Solution Structure of Human Cardiac Troponin C in Complex with the Green Tea Polyphenol, (−)-Epigallocatechin 3-Gallate*5

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Heart muscle contraction is regulated by Ca2+ binding to the thin filament protein troponin C. In cardiovascular disease, the myofilament response to Ca2+ is often altered. Compounds that rectify this perturbation are of considerable interest as therapeutics. Plant flavonoids have been found to provide protection against a variety of human illnesses such as cancer, infection, and heart disease. (−)-Epigallocatechin gallate (EGCg), the prevalent flavonoid in green tea, modulates force generation in isolated guinea pig hearts (Hotta, Y., Huang, L., Muto, T., Yajima, M., Miyazeki, K., Ishikawa, N., Fukuwara, Y., Wakida, Y., Tushima, H., Ando, H., and Nonogaki, T. (2006) Eur. J. Pharmacol. 552, 123–130) and in skinned cardiac muscle fibers (Liou, Y. M., Kuo, S. C., and Hsieh, S. R. (2008) Pflugers Arch. 456, 787–800; and Tadano, N., Yumoto, F., Tanokura, M., Ohtsuki, I., and Morimoto, S. (2005) Biophys. J. 88, 314A). In this study we describe the solution structure of the Ca2+-saturated C-terminal domain of troponin C in complex with EGCg. Moreover, we show that EGCg forms a ternary complex with the C-terminal domain of troponin C and the anchoring region of troponin I. The structural evidence indicates that the binding site of EGCg on the C-terminal domain of troponin C is in the hydrophobic pocket in the absence of troponin I, akin to EMD 57033. Based on chemical shift mapping, the binding of EGCg to the C-terminal domain of troponin C in the presence of troponin I may be to a new site formed by the troponin C-troponin I complex. This interaction of EGCg with the C-terminal domain of troponin C-troponin I complex has not been shown with other cardiotonic molecules and illustrates the potential mechanism by which EGCg modulates heart contraction.

Cardiovascular disease (CVD)2 is the number one cause of morbidity and mortality in western culture. In the United States, ~1 in 3 deaths in 2004 were caused by CVD (1). In heart failure, the ability of the heart to distribute blood throughout the body is perturbed, and there is a growing interest to develop drugs that directly regulate the response of the myofilament to Ca2+. Regulation of muscle contraction is triggered by Ca2+ binding to troponin. The troponin complex is situated at regular intervals along the thin filament, which is made up of two elongated polymers, f-actin and tropomyosin. The backbone of the thin filament is composed of actin molecules arranged in a double helix with tropomyosin wound around actin as a coiled-coil. Anchored at every seventh actin molecule is the heterotrimeric troponin complex, which consists of troponin C (TnC), troponin I (TnI), and troponin T (TnT). TnC is the Ca2+-binding subunit of troponin and has four EF-hand helix-loop-helix motifs. TnI is the inhibitory subunit of troponin. It regulates the actin-myosin cross-bridge formation by flipping between TnC and actin in a Ca2+-dependent manner. At low levels of cytosolic Ca2+, TnI is bound to actin, causing tropomyosin to sterically block the binding of the actomyosin cross-bridges. On the other hand, when Ca2+ concentration is high, TnI translocates from actin to TnC inducing tropomyosin to change its orientation on actin so that the actin-myosin interaction may occur. The subunit TnT fetters the troponin complex to the thin filament by way of its association with TnI (for reviews on contraction see Refs. 2–5).

The large number of structural studies on troponin and the thin filament has helped gain insight into the molecular mechanism of muscle contraction. TnC is a dumbbell-shaped protein that consists of terminal domains connected by an elongated flexible linker, as shown by solution NMR (6). The overall folds of the terminal domains of skeletal TnC (sTnC) and cardiac TnC (cTnC) are very similar (7–9). The apo state of the N-domain of sTnC (sNTnC) and cTnC (cNTnC) reveals that the domain is in a “closed” conformation, such that the hydrophobic core of the protein is buried (8, 10, 11). The overall folds of the terminal domains of skeletal TnC (sTnC) and cardiac TnC (cTnC) are very similar (7–9). The apo state of the N-domain of sTnC (sNTnC) and cTnC (cNTnC) reveals that the domain is in a “closed” conformation, such that the hydrophobic core of the protein is buried (8, 10, 11). The overall folds of the terminal domains of skeletal TnC (sTnC) and cardiac TnC (cTnC) are very similar (7–9). The apo state of the N-domain of sTnC (sNTnC) and cTnC (cNTnC) reveals that the domain is in a “closed” conformation, such that the hydrophobic core of the protein is buried (8, 10, 11).

Alternatively, cNTnC contains only one functional Ca2+-binding site, and its global conformation does not change as significantly as in sNTnC (11). Nonetheless, Ca2+ binding promotes the association of the switch region of cTnI (residues 147–163) with cNTnC. cTnI-(147–163) forms an α-helix when associated with cNTnC and has been elucidated by NMR in the solution structure of cNTnC-Ca2+-cTnI-(147–163) (12) and by the x-ray crystallography structure of cTnC3Ca2+-cTnI-(31–210) cTnT-(183–288) (13). The interaction of cTnI-(147–163) with cNTnC-Ca2+ is essential to draw the inhibitory (cTnI-(128–147)) and C-terminal (cTnI-(163–210)) regions of cTnI away from actin. cTnI-(128–147) is not visualized in the car-

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cTnC is Ca^{2+} interaction of cTnC. Drugs are safer than other currently prescribed medicines if they positively or negatively influence its function. These include EMD 57033 acts as a Ca^{2+} channel blocker. The Ca^{2+} binding of cTnC is also a suitable target for the development of therapeutic agents. The Ca^{2+} binding of cTnC has been shown to bind cTnC stereospecifically. The (−)-enantiomer is inactive (22). Because EMD 57033, EMD 57033 is associated in the hydrophobic cavity of cTnC. The C-domain of both sTnC and cTnC has two functional binding sites for Ca^{2+} and remains largely unstructured without Ca^{2+} bound. The folding of this domain occurs in the presence of Ca^{2+} (15, 16).

