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Protein lysine acylation and cysteine succination by intermediates of energy metabolism

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Abstract

In the last few years, several new protein post-translational modifications that use intermediates in metabolism have been discovered. These include various acyl lysine modifications (formylation, propionylation, butyrylation, crotonylation, malonylation, succinylation, myristoylation) and cysteine succination. Here, we review the discovery and the current understanding of these modifications. Several of these modifications are regulated by the deacylases, sirtuins, which use nicotinamide adenine dinucleotide (NAD), an important metabolic small molecule. Interestingly, several of these modifications in turn regulate the activity of metabolic enzymes. These new modifications reveal interesting connections between metabolism and protein post-translational modifications and raise many questions for future investigations.

Introduction

Protein post-translational modifications (PTMs) have been increasingly recognized to play important roles in various biological pathways via regulation of protein function. Walsh's 2005 book on PTMs provides an excellent introduction to the fascinating chemistry and biology for many PTMs (1). Since then, several new PTMs have been discovered. In addition, several known posttranslational modifications have been "rediscovered" - these PTMs were previously reported but were not widely recognized as important due to the lack of follow-up studies, or have been well known but new functions for them have been discovered. For example, protein lysine acetylation has been well known, but its function in regulating metabolic enzymes is only discovered recently. These new and rediscovered PTMs are the topics of discussion here. Most of these PTMs occur on lysine residues, including succinylation, malonylation, crotonylation, and myristoylation (Figure 1). One modification on cysteine residue, succination, will also be discussed (Figure 1).

A common theme of the new PTMs is that they all use small molecule intermediates in pathways of energy metabolism, such as succinyl-CoA in the tricarboxylic acid (TCA) cycle and acyl-CoA molecules in the β-oxidation pathway (Figure 2) (2–5). Furthermore, many of these modifications occur to metabolic enzymes and regulate the activity of the enzymes (3, 4, 6–9). The intimate connection between these PTMs and metabolism is of particular interest as it may provide new view points to understand cellular function. This review aims to provide brief summaries of these new PTMs and discuss some future directions and challenges. We will start with lysine acetylation because it is the best studied among these PTMs and knowledge about acetylation can greatly facilitate the investigation and discussion of other PTMs. Other PTMs will then be discussed in the order of increasing size and complexity.

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Lysine Acetylation

Acetylation is well known modification with important functions in regulating chromatin structure and transcription by modifying histones and transcription factors (1). However, the recent finding is that acetylation occurs to thousands of proteins (10). For example, many metabolic enzymes are acetylated and acetylation plays important roles in regulating metabolism. The regulation of metabolism will be the focus of the discussion here.

Lysine acetylation is known to be controlled by two opposing types of enzymes, acetyltransferases and deacetylases (Figure 3) (1, 11). For historical reasons, the protein lysine acetyltransferases are called histone acetyltransferases (HATs), which transfers acetyl group from acetyl-CoA to protein lysine residues (2). The protein lysine deacetylases consists of two classes. One is metal ion-dependent and are called histone deacetylases (HDACs) (12–14) while the other class is nicotinamide adenine dinucleotide (NAD)-dependent and are called sirtuins after the founding member of the class, yeast Sir2 (silencing information regulator 2) protein (15–19). There are 11 metal-dependent HDACs and seven sirtuins in mammals.

The first clue that acetylation may play important roles in metabolism is the proteomic identification of a large number of mitochondrial proteins, including metabolic enzymes, with acetyl lysine modifications (6, 8–10). This was achieved using pan-specific acetyl lysine antibodies to enrich acetyl lysine peptides followed by LC-MS/MS (liquid chromatography-tandem mass spectrometry) identification. However, the significance of lysine acetylation of metabolic enzymes was not clear until more functional studies were carried out (Table 1). Many of these studies were in done in conjugation with the study of sirtuins. Here we will focus on two proteomic studies that together provided an interesting systematic view of the interplay between metabolic status of cells and regulation of metabolism by lysine acetylation.

One such study was done in the bacterium *Salmonella* (8). Wang et al. profiled the protein lysine acetylation changes in *Salmonella* grown in different media (glucose versus citrate as the carbon source) and wild type (WT) versus CobB/PAT deletion strains. CobB is thought to be the NAD-dependent deacetylase and PAT is considered the acetyltransferase in *Salmonella*. The overall observation is that *Salmonella* cells have more acetylation when in minimal medium with glucose than in minimal medium with citrate. The acetylation of various enzymes (Table 1, entries 2–4) favors the metabolism of glucose by increasing activities of enzymes in glycolysis and TCA cycle. In contrast, lower acetylation level of various enzymes favors glyoxylate pathway and gluconeogenesis. Deletion of CobB, the deacetylase, or PAT (the acetyltransferases) affected the acetylation levels, the activities of the enzymes, and the metabolic flux controlled by these enzymes. These observations favor the hypothesis that Salmonella cells are able to sense the metabolic state of the cells and adjust their metabolism by controlling the acetylation of metabolic enzymes.

Another study was done in human cells (9). Zhao et al. identified 1047 proteins with acetyl lysine modifications in human liver and found that almost every enzyme in glycolysis, gluconeogenesis, the TCA cycle, the urea cycle, fatty acid oxidation, and glycogen metabolism was acetylated. They investigated four enzymes (Table 1), EHHADH (enoyl–coenzyme A hydratase/3-hydroxyacyl–coenzyme A dehydrogenase), MDH (malate dehydrogenase), ASL (argininosuccinate lyase), and PEPCK1 (phosphoenolpyruvate carboxykinase 1). It was discovered that EHHADH and MDH are activated by lysine acetylation, while the activity or protein level of ASL and PEPCK1 were decreased by lysine acetylation. EHHADH is involved in fatty acid oxidation. Its acetylation level and activity was increased by the presence of fatty acid in the cell culture medium. MDH is

involved in the TCA cycle. Its acetylation level and activity was increased by the presence of high concentration of glucose in the medium. ASL is involved in the urea cycle, which is also important for amino acid metabolism. The acetylation level was decreased and the activity was increased by the addition of extra amino acids in the medium. PEPCK1 is a key enzyme in gluconeogenesis. High glucose concentration promotes the acetylation and degradation of PEPCK1, thus leading to decreased gluconeogenesis. Interestingly, it seems that yeast PEPCK behaves differently from human PEPCK1, as it has been shown that acetylation of yeast PEPCK on K514 is important for its activity (20).

Guarente proposed an interesting model that nicely summarized the changes of acetylation level in different metabolic conditions (Figure 4) (35). Under high glucose concentration, NAD/NADH ratio is low due to high level of glycolysis, which converts NAD to NADH. The low NAD/NADH ratio decreases sirtuin activity and thus leads to high acetylation level on these metabolic enzymes. In contrast, under low glucose concentration, NAD/NADH ratio increases, leading to higher sirtuin activity and low acetylation level on proteins. He also hypothesized that protein lysine acetylation could serve as the energy store (35). Nutrient rich conditions favor protein acetylation and the acetyl group can be released by deacetylases under nutrient poor conditions, which can be metabolized via the TCA cycle to generate energy, similar to the synthesis of fat under nutrient rich conditions and the degradation of fat under fasting conditions. This model helps to memorize the change of acetylation levels at different metabolic conditions. However, in reality, other factors may also need to be considered, such as the concentration of acetyl-CoA and the activity of the acetyltransferases.

