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Reactive Acyl-CoA Species (RACS) modify proteins and induce carbon stress

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Abstract

In recent years, our understanding of the scope and diversity of protein post-translational modifications has rapidly expanded. In particular, mitochondrial proteins are decorated with an array of acyl groups that can occur non-enzymatically. Interestingly, these modifying chemical moieties are often associated with intermediary metabolites from core metabolic pathways. In this review, we describe biochemical reactions and biological mechanisms that activate carbon metabolites for protein post-translational modification. We explore the emerging links between the intrinsic reactivity of metabolites, non-enzymatic protein acylation, and possible signaling roles for this system. Finally, we propose a model of ‘carbon stress’, similar to oxidative stress, as an effective way to conceptualize the relationship between wide-spread protein acylation, nutrient sensing, and metabolic homeostasis.

Keywords

Carbon stress; Oxidative stress; Reactive acyl-CoA species; Reactive oxygen species; Post-translational modifications; Metabolism

Introduction

Both histone and non-histone proteins are subject to a wide range of post-translational modifications (PTMs), which, alone or in combination, shape the functional proteome. The chemical diversity of PTMs ranges from small, negatively charged moieties (e.g. phosphorylation) to large, hydrophobic molecules (e.g. palmitoylation), to complex, branched structures (e.g. glycosylation). Collectively, PTMs increase the diversity of

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chemical properties of protein side-chains beyond those imparted by each of the proteinogenic amino acids [1]. Furthermore, the dynamic addition and removal of these modifications provides the potential for precise control over protein localization, activation, repression, stability, degradation, or overall function.

A canonical paradigm in the field is transcriptional regulation by histone acetylation [2, 3]. Histone protein *N*-terminal tails have unique ‘writers’, known as histone/protein acetyltransferases, which place acetyl groups on lysine residues; ‘readers’, such as bromodomain-containing proteins which recognize and bind to acetylated lysines; and ‘erasers’, known as histone/protein deacetylase enzymes, which remove acetyl groups from proteins. Together, this system coordinates discrete transcriptional regulatory responses to maintain cellular homeostasis. This paradigm extends beyond protein acetylation, where PTMs such as phosphorylation, *O*-linked *N*-acetylglucosamine (O-GlcNAc), and crotonylation each have their own writers, readers, and erasers.

In recent years, the known landscape of PTMs has rapidly expanded. For example, lysine PTMs extend beyond acetylation and now include propionylation, succinylation, and glutarylation, to name a few [4]. Early studies on several protein acyl modifications revealed that mitochondrial proteins are commonly modified [5]. These modifications are thought to play key roles in controlling metabolic processes, and their dysregulation accelerates the development of several metabolic diseases [6]. Because of the established regulatory paradigm of PTM writers, readers, and erasers on histones and non-histone proteins, a similar concept was suggested for mitochondrial protein acyl modifications in which metabolic proteins are post-translationally modified, lysine hyperacylation reduces enzymatic activity, and deacylases restore enzymatic activity.

However, several recent studies support the idea that acyl modifications to mitochondrial proteins can occur stochastically and non-enzymatically [7–9]. Furthermore, not all mitochondrial protein modifications change protein function or overall metabolism in measurable ways (e.g. [10–12]). In light of these emergent concepts, we describe an alternative model by which PTMs could control mitochondrial metabolism. In this review, we consider the evidence for non-enzymatic protein acylation that is based on the intrinsic reactivity of metabolites, reactive species generated from carbon flux, and a possible signaling role for this system. We further propose that carbon stress, similar to oxidative stress, is an effective model to conceptualize the relationship between wide-spread protein acylation, nutrient sensing, and metabolic homeostasis.