Throughout the relaxation-contraction cycle, cTnC is Ca^{2+}-saturated with both Ca^{2+}-binding sites occupied (cTnC:Ca^{2+}) and is associated with the anchoring region of cTnI (cTnI-(34–71)). The crystal structure of cTnC:Ca^{2+}-cTnI-(31–210):cTnT-(183–288) shows cTnI-(34–71) is α-helical when bound with cTnC:Ca^{2+} (13). The interaction of cTnC:Ca^{2+} with cTnI-(34–71) is the primary site in which cTnC is tethered to the thin filament.

In light of the importance of the Ca^{2+}-dependent cTnI-cTnC interaction in the signaling of muscle contraction, the design of drugs that modulate this interaction would be useful in the treatment of heart disease. Compounds that target cTnC through modulation of the activity of cTnC are called Ca^{2+} sensitizers or desensitizers, depending on whether they positively or negatively influence its function. These drugs are safer than other currently prescribed medicines that alter the cytosolic Ca^{2+} homeostasis (such as milrinone and dobutamine), which may cause arrhythmia or death with prolonged usage.

The potential therapeutic advantage of Ca^{2+} (de)sensitizers has led to the development of a number of compounds that target cTnC. Compounds have been identified that elicit their activity through binding either cNTnC or cCTnC. Levosimendan and pimobendan are examples of molecules that increase heart muscle contractility through binding to cNTnC. Conversely, the molecule W7 decreases contractility via its interaction with cNTnC. For recent reviews on the molecular mechanism of these compounds and others see Refs. 17–19.

The discovery of small molecules that bind to cCTnC to elicit their Ca^{2+}-sensitizing effects suggests that cCTnC is also a suitable target for the development of therapeutics. The Ca^{2+} sensitizing, EMD 57033, is approved for the treatment of heart failure in dogs and binds to cCTnC:Ca^{2+} (20). In the NMR structure of cTnC:Ca^{2+:EMD 57033, EMD 57033 is associated in the hydrophobic cavity of cTnC:Ca^{2+} (21). The interaction of EMD 57033 with cTnC is stereospecific for the (−)-enantiomer and explains why the (−)-enantiomer is inactive (22). Because EMD 57033 has been shown to bind cTnC:Ca^{2+} concurrently with cTnI-(128–147) but not with cTnI-(34–71) (23), one postulate is that EMD 57033 acts as a Ca^{2+} sensitizer by weakening the interaction of cTnI-(34–71) with cTnC:Ca^{2+}, thus increasing the propensity of cTnI-(128–147) to bind cTnC:Ca^{2+} in vivo. The diluted cardiomyopathy (DCM) mutation, G159D, of cTnC has renewed interest in the role of the C-lobe for regulation in contraction. The mutation has been identified to decrease the sensitivity of the thin filament to Ca^{2+} (24).

The structure of the DCM phenotype of G159D might come from the modulation of the interaction of cTnC:Ca^{2+} with cTnI-(34–71) (25).

Green tea (Camellia sinensis) is one of the most widely consumed beverages in the world, and several epidemiological studies have linked the consumption of tea with a decrease in CVD (26, 27). (−)-Epigallocatechin gallate (EGCg) is a polyphenol that exists abundantly in unfermented teas and has been identified as a modulator of heart contraction through its interaction with cTnC (28–30). Here we use NMR spectroscopy to elucidate the three-dimensional structure of the cTnC:Ca^{2+}:EGCg complex. The solution structure reveals that EGCg binds to the hydrophobic core of cTnC inducing a small structural “opening.” We also use two-dimensional NMR spectroscopy to monitor the binding of EGCg to cTnC:Ca^{2+} and cTnC:Ca^{2+}:cTnI-(34–71). Because EGCg and cTnI-(34–71) can bind cTnC concurrently, the inotropic effect of EGCg may stem from its modulation of the cTnI-(34–71)-cTnC:Ca^{2+} interaction. The solution structure of cTnC:Ca^{2+}:EGCg provides insight into the mechanism in which EGCg might influence heart contraction. These results taken with previous research on the Ca^{2+} sensitizer EMD 57033 and the DCM mutation G159D bring into question the dogma that cNTnC is the exclusive site for regulation of contraction in cTnC.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—The expression vectors for cTnC-(91–161) and cTnI were designed, and the uniformly labeled 13C,15N-cTnC was isolated from Escherichia coli as described previously (31, 32). Unlabeled cTnI-(34–71), acetyl-AKKSKISASRKLQLKTLLQIAKQLELAAERREGEK-amide, was synthetically prepared by GL Biochem Ltd. EGCg was purchased from Sigma. All stock solutions of EGCg were prepared in 70–80 mM TCEP in aqueous solution or DMSO-d$_6$ (Cambridge Isotopes Inc.) at a concentration of ~100 mM. The solvents TCEP and DMSO-d$_6$ did not influence the interaction of EGCg with cTnC. The stock solutions were freshly prepared before any experiment was acquired, and during the titrations the stock solutions of EGCg were kept dark to prevent light-catalyzed degradation. The 500-μl NMR samples were prepared with 15N- or 13C,15N-labeled TnC in 5% D$_2$O, 2.5 mM 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (Chenomix standard), pH 6.7–6.9, 100 mM KCl, 8 mM CaCl$_2$, and 10 mM imidazole as a buffer. Samples of cTnC:Ca^{2+}:cTnI-(34–71) contained an ~1:1 ratio of cTnC:Ca^{2+}:cTnI-(34–71). All experiments run for the structure calculation contained 13C,15N-cTnC:EGCg at a ratio of ~1:4.