The effects of acetylation on the activity of enzymes are more difficult to generalize, but some trends can be seen (Figure 4). It seems that enzymes in glycolysis (GapA in Salmonella and PGAM1 in humans) are activated by acetylation under high glucose concentration. This suggests that under high glucose conditions, cells use acetylation to promote glycolysis. For most other enzymes, with the exception of yeast PEPCK, human EHHADH, and human MDH, acetylation decreases activity while deacetylation increases activity. Perhaps when nutrient is limited (NAD/NADH ratio is high), most non-glycolytic enzymes needs to be deacetylated and activated. A better picture will emerge in the future when the effects of acetylation of more metabolic enzymes are studied.

Lysine Formylation

Although formylation of methionine covalently linked to initiator tRNA is well known in the translation field, posttranslational lysine formylation from cellular proteins was first reported in 2007 (36, 37). Prior to this, it was known that the detoxification of trichloroethylene, a widely uses industrial solvent before 1970s, can lead to the generation of formyl lysine in an in vitro reaction with bovine serum albumin (Figure 5) (38). Formyl lysine was first found on linker histone H1 by mass spectrometry (MS) (36). Later, a proteomic study focusing on chromatin-associated proteins identified several core histones and non-histone proteins that contain formyl lysine (39). It should be noted that the mass of formyl lysine is almost the same as arginine or dimethyl lysine. Thus high resolution MS is required to confidently identify formyl lysine modifications (36). It has also been reported that silver staining of protein gels could lead to the artificial chemical formylation of protein lysine residues due to the presence of formaldehyde. Thus, silver staining should be avoided when identifying formyl lysine.

The function of protein lysine formylation is not clear at present. Interestingly, it was reported that lysine formylation increased when cells were treated with an enediyne DNA damaging reagent neocarzinostatin (37). Tritium labeling experiment showed that the formyl

group comes from the 5′-position of ribose in DNA. The explanation is that the neocarzinostatin induced DNA damage leads to the generation of formyl phosphate, which is the donor for lysine formylation (Figure 5). This observation implies that lysine formylation is secondary (and non-enzymatic) modification from oxidative DNA damage.

Another possible formyl donor is formyl tetrahydrofolate (THF, Figure 5), which is used to form formyl methionyl-tRNA. Formyl-THF can be formed from oxidation of methylene THF or from the ligation of formate to THF by formyl-THF synthetase (40). The formyl-THF synthetase reaction is ATP-dependent and goes through a formyl phosphate intermediate. It is possible that formyl-THF can be used by a transformylase to formylate protein lysine residues. DNA repair will likely require the activation of thymidylate synthesis and thus folate metabolism, which would be consistent with the increased formyl-THF production and increased lysine formylation.

Regardless of how formylation occurs (enzymatically or non-enzymatically), it will compete with other lysine modifications, such as acetylation and methylation, which are known to play important roles in transcriptional regulation. Thus the biological effects of formylation may be complex. Due to the similarity to acetyl lysine, we suspect that formyl lysine can be removed by known deacetylases, including the metal dependent HDACs and NAD-dependent sirtuins, just like some deacetylases are known to be able to remove propionyl and butyryl lysines as discussed below.

Lysine propionylation and butyrylation

Lysine propionylation and butyrylation was first reported by Zhao and coworkers in 2007 (41). Using a pan-specific acetyl lysine antibody, propionyl and butyryl lysine H4 peptides were pulled out and identified from HeLa cells (propionylation: histone H4 K5, K8, and K12; butyrylation: histone H4 K5 and K12). These modifications are structurally similar to the well-known lysine acetylation, as they are pulled out by the acetyl lysine antibody. Histone H3 was also reported to be propionylated on K23 (42). The levels of H3 K23 propionylation are different in different types of leukemia cells and are also regulated by cell differentiation (42). Yeast histones were also found to contain propionyl and butyryl lysine (43). Lysine propionylation was also reported to occur in bacteria (44). The *Salmonella* propionyl-CoA synthetase was found to be propionylated on K592 when the cells were grown with propionate as the carbon source, which inhibits its activity.

It seems that in both bacteria and mammals, the levels of propionyl and butyryl lysine can be controlled by the same set of enzymes that control acetylation and deacetylation. In human cells, the known acetyltransferases, p300 and CBP, are able to catalyze propionyl and butyryl transfer in vitro using purified proteins (41) and in cells (45). In *Salmonella*, the propionylation of propionyl-CoA synthetase has been shown to be catalyzed by the previously identified acetyltransferases, PAT (44, 46). It is known that the NAD-dependent deacetylases, sirtuins, can remove propionyl and butyryl groups (47, 48). For example, in *Salmonella*, CobB can regulate the propionylation of propionyl-CoA synthetase (44). This explains why CobB deletion strain has a growth defect when grown on propionate because propionylation inactivates propionyl-CoA synthetase. Based on available quantitative information about the abundance of propionyl lysine, it seems that propionylation is ≥ 10 times less abundant than acetylation. The number of propionyl/butyryl lysine peptides identified is also much lower than the number of acetyl peptides identified. In cells the propionyl/butyryl-CoA concentration is generally ~10 time lower than that of acetyl-CoA (49), which is consistent with lower abundance of propionyl/butyryl lysine. One reference was often cited to suggest that the concentration of propionyl and butyryl-CoA are much higher (close to acetyl-CoA concentration) (50). However, the values reported in this

reference were measured from rats injected with propionate and butyrate, respectively, and are not normal physiological values.

Most of the evidence are consistent with the idea that protein lysine propionylation and butyrylation are similar to acetylation and are regulated by the same set of enzymes. However, one observation does point out a potential distinct function of lysine propionylation. Zhang and coworkers measured the levels of acetylation and propionylation on histone H3 K23 in three different cell lines, HL60, THP1, and U937 (42). The levels of acetylation were essentially the same in all three cell lines, but the level of propionylation is about 10 times higher in U937 cells than in HL60 and THP1 cells. This could suggest that propionylation is regulated differently from acetylation. An alternative explanation is that the U937 cells have a higher propionyl-CoA concentration. Propionyl-CoA is mainly generated from the β-oxidation of odd chain fatty acids. The major metabolic pathway to degrade propionyl-CoA is to convert it to methylmalonyl-CoA via a carboxylation reaction and then to succinyl-CoA via a cobalamin-dependent rearrangement reaction. If U937 cells have a less efficient propionyl-CoA metabolic pathway, it may accumulate more propionyl-CoA and thus lead to more lysine propionylation.