Ancient Reactive Metabolites

A model for the origin of biochemistry, and therefore of all life, focuses on prebiotic analogues of core biochemical reactions [13]. These hypothetical chemical reactions likely led to the synthesis of purines and pyrimidines, thereby generating the building blocks for nucleic acids and DNA that permitted the evolution of genes. An inexhaustive list of probable candidate compounds for central involvement in prebiotic chemistry include: acetate, acetyl thioesters, acetyl phosphate, carbamate, carbamoyl phosphate, carbon monoxide, carbonyl sulfide, carboxy phosphate, cyanide, formate, formyl phosphate, metal

sulfides, or methyl sulfide [13–15]. At first glance, this list (Fig. 1, Key Figure) seems unremarkable, with chemicals that are generally not central to clinical biochemistry. Instead, important roles are highlighted for glucose, amino acids, lipids, adenosine triphosphate (ATP), acetyl-CoA, malonyl-CoA and other intermediates of mammalian biochemical pathways. However, these complex macromolecules must have assembled from reactive molecular building blocks. Carbon precursors with enough reactivity to assume this role include acetyl-thioesters or acetyl-phosphate, with the latter possibly serving as the universal energy currency prior to the appearance of ATP. Energy-rich nitrogen might have entered metabolism as ammonia, followed by the synthesis of carbamoyl phosphate. Together, these simple chemical intermediates could have produced the more complex macromolecules that are familiar to contemporary biochemists; indeed, several of these reactive precursors can still be found as intermediates in biochemical and metabolic pathways of modern biochemistry, such as nucleotide synthesis.

High, intrinsic reactivity of complex biomolecules would present a liability for carrying out cellular functions. Intra- and inter-cellular communication, or long-range communication between distant organs, all require stable molecules that do not readily react with the surrounding complex milieu. Carbon bonds are stable with low intrinsic reactivity, making them useful backbones for building complex molecules. However, too little reactivity presents a problem for building new molecules, because the energy required to make or break new bonds is prohibitively high. Thus, activation of stable bonds, such as carbon-carbon bonds, is required for building the proteins, lipids, carbohydrates, and nucleic acids needed for life.

Activated Metabolites

The concept of carbon activation was pioneered during a series of studies on the mechanism of sulfonamide acetylation in pigeon liver extracts by Fritz Lipmann [16]. This work led to discovery of a new cofactor that activated acetate which Lipmann named coenzyme A (CoA) – with the ‘A’ standing for activation (of acetate); acetyl-CoA was produced by a reaction of ATP with acetate and this new coenzyme, which was the activated carbon species that acetylated sulfonamide [17]. Further studies showed that CoA is composed of adenosine-5'-phosphate pantothenic acid and a sulfhydryl moiety (Fig. 2); the biochemical importance of acetyl-CoA became evident from studies implicating it in the acetylation of choline, in the synthesis of citrate and acetoacetate, and in pyruvate metabolism [18]. Lipmann's work on the coenzyme was eventually awarded the Nobel Prize in Physiology or Medicine in 1953 “for his discovery of co-enzyme A and its importance for intermediary metabolism”, which he shared that year with Hans Krebs “for his discovery of the citric acid cycle”.

Coenzyme A is now considered the quintessential activating coenzyme for breaking down large biomolecules and building new ones. CoA activates carbon through its thioester bond with a carbonyl group, which generates a new bond that links carbon with a good leaving group. The energy catalyzing this activation is generated through the degradation of a substrate, such as occurs with the enzyme pyruvate dehydrogenase, or by activation with ATP. Indeed, acetyl-CoA produced from pyruvate oxidation is a canonical example of central carbon metabolism requiring activated metabolites. Acetyl-CoA subsequently enters

the citric acid cycle, producing citrate that undergoes catabolic reactions to fuel oxidative phosphorylation. Within and beyond the citric acid cycle, CoA activates acetyl groups for use in numerous metabolic processes [19].

Furthermore, CoA is not limited to activating acetyl groups and can activate other, longer carbon chains (Fig. 2). For example, early work on microbial lipid oxidation by Horace Barker demonstrated that oxidation of butyrate in extracts of *C. kluyveri* occurs by a mechanism in which butyrate is first converted to butyryl-CoA, and the 4-carbon intermediates involved in its oxidation each exist as CoA derivatives [18]. Then the last of these to be formed, acetoacetyl-CoA, is cleaved by reaction with a molecule of free CoA to form two molecules of acetyl-CoA, which in the presence of phosphate is converted by phosphotransacetylase to acetyl-phosphate and CoA; these early findings in bacteria were subsequently shown to be conserved in animals. Indeed, it is now well-accepted that CoA activation of carbon is a major mechanism that organisms use for metabolism. Conversely, CoA activation of carbon is also used in the synthesis of long carbon chains. Malonyl-CoA permits the transfer of carbon moieties to the enzyme fatty acid synthase, forming the sixteen-carbon fatty acid palmitate, which then can be further coupled to CoA for reactions directed towards storage, oxidation, or transport.