**Stability of EGCg in Aqueous Solution**—Certain steps had to be taken when working with EGCg, because it is rapidly oxidized in aqueous solution. For most of the experiments, TCEP was used to keep EGCg reduced for the duration of the longer three-dimensional experiments (4–5 days). TCEP has been shown to be an adequate reducer of ascorbic acid (33). For some of the shorter experiments, the sample was simply flushed with Ar$_2$ gas prior to the addition of EGCg. Titrations with EGCg were all initially done with no TCEP present and then repeated with TCEP. This confirmed that TCEP had no influence on the interaction of EGCg with cTnC (data not shown). To monitor sample stability, 1H,15N HSQC spectra were acquired before and after each long three-dimensional experiment. In cases
where degradation of EGCg was witnessed, the solution first began to change from clear to a brownish hue, and eventually, precipitate started to form at the bottom of the NMR tube. In this stage the $^1$H,$^{15}$N HSQC spectrum revealed a slight recession of the amide correlation peaks toward the unbound chemical shifts of cCTnC. In addition to the visual cues of EGCg degradation, one-dimensional $^1$H NMR spectra were used to monitor the transition of EGCg from its reduced form to the oxidized state. When EGCg was oxidized, additional peaks began to appear in the spectrum, and peaks representative of the reduced form of EGCg decreased in intensity (data not shown). Fig. 2 shows the chemical structure and one-dimensional $^1$H NMR spectrum of 1.1 mM EGCg in DMSO-$_d_6$.

**Titrations of EGCg to cTnC, cCTnC, and cCTnCcTnI**—All NMR samples contained 500 µl of aqueous NMR buffer (see under “Sample Preparation”). The pH was adjusted with NaOH when necessary. EGCg concentration was determined by amino acid analysis and tyrosine absorption at 280 nm. Protein and EGCg concentrations were corrected for the dilution factor. The stock solutions of EGCg were prepared in DMSO-$_d_6$ or in an aqueous TCEP buffer. TCEP was used to keep EGCg reduced during the titration. The pH was adjusted with NaOH when necessary. EGCg concentration was determined by weight and by comparing EGCg peak heights or integrals with the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt in the one-dimensional $^1$H NMR spectrum. EGCg was titrated into a 0.45 mM $^{15}$N-cTnC NMR sample to final EGCg concentrations at each step of 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.79, 0.99, 1.19, 1.57, and 2.53 mM. EGCg was titrated into a 1.13 mM $^{15}$N-cCTnC NMR sample to final EGCg concentrations at each step of 0.13, 0.25, 0.37, 0.50, 0.61, 0.86, 1.22, 1.57, 2.38, 3.16, and 4.23 mM. EGCg was titrated into a 0.35 mM $^{15}$N-cCTnCcTnI (34–71) NMR sample to final EGCg concentrations at each aliquot of 0.18, 0.36, 0.71, 1.42, 2.14, 2.90, and 4.12 mM. EGCg was titrated into a 80 µM $^{15}$N-cCTnC NMR sample to final EGCg concentrations at each step of 8, 17, 25, 32, 41, 49, 57, 65, 83, 98, 113, 143, 218, 293, 378, 443, 578, and 713 µM.

**NMR Spectroscopy**—Most NMR experiments were collected on either a Varian Inova 500-MHz or a Unity 600-MHz spectrometer. All data were collected at 30 °C. Both spectrometers have triple resonance HCN probes and z-pulsed field gradients. The titration of EGCg to 80 µM $^{15}$N-cCTnC was done at 25 °C on a Varian Inova 800-MHz equipped with a cryogenic probe. The supplemental Table 1 lists the experiments acquired for this work.

**Data Processing and Peak Calibration**—All two- and three-dimensional NMR data were processed with NMRPipe (34). One-dimensional NMR spectra to assign EGCg in DMSO-$_d_6$ and address EGCg degradation were processed using VNMRJ (Varian Inc.) Assignment of chemical shifts was carried out in NMRView (35), and backbone assignments were aided with the software package SmartNotebook (36). Intramolecular NOEs of EGCg measured from two-dimensional NOESY acquired in D$_2$O were categorized as strong (1.8 – 4.0 Å), medium (1.8 – 5.0 Å), and weak (1.8 – 6.0 Å). Intermolecular NOEs were categorized as strong (1.8 – 4.0Å), medium (1.8 – 5.0 Å), and weak (1.8 – 6.0 Å). Intramolecular NOEs of cCTnC were calibrated automatically with the CYANA standard procedure, with upper bounds set to 6 Å. The chemical shifts that were assigned in NMRView were converted to CYANA nomenclature for NOE calibration. After the CYANA refinement, the final restraints were converted to XPLOR-NIH nomenclature. Because CYANA calculates distances differently than XPLOR-NIH, NOE restraints were loosened by 1 Å.

**Generation of EGCg Structure File for Structure Refinement**—The structure file of EGCg was generated for XPLOR-NIH by the PRODRG2 webserver (37). This on-line resource converts the chemical structure of a small ligand into PDB format. Following PDB conversion, XPLOR2D was used to generate XPLOR-NIH compatible structure files of EGCg.

**Structural Calculation**—Structures were initially generated using the program CYANA 2.1 (38–40). Unambiguous restraints were assigned manually and were forced to keep their assignments during the first four runs of CYANA calculations, after which they were open for automatic assignment with the “NOEassign” command of CYANA (41). Distance restraints were calibrated with CYANA standard procedure using upper limits of 6 Å. Dihedral angle restraints from TALOS (42) were used as well as 12 distance restraints from x-ray crystallographic data of chelating oxygen atoms to the two Ca$^{2+}$ ions. CYANA was used to calculate 100 structures, of which the 30 conformers with the lowest target function were used to further refine the structure. The 30 conformers were averaged in X-PLOR-NIH and used as a template structure in the simulated annealing protocol, with 10,000 high temperature steps and 6000 cooling steps. After the structure of cCTnC was well defined, the binary cCTnC$^{2Ca^{2+}}$-EGCg structure was solved in a similar manner, starting with an extended conformation of cCTnC. The calculations contained 10 intramolecular EGCg NOE restraints and 18 intermolecular NOE restraints. The final ensemble discussed in this study is represented by the 30 lowest energy structures of the 100 calculated (see Table 1 for statistics). The final refined ensemble has been deposited in the Protein Data Bank with the accession code of 2kdh.