One research direction that can potentially establish propionylation and butyrylation as having distinct function from that of acetylation is to investigate whether they can induced more potent or different effects. For example, it is well known that histone lysine acetylation can be recognized by bromo domains (51). It was shown that the bromo domains of Brd4 (bromo domain-containing protein 4) bind propionyl and butyryl lysines less tightly than acetyl lysine (52). It would be interesting to find out whether some bromo domains bind propionyl or butyryl lysine better than acetyl lysine.

Lysine crotonylation

Lysine crotonylation was recently discovered on histones (53). Zhao and coworkers studied the modifications of human and mouse histone proteins using MS and found that some peptides have a modification that increases the mass of the peptide by 68 Dalton. One of the peptides that has this modification is the human H2B K5 peptide. The mass increase is consistent with lysine crotonylation. LC-MS/MS of synthetic crotonyl H2B K5 peptide confirms that the modification is lysine crotonylation. A total of 28 human histone peptides were found to have lysine crotonylation in this study. Among them, 19 of them were confirmed by feeding experiments with deuterium-labeled crotonate.

A pan-specific crotonyl lysine antibody was developed (53). This antibody was demonstrated to be able to recognize crotonyl lysine, but not acetyl, propionyl, butyryl, or free lysine. Using this antibody, it was demonstrated that several other eukaryotic species also have lysine crotonylation. The antibody was also used in ChIP-seq (chromatin immunoprecipitation-sequencing) experiments to detect the genomic localization of the histone lysine crotonylation in human fetal lung fibroblast IMR90 cells and mouse sperm cells. Interestingly, it seems that that histone lysine crotonylation was associated with mainly promoter or predicted enhancer regions. An increase in crotonylation was observed during spermatogenesis, which coincides with a previously reported histone hyperacetylation. It is not clear whether dedicated enzymes exist to control crotonylation or the same enzymes for lysine acetylation can also regulate crotonylation.

Crotonyl-CoA, the presumed crotonyl donor, can be generated in cells from several different pathways, including the fatty acid β-oxidation pathway, and the lysine degradation pathway (Figure 2). In the fatty acid β-oxidation pathway, many other acyl-CoA molecules could also potentially be used to modify protein. For example, crotonyl-CoA will be converted to 3-hydroxybutyryl CoA. If 3-hydroxybutyryl CoA is used to modify proteins, 3-hydroxybutyryl

lysine will be formed. It should be noted that in two of the sample preparation methods used to identify lysine crotonylation, propionic anhydride was used to propionylate free lysine and the N-termini of proteins/peptides. It is not clear whether the reaction conditions will also lead to the propionylation of hydroxyl group of 3-hydroxybutyryl lysine. If that did happen, it could further lead to an elimination reaction and generate crotonyl lysine (Figure 6). Thus, future studies should investigate whether 3-hydroxy butyrylation also occurs in cells.

Another note about the chemical propionylation method is that although it helps to increase the sequence coverage of histones, it also prevents the identification of proteins that are naturally propionylated. It also makes the identification of butyryl lysine difficult because it has the same mass as propionylated methyl lysine. That is probably why no butyryl lysine was identified in the study.(53) An alternative reason is that butyryl lysine is low in abundance compared to crotonyl lysine.

Lysine malonylation and succinylation

Lysine malonylation and succinylation were discovered in two different ways. One was with mass spectrometry, similar to the discovery of crotonylation, by Zhao and coworkers (4, 7). The other way was through the study of the enzymatic activity of Sirt5, one of the sirtuin enzymes in mammals (3). There are seven sirtuins in mammals. Three of them, Sirt1, Sirt2, and Sirt3, have readily detectable deacetylase activities. Many in vivo deacetylation substrate proteins have been identified for them (19). The other four mammalian sirtuins, Sirt4-7, however, have very weak deacetylases activities (in certain cases too weak to be detectable) *in vitro* (54–58). Furthermore, knocking out several of these sirtuins also failed to lead to global changes in protein acetylation *in vivo* (59). We have been interested in understanding why these sirtuins have weak deacetylase activity. A breakthrough was made the crystal structure of human Sirt5 was obtained in complex with a thioacetyllysine peptide and a CHES buffer molecule (3). CHES has a negatively charged sulfonate group, which was bound by Tyr102 and Arg105 of Sirt5 in the structure. The sulfonate group of CHES is close to the bound thioacetyllysine (Figure 7). These structure features led to the hypothesis that Sirt5 may recognize negatively charged acyl lysine modifications, such as malonylation and succinylation, which was proved to be true by *in vitro* enzymology studies. Biochemical and MS studies also confirmed the existence of lysine succinylation and malonylation in mammalian cells (3).

It should be pointed out that although these studies were probably the first to discover lysine malonylation, they were not the first to discover lysine succinylation. In 2004, it was reported that homoserine transsuccinylase was succinylated on a catalytic lysine residue (60). It was reported in 2006 that protein lysine succinylation could occur during lipid oxidation process under oxidative stress (61). In 2009, Walsh and coworkers discovered that certain thiocillin peptides were succinylated on lysine residues when expressed in *Bacillus cereus* (62). The new finding from the recent studies is that lysine succinylation is more abundant than previously imagined. Zhao and coworkers identified 14 proteins in *E. coli* that have succinyl lysine modification (4). Our study in mammalian cells also suggested that lysine succinylation is very abundant (3). First, most mammalian mitochondrial proteins we purchased from Sigma were shown to have succinyl lysine modifications. Second, the number of succinyl lysine residues in a given protein is close to the number of acetyl lysine residues in that protein. For example, in bovine glutamate dehydrogenase (GDH), we identified 9 succinyl lysine residues, while 16 acetyl lysine residues (3). In contrast to the abundance of lysine acetylation and succinylation, malonylation seems to be lower in abundance. For example, we only identified 3 malonyl lysine residues in GDH.

Most of the succinylated and malonylated proteins are metabolic enzymes. Therefore, it is conceivable protein lysine succinylation and malonylation can regulate the activity of metabolic enzymes, similar to lysine acetylation. One example is CPS1, which was reported to be a Sirt5 target (63). Using MS, we found that Sirt5 regulates the succinylation level of CPS1 on Lys1291, but not the acetylation level of this residue (3). This finding provide evidence that Sirt5's desuccinylase activity is functioning *in vivo*, but clearly more studies are needed to identify more Sirt5 targets and understand how the activity of the target proteins are affected by the desuccinylase/demalonylase activity of Sirt5.

The fact that there is a dedicated enzyme, Sirt5 (3), to remove malonyl and succinyl groups suggests that lysine malonylation and succinylation is distinct from acetylation and may play important roles in regulating physiological processes. Chemically, malonylation and succinylation are unique among all the acyl lysine modification due to the presence of the negatively charged carboxylate group. The carboxylate group can interact with arginine residues, which is similar to the phosphate group found in protein phosphorylation (1). Phosphorylation is known to be able to alter protein structure and regulate protein activity. This chemical similarity of succinyl and malonyl to phosphate suggests that lysine malonylation and succinylation could have unique functions that acetylation could not have. This hypothesis needs to be tested in future research.

