Lipid Modifications of Proteins; Ubiquitination, SUMOylation; Hydroxylation, Glycosylation

It is tempting to call this lecture "the beginning, the middle and the end", since the three types of lipid modification to be discussed occur respectively at the N-terminus, in the middle, and at the C-terminus of proteins. They were a rather hot item in the early 1990s, not least because at least prenylation is found on all G-proteins, the heterotrimeric proteins - one each of three different kinds of subunit - involved in transmission of signals from membrane receptors to cytoplasmic systems, including the vision G protein transducin and the *ras* proteins which are oncogenic in slightly mutated form. Other proteins involved in vision, rhodopsin kinase and a cGMP-specific phosphodiesterase which is activated by the α subunit of transducin, are also farnesylated. A general review of all three types of modification on G-proteins is by Casey, *Current Opinion in Cell Biology* **6:**219-225 (1994); a review of isoprenylation, focusing on subsequent processing and the function of prenylated proteins, is by Rando (*Biochim. Biophys. Acta* **1300:**5-16 (1996). And prenylation is reviewed by Zhang and Casey in *Ann. Rev. Biochem.* **65:**241-269 (1996).

The handout summarizes, in a little more detail than I would deliver orally, information about protein N-**myristoylation**, from a review article by J.I. Gordon, in the *Journal of Biological Chemistry*, 1991. A more recent review by Gordon is in *Trends in Cell Biology* **7:**14 (1997). Myristoylation is fairly specific for the C_{14} length of fatty acid, although variant fatty acids with oxygen or sulfur substituted for a CH_2 - oxa- and thia-acids, which are considerably more polar, will readily substitute for myristate. These acids are readily taken up by cells and converted to the acyl-CoA derviatives, and substitution for myristate on proteins results in less hydrophobic proteins which do not go to expected cell sites or properly associate with other proteins, and they have been proposed as drugs against both HIV-1 and the opportunistic fungal pathogens frequently infecting AIDS patients. However, I have heard nothing more about this lately. Many of the G proteins involved in signal transduction are myristoylated on the α subunit, and the modification is necessary for binding to the β and γ subunits. Transducin can also be substituted with lauryoyl, the C_{12} fatty acid, or one of two unsaturated C_{14} acids.

Myristoylation is specific for N-terminal glycine, no other N-terminal amino acid is so modified. The activity of the enzyme, myristoylCoA:protein N-myristoyltransferase, is affected by at least the first eight amino acids of the protein's sequence. The enzyme has an ordered sequential mechanism; binding of myristoylCoA results in a large conformational change, seen in a decrease of the pI from 8.15 to 6.7 and quenching of tryptophan fluorescence. Addition of the peptide/protein substrate results in progress of the reaction and return of the enzyme to the native conformation and characteristics. This change is not due to formation of an acyl-enzyme intermediate, as it happens even with the non-hydrolyzable substrate analog *S*-(2-oxo)pentadecylCoA.

Myristoylation has been characterized as an electrostatic switch – phosphorylation prevents myristoylated proteins going to their usual membrane site, because it reduces the positive charge on an area of the protein which otherwise would interact with the negatively charged head groups of phospholipids in the membrane. In this metaphor, myristoylation is the switch, phosphorylation is the switching hand.

Palmitoylation refers more generally to *S*-acylation, formation of thioesters on cysteine, since the modification can be with other fatty acids. There is also *N*-palmitoylation, on the amino terminus or on lysine residues, and *O*-palmitoylation on serine or threomine; but *S*-acylation is the common modification. It can occur anywhere in the peptide sequence - near the C-terminal in *ras* proteins, which must first be prenylated, near the N-terminus in the α subunits of G proteins, which may be *N*-myristoylated; the other modification occurs first. Very often palmitoylation occurs on both cysteines of a –CC- or C-X-C- sequence, or on cysteines a little further apart. When there is *N*-palmitoylation on the amino terminus it mat occur first as *S*-palmitoylation of cys-2, then be transferred to the amino group. No sequence motif directing palmitoylation has yet been identified. A wide variety of proteins are

palmitoylated, mostly plasma membrane proteins including many receptors, but also the cytoskeleton proteins ankyrin and vinculin, and the α subunits of many G proteins.