In addition to CoA conjugation, the creation of phosphoester bonds between a carbonyl group and phosphate also provides activating energy (Fig. 1). Acyl-phosphates are commonly used in bacterial species for carbon activation, and can be found in a few discrete cases in mammalian metabolism [20]. For example, glutamine synthase generates gamma-glutamyl phosphate as a reaction intermediate, which is considered ‘activated glutamate’ [21]. Similarly, asparagine synthase generates an aspartyl acyl-phosphate intermediate; but in this case, the ‘activated aspartate’ is conjugated to a reactive acyl-AMP [22]. Perhaps because of their high reactivity, acyl-phosphates are more often found as reaction intermediates, as opposed to intermediary metabolites.

Anabolic and catabolic processes in mitochondria require the formation of reactive intermediate species for chemical reactions. The foregoing concepts of ancient, reactive biochemical intermediates predict that reactive intermediates have no discrimination between modification of a substrate in an enzyme and modification of proteins, such as the enzymes that catalyze the production of the intermediates. Thus, reactive compounds that are candidates to have started life itself, and are found in modern biochemical reactions, are likely reactive enough to non-enzymatically modify proteins.

Reactive Acyl-CoA Species and Other Reactive Activated Carbon Species (RACS)

Historically, acetyl-CoA was known as an effective non-enzymatic acetylating agent in studies with recombinant or purified proteins [23–26]. In the past 5 years, the concept of non-enzymatic protein acetylation, especially of mitochondrial proteins, has gained traction as a mechanism to explain *in vivo* protein acetylation. This process was shown to be more favorable in the slightly elevated pH environment of the mitochondrial matrix [27]. The elevated pH of the mitochondria (pH = 7.9–8.0) is predicted to create a larger proportion of

deprotonated amine groups on lysines; however, it is important to note that the pKa of the ϵ -amino group of a free lysine is 10.53 at 38 °C [28], still greater than two orders of magnitude higher than the predicted mitochondrial pH. However, the pKa of the ϵ -amino group of a lysine in the context of a folded, functional protein could be significantly lower [29, 30]. When deprotonated, the lone pair of electrons on the free amine can perform a nucleophilic attack on the carbonyl carbon of acetyl-CoA. Supporting this idea, the incubation of liver mitochondrial protein with acetyl-CoA at pH 8.0 leads to abundant acetylation in a time and concentration dependent manner, whereas incubation of protein with acetyl-CoA at lower pH does not lead to acetylation [9]. This mechanism extends to other straight-chain acyl-CoA species, including propionyl-CoA, and butyryl-CoA, which similarly modify BSA at basic pH but not acidic pH [9]. More recently, one study described *S*-acetylation as an intermediate to intra- or inter-molecular *N*-acetylation. This mechanism may provide direction on what acylation events have evolved to have a greater functional impact [31]. All together, these studies support the idea that protein acylation can arise non-enzymatically from reactive acyl-CoA species (RACS).

Another mechanism of protein acylation applies to a specific subset of activated carbon metabolites. Dicarboxylic acyl-CoAs with a backbone of 4–5 saturated carbons and a negatively charged carboxylate undergo intramolecular catalysis (i.e. self-hydrolysis) to form a reactive anhydride species and free CoA [8]. This class of metabolites includes succinyl-CoA, glutaryl-CoA, and other dicarboxylic acyl-CoAs with similar structures (Fig. 2). The resulting anhydride is highly reactive and reacts with free amines on lysine residues to form acyl-lysine modifications. Thus, reactive acyl-CoA species that undergo intramolecular catalysis are more reactive than other acyl-CoA species, more readily hydrolyze, and are more potent protein-modifying agents.