Succination of cysteine

Certain pathological conditions can lead to the increase in cellular fumarate concentration. This includes cancer with fumarate hydratase (FH)-deficiency(64, 65) and diabetes (5). Fumarate can react with cysteine residues in certain proteins in a Michael addition reaction, leading to the production of *S*-(2-succinyl)cysteine (Figure 8A). This modification has been called cysteine succination to differentiate from modifications in which succinyl is attached via one of the carbonyl groups (5).

Cysteine succination was discovered in the process to quantify the amount of S-(carboxymethyl) cysteine (CMC), the putative product of reaction of glyoxal or glycolaldehyde with cysteine (66). To quantify CMC relative to the amount of free cysteine, proteins were treated with *N*-ethyl maleimide, which after acid hydrolysis will generate *S*-(2-succinyl)cysteine. Unexpectedly, it was discovered that *S*-(2-succinyl)cysteine was already present without *N*-ethyl maleimide treatment (66). The amount of *S*-(2-succinyl)cysteine increases with age and diabetes. It was demonstrated that certain cysteine residues in proteins, such as GAPDH and adiponectin, can be chemically succinated when incubated with fumarate at physiological pH. Thus, it is believed that cysteine succination occur non-enzymatically and is irreversible. However, succination occurs to certain specific cysteine residues with lower *pKa* values in proteins, such as catalytic cysteine residues in enzymes. Increased succination of GAPDH can explains the loss of activity in muscles of streptozotocin-diabetic rats (67) and increased succination of adiponectin may explain the decreased secretion of adiponectin from adipose tissue in type 2 diabetes (68, 69).

The mechanism behind the increase in fumarate in diabetes may be complicated, despite the model that has been proposed (5). In contrast, the increase in fumarate in FH-deficient cancer cells (64, 65) is much easier to understand. FH is an enzyme in the TCA cycle that converts fumarate to malate. Deficient in FH thus will lead to accumulation of fumarate. FH deficiency predispose to hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome (70). One mechanism proposed for FH-deficiency induced tumorigenesis is that the accumulated fumarate and succinate inhibits prolyl hydroxylase and thus stabilizes HIF-1α (64, 65), which is a transcription factor that can activate the transcription of many genes required for tumorigenesis. However, recently, two reports suggested another

mechanism that involves protein succination (71, 72). It was demonstrated that HLRCC tumors have increased protein succination (73). The succinated protein that has been demonstrated to be important in controlling tumorigenesis is Keap1 (Kelch-like ECH-associated protein 1). Keap1 is thought to be part of an E3 ubiquitin ligase complex that ubiquitinylates the transcription factor Nrf2 (nuclear factor 2), which controls the induction of genes that help to deal with oxidative and electrophilic agents (74). Keap1 has several cysteine residues that are important for its function in regulation Nrf2.(75) Some of these cysteine residues are succinated in FH-deficient cancer cells (71, 72). It is believed that succination of these cysteine residues inactivates Keap1, leading to the stabilization of Nrf2 and tumorigenesis (71, 72).

Cysteine succination is considered to occur non-enzymatically, similar to many other modifications of cysteine, such as nitrosylation and oxidation to sulfinic acid (1). Cysteine succination is also thought be irreversible. However, it is conceivable that succination can be reversed, especially if the original cysteine residue has a lower *pKa* and thus a good leaving group, and the fumarate generated can be quickly consumed (Figure 8B).

Lysine long chain fatty acylation

Protein fatty acylation, including myristoylation on N-terminal glycine residues and palmitoylation on cysteine residues (1), are well known. They play important roles in regulating signal transduction process and membrane trafficking. In contrast, fatty acylation of lysine residues, although reported, have been largely ignored for two decades. However, recent work from our laboratory suggested that lysine fatty acylation may be more abundant than previously thought (76). The work was about the enzymatic activity of a *Plasmodium falciparum* sirtuin protein, PfSir2A. This enzyme has been shown to have deacetylase activity, but the activity was hundreds fold weaker compared with human Sirt1 (77, 78). Our discovery that Sirt5 prefers to remove succinyl and malonyl groups from protein lysine residues prompted us to investigate whether PfSir2A can also remove acyl groups other than acetyl. Surprisingly, we found that PfSir2A is much better at removing long chain fatty acyl groups, such as myristoyl. The catalytic efficiency of demyristoylation is hundreds fold better than that for deacetylation. The increased efficiency of demyristoylation comes from both increased k_{cat} and decreased K_m values. The crystal structure of PfSir2A in complex with a myristoyl peptide reveals that the enzyme has a long hydrophobic pocket that can accommodate the myristoyl group.

The enzymology and structural data suggest that PfSir2A's physiological function may be achieved via demyristoylation. It is known to be involved in antigenic variation in the malaria parasite, a process in which the parasite periodically changes its surface antigen presentation to evade the host's immune system (79, 80). It was thought that PfSir2A achieve this function by deacetylating histones. It is possible that this function is actually achieved by demyristoylation of histones or other proteins. Consistent with the hypothesis, long chain fatty acyl lysine modifications was detected in *P. falciparum* proteins using an enzymatic assay (76).

Several proteins are known to be myristoylated on lysine residues. These include the *Escherichia coli* Hemolysin HlyA (81), TNF-α and IL-1a in mammalian cells (82, 83), and cytochrome c oxidase in *Nerospora crassa* (84). Recently, several chemical proteomic approaches has been developed to label and identify N-terminal myristoylated protein and cysteine palmitoylated proteins (85–88). However, it should be noted that in most of these approaches, the residues that are fatty acylated cannot be identified by MS. Thus, it is likely that some of the identified proteins actually are fatty acylated on lysine residues, instead of N-terminal glycine or internal cysteine residues. The fact that eukaryotic cells have sirtuins

that can remove long chain fatty acyl groups suggest that lysine fatty acylations are more abundant than previously known and they likely play important roles in physiology.

Unaddressed questions and future directions

The discoveries of the PTMs discussed above are fairly recent with most of the studies published within the last five years or so. It is amazing how fast the field has progressed, thanks to the availability of many modern technologies, such as MS. These discoveries also raised many interesting questions and posed new challenges for existing technologies. Some of these questions and challenges are discussed below.

Improved analytical chemistry for the detection of new modifications

The identification of new modifications calls for corresponding analytical methods to detect and analyze them. At present, mass spectrometry is the major analytical method for the detection of protein modifications. However, many improvements are needed for the detection of new modifications. First, most routine protein MS method can only detect modifications that the users tell the software to look for. Thus, completely new modifications are not easy to identify. Certain search algorithm are available that helped the identification of certain modifications (89). However, the search results may give many unexplainable modifications and figuring out the structures of those modifications are not straightforward. Second, some modifications may be low abundance under the conditions used and thus are difficult to detect. Therefore, methods that can be used to enrich these modifications are needed. Third, certain modifications may be difficult to detect due to their distinct chemical properties. For example, long chain fatty acyl groups are very hydrophobic and peptides with the modifications may be difficult to isolate from the crude mixture, or difficult to resolve on HPLC. Antibodies for such modification are also difficult to obtain, as no antibodies for *N*-terminal glycine myristoylation or cysteine palmitoylation are available. Chemical tagging methods recently developed are useful for labeling and identifying proteins with acyl lysine modifications.(85–88, 90, 91) Most of these methods utilize alkyne-tagged acyl groups that allow installation of affinity or fluorescent probes via click chemistry to facilitate the detection, isolation, and identification of modified proteins. However, for long chain fatty acyl lysine modifications, directly identifying the residues modified using MS is still difficult.