**RESULTS**

Two-dimensional $^1$H,$^{15}$N HSQC and $^1$H,$^{13}$C HSQC NMR experiments were used to monitor the binding of EGCg to cTnC$^{3Ca^{2+}}$, cCTnC$^{2Ca^{2+}}$, and cCTnC$^{2Ca^{2+}}$cTnI (34–71). The HSQC experiment correlates backbone or side chain $^1$H with $^{15}$N or $^{13}$C nuclei, so that each cross-peak in the spectrum represents an individual $^1$H-$^{15}$N (or $^1$H-$^{13}$C) from the $^{12}$C,$^{15}$N-labeled protein. Typically, when a ligand binds a labeled protein, the chemical shifts of individual cross-peaks in the spectrum move as a function of the ligand concentration. The changes in chemical shifts can be quantified to derive ligand stoichiometry and affinity, as well as be used to approximate the ligand-binding site on the protein, an approach commonly referred to as chemical shift mapping.

**Effect of EGCg on cTnC$^{3Ca^{2+}}$**—EGCg was titrated into cTnC$^{3Ca^{2+}}$ to assess its primary binding site. The backbone of EGCg-free cTnC$^{3Ca^{2+}}$ was assigned by the three-dimensional CBCACONNH and HNCA CB NMR experiments, and well resolved amide resonances in the $^1$H,$^{15}$N HSQC spectrum...
Structure of Cardiac Troponin C Bound to EGCg

The binding curves that were globally fit are shown in supplemental Fig. 1B. The binding of EGCg to cTnC-3Ca$^{2+}$ was fit to a 1:1 stoichiometry, with a best fit $K_D$ of 1.12 mM. The $K_D$ value was also calculated by averaging the normalized individual chemical shifts as a function of the ligand to protein ratios and fitting using xcrvfit. This approach yielded a $K_D$ of 1.1 ± 0.12 mM. The observation that EGCg targets the C-terminal domain of cTnC focused our subsequent structural analysis to cTnC.

Effect of EGCg on cCTnC-2Ca$^{2+}$—

The assigned $^1$H,$^1$N HSQC and $^1$H,$^1$C-HSQC NMR spectrum of cTnC-2Ca$^{2+}$ was used to observe the binding of EGCg to cCTnC-2Ca$^{2+}$ (Fig. 1, $a$ and $b$). The $^1$H,$^1$N HSQC spectrum of cTnC-2Ca$^{2+}$ has been previously assigned (21, 44), and the $^1$H,$^1$N HSQC and $^1$H,$^1$C HSQC spectra of the cCTnC-2Ca$^{2+}$-EGCg complex were assigned using three-dimensional CBCACONH and HNCACB NMR spectra. Because the resonance perturbations of cTnC are in fast exchange, the chemical shifts could be easily followed throughout the titration. The chemical shift of each assigned amide peak was recorded for every titration point, and a total chemical shift was normalized for each resonance. A global fitting approach using the program xcrvfit was used to determine the $K_D$ value that best fit Reaction 2,

cCTnC-2Ca$^{2+}$ + EGCg ↔ cCTnC-2Ca$^{2+}$-EGCg

REACTION 2

The binding curves that were globally fit are shown in Fig. 1c. The binding of EGCg to cCTnC-2Ca$^{2+}$ was fit to a 1:1 stoichiometry, with a best fit $K_D$ of 1.10 mM. The $K_D$ value was also calculated by averaging the normalized individual chemical shifts as a function of the ligand to protein ratios and fitting using xcrvfit. This approach gave a $K_D$ of 1.09 ± 0.08 mM. Given EGCg binding to the C-terminal domain of cTnC-3Ca$^{2+}$ and to cTnC-2Ca$^{2+}$ induced effectively identical chemical shift changes and dissociation constants, we concluded that EGCg binds both the C-terminal domain of cTnC-3Ca$^{2+}$ and cTnC-2Ca$^{2+}$ in a similar fashion.

The dissociation constant was significantly higher than previous groups reported for EGCg (28). To investigate whether...
Structure of Cardiac Troponin C Bound to EGCg

there were concentration-dependent effects of EGCg, such as aromatic stacking, we lowered the protein concentration to 80 μM and repeated the titration. The data gave a best fit $K_d$ of 385 μM, which indicates that EGCg is undergoing a competing equilibrium in aqueous solution. Therefore, the observed or apparent $K_d$ value is an upper limit of the actual dissociation constant. The chemical shift changes were identical in both the low and high concentration titrations, suggesting that the location of the binding site of EGCg is independent on cCTnC concentration.

A number of cCTnC·2Ca$^{2+}$·1H,15N and 1H,13C cross-peaks underwent significant chemical shift perturbations during the EGCg titration. Chemical shift mapping of the amide peaks was used to identify specific regions of the protein that underwent large perturbations induced from EGCg binding to localize the binding site of EGCg (Fig. 1d). The amide resonances of residues on the loop connecting helices F and G (Thr$^{124}$–Thr$^{129}$) underwent the largest changes in chemical shifts, suggesting a close proximity of the ligand to their backbone and side chain nuclei or a large change of conformation or dynamics in the loop. This may reflect a change in the positions of helices F and G relative to one another upon formation of the EGCg-cCTnC complex. Other residues of cCTnC that had significant backbone chemical shift perturbations were residues Met$^{120}$, Leu$^{121}$, Glu$^{122}$, and Ala$^{123}$ of helix F, residues Leu$^{136}$, Gly$^{140}$, and Asp$^{141}$ of helix G, and residues Met$^{157}$, Lys$^{158}$, Gly$^{159}$, Val$^{160}$, and Glu$^{161}$ of helix H. Amide and methyl resonances of residues on the β-sheet (Tyr$^{111}$, Ile$^{112}$, Asp$^{113}$, Arg$^{147}$, Ile$^{148}$, and Asp$^{149}$) did not undergo any significant chemical shift changes. This suggests that the binding of EGCg is near the opening of the hydrophobic cleft of cCTnC, rather than deep within the pocket. To obtain a more detailed knowledge of the interaction of cCTnC with EGCg, the solution structure of cCTnC·2Ca$^{2+}$·EGCg was determined by NMR spectroscopy.