As one example, this concept was tested both *in vitro*, by adding increasing amounts of acyl-CoA to purified protein, and *in vivo*, in the mouse model for HMG-CoA lyase (HMGCL) deficiency, where the enzyme HMGCL is not present to breakdown HMG-CoA. Protein ‘HMGylation’ is elevated 4–5-fold in HMGCLKO mouse livers [8]. Mitochondrial proteins are particularly susceptible to modification, perhaps because the self-hydrolysis reaction is more likely to occur at higher than neutral pH, as is found in the mitochondrial matrix; and because the higher the concentration of the reactive metabolites could lead to a greater the number of modifications.

Beyond reactive acyl-CoA species, the concept of RACS extends to other reactive activated carbon species. 1,3-bisphosphoglycerate (1,3-BPG) is a reactive acyl-phosphate metabolite found in the glycolytic pathway (Fig. 1). The production of 1,3-BPG by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) leads to 3-phosphoglyceryl-lysine (pgK) modifications on GAPDH [32]. Interestingly, 1,3-BPG concentrations increase when cells are exposed to high glucose levels and this increase is positively correlated with pgK modifications on GAPDH as well as other proteins. These data suggest pgK modifications are produced as a direct result of the flux through the glycolytic pathway that generates this reactive metabolite. Additionally, a recent study showed that amino acids that are activated by aminoacyl tRNA synthetases to form reactive aminoacyl-adenylates can modify proteins [33]. This study found wide-spread lysine modification by amino acids, covering all twenty

proteinogenic residues. The acyl-phosphate linkage of activated amino acids was permissive for protein aminoacylation, and could play an important signaling role for sensing amino acid levels.

As a final example, fumarate is a reactive activated carbon species that can modify proteins under conditions of elevated metabolite levels. Unlike acyl-CoA or acyl-phosphates, the reactivity lies within a carbon-carbon double bond, which reacts with proteins to form a covalent modification called ‘succination’ [34, 35] through Michael addition of cysteine to a double bond. One example of succination, is the modification of respiratory chain proteins that occurs when fumarate hydratase (FH) is inactivated. Following loss of FH, levels of both fumarate and succination increase, particularly on the mitochondrial protein succinate dehydrogenase (aka complex II) [36]. *In vitro* studies show that incubation of complex II with fumarate results in a dose dependent, non-competitive inhibition of complex activity. Thus, the inherent reactivity of RACS means that they have a strong propensity to modify proteins based on their concentration and proximity.

The Carbon Stress Model

Despite progress in understanding the biochemical bases for non-enzymatic protein modifications, a major remaining challenge is integrating the seemingly abundant and complex landscape of protein post-translational modifications with the functional relevance and control of metabolism. In some cases, protein hyperacylation can be associated with altered flux through metabolic pathways or reduced enzymatic activities; however, in other cases, no such alterations can be measured. If RACS are intrinsically reactive, then a stochastic model of reactivity with proteins might be expected. In this paradigm, some, but not all, protein modifications would be influential. To reconcile these observations and emerging notions, we propose a model whereby RACS induce ‘carbon stress’, in a similar way that reactive oxygen species (ROS) induce oxidative stress (Fig. 3).

Initially thought to be only mediators of protein damage, ROS are now appreciated to have important signaling roles. ROS-mediated signaling events occur primarily through modification of thiol residues, creating sulfenic groups that can modulate protein interactions, activity, and overall protein function (Fig. 3). As a canonical example, ROS signaling is a key cellular mechanism to respond to growth factors [37]. However, ROS-mediated signaling can transition to cellular damage when uncontrolled [38]. Similar to ROS, RACS may have a signaling role in some contexts, but may be damaging when uncontrolled.

The carbon stress model posits that the stochastic, non-enzymatic nature of some protein acylation events could impart signaling-like effects by specifically tuning up or down select metabolic pathways in response to changing metabolite concentrations. Indeed, protein post-translational modifications have been shown to influence protein interactions, activity, and overall protein function, similar to ROS. In this setting, carbon stress would be dependent upon the concentration of metabolites generated from central carbon metabolism. At lower concentrations, RACS might modify select residues, resulting in a signaling response,

whereas at higher concentrations a greater number of residues might become modified and have a more potent and broader impact on pathway and cellular function.