Stoichiometry of different acyl lysine modifications

Most publications on protein lysine acetylation did not report on the percentage of acetylation. A few reports did provide such quantitative information using standard peptides combined with iTRAQ (9, 28). Now that many more acyl lysine modifications are identified that can occur on the same lysine residue, a new question emerges: what is the stoichiometry of different acyl modifications that occur on the same lysine residue? This question is of particular interest from several considerations.

First, MS typically can identify many acetyl or succinyl lysine residues on one protein. However, typically only some of these modified residues are important for the activity of the protein and are regulated by sirtuins. Is it possible that modified residues that do not affect the activity simply because the stoichiometry of the modification is very low while those that do affect the activity have a higher abundance? Measuring the stoichiometry of all the modifications will help to address this question.

Second, considering the connection between metabolism and protein modifications, does the stoichiometry of the different acyl lysine modifications correlate with the abundance of the different acyl-CoA molecules? This question is also relevant for finding out whether these modifications occur enzymatically or non-enzymatically, as will be discussed further below.

Investigating the specificity of existing acyl lysine antibodies

Most acetyl lysine antibodies were developed before the new acyl lysine modifications were discovered and thus whether they are truly specific for acetyl lysine or they can also recognize some of the new acyl lysine modifications need to be further investigated. Although this may be a tedious job, it is of particular importance because acyl lysine antibodies are typically used in high-throughput studies, such as ChIP-seq (92), to investigate the function of the acyl lysine modifications. If the antibodies used are not specific, the conclusions of such studies will be unreliable. This problem is not limited to lysine acylation, as a recent survey of ~200 antibodies against different histone modifications revealed that ~25% did not pass the specificity test (93). This problem may be even worse considering that the modifications tested did not include any of the newly identified modifications.

Physiological functions of newly discovered modifications

For all the new modifications discovered, the key question is what the physiological functions of these modifications are. This question is naturally connected to whether there are specific enzymes that catalyze the addition/removal of the modifications. Cysteine succination appears to be non-enzymatic and irreversible. Other modifications discussed above, can be enzymatic and reversible. Finding the enzymes that are specific for these modifications are important because disrupting these enzymes will allow the dissection of the function of the modifications. This point can be easily appreciated from what have been learned about lysine acetylation and deacetylation – most of these would not have been possible if the acetyltransferases and deacetylases were not known.

One enzyme specific for the newly identified modifications is Sirt5, which is specific for removing succinyl and malonyl groups (3). Sirt5 thus will be important for understanding the function of lysine succinylation and malonylation. Another one is PfSir2A, which is specific for removing long chain fatty acyl groups (76). Although the physiological function of this activity has not been directly proven, studying this enzyme will certainly provide important insights about the function of lysine long chain fatty acylation.

In contrast to enzymes that can remove different acyl groups, it is not clear whether there are enzymes that catalyze the addition of different acyl groups. Several observations have prompted the hypothesis that acyl lysine modifications, including acetylation of metabolic enzymes, may occur non-enzymatically. First, it is known that acyl-CoA molecules can slowly react with protein lysine residues to form a small amount of acyl lysine modifications *in vitro*. It is possible the same could occur in cells. Second, for a given protein, MS can identify many acetyl lysine modifications, however, only a few of them (Table 1) seems to be important for the activity of the protein and can be removed by sirtuins. Those that do not affect the activity of the protein (and presumably occurs at very low levels) may occur by non-enzymatic pathways.

On the other hand, there are also observations suggesting that there may be enzymes catalyzing the formation of acyl lysine modifications. First, it has been shown that in *Salmonella*, PAT can catalyze the acetylation of metabolic enzymes. Recently, the mammalian mitochondrial acetyltransferase, GCN5L1 (94), has also been reported. Thus, at least some of the acetylation events are enzymatic. Second, for metabolic enzymes that have multiple acetyl lysine identified, typically only one or a few of them are important and account for most of the detectable acetylation on Western blot. It is reasonable to postulate that the acylation of these acyl lysine resides are enzymatic while those that are much less abundant and not important for activity occur non-enzymatically.

To conclusively address this question, the responsible acyltransferases must be identified and characterized. At this point, the acetyltransferases, PAT and GCN5L1, responsible for the modification of metabolic enzymes are the best candidates to study. Understanding how these acetyltransferases are regulated may provide important information that can lead to the identification of other acyltransferases or help to better understand the regulation of metabolism by acetylation/deacetylation. For example, PAT has an acetyl-CoA synthetase (ACS) domain in addition to the acetyltransferase domain. What is the function of the ACS domain? Could it regulate the activity of the acetyltransferase domain? These kinds of questions will have to be addressed in order to fully understand the function of PAT and protein acetylation. Toward this direction, another protein lysine acetyltransferase, PAT from Mycobacterium, is worth mentioning (95, 96). The Mycobacterium PAT protein has an N-terminal cyclic AMP (cAMP) binding domain. The binding of cAMP activates the acetyltransferase activity, suggesting that protein lysine acetylation in mycobacterium is regulated by cAMP signaling. In *E. coli*, cAMP signaling may also regulate protein acetylation (97).

For those modifications that show important physiological function, it will be interesting to investigate how they affect the function of the proteins. For this purpose, it will be necessary to examine the activity of the proteins with and without the modifications on specific residues. Thus, methods to site-specifically install modified residues into proteins will be very useful. Chemical semi-synthesis methods have been developed to incorporate acetyl lysine analogs into proteins.(98–100) This method is easy to adopt but requires native chemical ligation (which requires the modification to be close to the N or C terminal of proteins) or the replacement of the modified lysine to cysteine. Methods to genetically incorporate acetyl lysine residues into proteins in *E. coli* has also been developed (101, 102) and used to study the acetylation of histones (103), p53 (104), and cyclophilin A (105). This method is more difficult to develop but have a lot of potential. Efforts are currently being invested to develop similar systems that can be used in higher eukaryotic cells (106).

Obtaining a better picture for the connections between metabolism and protein modifications

All the modifications discussed here use common metabolites as donor molecules for the modification. The removal of some of the acyl lysine residues requires NAD, which is another metabolic small molecule. Many of the modifications also occur on metabolic enzymes and regulate the activity of metabolic enzymes. Thus these modifications can be mechanisms that cells sense the metabolic state to regulate cellular metabolism. Future studies are needed to further test this hypothesis. There are several other systems known to function like this. For example, the mammalian target of rapamycin (mTOR) system is known to sense the availability of nutrients and grow factor signaling to phosphorylate proteins to regulate cell grow and proliferation.(107) The AMP-activated protein kinase (AMPK) pathway is known to sense the increase in AMP/ATP ratio and phosphorylate proteins to increase ATP production.(108) In the future, it will be interesting to investigate the difference, similarity, and connections among these different systems(109) when more details about these systems are available.

