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Post-translational modifications of ATP synthase in the heart: biology and function

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Abstract The ATP synthase complex is a critical enzyme in the energetic pathways of cells because it is the enzyme complex that produces the majority of cellular ATP. It has been shown to be involved in several cardiac phenotypes including heart failure and preconditioning, a cellular protective mechanism. Understanding the regulation of this enzyme is important in understanding the mechanisms behind these important phenomena. Recently there have been several post-translational modifications (PTM) reported for various subunits of this enzyme complex, opening up the possibility of differential regulation by these PTMs. Here we discuss the known PTMs in the heart and other mammalian tissues and their implication to function and regulation of the ATP synthase.

Keywords ATP synthase · Post-translational modification · Heart

Heart failure is a major health concern and has a complex and diverse etiology, the mechanisms of which are still under intense study. The energetics of the heart, and thus the mitochondria, are integrally involved in the causes and phenotype of heart failure (Marin-Garcia and Goldenthal 2008, Murray et al. 2007). Mitochondria and modulation of their energetic pathways have also been implicated in preconditioning (PC), a phenomenon by which certain triggers activate a cardioprotective state that minimizes heart damage during ischemic events (for reviews see: (Foster et al. 2008, Halestrap et al. 2007, Murphy and Steenbergen 2008). There have been several reports of the modulation of the mitochondrial ATP synthase complex in various cardiac phenomena (for reviews see: (Das 2003, Grover et al. 2008). Specifically, it has been observed that there is an increase in ATP hydrolysis by the ATP synthase complex in ischemic myocardium (Grover et al. 2004) which is hypothesized to contribute to the overall depletion of the cellular ATP pool during ischemia. Two cardioprotective drugs, diazoxide and adenosine, have been shown to cause the down-modulation of activity (Comelli et al. 2007) and post-translational modification (PTM) (Arrell et al. 2006) of this enzyme complex, respectively. The PTM was found to be phosphorylation of the β subunit of the complex at up to five amino acid residues. Given this enzyme's involvement in these cardiac phenomena, it is critically important to understand the ways in which the ATP synthase complex can be regulated in the heart.

The mitochondrial ATP synthase complex, the primary site of ATP synthesis in myocytes, is a large multi-subunit protein complex comprised of a membrane (F_o) domain and soluble (F_1) domain. The F_o domain contains both the membrane spanning a and c subunits that are responsible for proton translocation, as well as several other subunits that are a part of the peripheral stalk of the enzyme and are involved in regulation of structure and function. The F_1 domain contains the α, β, δ, γ, and ε subunits that are involved in both catalysis and regulation. Much is known about the structure and function of this

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enzyme and its subunits (Ackerman and Tzagoloff 2005, Feniouk and Yoshida 2008, Pedersen 2007), but until recently there was essentially no knowledge regarding the PTM-dependent modulation/regulation of the protein subunits in the context of mammalian systems.

The recent developments of PTM-specific mass spectrometry-based methods (MS), as well as selective dyes and antibodies, have allowed for the detection of a surprising number of PTMs in subunits of ATP synthase. Examples include phosphorylation, acetylation, trimethylation, nitration, s-nitrosylation and tryptophan oxidation (Tables 1 and 2). While some of these PTMs have been associated with specific biological processes or diseases, the biggest ongoing challenge is to find direct functional implications for these PTMs. Studies are needed to examine the link between each PTM and the resulting function. This review will discuss the ATP synthase subunit PTMs found in the heart (Table 1) and briefly compare these with PTMs found in other mammalian tissues (Table 2).

Phosphorylation

Phosphorylation is one of the most commonly and extensively studied PTMs in mammalian systems (Sefton and Shenolikar 2001). Several mitochondrial proteins (e.g. pyruvate dehydrogenase) have been known to be the targets of phosphorylation-specific regulation for over a decade. Until recently, it was suspected that the global mitochondrial proteome was not highly phosphorylated, but with the recent advent of new technologies for studying the phosphorylation of proteins, a new picture has emerged of an extensively phosphorylated and dynamically regulated mitochondrial proteome (Agnetti et al. 2007, Foster et al. 2008, Pagliarini and Dixon 2006) and see Foster et al. in this journal). Several studies have made use of these new technologies to study the phosphorylation of the ATP synthase complex.