Palmitoylation *can* take place from acyl-CoA, without any enzyme, on a membrane surface, but this is not likely to be significant at physiological concentration of free acyl-CoA, since most acyl-CoA is bound to acyl-CoA binding protein. In yeast two acyltransferases, transferring palmitoyl from palmitoyl-CoA to protein, have been found: the Erf2/Erf4 complex acylates Ras2, the enzyme Akr acylates Yck2. They are membrane-associated, acting on proteins already in the membrane (often thanks to prior prenylation or myristoylation). Both have the sequence As—His-His-Cys before a cysteine-ruch region. Other proteins with similar sequences are known, may also be palmitoyltransferases.

Palmitoylation helps proteins get *into* membranes, and directs them to so-called 'lipid rafts', areas of membrane rich in sphingolipids and cholesterol (and acylated proteins). Myristoylation or prenylation seems to get proteins to spend *some* of their time in the membrane, and allows them to be palmitoylated; once palmitoylated they *stay* in the membrane. Plamitoylation occurs in the 'early secretory' pathway, directs the proteins to membranes, whether external or internal. For instance, the Ras proteins are signal receptors, both on the outside of the cell and in internal membranes. Normally palmitoylated G_α proteins which have been mutated cys∅ser so that they can't be palmitoylated fail to associate normally with membranes.

Palmitoylation is reversible, by a plasma membrane deacylase, both contitutively and in response to signals; in this it differs from prenylation and myristoylation. A protein may be palmitoylated and depalmitoylated many times during its lifetime. In G proteins, G_α, palmitoylated and *N*-myristoylated, dissociates from the other two subunits, $G_{\beta\gamma}$, when in response to a signal it binds GDP rather than GTP. Free G_α, is then depalmitoylated.

Prenylation of proteins - substitution with the C_{15} isoprenoid farnesyl or the C_{20} isoprenoid geranylgeranyl - was discovered by the effects of the HMGCoA reductase inhibitor lovastatin on cell cycling and tumor growth *in vivo*. A similar substitution occurs on fungal pheromone peptides such as the yeast **a** mating factor. The first protein found to be prenylated was the nuclear envelope protein lamin B. It occurs also on the *ras* oncogene protein products, lamin A, the γ subunit of "G-proteins" , and other *ras*-related small GTP-binding proteins. Most G_γ subunits are geranylgeranylated, but the one in visual transduction is farnesylated. G_γ subunits which cannot be prenylated still bind to the β subunit, but the βγ complex cannot interact functionally with either the α subunit or with adenyl cyclases it would normally regulate. Some of these proteins, but not all, are directed to cell membranes by the modification; the interaction of *ras*2 with adenyl cyclase is stimulated 100x by farnesylation.

There are basically three parallel enzyme systems involved: farnesyltransferase, geranylgeranyltransferase I and geranylgeranyltranferase II.

Farnesyltransferase and geranylgeranyltransferase I modify proteins on the cysteine SH of a C-terminal sequence CAAX, where A,A are neutral aliphatic amino acids and X is methionine, serine, or rarely cysteine, alanine, or glutamine for farnesylation, leu for geranylgeranylation. The result is a prenyl thioether, not reversible. These two enzymes share a common α subunit (called RAM2 in yeast), mol. wt. 44 kDa, but have different β subunits (the farnesyltransferase β subunit, mol. wt. 48.6 kDa, is RAM1 in yeast); the β subunits have the specificity for the protein substrate. The two β subunits are about 30% identical in sequence. The farnesyltransferase is dispensable in yeast (mutants are viable but temperature-sensitive) while geranylgeranyltransferase I is necessary. The enzymes require Mg^{++} and Zn^{++} for activity. The zinc is tightly bound and activates the substrate SH for reaction by deprotonating it, while Mg^{++} is required at millimolar levels for farnesyltransferase activity and coordinates to the pyrophosphate part of the prenylpyrophosphate; oddly, Mg^{++} is not required by geranylgeranyltransferase I. Heptapeptides are big enough to be substrates, while CAAX tetrapeptides

with aromatic amino acids at the second A position are inhibitors. A mutant (ser-159∅gln) farnesyltransferase farnesylates proteins which normally are substrates for geranylgeranyltransferase. Normal (non-oncogenic) function of *ras* proteins is specific for farnesylation, inhibited by geranylgeranylation.