Structure of cCTnC·2Ca$^{2+}$·EGCg—The solution structure of cCTnC·2Ca$^{2+}$·EGCg was determined using the NMR experiments listed in supplemental Table 1 (see references for experiments therein). Dihedral angle restraints were calculated from chemical shifts with the program TALOS (42). The chemical shifts corresponding to backbone atoms of cCTnC in the cCTnC·2Ca$^{2+}$·EGCg complex were assigned using the two-dimensional 1H,15N HSQC and the three-dimensional CBCA-COHNH and HNCACB NMR spectra. The two-dimensional 1H,13C-HSQC and the three-dimensional HCCCH-TOCSY, CCONH, and HCCONH NMR spectra were acquired to assign side chain resonances. The 15N-edited HNHA and HNHB experiments were acquired to assign Hα and Hβ resonances. Distance restraints for cCTnC were determined using 1H-NOESY HSQC and 15N-NOESY HSQC NMR spectra. Resonances for aromatic residues of cCTnC were assigned using the two-dimensional NOESY NMR spectrum in D$_2$O (Fig. 2). 13C/15N-edited/filtered experiments (two-dimensional and three-dimensional) were run to assign intermolecular NOEs between EGCg and cCTnC to identify the binding site and orientation of EGCg when bound to cCTnC·2Ca$^{2+}$. 12 Ca$^{2+}$ distance restraints from crystallographic data were incorporated into the structure determination as described previously (21). There were a total of 1053 structural restraints used in the structure determination, including 899 intramolecular cCTnC distance restraints, 18 intermolecular distance restraints, 10 intramolecular EGCg distance restraints, 12 Ca$^{2+}$ distance restraints, and 114 dihedral restraints. Table 1 contains a list of the structural statistics for cCTnC·2Ca$^{2+}$·EGCg.

Plots of the intermolecular NOEs between EGCg and cCTnC·2Ca$^{2+}$ are shown in Fig. 3a. To assign the intermolecular NOEs, both the three-dimensional and two-dimensional versions of the 13C/15N-edited/filtered experiment were acquired. The two-dimensional experiment was acquired because it provides a better signal-to-noise ratio than the three-dimensional experiment, and the 13C-edited three-dimensional experiment was run to confirm the two-dimensional assignments. The NOE restraints observed between EGCg and cCTnC·2Ca$^{2+}$ are between a number of hydrophobic residues that line the hydrophobic pocket. Residues that have NOEs to EGCg include Met$^{157}$ and Val$^{160}$ on the terminal end of helix H, Leu$^{121}$ and Met$^{120}$ on helix F, and Leu$^{136}$ on helix G, all of which point toward the hydrophobic pocket of cCTnC. The tetrahydrofuran ring of EGCg contains four hydrogen atoms (H01, H02, H15, and H33), all of which make significant contacts to cCTnC. The two hydrogen atoms on the benzenediol ring, on the other hand, do not make any NOE contacts to cCTnC, hence its loosely defined orientation. Fig. 3b depicts the specific observed NOEs between cCTnC residues and EGCg hydrogen atoms. There were no intermolecular NOEs observed between EGCg and the hydrophobic residues of the β-sheet (Ile$^{144}$ or Ile$^{112}$), indicating that EGCg is not buried deep in the hydrophobic pocket as is EMD 57033. Also, no NOEs were observed between EGCg and any residues along the loop between helices F and G, even though these residues had the greatest chemical shift perturbation (Fig. 1). The large perturbations of the loop residues could result from a proximity to the aromatic polypehlor rings of EGCg or from a change in the conformation or dynamics of the loop upon binding EGCg.

The ensemble of the 30 lowest energy structures of EGCg in complex with cCTnC·2Ca$^{2+}$ is depicted in Fig. 4, a and b. The ensemble of EGCg is shown in Fig. 4c, and the lowest energy structure of cCTnC·2Ca$^{2+}$·EGCg is shown in schematic representation in Fig. 4d. The overall fold of cCTnC is similar to that which has been previously described for cCTnC. There are four well defined helices as follows: helices E, F, G, and H. The two Ca$^{2+}$-binding sites (sites III and IV) are between helices E and F and G and H. There is a short twisted anti-parallel β-sheet that joins the two EF-hands. The r.m.s.d. of cCTnC for the backbone atoms of the well defined residues is 0.80 ± 0.14 Å. The well defined regions (r.m.s.d. < 1 Å) involve residues 94–122, 132–143, and 145–157. These regions include residues from helices E, F, G, and H as well as those of the Ca$^{2+}$-binding loops and anti-parallel β-sheet. The N and C termini as well as the interhelical F-G loop of cCTnC·2Ca$^{2+}$ had fewer structural
restraints, and hence had a larger r.m.s.d. in the ensemble. Given the rotational freedom of EGCg around the galloyl (ring B') and pyrogallol (ring B) moieties, there was significant mobility in the ensemble of EGCg (Fig. 4c). In addition to the rotational freedom of the trihydroxyphenyl rings, the pairs of hydrogen atoms on the rings (H39/H41 and H12/H4) are chemically equivalent, and so it was not possible to differentiate NOE contacts within the pairs. Nonetheless, the 10 intramolecular NOEs of the bound EGCg and the 18 intermolecular NOEs between EGCg and cCTnC:2Ca^{2+} positioned the three functional moieties of EGCg with reasonable precision. The galloyl moiety is positioned near the loop connecting helix F and helix G. The large chemical shift perturbations of the F-G loop residues (Fig. 1) could in part result from a propinquity to the gal-

FIGURE 2. Assignment of EGCg. a, chemical structure of EGCg. The benzenediol is labeled as ring A, the pyrogallol ring as B, the galloyl moiety as B', and ring C is the tetrahydropyran moiety. The hydrogen atoms attached to carbon atoms are also labeled. b, assigned one-dimensional $^1$H NMR spectrum of EGCg in DMSO-$d_6$. c, a few strip plots from the two-dimensional NOESY with resonances assigned that belong to EGCg in complex with cCTnC:2Ca^{2+}. The data were acquired in D$_2$O as to remove amide signals that predominate this region of the two-dimensional NOESY spectrum in H$_2$O. Details of the experiment are outlined in Table 1.
loyl trihydroxyphenyl ring. The pyrogallol ring rests near the C terminus of cCTnC, which explains the large chemical shift perturbations of helix H residues Met$^{157}$–Glu$^{161}$ during titration with EGCg (Fig. 1). The fused tetrahydropyran (ring C) and benzenediol (ring A) rings lie near the surface of helix E that faces the hydrophobic core of cCTnC-2Ca$^{2+}$.