One such new reagent is the commercially available phosphorylation targeted dye ProQ Diamond (Invitrogen). This dye has been used both to observe the phosphorylated proteins present in the mitochondria (Murray et al. 2004, Schulenberg et al. 2004) and in the ATP synthase complex specifically (Murray et al. 2004). Hopper et al. used this dye in conjunction with 2D gels and MS identification of proteins to visualize the phospho-proteome of the mitochondrial matrix (Hopper et al. 2006). Through this study, they observed that the α, β, γ, c, and O subunits of the ATP synthase complex had ProQ Diamond positive signal in 2D gels of mitochondria from pig hearts. The α and β subunits were also observed to react with the ProQ Diamond dye in ATP synthase complex immuno-precipitated from bovine heart mitochondria (Murray et al. 2004). While this dye is designed to bind to the negative charge of the phosphate group, it can also bind to other negatively charged moieties, such as sequential negatively charged amino acid residues. Therefore, datasets generated using this dye can include false positives which must be eliminated using other analyses. To improve the specificity of their study, Hopper

Table 1 Post-translational modifications of the ATP synthase complex in heart tissue

Subunit	Modification	Method	Residue	Organism	Reference
α	Nitration	3-NT Ab[a]		Mouse	(Liu et al. 2009)
	S-Nitrosylation	BS, MS		Mouse	(Sun et al. 2007)
	Phosphorylation	ProQ		Pig	(Hopper et al. 2006)
	Phosphorylation	ProQ		Cow	(Murray et al. 2004)
β	Phosphorylation	MS	S106, T107, T262/S263, T312, T368	Rabbit	(Arrell et al. 2006)
	Phosphorylation	ProQ		Pig	(Hopper et al. 2006)
	Phosphorylation	ProQ		Cow	(Murray et al. 2004)
γ	Phosphorylation	pY Ab & MS	Y44 or Y52	Cow	(Di Pancrazio et al. 2006)
	Phosphorylation	ProQ		Pig	(Hopper et al. 2006)
a	Tryptophan oxidation	MS	W48	Human	(Taylor et al. 2003)
c	Trimethylation	MS	K43	Cow	(Chen et al. 2004)
	Phosphorylation	ProQ		Pig	(Hopper et al. 2006)
d	Tryptophan oxidation	MS	W54, W13, W153	Human	(Taylor et al. 2003)
O	Phosphorylation	ProQ		Pig	(Hopper et al. 2006)
g	Tryptophan oxidation	MS	W31	Human	(Taylor et al. 2003)

[a] Abbreviations: 3-NT Ab; 3-nitrotyrosine Ab, BS; biotin switch, MS; mass spectrometry, ProQ; ProQ Diamond phospho-protein stain, pY ab; phospho-tyrosine antibody

Table 2 Post-translational modifications of the ATP synthase complex in other mammalian tissues

Subunit	Modification	Method	Residue	Organism/Tissue	Reference
α	Phosphorylation	MS[a]	S76	Mouse/brain	(Vosseller et al. 2005)
	Acetylation	MS	K132, K230, K239, K261, K305, K427, K498, K531, K359	Mouse/liver	(Kim et al. 2006)
β	Phosphorylation	MS	T213	Human/muscle	(Hojlund et al. 2003)
	Phosphorylation	MS	S529	Mouse/liver	(Lee et al. 2007)
	Acetylation	MS	K133, K259, K522	Mouse/liver	(Kim et al. 2006)
δ	Phosphorylation	P^{32}, pY Ab		NIH3T3 and kidney cells	(Ko et al. 2002)
	Phosphorylation	P^{32}		Cortical neurons	(Zhang et al. 1995)
γ	Acetylation	MS	K79, K90, K115	Mouse/liver	(Kim et al. 2006)
b	Acetylation	MS	K115, K118, K131, K162, K221, K225, K233	Mouse/liver	(Kim et al. 2006)
c	Trimethylation	MS	K43	Human/brain	(Chen et al. 2004)
d	Acetylation	MS	K63, K78, K85, K99, K117, K149	Mouse/liver	(Kim et al. 2006)
e	Phosphorylation	MS	Y32	Rat/brain	(Lewandrowski et al. 2008)
	Phosphorylation	MS	Y32	Mouse/brain	(Ballif et al. 2008)
g	Acetylation	MS	K54, K66	Mouse/liver	(Kim et al. 2006)
O	Acetylation	MS	K60, K70, KK159, K162, K172, K176, K192	Mouse/liver	(Kim et al. 2006)
8	Acetylation	MS	K54	Mouse/liver	(Kim et al. 2006)
F6	Acetylation	MS	K84, K99	Mouse/liver	(Kim et al. 2006)