To certain extent, these various modifications that regulate different biological processes speak for the importance of metabolism. Traditionally, it has been viewed that signaling pathways regulate metabolism by transcriptional control and protein modifications (mainly phosphorylation). These PTMs that use metabolic intermediates suggest that metabolism perhaps can directly regulate signaling pathways by changing the modification and activity of important signaling proteins. Such reverse regulation may be very abundant given the large number of proteins that are known to be acetylated (10). Consistent with this idea, it has been suggested that acetyl-CoA is a critical metabolic signal for cell growth and

proliferation in yeast (110). Understanding how metabolism is regulated and how metabolism regulates other cellular processes is extremely interesting and important for human health. This is a truly inter-disciplinary area that will require the close collaboration between chemists and biologists. Chemistry, such as new analytical method, new chemical tools, and chemical thinking, will play important roles in addressing the challenges in the field.

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Keywords

Lysine acylation	modification of protein lysine residues by various acyl groups, such as acetyl and succinyl
Sirtuins	a class of enzymes that are homologous to yeast Sir2 protein. Sirtuins can use nicotinamide adenine dinucleotide (NAD) to remove acyl groups from protein lysine residues
Energy metabolism	the transformation of various carbon sources (e.g. glucose, fatty acid, and amino acids) for the production ATP or biosynthetic intermediates
Protein post-translational modifications	changes in protein chemical structure after proteins are synthesized
Cysteine succination	a modification of protein cysteine residue resulting from a Michael reaction of cysteine side chain with fumarate
Glycolysis	the pathway that covert glucose to pyruvate, which is then converted to acetyl-coenzyme A and enters the tricarboxylic acid cycle. Glycolysis coverts NAD to NADH and generates ATP mainly in the cytosol
TCA cycle	the tricarboxylic acid cycle, or the Krebs cycle, or the citric acid cycle. Through this cycle, acetyl-CoA is converted to carbon dioxide (CO_2) and the energy released is eventually used to synthesize ATP. Intermediates in the pathway can also be used for biosynthesis purpose
Gluconeogenesis	the pathway that synthesizes glucose under low glucose conditions. Such a pathway exists because glucose is the precursor for many other biosynthesis pathways or is the preferred energy source for the mammalian brain
Fatty acid β-oxidation	the pathway that oxidizes fatty acyl-CoA to generate acetyl-CoA (propionyl-CoA can be generated for fatty acids with odd number of carbon atoms). Intermediates in this pathway include enoyl-CoA, β-hydroxyacyl-CoA, and β-ketoacyl-CoA
Fatty acid biosynthesis	the pathway that condenses malonyl-CoA and acetyl-CoA (or propionyl-CoA) to generate long chain fatty acyl-CoA. Intermediates in this pathway include enoyl-CoA, β-hydroxyacyl-CoA, and β-ketoacyl-CoA

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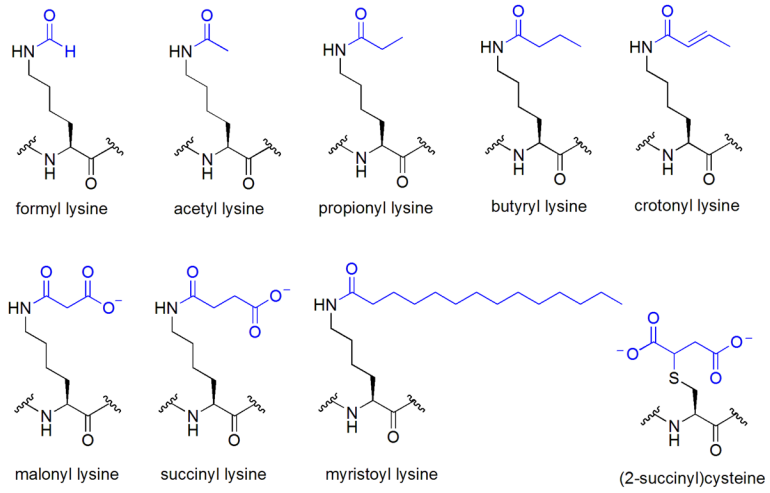

Figure 1.
Structures of PTMs that will be discussed. The post-translationally added parts are colored in blue.

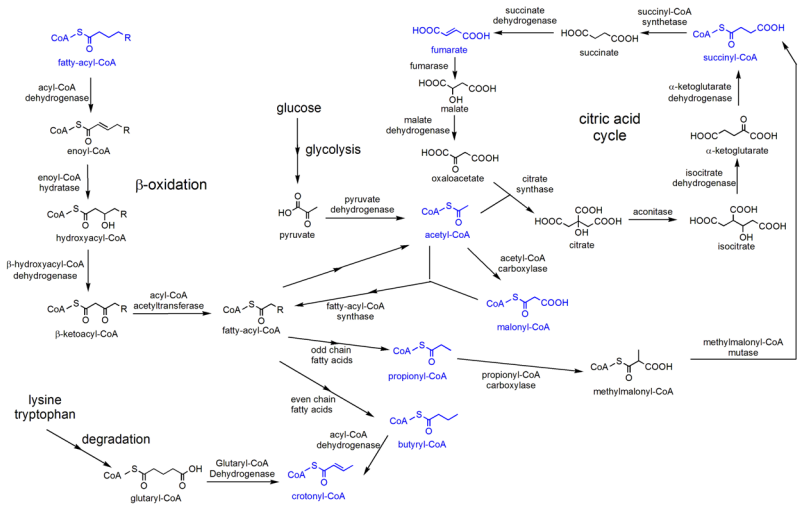

Figure 2.
Metabolic pathways that provided the donors for the PTMs shown in Figure 1, except for lysine formylation.

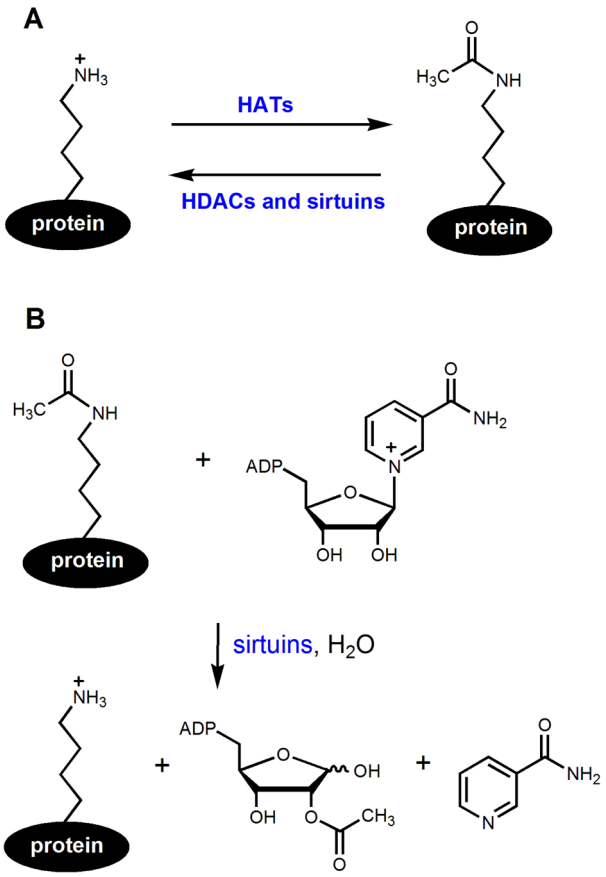

Figure 3.