**Comparison of Solution Structures** cCTnC-2Ca$^{2+}$ and cCTnC-2Ca$^{2+}$-EGCg—The solution structure of cCTnC-2Ca$^{2+}$-EGCg adopts a similar fold to other structures determined for cCTnC (6, 13, 21). The differences in the structures determined for cCTnC are all primarily the result of varying degrees of “openness,” in which the helices are spread out from the hydrophobic core of the protein. The degree in which cCTnC is open is described by the inter-helical angles between helices E and F and between helices G and H. The closer the inter-helical angles are to 90°, the more open the structure is. Inter-helical angles were calculated using the program interhlex (K. Yap, University of Toronto).

When the structure of cCTnC-2Ca$^{2+}$-EGCg was overlaid with the NMR structure of cCTnC-2Ca$^{2+}$ (6), a minor perturbation in the structure was observed. Between the two structures, an r.m.s.d. of 1.53 Å for backbone atoms of helices E–H was observed (Fig. 5, a and b). Notable differences between the structures include helix E, helix G, and helix H that are positioned slightly away from the core of the protein when compared with cCTnC-2Ca$^{2+}$. The E-F inter-helical angle of cCTnC-2Ca$^{2+}$ is 112°, and the G-H angle is 117°. The E-F inter-helical angle of cCTnC-2Ca$^{2+}$-EGCg is 105°, and the G-H inter-helical angle is 113°, revealing that the overall outcome of EGCg binding is a more open conformation of cCTnC.

The solution structure of cCTnC-2Ca$^{2+}$ bound to EMD 57033 has also been solved (21). When cCTnC-2Ca$^{2+}$-EGCg was overlaid with cCTnC-2Ca$^{4+}$-EMD 57033, an r.m.s.d. for the backbone atoms for the helices is 1.84 Å (Fig. 5, c and d). The inter-helical angles of the E-F and the G-H helices of cCTnC-2Ca$^{2+}$-EMD 57033 are 96 and 118°, respectively. The largest difference between the structures is in the positions of the G and F helices. In the EMD 57033-bound structure, the G helix is shifted nearer to the core of cCTnC (as in the unbound form of cCTnC-2Ca$^{2+}$). In contrast, helix F is further from the core of cCTnC in the EMD 57033-bound structure when compared with the EGCg-bound structure of cCTnC-2Ca$^{2+}$. The location of the two ligands is similar, with both drugs binding the core of the protein; however, EMD 57033 is buried deep within cCTnC, and EGCg remains near the surface of the opening of cCTnC. The methyl on the thiadiazinone ring of EMD 57033 makes several NOE contacts with Ile$^{148}$ and Ile$^{112}$ of the β-sheet. In the case of EGCg, the ring protons all make contacts exclusively to hydrophobic residues that line the surface of cCTnC.

The region of the crystal structure of the cardiac troponin complex (13) corresponding to cCTnC-2Ca$^{2+}$-cTnI-(34–71) was also overlaid with cCTnC-2Ca$^{2+}$-EGCg and gives an r.m.s.d. of 1.45 Å for the backbone atoms of the helix residues (Fig. 5, e and f). In accordance with the good agreement in r.m.s.d., the inter-helical angles of cCTnC in the cCTnC-2Ca$^{2+}$-cTnI-(34–71) complex also resemble the cCTnC in the cCTnC-2Ca$^{2+}$-EGCg complex; the E-F inter-helical angle is 100°, and the G-H inter-helical angle is 114°. Therefore, EGCg induces a conformational change in cCTnC most closely akin to that caused by cTnI-(34–71). The helices of cCTnC bound to cTnI-(34–71) are depicted in the schematic representation given in Fig. 6.
The binding of EGCg to cardiac troponin C (cTnC) occurs with a 1:1 stoichiometry, with a best fit reaction for the cTnI-(34–71)-bound form of cTnC. The orientation of cTnC is the same as in the cTnI-(34–71) complex. The methyl region of the 1H,13C-HSQC NMR spectra of cTnC and the backbone of cTnI-(34–71) occupy the same surface of cTnC. To test how this steric clash affects EGCg binding, we titrated EGCg into the cTnI-(34–71) saturated cTnC complex. The amide resonances that were most perturbed upon EGCg binding were used to determine the dissociation constant that best fits Reaction 3.

\[
\text{cTnC-2Ca}^{2+} - \text{cTnI-(34–71)} + \text{EGCg} \leftrightarrow \text{EGCg-cTnC-2Ca}^{2+}-\text{cTnI-(34–71)}
\]

**REACTION 3**

The binding curves that were globally fit are shown in Fig. 6c. The binding of EGCg to cTnC was fit to a 1:1 stoichiometry, with a best fit dissociation constant of 1.8 mM, about 1.5-fold weaker than the dissociation constant for EGCg binding to cTnC. The dissociation constant value was also calculated by averaging the normalized individual chemical shifts as a function of the ligand to protein ratios and fitting using xcrvfit. This approach gave a dissociation constant of 1.64 ± 0.24 mM.

The perturbation of 1H,15N cross-peaks was less than that for the binding of EGCg to cTnC alone, and the perturbed residues correspond to several isolated regions of cTnC. This made chemical shift mapping difficult to interpret, forestalling the localization of the binding surface of EGCg. 1H,13C-HSQC NMR spectra of cTnC and cTnI-(34–71) complex were also acquired during the EGCg titration to cTnC. The methyl region of the 1H,13C-HSQC NMR spectra is shown in Fig. 6b. Two or three regions of the protein experienced large chemical shift changes in the 1H,13C-HSQC NMR spectra as follows: near the E-H helix interface, along the F helix, and E-F loop.