[a] Abbreviations: MS; mass spectrometry, ^{32}P; ^{32}P radio-labeling of proteins, pY ab; phospho-tyrosine antibody

et al. also used a ^{32}PγATP labeling strategy which only observed the γ subunit of ATP synthase subunit to be phosphorylated (Hopper et al. 2006). This phosphate radio-labeling of proteins is the gold-standard for proving phosphorylation, but is only capable of labeling residues which have a high rate of phosphate turnover in the system. Thus, the ^{32}P experiments do not prove that the α, β, c, and O subunits observed with ProQ diamond are not phosphorylated, only that they do not have a high rate of turnover in the porcine heart mitochondria under the conditions that Hopper et al. examined. Another possible method of confirming phosphorylation is the use of phosphatases to remove the phosphate group. These can be used in conjunction with ProQ diamond; an observed reduction in ProQ signal with phosphatase treatment is a good indicator that the ProQ diamond signal is the result of phosphorylation. However, given the selectivity of most phosphatases for specific proteins, a negative result does not prove that the protein is not phosphorylated.

Another method for detecting phosphorylation is the use of phospho-specific antibodies, which have improved in specificity in recent years. Through the use of a phospho-tyrosine antibody, Di Pancrazio et al. (2006) were able to show that, in bovine hearts, the monomeric form of the ATP synthase complex contains a phosphorylated form of the γ subunit that is not present in the dimeric form of the complex. They were able to use MS to confirm that the phospho-tyrosine reactive band corresponded to the γ subunit. The authors were also able to show that the phosphorylated residue of the γ subunit was contained within a peptide fragment containing both Y44 and Y52 (Di Pancrazio et al. 2006). This study is very interesting as it points to the possible regulation of dimer formation by phosphorylation. ATP synthase dimer and oligomer formation can change the function of the enzyme complex and the structure of the cristae of the inner mitochondrial membrane, thereby affecting overall mitochondrial structure and function (Wittig and Schagger 2009). Thus, understanding the regulation that causes the ATP synthase complex to transition between its monomeric and higher order forms is not only essential for understanding the complex, but the whole mitochondria.

Phospho-tyrosine antibodies were also used by Ko et al. to confirm that the platelet-derived growth factor (PDGF) induced phosphorylation of the δ subunit (Ko et al. 2002). This group also used ^{32}P labeling to show that the δ subunit could be differentially phosphorylated *in vitro* by mitochondrial extracts that had been isolated from either untreated NIH3T3 cells or from PDGF-treated NIH3T3 cells. A similar method had previously been used by Zhang et al. to show the phosphorylation of the δ subunit in cortical neurons (Zhang et al. 1995). Ko et al. also confirmed this finding *in vivo* using the phospho-tyrosine antibody to immuno-precipitate (IP) phosphory-

lated proteins in kidney cells treated with PGDF (Ko et al. 2002). They were able to observe that phosphorylation of the δ subunit of the ATP synthase complex occurs in as little as five minutes following PDGF treatment. Although this report does not provide evidence that the phosphorylation of the δ subunit has a functional affect on the ATP synthase complex, it is a striking finding because it suggests that the cellular signaling pathways extend into the mitochondrial matrix and are capable of very quickly modifying proteins in the inner mitochondria.

Mass spectrometry has undergone a number of revolutionary developments since the mid 1990s which have led to its extensive application in the study of all PTMs, including phosphorylated proteins (Paradela and Albar 2008). Several strategies exist to enrich the phosphopeptides prior to MS, including immobilized metal affinity chromatography (IMAC), titanium diazoxide columns (TiO_2), the sequential combination of IMAC and TiO_2 (called SIMAC) and immuno-precipitation with phospho-specific antibodies (for a review of these strategies see: (Thingholm et al. 2009). Enriching phosphopeptides prior to MS decreases the interference of non-phosphopeptides, allowing for more sensitive MS (Thingholm et al. 2009). Using IMAC enrichment coupled with MS, our lab has shown that the β subunit could be extensively phosphorylated in the rabbit heart (Arrell et al. 2006). In this study we were able to show that the β subunit was modified by preconditioning rabbit myocytes with adenosine and that this subunit contained at least five phosphorylated amino acids: S106, T107, T262/S263 (an unambiguous assignment of which residue was phosphorylated could not be made), T312 and T368. This was the first study to show that cardiac intervention could cause *in situ* phosphorylation of the ATP synthase complex. MS has also been used to show phosphorylation of the β subunit of the ATP synthase complex in other mammalian tissues. This includes the observation by Hojlund et al. that T213 could be phosphorylated in human skeletal muscle tissue and that this phosphorylation may play a role in modulation of the ATP synthase complex in Type 2 diabetes (Hojlund et al. 2003). In a broad-based mitochondrial phospho-proteome study, Lee et al. also found that the C-terminal serine of the β subunit (S529) was phosphorylated in mouse liver.