If true, then we might predict that enzymes handling reactive acyl-CoAs would be most susceptible to modification by the metabolites they handle. As one example, we previously found the methylcrotonyl-CoA carboxylase complex (MCCC) was modified by a number of acyl species, but most predominantly by the activated carbon metabolites formed in the leucine catabolic pathway, of which MCCC is a part [39]. Modifications to MCCC are removed by the mitochondrial protein deacylase sirtuin 4 (SIRT4) and knocking out SIRT4 leads to MCCC hyperacylation. In this state, MCCC has lower activity and complex formation is impaired [39]. Beyond MCCC activity, leucine catabolic flux is also lower. This example suggests that enzyme acylation by RACS generated in the same metabolic pathway could provide a negative feedback loop that temper the activity of the enzyme and contribute to metabolic homeostasis.

In a second example, when GAPDH is modified by the reactive metabolite it handles, as described above, its activity is reduced [32]. Specifically, pgK-modified GAPDH has a higher K_M when compared to the unmodified enzyme, indicating impaired substrate binding. Enolase, another enzyme within the glycolytic pathway, also is modified with pgK in cells exposed to elevated glucose levels. When the modified residues are mutated to mimic the modification, the activity of enolase is nearly ablated. While these examples support the idea that RACS generated in a discrete metabolic pathway can modify proteins in the same pathway, further studies to directly test the relationship between reactive substrates or products, and the activity of enzymes that handle them, are required to determine the broad applicability of this concept.

Beyond the simple relationship between RACS, the enzymes that handle them, and subsequent protein modifications, large-scale proteomic studies have shown that hundreds of enzymes that do not handle RACS are also modified. While some of these modification events are likely stochastic, others might be key signaling nodes. In one recent study, the enzyme methylmalonyl-CoA mutase (MUT) was uniquely suppressed by the reactive metabolites itaconyl-CoA (Fig. 2) and/or citramalyl-CoA made from adjacent pathways [40]. Mutation or ablation of the enzyme Citrate LYase subunit Beta-Like (CLYBL) leads to an accumulation of itaconyl-CoA and/or citramalyl-CoA, which leads to a cell autonomous defect in mitochondrial vitamin B₁₂ metabolism. While the exact mechanisms of inhibition are not yet known, increased concentration of RACS impairs the MUT enzyme, and its ability to regenerate adenosylcobalamin (AdoCbl, the active form of coenzyme B₁₂) as an obligate cofactor of MUT. Lower AdoCbl levels break the catalytic cycle of MUT, and prevent the conversion of methylmalonyl-CoA to succinyl-CoA. Determining whether this is a stochastic consequence of elevated reactive activated carbon species, or whether reduced B₁₂-dependent MUT activity contributes to autocrine or paracrine metabolic signaling, is an important area of future investigation. Indeed, testing the difference between these two scenarios remains a major challenge in the field.

Outlook

When considered together, the above concepts point to an overall model where intrinsic reactivity of activated carbon species can provide a signaling mechanism for the cell to sense the acyl-CoA load, and overall carbon flux. During high pathway flux, either from temporary nutrient shifts or from sustained metabolic changes, this mechanism could contribute to negative feedback on the system by reducing the activity of metabolic enzymes. In times of excess RACS, this mechanism might surpass its signaling capacity and stress metabolic and cellular function.

The major challenge moving forward will be to directly test these models and clarify the role of RACS in carbon stress and metabolic homeostasis (see Outstanding Questions). One possible place to begin investigating these hypotheses is to assess the acylation status of enzymes that handle reactive acyl-CoAs. As described in the examples above, relationships between enzymes with acyl modifications corresponding to their substrates have been identified, but testing for direct evidence of a regulatory role of such modifications remains incomplete [39, 41]. Specifically, manipulating carbon flux coupled with high-resolution proteomics will be crucial for testing whether enzymes that generate RACS are more susceptible to protein acylation. Additionally, recent studies using ^{13}C -carbon isotope tracing have allowed tracking acyl groups from discrete metabolite pools onto specific proteins [42–44]. Continued development of these cutting-edge technologies will allow direct relationships between carbon metabolites and protein modifications to be interrogated.