While steady state kinetics suggested that the mechanism was random sequential, and both substrates bind independently, isotope partitioning and pre-steady state kinetics have shown that the prenyl pyrophosphate binds first and very tightly, with a conformational change in the protein. This complex then reacts rapidly with the protein substrate; product release is the rate-limiting step, as is not surprising considering that all the bulk and interaction are on one product. The prenyl pyrophosphate binds to the β subunit, but the site for the protein substrate and for catalysis seem to involve both subunits: mutation of lys^{164} to asn in the α subunit resulted in a protein which could dimerize and bind substrates, but was catalytically inactive.

Because of the requirement of many oncogenic *ras* proteins for prenylation to be active in transformation, there is much interest in inhibitors of prenyltransferase activity as possible anticancer agents.

Geranylgeranyltransferase II, GGPT II, prenylates one or usually both cysteines of C-terminal Cys-Cys-COOH or Cys-X-Cys-COOH sequences. It has two subunits, 50 and 38 kDa, similar to those of the other enzymes. Activity also requires a 95 kDa protein now called RepI (for Rab Escort Protein), which binds the protein substrate, presents it to the GGPT II, and rermains tightly bound to it in absence of detergents. The protein substrates are almost exclusively of the Rab family of Ras-related G proteins. Peptides are not substrates, presumably because they do not bind to RepI. Lack of RepI in the retina causes a retinal degeneration called choroidemia; but there is some geranylgeranylation without it and elsewhere in the body, due to a second protein Rep2.

Prenylation by FPTase or GGPTase I is followed by removal of the three amino acids C-terminal to the modified cysteine, by carboxypeptidase Y (in yeast), usually by a membrane-bound protease which may remove them as a tripeptide, though there is also a cytoplasmic metallopeptidase. The COOH of the cysteine is then converted to a methyl ester by transfer of CH_3 from S-adenosyl-methionine. This methylation makes the product - for instance the photoreceptor G protein transducin - much more membrane-associating, especially if the prenyl is farnesyl - the *n*-octanol/water partition factor increases from 6.8 to 719 on methylation of acetylfarnesylcysteine, but only from 575 to 1450 for acetyldigeranylcysteinylcysteine. However, methylation of farnesylated transducin improves its membrane association only about 25% in light, where rhodopsin is activated - the rhodopsin and the myristoylated α subunit ensure it is membrane-bound anyway. On the other hand, processes regulated by the βγ complex without the α subunit, such as activation of phosphoinositol-3-kinase and phospholipase C-β, require the methylation, whether by favoring membrane binding or directly affecting association with these proteins is not known.

Methylation of farnesyl- and geranylgeranylproteins and mating factor **a** are all carried out by the same membrane-bound enzyme. The geranylated CysCys sequence is not methylated, unlike all other prenylated C-termini including Cys-X-Cys. A specific enzyme for removal of the methyl group has now been identified (Tan and Rando, *Biochemistry* **31:**5572-5578 [1992]), and some typically is lost during purification.

The protease is inhibited by N-blocked farnesylcysteine analogs, with an aldehyde (K_i 1.95 μM), a CH_2-peptide such as CH_2NH-val-ile-met, K_i 86 nM, or -CH(OH)CH_2CONH-val-ile-met, K_i 64 nM. The methyltransferase doesn't even require the blocked amino group, it works on 3-farnesylthiopropionate and is inhibited by farnesylthioacetate. Various farnesylcysteine analog compounds have effects on cell processes, perhaps by competing with isoprenylated proteins in signal transduction processes.

Other methylations of protein carboxyls include that of the γ-COOH of glutamate in bacterial chemoreceptors, of L-isoaspartyl and D-aspartyl residues formed during aging (it leads to reversion to L-aspartyl), and of C-terminal leu in a bovine brain protein. This last turns over rapidly, $t_{1/2}$ = 180 min.

Some other modification reactions:

ADP-ribosylation: the toxin of the pathogen *Corynebacterium diphtherium*, actually coded for in a virus of this bacterium, transfers ADP-ribose from NAD^+ to a modified amino acid called diphthamide, found only in the critical elongation fractor EF-2. Diphthamide is a histidine with an alkyl group on the C-2 between the nitrogens. The alkyl is α-trimethylaminobutyramide, attached y its 4 position. Diphtheria toxin ADP-ribosylates one of the ring nitrogens of the imidazole ring, inactivating EF-2 for protein synthesis.