In addition to the β subunit, MS studies have shown that other subunits of ATP synthase are also phosphorylated. For example, S76 of the α subunit of ATP synthase was observed to be phosphorylated in a global analysis of proteins from a mouse brain lysate (Vosseller et al. 2005). Two other independent large-scale phosphoproteomic experiments, one on rat brain mitochondria (Lewandrowski et al. 2008), and one on whole mouse brain lysate (Ballif et al. 2008), also observed the same phospho-tyrosine residue (Y32) on the e subunit using MS. One drawback of these large studies is that there is no indication of the pathways or physiology that these residues may be involved in; the need for follow-up experiments is clear.

Oxidative/nitrative modifications

Recent evidence suggests that there are several oxidative protein modifications that can be important and potentially regulatory modifications involved in heart failure (Kuster et al. 2006, Sawyer et al. 2002). In the mitochondria a rigorous investigation by Taylor et al. has shown that the tryptophan residues in ATP synthase complex can be targets of oxidative modification (Taylor et al. 2003). In this study, they used MS to examine the extent of tryptophan oxidation in normal human hearts, and found that the ATP synthase complex was one of the "hot spots" for this type of oxidation in the mitochondria. They identified modified residues on the a, d, and g subunits (see Table 1), but did not assign the modification of a specific amino acid residue. A hallmark of heart failure is an increase in the level of reactive oxygen species (ROS) (Tsutsui 2001). Since these oxidative modifications are present in the healthy heart, it is likely that the ATP synthase complex is also a "hot spot" for oxidative damage in the diseased heart.

In contrast to oxidative modifications, the ATP synthase complex has also been shown to be modified by nitrosyl groups (S-Nitrosylation). Specifically, the α subunit has been observed to be S-Nitrosylated in response to GSNO treatment of a mouse heart membrane fraction (Sun et al. 2007). Although this modification has also been extensively studied on Complex I subunits (Burwell et al. 2006), the observation that the α subunit is S-Nitrosylated is the only published direct link between the ATP synthase complex and nitric oxide signaling. As such, this needs to be followed up in an *in vivo* system.

A large study that looked at protein tyrosine nitration in mouse heart showed that the mitochondria contained a large proportion of the nitro-tyrosine modified proteins (Liu et al. 2009). This group showed that a 3-nitrotyrosine antibody reactive spot contained the α subunit and that this nitration was increased following ischemia/reperfusion (I/R) injury. This could be either an indication of PTM signaling or damage to the ATP synthase complex caused by the stress of I/R. The next step will be to identify the sites of modification and determine any functional associations.

Lysine modifications

Several subunits of the ATP synthase complex have also been observed to contain lysine modifications, including

methylation and acetylation. In 1994, Katz et al. observed the trimethylation of the c subunit in lysosomal storage bodies in the brains and kidneys of a dog model of ceroid lipofuscinosis (Katz et al. 1994). For several years, this was thought to be a modification specifically found in the storage bodies found in this family of ceroid lipofuscinosis diseases (including Batten disease) and not one found in mitochondria under normal physiological conditions. However, in a thorough MS-based analysis, targeting the ATP synthase subunit c from normal and diseased human brain (Batten disease) and healthy bovine heart, Chen et al. showed that this modification is present in both storage bodies of diseased tissues and in mitochondria from healthy tissues (Chen et al. 2004). The functional implication of this trimethylation in the healthy or diseased heart is, as of yet, not understood.

Protein acetylation has been extensively studied in the context of chromosome remodeling and histone modifications (Shahbazian and Grunstein 2007, Smith and Denu 2009). Surprisingly, a large proteomic study of protein acetylation in mouse liver revealed that more than 20% of mitochondrial proteins contained acetylated lysine residues (Kim et al. 2006). This long list of acetylated mitochondrial proteins included nine subunits of the ATP synthase complex (α, β, γ, b, d, g, O, 8 and F6 subunits). The observation that many of the acetylated residues on these subunits were affected by fasting/feeding of the mice indicates that these PTMs may be dynamic depending on the cellular environment/signaling pathways. Again, it is not known what the function of these modifications is, or if they are present on these subunits in other tissues, such as the heart.

Conclusions

An increasing number of PTMs to the mitochondrial ATP synthase are being reported, due in part to new technologies and approaches. However, few of the actual modified amino acid residues have been identified. Such residue identifications are essential to directly test the functional consequences of these PTMs. There is also a great need to elucidate the enzymes responsible for the addition/removal of the PTMs and place them within the broader context of the cellular signaling pathways. Finally, we need to understand these quantitative changes with disease and determine whether common beneficial/detrimental mechanisms are present. This will hopefully lead to the development of therapeutics to manipulate those PTMs with important functions in disease.

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