Unraveling Molecular Heterogeneity of Phosphorylated Human Cardiac Troponin I by Top Down Electron Capture Dissociation/Electron Transfer Dissociation Mass Spectrometry

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Overview

• Top down ECD and ETD mass spectrometry (MS) has been employed here for characterization and quantitative determination of phosphorylation in cardiac troponin I (cTnI) purified from human heart tissue.

• ECD of monophosphorylated cTnI localized Ser22, a well known phosphorylation site, and Ser76/Thr77, a novel site with partial occupancy.

• ETD MS^3 of b_{31} (both mono and di-phosphoryalted) generated from CAD of diphosphorylated cTnI indicates Ser23 is occupied only in diphosphoryated cTnI, suggesting an ordered/sequential phosphorylation.

• Top down MS of cTnI provides unparallel opportunity for characterization and quantification of phosphorylated isomeric protein forms to explore interdependencies between phosphorylation sites by different kinases .

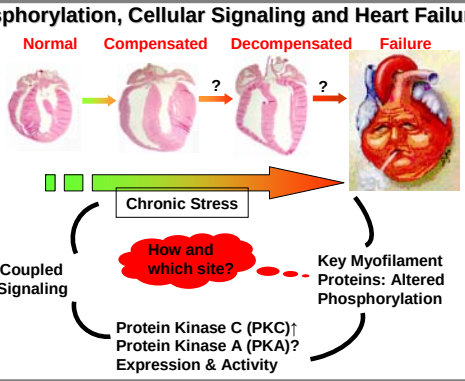
Introduction

Cardiac troponin I (cTnI, 24 kDa) is the inhibitory subunit of the key thin filament troponin-tropomyosin regulatory complex playing a critical role in Ca^{2+}-mediated regulation of skeletal and cardiac muscle contraction and relaxation[1,2]. Phosphorylation of cTnI is a critical cell signal regulating contractility that is particularly important in modulating cardiac function[3,4]. Altered phosphorylation of cTnI may contribute causally to cardiac dysfunction in the transition from compensated hypertrophy to heart failure. Regulated by protein kinase A (PKA), protein kinase C (PKC), p21-activated kinase (PAK), protein kinase G (PKG), and protein kinase D (PKD), cTnI has five well-known serine/threonine phosphorylation sites[3-5] and potentially other unidentified sites. cTnI has twelve serine, eight threonine and three tyrosine residues which makes it difficult for determination of the specific phosphorylation sites especially due to its high p*I* (~9.5). Here we applied top down electron capture dissociation (ECD)[6-8] and electron transfer dissociation (ETD)[9] mass spectrometry for characterization and quantitative determination of phosphorylation in human cTnI.

Methods

Human cTnI, purified from human heart tissue, was analyzed using a hybrid linear ion-trap/FTICR (LTQ FT) and a LTQ mass spectrometers (Thermo Scientific) after a quick desalting step. Multiply protonated precursor ions were guided into the FTICR cell and individual charge states were isolated and further fragmented using ECD, ETD, infrared multiphoton dissociation (IRMPD), and collisionally activated dissociation (CAD). ECD was performed via an electron dispenser operated at 1.1 A with an electron energy of 0.1 to 0.3 eV. ETD used a 100 ms reaction time and $2e^5$ anions of fluoranthene produced by a negative chemical ionization source attached to the back of the linear trap. FTMS data was derived from an average of 50 - 500 transient signals. All spectra were processed using Xtract (Thermo Scientific). Fragment mass sequence assignments were made using ProsightPC software (Thermo Scientific) and manually validated.

Phosphorylation, Cellular Signaling and Heart Failure



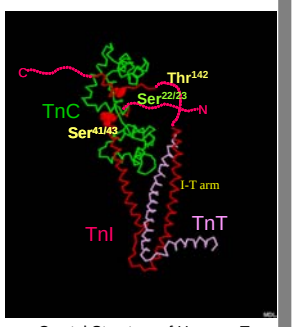
Altered Phosphorylation of cTnI, a Possible contributing Factor in Heart Failure

- cTnI is the inhibitory subunit of troponin/ tropomysin thin filament regulatory complex
- cTnI becomes depleted in ischemic, infarcted and failing myocardium
- cTnI is a useful serum biomarker for diagnosis of heart diseases.