Ubiquitination: ubiquitin is a small protein, 76 amino acids after removal of some C-terminal amino acids by activating proteases, which plays a critical role in targeting cellular proteins for proteolytic disposal. Ubiquitination starts by formation of a thioester bond between its C-terminal glycine COOH and a cysteine of a protein called E1 or UBA. This requires ATP, hydrolyzed to AMP and PP_i; an acyl adenylate between the C-terminal and the AMP is an intermediate. It is then passed to a cysteine on another protein, E2 or UBC, and then to a lysine residue of a target protein, a reaction catalyzed by one of a number of ligating enzymes E3. A protein can have many ubiquitins attached, either on different lysines or on the first ubiquitin. A polyubiquitinated protein is then recognized by a large complex protease, mol. wt. 10^6 daltons, called a proteasome, and degraded. But histone H2A is ubiquitinated without proteolysis, and ubiquitination of some DNA-binding regulatory proteins, for instance Met4 which regulates methionine biosynthesis, controls their interactions with promoter regions as well as proteolysis.

SUMOylation: SUMO, short for short ubiquitin-like modifier, also called sentrin, is another small protein, attached to proteins like ubiquitin, but it does not lead to proteolysis – indeed SUMOylation can block ubiquitination. There are 3 SUMO proteins, 1, 2 and 3, in mammals, even more in plants (8 in *Arabidopsis*), only 1 in yeast. Mammalian SUMO2 and 3 are 95% identical and are believed to have the same functions, while SUMO1 is less similar. Most SUMO1 is on proteins at any time, while most SUMO2 and 3 isn't, only gets attached in response to stresses. They are about 100 amino acids long after activation by C-terminal trimming by proteases or carboxypeptidases. While there is little sequence homology with ubiquitin, they fold the same way, with an extra 22 amino acids at the amino terminus, though in yeast this can be deleted with little effect on function. SUMOs are found in all eukaryotes and are required for viability of eukaryotic cells. They have been found attached to more than 60 cell proteins, though often only a small fraction is SUMOylated, and they are deSUMOylated immediately upon cell lysis, which is why this modification was discovered only in 1996.

The process is similar to ubiquitination. The enzyme E1, a heterodimeric complex also called AOS1 /UBA2, catalyzes formation of an acyl adenylate at the C-terminus of the SUMO protein. A cysteine of E1 then attacks this, forming a thioester with the C-terminus, releasing AMP. Finally the SUMO is transferred to an E2 component Ubc9 – there is only one of these, and it doesn't accept ubiquitin. The SUMOylated Ubc9 can interact with most known substrates for SUMOylation, and can SUMOylate them on its own, but the transfer is facilitated by an E3 enzyme: one of a group of proteins called PIAS proteins, or Siz1 and 2 in yeast, different ones for different substrates, or the large vertebrate nuclear pore protein RanBP2, or a protein called Pc2 involved in transcriptional repression. The target for transfer is a tetrapeptide sequence: a hydrophobic amino acid, the lysine where transfer occurs, any amino acid, and a glutamate. If the SUMO transferred is SUMO-2 or 3, more can be added, as these proteins have such a sequence around their own lys-11; SUMO-1 terminates such a chain. But this is not generally observed. SUMOylation can be affected by modification of lysine by acetylation or methylation, or phosphorylation of a nearby serine; and besides specificity of particular SIZ/PIAS proteins for particular substrates, there may be other protein cofactors involved.

SUMOylation has regulatory effects, especially on proteins binding to promoter sequences, where it generally reduces transcription, and is a hot topic right now. It occurs mostly in the nucleus, or sometimes when a protein is entering the nucleus, though it can also occur in the cytoplasm. It often affects subcellular location or protein stability – the latter of course if it competes with ubiquitination. SUMO residues can be taken off again, by what are called Ulp-1 or Ulp-2 proteases in yeast, really isopeptidases since the link to lysine ε-amino groups are termed isopeptides; thus the regulation is reversible. Mammals have seven of these proteases. They are also used for activation of SUMOs, which are synthesized with a short extra C-terminal peptide which must be cleaved off.