Finally, identifying the key nodes that are influenced by acylation and measuring the functional consequences of protein hyperacylation are the biggest challenges of working in this field. In some cases, protein acylation has been shown to negatively impact the enzymatic function, supporting the idea that high flux results in modifications to check the activity of the pathway. In other cases, subtle or no effects on protein function can be found. In rare cases, increases in enzyme activity is observed. We suggest that one way to identify common signaling nodes is by comparing acylation sites that are modified by numerous PTMs in multiple systems. These commonly modified sites, especially those outside of pathways that directly generate RACS, could indicate a nexus of signaling from multiple pathways. Further investigation into the proteins within mitochondria where RACS are produced and identifying commonly acylated proteins could determine the acylation sites that mediate signaling vs. those that lead to damage. Ultimately, taking cues from the oxidative stress model to interrogate the carbon stress model of protein acylation has the potential to integrate RACS, wide-spread protein acylation, nutrient sensing, and metabolic homeostasis.

Conclusions

If the primordial, intrinsic reactivity of intermediary metabolites was obligatory for the origin of biochemistry and subsequent development of complex macromolecules, then the major teleological question remaining is whether evolution ‘took advantage of’ the fact that these processes were occurring, and therefore encoded information into the system about the metabolic state via non-enzymatic post-translational modifications (*a la* signaling) or

whether evolution simply developed buffering and detoxification mechanisms to deal with them (*a la* damage). What is certain though, is that our understanding of the scope and complexity of protein modifications will continue to expand. With these discoveries, continued investigation in this emergent field will lead to unexpected and exciting new mechanisms of metabolic control, nutrient sensing, and organismal homeostasis in health and disease.

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Highlights

- Several key intermediary metabolites have high intrinsic reactivity
- A wide-range of reactive acyl-CoA species are able to modify proteins non-enzymatically
- High flux through some metabolic pathways lead to cellular increases in RACS and result in protein modifications that can impair protein function.
- Similar to reactive oxygen species, reactive acyl-CoA species may have a signaling role in some contexts, but may be damaging when uncontrolled.

- Is 'Carbon Stress', similar to oxidative stress, a useful model for explaining pathway regulation and signaling by nutrient flux?
- What are the molecular mechanism by which RACS signal?
- During high pathway flux, are acyl-CoA handling enzymes susceptible to modification by RACS?
- Do RACS play a signaling role by inhibiting proteins during high pathway flux, thereby providing a negative feedback mechanism?
- Are there signaling nodes within or between pathways where multiple RACS converge to modulate metabolism?
- When concentrations of RACS are high, do they stress the cell by indiscriminately disrupting protein function?

Fig. 1.

Ancient reactive metabolites predicted to be biochemical precursors of modern biochemistry, including: 1,3-bisphosphoglycerate (1,3-BPG), Acetyl-phosphate (Acetyl-P), Acetyl-Sulfide, Adenosine Triphosphate, Carbamoyl-phosphate (Carbamoyl-P), Carbonyl-Sulfide, Carboxy-phosphate (Carboxy-P), Cyanide, Glucose-1-phosphate (Glucose-1P), Glucose-6-phosphate (Glucose-6P), Metal-Sulfide, Methyl-Sulfide, Phosphoenolpyruvate (PEP); stylized using standard Corey-Pauling-Koltun (CPK) coloring convention: black, carbon; red, oxygen; blue, nitrogen, yellow, sulfur; purple, phosphorus; gray, metal; thin lines, single bond; medium lines, double bond; thick line, triple bond; hydrogens omitted for clarity

Fig. 2. Modern reactive metabolites. A. Coenzyme A structure depicted using standard CPK coloring convention (see Fig. 1 for key), hydrogens omitted for clarity; B. Prototypical mechanism by which an acyl-CoA synthase enzyme activates fatty acids into fatty acyl-CoAs; C. Reactive acyl-CoA species known or predicted to modify proteins, with corresponding rates of spontaneous hydrolysis as one indicator of reactivity of each acyl group from CoA at room temperature [8].

Fig. 3.

RACS and ROS could have similar signaling roles and damaging consequences. Protein oxidation is known to alter protein-protein interactions, inactive proteins, and in some settings, cause irreversible damage. Similarly, protein acylation can alter protein-protein interactions, reduce enzyme activity, cause protein damage, and modify proteins outside of the pathway where the acyl-species was generated. In each case, the system would be predicted to have an appropriate cellular response.