The amide resonances that were most perturbed upon EGCg titration are mapped onto the structure of cTnC.
Structure of Cardiac Troponin C Bound to EGCg

Figure 6: Titration of cCTnC\(2Ca^{2+}\)-cTnl(34–71) with EGCg. Two-dimensional \(^1\)H,\(^{15}\)N HSQC (a) and \(^1\)H,\(^{13}\)C-HSQC (b) spectra arising from backbone and side chain amide groups (a) and side chain methyl groups (b) are overlaid for a series of EGCg additions. Each titration point represents the titration points described under "Experimental Procedures." The titration was made into \(^{13}\)C,\(^{15}\)N-labeled cCTnC\(2Ca^{2+}\)-cTnl(34–71), and both the \(^1\)H,\(^{15}\)N HSQC and \(^1\)H,\(^{13}\)C-HSQC spectra were acquired at each titration point. Assignments of some of the cross-peaks are labeled. The multiple contours (●) represent the initial point in the titration, with no EGCg added, and the open contours (○) represent the end point in the titration for a given residue. b, red contours represent cross-peaks with negative intensity, a feature of the constant time \(^1\)H,\(^{13}\)C-HSQC experiment. The direction that the peaks shift is indicated by arrows, for example see Met120. c, curves represent a number of residues affected by ligand binding, as shown in a. The curves were fit as a function of normalized total chemical shift perturbation versus [EGCg]total/[cCTnC\(2Ca^{2+}\)-cTnl(34–71)]total. d, cCTnC\(2Ca^{2+}\)-cTnl(34–71) complex is shown in lime green with cCTnC\(2Ca^{2+}\) and cTnl(34–71) shown in schematic representation. Chemical shift perturbations of the backbone amide resonances induced by EGCg binding to cCTnC\(2Ca^{2+}\)-cTnl(34–71) are colored in red for residues that shifted greater than the mean shift of all residues of cCTnC. Total chemical shift changes are calculated in hertz as follows: \(\Delta \delta = (\Delta \delta ^{1} \text{H})^2 + (\Delta \delta ^{15}\text{N})^2\). Because hertz is used instead of parts/million, a correction factor of 1/5 for the \(^{15}\)N dimension is not used.

(34–71) (Fig. 6d) from the core troponin structure (13). It seems that EGCg binds in the proximity of helix F. Further evidence of EGCg binding near helix F is given when the methyl region of the \(^1\)H,\(^{13}\)C-HSQC spectrum was monitored during the titration of cCTnC\(2Ca^{2+}\)-cTnl(34–71) with EGCg. The terminal methyls of the F helix residues, Leu114 and Ile119, underwent large chemical shift perturbations (Fig. 6b). It may be that EGCg binds to the interface between cTnl and cCTnC near helix F or simply to the side of the protein near the F-G loop. There were also perturbations of amide resonances near the N terminus of helix H toward the \(\beta\)-sheet and of residues on the \(\beta\)-sheet. These perturbations may be caused by direct contact with EGCg or from a conformational change in the structure of cCTnC\(2Ca^{2+}\)-cTnl(34–71) necessary to lodge EGCg.

DISCUSSION

Common treatment schemes of heart failure modify levels of cytosolic Ca\(^{2+}\). This provides immediate improvement in heart function, but it can lead to serious side effects if used for an
Structure of Cardiac Troponin C Bound to EGCg

extended period of time. Drugs that alter the Ca\(^{2+}\) sensitivity of the thin filament, rather than the cytosolic Ca\(^{2+}\) concentration, provide a safer alternative. There are compounds that increase or decrease the sensitivity of the thin filament through interacting specifically with troponin. An increase in Ca\(^{2+}\) sensitivity would be beneficial for the treatment of heart failure, whereas the use of Ca\(^{2+}\) desensitizers may provide protection against the development of hypertrophic cardiomypathy (HCM). HCM is identified by an enlargement of the heart muscle and a decrease in chamber volume of the ventricles. Patients with HCM often suffer from shortness of breath and angina but may also eventually experience heart failure, arrhythmia, and sudden death. The treatment of HCM has been traditionally pursued with the use of negative inotropes that block neurohormones, target pathological load on the heart, or block calcium channels (for reviews on HCM and therapies, see Refs. 46–49).

The use of Ca\(^{2+}\) desensitizers would provide another treatment option, because Ca\(^{2+}\) desensitizers do not disrupt the cytosolic Ca\(^{2+}\) homeostasis or hormone levels. A compound that has demonstrated the ability to inhibit cardiac muscle activation is W7. In skinned rabbit psoas fibers, W7 was shown to inhibit the striated muscle activation (50). Silver et al. (51) also indicated that W7 inhibited ATPase activity and proposed this deactivation occurred through interaction with cTnC. NMR has been utilized to show that W7 binds both the C- and N-terminal domains of cTnC in the absence of cTnI (43); however, in the presence of cTnI-(34–71) and cTnI-(128–163), W7 associates exclusively in the N-terminal domain of cTnC (52). Similarly, EGCg has been identified by the preliminary study of Tadano et al. (30) and by the recent work of Liu et al. (28) to reduce the Ca\(^{2+}\) sensitivity of myofibrillar ATPase activity in cardiac myofibrils. Contrary to W7, however, we illustrate by NMR that EGCg binds to the C-domain of cTnC preferentially. This observation has also been shown by fluorometry and NMR spectroscopy (28, 30).

Structural biological has supplemented the understanding of cardiac muscle contraction and has revealed interesting therapeutic opportunities with cTnC as the primary target. In this study we used NMR spectroscopy to define the molecular details of the interaction between EGCg and cTnC. Analogous to other ligands, we found that EGCg binds in the hydrophobic cavity of cTnC-Ca\(^{2+}\). In addition to the EGCg-cTnC interaction, we found that EGCg also binds to the cTnC-Ca\(^{2+}\)-cTnI-(34–71) complex. Both of these observations have also been described by others, and it is thought that it is these interactions with the C-domain of cTnC that is responsible for the activity of EGCg (28, 30). Two-dimensional HSQC NMR spectra were acquired, and chemical shift changes of \(^{15}\)N,\(^{13}\)C-labeled cTnC were followed during the EGCg titration into solutions containing cTnC-Ca\(^{2+}\) or cTnC-Ca\(^{2+}\)-cTnI-(34–71). Because two-dimensional HSQC NMR spectroscopy provides information regarding the chemical environment surrounding individual nuclei in the protein, we were able to identify specific residues that were affected by EGCg. This branded residues that are proximal to EGCg in the protein-ligand complex or that experience conformational changes upon ligand binding. The \(^{1}H,^{13}C\)-HSQC NMR experiment was utilized to elucidate nearby residues via side chain resonance perturbations, and thus gain insight into the binding location of EGCg to cTnC.