(**A**) Protein lysine acetylation and deacetylation are controlled by acetyltransferases (HATs) and deacetylases (HDACs and sirtuins). (**B**) The NAD-dependent deacetylation reaction catalyzed by sirtuins.

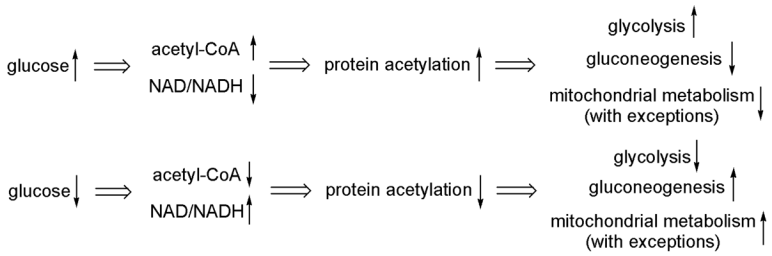

Figure 4.
General model of how metabolic enzyme acetylation changes according to glucose concentration.

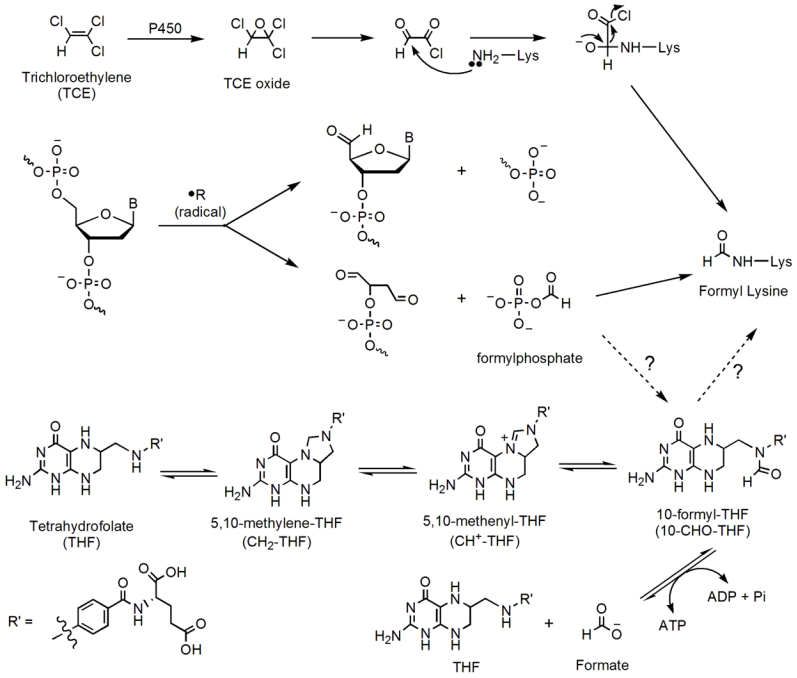

Figure 5.

Different pathways for lysine formylation. Formyl-THF could be the formyl donor for a hypothetical enzymatic pathway.

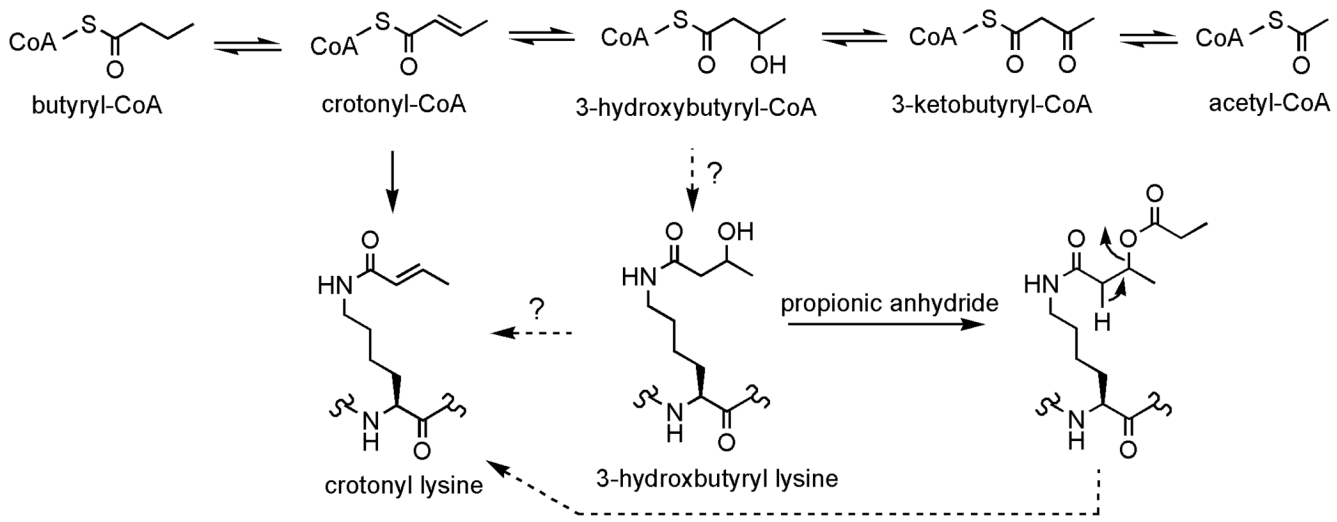

Figure 6.
Possible pathways for lysine crotonylation.

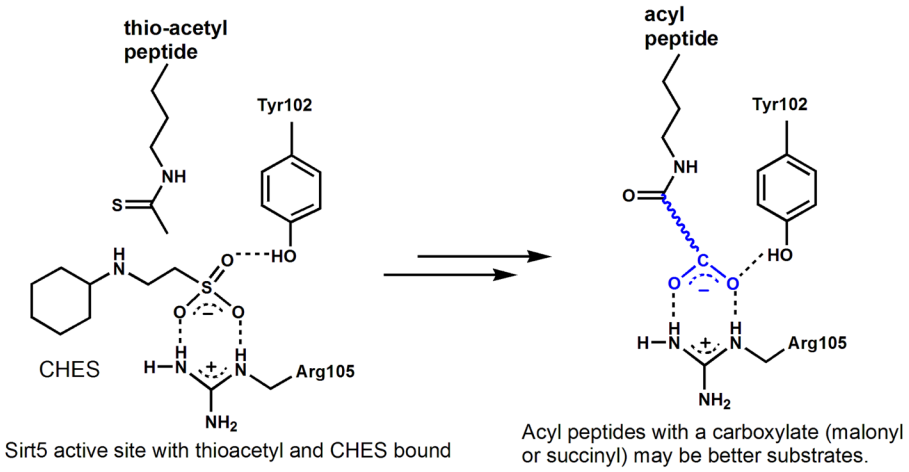

Figure 7.