The *Annual Review of Biochemistry* article goes on about functions of SUMOylation, but observes that 1) in most cases only a small percentage of its substrates is SUMOylated, 2) it is often difficult to demonstrate that abolition of a SUMOylation site on a receiving protein, by mutation of the lysine to something else, really has much biological effect.

Hydroxylation, Glycosylation

You can read about analysis of glycosylation in Rosenberg, pp. 208-224. It occurs to me that undergraduates who want to write a review paper could review one of the post-translational modifications, starting with the handout of tables of contents of Methods in Enzymology and reading more recent papers.

Among additive reactions, it is convenient first to mention **hydroxylation**. 4-Hydroxyproline, δ-hydroxylysine and to a much lesser extent 3-hydroxyproline are characteristic, essential components of collagen and other structural proteins, formed by specific enzyme systems requiring α-ketoglutarate and oxygen as substrates and Fe^{++} and a reducing agent, usually ascorbate - this is why you need fruit or fresh vegetables to prevent the breakdown of structural tissues known as scurvy. This hydroxylation occurs outside the cell. There is also β-hydroxylation of aspartic acid and asparagine in some of the blood clotting proteins, This too is carried out by a dioxygenase requiring α-ketoglutarate. Glycosylated β-hydroxy-phenylalanine and tyrosine have also been observed, though the unglycosylated amino acids have not been observed.

Glycosylation occurs mainly on the NH_2 of asparagine and on the hydroxyls of serine and threonine, though it is also seen on hydroxyproline and hydroxylysine, on the SH of cysteine, and as I just mentioned at the β position of phenylalanine and tyrosine. Glycosylated hydroxyamino acids generally bear only one or two carbohydrate residues, but asparagines bear large branched chains, containing in extreme examples up to 300 mannose residues. The basic core on this contains, reading out from the protein, two N-acetylglucosamine residues in β-1∅4 linkage, a mannose linked β-1∅4 to the acetylglucosamine, and two more mannoses in α-1∅3 and α-1∅6 linkage to the base mannose. This structure is synthesized on a membrane-bound lipid called dolichol pyrophosphate, indeed what is synthesized is somewhat larger, with a glucosyl-glucosyl-glucosyl-mannosyl on the 1∅3 linked mannose and two mannobiose units on the 1∅6 linked mannose. This whole thing is transferred to the asparagine while the protein is still being synthesized in the endoplasmic reticulum. The new glycoprotein goes to a subcellular organelle called the Golgi apparatus, where the additional sugars mentioned are trimmed off and new ones are put on. These may be just more mannose, or may include N-acetylglucosamine, galactose, fucose, and sialic acid. The terminal sugar residues are tissue-specific and cell type specific. What the carbohydrates are is generally studied by treatment with a variety of specific glycosidases, followed by derivativization and gas chromatography or hplc of the free carbohydrates. Whether particular proteins are glycosylated can be determined by SDS gel electrophoresis followed by periodate oxidation of the carbohydrates and reaction of the aldehydes with pararosaniline, the basic fuchsin method of Fairbanks et al. which I gave you when talking about electrophoresis; or for cells in tissue culture by adding an inhibitor of glycosylation such as tunicamycin and noting what bands increase their mobility in electrophoresis.

What determines where and what proteins are glycosylated? Modification generally is determined by local sequence: N-glycosylation occurs on asparagines in the sequence Asn-X-Ser/Thr; collagen hydroxylation occurs at the middle amino acid of the sequence X-Pro/Lys-Gly; protein kinases which phosphorylate serine or threonine act on the sequence Arg-X-Y-(Z)-Ser/Thr, where X, Y and sometimes Z are unspecified amino acids.

Why are only *some* molecules of a population of protein molecules modified? For instance, pancreatic ribonuclease is often glycosylated, at one or more of six sites. In some species none are glycosylated, in some all, in some only some of the enzyme is glycosylated; how does this happen? What does it mean? Is it adjusted according to circumstances, and if so, what circumstances?