The solution structure of cTnC-Ca\(^{2+}\)-EGCg was determined to unravel the mode of action of EGCg. It was found that EGCg binds to the hydrophobic pocket of cTnC-Ca\(^{2+}\) as do other ligands of cTnC, such as the anchoring region of cTnI (cTnI-(34–71)) and the cardiotoxic drug (EMD 57033) (13, 21). Unlike cTnI-(34–71) and EMD 57033, EGCg binds closer to the surface of the hydrophobic pocket rather than deep within the core of cTnC. EGCg was shown to open the core of cTnC-Ca\(^{2+}\) in a similar manner as cTnI-(34–71) does. It was also seen that EGCg occupies the same binding site as cTnI-(34–71) (Fig. 5, e and f), which might suggest the mode of action of EGCg. To address the possibility that EGCg may compete with cTnI-(34–71) for binding to cTnC, the interaction of EGCg with cTnC-Ca\(^{2+}\)-cTnI-(34–71) was measured.

We found that EGCg induced chemical shift perturbations of cTnC-Ca\(^{2+}\)-cTnI-(34–71); however, the overall magnitude of the perturbations, when compared with cTnC-Ca\(^{2+}\), appear smaller, and the affinity of EGCg for the complex is decreased. Possible reasons for the lessened affinity of EGCg for cTnC-Ca\(^{2+}\)-cTnI-(34–71) are EGCg and cTnI-(34–71) compete for the same binding site on cTnC-Ca\(^{2+}\) or there is a new binding site for EGCg in the cTnC-Ca\(^{2+}\)-cTnI-(34–71) complex. The chemical shift changes induced by EGCg on the cTnC-Ca\(^{2+}\)-cTnI-(34–71) complex do not indicate a dissociation of cTnI-(34–71) from cTnC-Ca\(^{2+}\), but rather suggest the formation of a ternary complex, cTnC-Ca\(^{2+}\)-cTnI-(34–71)-EGCg. The smaller chemical shift changes suggest less of a structural perturbation of the troponin C-I complex than of cTnC.

The affinity of EGCg for cTnC has been measured by intrinsic tyrosine fluorescence quenching to be 3–4 μM for EGCg to cTnC (28). At the higher concentrations typically used for NMR spectroscopy, we found that EGCg bound to cTnC with an \(K_d\) of 1.1 ± 0.12 mM, to cTnC with a \(K_d\) of 1.09 ± 0.08 mM, and to cTnC-cTnI-(34–71) with a \(K_d\) of 1.64 ± 0.24 mM. It has been shown that aromatic stacking of EGCg occurs in aqueous solution (53, 54), and this additional equilibrium would confound accurate \(K_d\) determination. Prompted by these reports, we repeated the titration of EGCg into cTnC-Ca\(^{2+}\) at a lower concentration. Our results show that as we decrease the concentration of cTnC, the apparent \(K_d\) was decreased as well (from 1.09 mM to 385 μM). This enhanced affinity supports the notion of EGCg stacking in vitro, and it explains the weaker affinity we measured when compared with fluorescence spectroscopy.

The perturbation of the cTnC-cTnI-(34–71) interaction by EGCg could weaken the anchoring of cTnC to the thin filament and thus decrease the sensitivity of the thin filament for Ca\(^{2+}\). There are compounds that have been identified to bind the N-terminal domain of cTnC and function by modulating the interaction of cTnC and cTnI. Levosimendan is expected to work by increasing the affinity of cTnI-(147–163) for cTnC, thus increasing the Ca\(^{2+}\) sensitivity of the thin filament through an indirect mechanism (55). There has been renewed interest in the role of the so-called structural domain of cTnC (cTnC) in regulation of contraction. EMD 57033 is a drug that...
has been shown to interact exclusively with cCTnC. EMD 57033 may act as a Ca\(^{2+}\) sensitizer by modulating the interaction of cCTnC and cTnI-(34–71). Small compounds that bind to and inhibit or strengthen the interaction of cCTnC for cTnI-(34–71) may have a pronounced effect on contraction rate or force and Ca\(^{2+}\) sensitivity.

With the aid of this solution structure it might be possible to design new agents using EGCG as a lead compound. The strategic methylation of some of the hydroxyl groups on the polyphenol rings, for example, could potentially increase the potency of EGCG. In fact, it has been shown that catechin and epicatechin are O-methylated in rat small intestine (56), and the \textit{in vivo} mechanism of EGCG on thin filament activity may include these substituted metabolites. The concept of chemical modification of natural products to improve their effectiveness is common, and there are many examples of pharmaceuticals currently prescribed that are derived from natural products (for a review see Ref. 57). The potential role of EGCG as a Ca\(^{2+}\) desensitizer is particularly interesting in regards to treatment for HCM, because there is evidence that reactive oxygen species may be one of the causes of cardiac hypertrophy (49). Because EGCG is a known scavenger of radicals, it may help treat and/or prevent HCM by sequestering reactive oxygen species as well as by inhibiting ATPase activity.

The data presented in this work provide evidence to support the notion that cTnC is one of the primary targets for EGCG in the myofilament, the effective binding site is in the hydrophobic pocket of cCTnC, and the binding induces an opening of the domain. We describe the interaction of EGCG with the cCTnC\(\cdot\)2Ca\(^{2+}\)\cdot cTnI-(34–71) complex, indicating a possible mechanism in which EGCG modulates contraction. EGCG may compete with cTnI-(34–71) and weaken the anchoring of cTnC to the thin filament. This has been postulated for the DCM mutation G159D. The G159D mutation is in the cCTnC and has been shown to weaken the affinity of cTnI-(34–71) for cCTnC (25). EGCG may work in a similar manner, protecting the heart from the development of hypertrophy.

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