Schematic representation of Sirt5 active site structure with thioacetyllysine and CHES bound. This structure led to the hypothesis that Sirt5 is a demalonylase or desuccinylase.

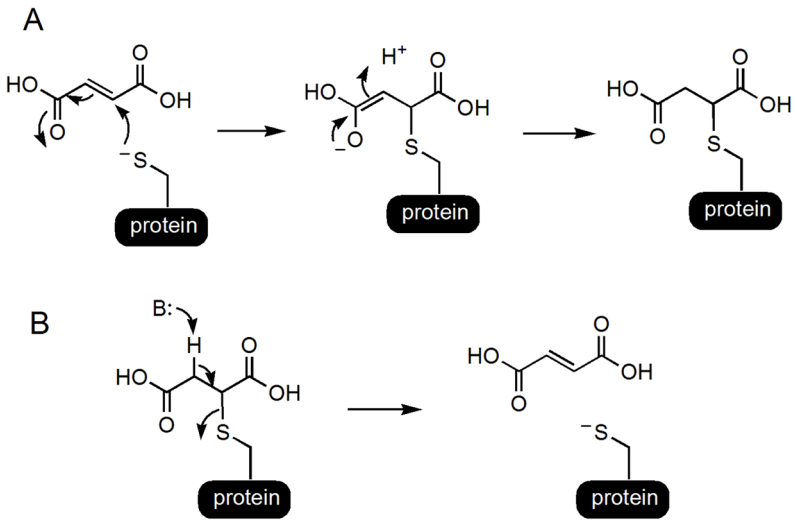

Figure 8.
Cysteine succination. (**A**) Michael reaction for the formation of (2-succinyl)cysteine. (**B**) A general base can potentially promote the reversal of (2-succinyl)cysteine.

Table 1

Metabolic enzymes that are regulated by acetylation/deacetylation. Enzymes that are activated by acetylation are colored blue.

Enzymes	Function of enzyme	Effect of acetylation on enzyme activity	Key acetyl lysine residues identified	Effects of metabolic condition on acetylation	Acetyl-transferase involved	Deacetylase involved
Salmonella ACS(21)	Acetyl-CoA synthetase, converting acetate to acetyl-CoA, important for growth on acetate as the carbon source	Acetylation decrease activity	K609		PAT	CobB
Salmonella GapA(8)	Glyceraldehyde phosphate dehydrogenase, important for glycolysis and gluconeogenesis	Acetylation increase glycolysis but inhibits gluconeogenesis	K331	More acetylation in glucose than in citrate	PAT	CobB
Salmonella AceA(8)	Isocitrate lyase. Converts isocitrate to glyoxylate and succinate; important for gluconeogenesis when grow in citrate as the carbon source	Acetylation decreases activity	K308	More acetylation in glucose than in citrate	PAT	CobB
Salmonella AceK(8)	Isocitrate dehydrogenase (IDH) kinase/phosphatase, phosphorylation of IDH by AceK inhibits IDH activity, which is important for the TCA cycle.	Acetylation inhibits activity and thus lead to increased IDH activity	K72, K83 K553	More acetylation in glucose than in citrate	PAT	CobB
Yeast PEPCK(20)	Phosphoenolpyruvate carboxykinase, an enzyme in gluconeogenesis	Acetylation is important for activity	K514	It was presumed that starvation increases acetylation, but not experimentally demonstrated	NuA4	Sir2
Human EHHADH(9)	Enoyl–coenzyme A hydratase/3-hydroxyacyl–coenzyme A, functions in fatty acid beta oxidation	Acetylation increases activity	K165, K171, K346, K584	More acetylation when cells were cultured with fatty acids		
Human MDH(9)	Malate dehydrogenase, TCA cycle enzyme	Acetylation increases activity	K185, K301, K307, and K314	High concentration of glucose increased acetylation		
Human ASL(9)	argininosuccinate lyase, urea cycle enzyme	Acetylation decreases activity	K69, K288	Extra amino acids (Asp and Glu) decreased acetylation		
Human PEPCK1(9, 22)	Phosphoenolpyruvate carboxykinase1, an enzyme in gluconeogenesis	Acetylation leads to protein degradation	K70, K71, K594	High conc. of glucose increased acetylation and high conc. of amino acids in glucose-free medium decreased acetylation	P300(22)	Sirt2(22)
mouse ACS1(23)	Cytosolic acetyl-CoA synthetase, converting acetate to acetyl-CoA	Acetylation decreases activity	K661			Sirt1
HMGCS1(24)	Cytosolic 3-hydroxy-3-methylglutaryl-CoA synthase, important for cholesterol biosynthesis and isoprene lipid biosynthesis	Not reported	Not experimentally determined			Sirt1
Human PGAM1(25)	Phosphoglycerate mutase-1, interconvert 3-phosphoglycerate to 2-phosphoglycerate	Acetylation increases activity	K251, K253, K254	Glucose increased acetylation		Sirt1
Mouse/Human ACS2(23, 26)	mitochondrial acetyl-CoA synthetase, converting acetate to acetyl-CoA	Acetylation decreases activity	K635(mouse) K642(human)			Sirt3
Mouse/Human MnSOD(27, 28) (29)	Mitochondrial Mn-dependent superoxide dismutase, which converts superoxide to dioxygen and hydrogen peroxide. Important for removing reactive oxygen species	Acetylation decreases activity	K53(29), K68(28), K89(29), K122(27), K130(28). Conclusions about which acetyl lysine is important differ in the three references.	Calorie restriction induced deacetylation(28)		Sirt3

Enzymes	Function of enzyme	Effect of acetylation on enzyme activity	Key acetyl lysine residues identified	Effects of metabolic condition on acetylation	Acetyl-transferase involved	Deacetylase involved
SDH(30, 31)	Succinate dehydrogenase (complex II), a multi-subunit enzyme responsible for oxidizing succinate to fumarate and transferring electrons to ubiquinone	Acetylation decreases activity	Several, but not clear which ones are important for activity			Sirt3
IDH2(32)	$NADP^+$-dependent isocitrate dehydrogenase in mitochondria. Converts isocitrate to a-KG and generates NADPH, which is important for removal of oxidative stress	Acetylation decreases activity				Sirt3
HMGCS2(33)	mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase, important for ketone body production in the liver during fasting	Acetylation decreases activity	K310, K447, and K473. Regulated by Sirt3. The importance was not tested	Calorie restriction induced deacetylation		Sirt3
Mouse LCAD(34)	Long chain fatty acid dehydrogenase, important for the beta oxidation of long chain fatty acid	Acetylation decreases activity	K42 was determined to be the most important one	Calorie restriction induced deacetylation		Sirt3