Some papers I read years ago provide some answers. Beintema (J. Mol. Evolution **24:**118-120 (1986) discusses mammalian ribonucleases, which may be glycosylated at any of six locations in the protein, all surface bends of the polypeptide chain, if the sequence is asn-X-ser/thr as required for N-glycosylation. However, no species has CHO attached at more than three of these points, and only pig pancreatic and human urinary RNAse have that much. Glycosylation may be limited either absolutely by not having the Asn-X-ser/thr sequence, or partially by having a relatively inefficient glycosylation apparatus, at least for pancreatic ribonuclease; in man pancreatic RNAse is only partially glycosylated and only at one of three possible sites, while urinary RNAse is fully glycosylated at all three sites. Thus, whatever the evolutionary value of glycosylation may be, it can be modulated from tissue to tissue by the efficiency of the apparatus. (He doesn't seem to have looked at urinary RNAse in other species.) He points out that animals with what he calls 'stomach fermentation' , including the ruminants, have relatively little glycosylation, at most one site glycosylated and that usually only partially, while species with 'cecal fermentation', breakdown of complex carbohydrates from plants largely in the large intestine, are more fully glycosylated, fully at three sites in the pig and two in the horse (plus partially at a third site). The suggestion is that in these species it is important to protect the RNAse from absorption in the gut = small intestine so that it can hydrolyze bacterial RNA in the large intestine.

The significance of glycosylation is explored more generally by West (Mol. Cell. Biochem. **72:** 3-20 (1986). The general question is whether glycosylation has a general, non-specific role such as generalized protection of extracellular proteins, without much dependence on the exact set of sugars attached, or has specific functions dependent on the interaction of specific CHO side chains with specific receptors. The answer seems to be that the general function came first, and probably still is all for a majority of proteins, but various specific functions evolved later.

Glycosylated proteins are resistant to protease attack, heat denaturation, and perhaps, our Dr. Strumeyer suggested, to tannins - in general, to the harsh life outside the cell. Glycosylation is particularly heavy on proteins of the surface of cells which face a non-cellular environment, as those on the surface of blood vessels; the non-specific theory of glycosylation suggests that the surface properties are largely controlled by it, particularly by the O-sulfated, negatively charged carbohydrates of the glycosaminoglycans. To quote West, "the collective oligosaccharide density on cell surface proteins is sufficiently great so as to invite comparison with the carbohydrate density of the matrix space. This observation has led to the suggestion that cell surface carbohydrate might also establish a convection and diffusion-limited region around the cell in an area thick enough to be named, in certain cell types, the glycocalyx or fuzz. The polyanionic nature of the carbohydrate found on some cell surfaces probably results in marked cation and pH differences in this region relative to the surrounding medium." Carbohydrate heterogeneity may even modify protein functions to broaden their pH and ionic strength optima.

Probably the strongest argument for non-specificity of glycosylation is that preventing it, by inhibitors such as tunicamycin or by mutation knocking out enzymes in the glycosylation pathway, generally has little effect on the cells in culture; they remain viable, secrete the non-glycosylated proteins, adhere, proliferate, and remain able to cause tumors. Of course, transformed tumor cells probably don't need sophisticated functions characteristic of differentiated cells specific to a particular tissue location. But elimination of specific glycoantigens in the cellular slime mold *Dictyostelium*

discoideum doesn't seem to affect the normal life cycle of the organism. One has to argue either that the carbohydrate has a *very* non-specific role or that it becomes important only in an environment more natural than the laboratory.

Evidence for specific roles for protein glycosylation is difficult to summarize, particularly because few generalizations hold up. For instance, *some* proteins which normally go to lysosomes don't when they lack mannose-6-phosphate residues - but others are unaffected, and some of the altered enzymes still make it to the lysosomes. The rate of secretion of some proteins, but not of others, is affected by inhibition of their glycosylation. Most polypeptide hormones stored in vesicles of pituitary cells are sulfated, while those not stored aren't. There is a cell surface protein called ligatin, which recognizes a mannose-6-phosphate-1-glucose structure and probably binds glycoproteins bearing this structure to the cell surface, since glucose-1-phosphate can elute them off. It is not known whether this recognition serves in routing such proteins to the cell surface, or in binding proteins that had been secreted.

I shall not go on, since further examples are generally vaguer or with less context; you can look for a more recent review if you need to know that state of knowledge in this area. It is certainly a fertile area for research.

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