PKA phosphorylation of cTnI
- decreases Ca^{2+} sensitivity
- increases ATP hydrolysis by actomyosin
- narrows twitch, faster re-lenthening
- increases myofilament requirements for ATP & Ca^{2+}

PKC phosphorylation of cTnI
- increases Ca^{2+} sensitivity
- reduces ATP hydrolysis by actomyosin
- prolongs twitch, slower re-lenthening
- reduces myofilament requirements for ATP & Ca^{2+}

5 Known Phosphorylation Sites



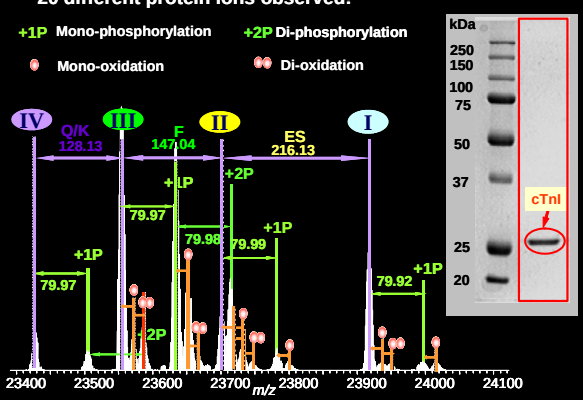
Crystal Structure of Human cTn

cTnI: 12 Ser, 8 Thr, 3 Tyr Possible new phosphorylation site? Maeda and coworkers (2002)

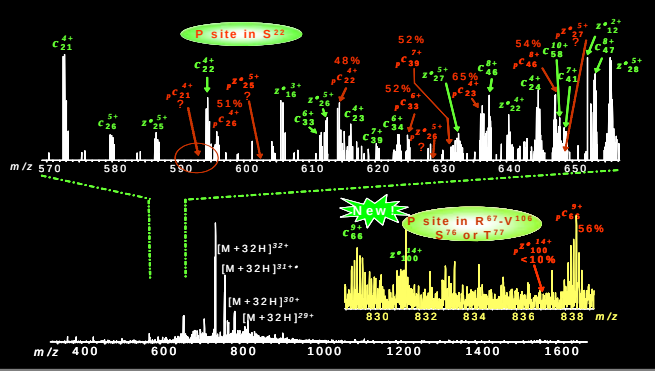
ESI/FTMS Analysis of Human cTnI

~ 20 different protein ions observed!

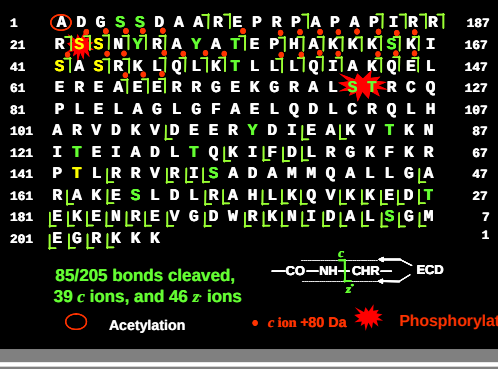
+1P Mono-phosphorylation +2P Di-phosphorylation
Mono-oxidation Di-oxidation



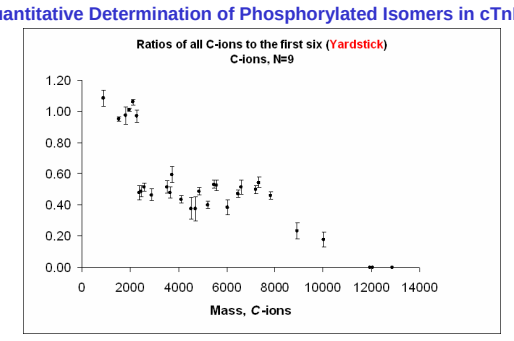
Electron Capture Dissociation (ECD) of cTnI Mono-phosphorylated Isoform III ($^{2+}$)



Locating Monophosphorylation Sites in Human cTnI



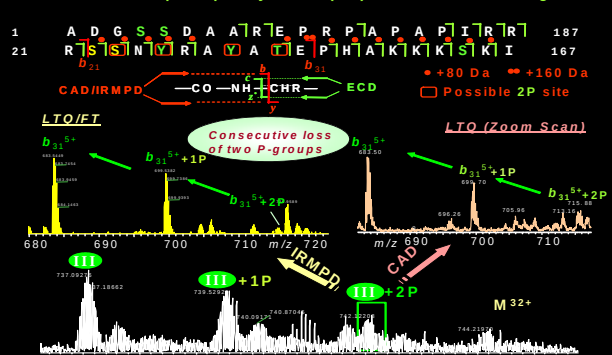
Quantitative Determination of Phosphorylated Isomers in cTnI by ECD



P occupancy of Ser22 = 45-55% P occupancy of Ser76/Thr77= 45-55%

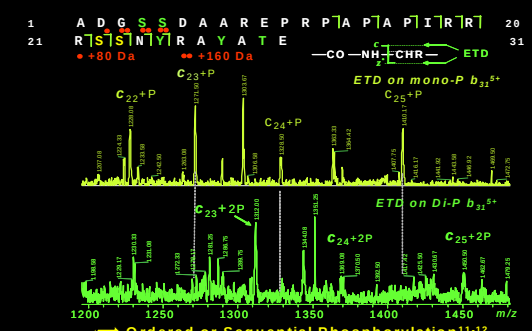
IRMPD/CAD of Di-phosphorylated Isoform III

