser effects, it is only appropriate to construct a relatively low resolution model of the complex, without defining close contacts between specific residues and individual drug protons. However, from the docking procedure it is obvious which protein side chains are likely to participate in drug binding.

Excision of a "shell" of ~9 Å radius about the docked bepridil reveals more clearly the residues most likely to interact with the drug (Fig. 5B). The residues which line the hydrophobic cleft include Phe-27, Phe-77, Met-45, Met-60, Met-81, Met-85, Val-64, Cys-84, and Lys-92. A second sphere of residues, not in direct contact with the drug but sufficiently close to be affected, comprises Phe-20, Phe-24, Ile-36, Leu-41, Leu-57, Ile-61, Met-80, and Val-82 inter alia.

This model is consistent with several features of the NMR titration. Signals assigned to Phe-24, Phe-27, and Phe-77 broadened and shift; the broadening is consistent with a reduction in mobility, and the chemical shift changes may be influenced by ring currents from aromatic rings on the drug. Signals due to Phe-74 also shift downfield, and although the model suggests that this residue is not directly involved in the binding site, it also shows that it is possible that changes in the orientation of Phe-77 could be transmitted to Phe-24 and Phe-74 inter alia, resulting in the chemical shift perturbations.

Four methionine methyl signals, M1, M4, M5, and M7, are affected by the complexation with the drug. Of these, only M1 has been assigned to either Met-81 or Met-157 on the
The bepridil-cTnC model shows that Met-45, Met-60, Met-81, and Met-85 are part of the hydrophobic pocket and are in close proximity to the bound drug. This makes the assignment of M1 to Met-81 very likely.

Similarly, the upfield-shifted methyl groups, T2 and T3, have been assigned to Leu-41 and Ile-36. T2 shifts prominently upfield with negligible further broadening. Ile-36 is close to and shielded by Phe-24 and Phe-77, thus it is possible that the changes in orientation in Phe-77 on binding the drug are mirrored by changes in chemical shift of signals tentatively assigned to this residue.

Although the majority of perturbed resonances are associated with the N-terminal domain, some C-terminal signals also undergo some changes, in particular those due to Phe-153. In this domain Phe-104, Phe-153, Phe-156, and Met-157 form a distinct hydrophobic cluster, and it is possible that some weak bepridil binding occurs here. On the other hand, it is conceivable that the few changes definitively ascribable to the C terminus may be a result of conformational changes transmitted along the α-helical sequence linking this with the N terminus.

The increase in dispersion of the signals A1 to A4, which are assigned to α-protons in the β-sheet regions of the protein, is significant. These residues are part of the calcium binding regions of the protein, and furthermore are distant from the proposed drug-binding site. Thus, chemical shift changes are most likely due to binding-induced conformational changes rather than contact with the bound drug. These conformational changes probably influence the affinity of the protein for calcium, and are hence the basis of bepridil’s calcium-sensitizing properties. Unfortunately, we have not been able to model these changes in detail as yet. The CD results, in contrast to NMR, provide direct evidence for an increase in α-helicity of the protein. The increase in α-helical content observed in CaM when several peptides bind has already been referred to. The propensity for linear peptides to form α-helices is increased when they are incorporated into a hydrophobic environment such as model lipid membranes or non-aqueous solvents (Inagaki et al., 1989). This effect is presumably due to the preference, in the latter environments, to form internal hydrogen bonds, rather than to hydrogen bond with the solvent exterior. Thus, it is conceivable that the binding of a hydrophobic drug to cTnC, and concomitant displacement of bound solvent molecules, might cause a rearrangement of the hydrogen bond network in favor of α-helix formation.

Comparison with Drug Binding to Homologous Proteins—
The interactions of hydrophobic and amphiphilic ligands with calmodulin and sTnC have been the subjects of numerous investigations (Reid et al., 1990, and references cited therein). The results are consistent with the existence of hydrophobic regions on the proteins, exposed by calcium chelation, which interact with complementary regions on the ligands. Binding of these compounds may also be favored by charge interactions between the numerous carboxylate side chains on the protein and cationic groups on the ligands. Many compounds also appear to display a propensity to interact with both N- and C-terminal domains of the protein, either independently, as in the case of smaller ligands such as trifluoperazine, or, possibly, simultaneously, as exemplified by larger peptides.
and the calmodulin antagonist calmidazolium. The salient features of such small molecule interactions are typified by recent models of calmidazolium analogue (Reid et al., 1990) and trifluoperazine (Strynadka and James, 1988) complexes with calmodulin.

Our model building predicts the existence of analogous hydrophobic areas on both domains of cTnC. NMR results are consistent with an interaction scheme for bepridil similar to other hydrophobic drug binding to CaM and sTnC and involving homologous protein residues. The strong preference of bepridil toward the N-terminal domain of cTnC may be explained on the grounds of the very different overall shape of the hydrophobic sites on each domain, well conveyed by Fig. 5A. The N-terminal bepridil-binding site is an extensive and capacious pocket, in contrast to the C-terminal site which is much shallower and smaller. This difference between the two putative sites in cTnC is much more marked than in the case of CaM. We suspect this feature could provide a useful basis for rational design of novel cardiac-specific calcium-sensitizing agents.

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