- Localized di-phosphorylation (2P) in Ser22-Glu31 region



ETD *on Mono- and Di-phosphorylated* b_{31}^{5+} (MS^3)

- Localized Di-phosphorylation (2P) Site at Ser23



Ordered or Sequential Phosphorylation[11-12]

Potential Physiological Implication of the New P-Site

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Mouse cTnI     61 EMEREAEERRGEKGRVLSTRCQPLELDGLG     90
Rat cTnI       61  MEREAEERRGEKGRVLSTRCQPLVLDGLGF    90
Rabbit cTnI    61  LEREAEERRGEKGRALSTRCQPLELAGLGF    90
Bovine cTnI    61 ELEREAEERRGEKGRALSTRCQPLELAGLG     90
Human cTnI     61   EREAEERRGEKGRALSTRCQPLELAGLGFA   90
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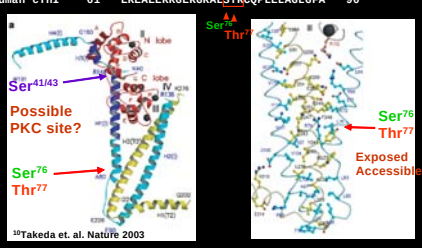

Bovine Ser78 is phosphorylated in vitro by PKC[11].

Conclusions

• Top down ECD and ETD MS has been directly applied here for characterization and quantitative determination of phosphorylation in cTnI purified from human heart tissue.
• High Resolution FTMS spectra of cTnI revealed high degree of heterogeneity: C-terminal truncation, acetylation, oxidation and phosphorylation
• ECD of monophosphorylated cTnI localized Ser22, a well known phosphorylated site, and Ser76/Thr77, a novel site with partial occupancy (45-55%). This is the first evidence of identifying Ser76/Thr77 as in vivo phosphorylation site in human cTnI. Phosphoryaltion of Ser76/Thr77 could potentially extend an alpha helix altering the rigidity of a lever arm in the heterotrimeric protein complex.
• IRMPD/CAD data localized a double phosphorylation site between Ser22 and Glu31. ETD MS^3 of b_{31} (both mono and di-phosphoryalted) generated from CAD of diphosphorylated cTnI indicates Ser23 is occupied only in diphosphoryated cTnI, indicating an ordered or sequential phosphorylation[11-12].
• Top down MS of cTnI provides unparallel opportunity for characterization and quantification of phosphorylated isomeric protein forms thus allowing to explore interdependencies between phosphorylation sites by different kinases and their relevance to physiological function[13].

1. Walker JW. *J. Mol. Cell. Cardiol*. 2006; 40: 446-450. 2. Kobayashi T, Solaro RJ. *Ann. Rev. Physiol*. 2005; 67: 39-67. 3. Haworth RS, Cuello F, Herron TJ, Franzen G, Kentish JC, Gautel M, Avkiran M. *Circ. Res*. 2004; 95:1091 4. Layland J, Solaro RJ, Shah AM. *Cardiovasc. Res*. 2005; 66: 12-21. 5. Kobayashi T, Yang XF, Walker LA, Van Breemen RB, Solaro RJ. *J. Mol. Cell. Cardiol*. 2005; 38: 213-218. 6. Ge Y, Lawhorn BG, ElNaggar M, Strauss E, Park JH, Begley TP, McLafferty FW. *JACS*. 2002; 124: 672-678. 7. Zabrouskov V, Han XM, Welker E, et al. *Biochemistry*. 2006; 45: 987-992. 8. Pesavento JJ, Mizzen CA, Kelleher NL. *Anal. Chem*. 2006; 78: 4271-4280. 9. Coon JJ, Ueberheide B, Syka JEP, Dryhurst DD, Ausio J, Shabanowitz J, Hunt DF. *PNAS* 2005; 102: 9463-10.Takeda S, Yamashita A, Maeda K, Maeda Y, *Nature*, 2003, 424: 35-41. 11. Noland TA, Raynor RL, Kuo JF. *J. Biol. Chem*. 1989; 264: 20778-20785. 12. Perry SV, Patchell VB, Levine BA, Quirk PG. *J. Muscle Res. Cell Motil*. 1996; 17: 151-151. 13. Keane NE, Quirk PG, Gao Y, Patchell VB, Perry SV, Levine BA. *Eur. J. Biochem*. 1997; 248: 329-337. 14. Zhang R, Zhao JJ, Potter JD. *J. Biol. Chem*. 1995; 270: 30773-30780.

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