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Heterogeneous N-terminal acylation of retinal proteins

J.C. DeMar Jr[a, b, *], D.R. Rundle[f], T.G. Wensel[a], R.E. Anderson[c, d, e, f, g]

[a]*Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030 U.S.A.*
[b]*Current address: USDA, ARS, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030 U.S.A.*
[c]*Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 U.S.A.*
[d]*Department of Ophthalmology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 U.S.A.*
[e]*Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 U.S.A.*
[f]*Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104 U.S.A.*
[g]*Dean A. McGee Eye Institute, Oklahoma City, Oklahoma 73104 U.S.A.*

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* Corresponding author.

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1. Introduction

Fatty acylation of proteins is becoming increasingly recognized to have a critical role in many biochemical processes. Foremost among these processes, fatty acylation has been shown

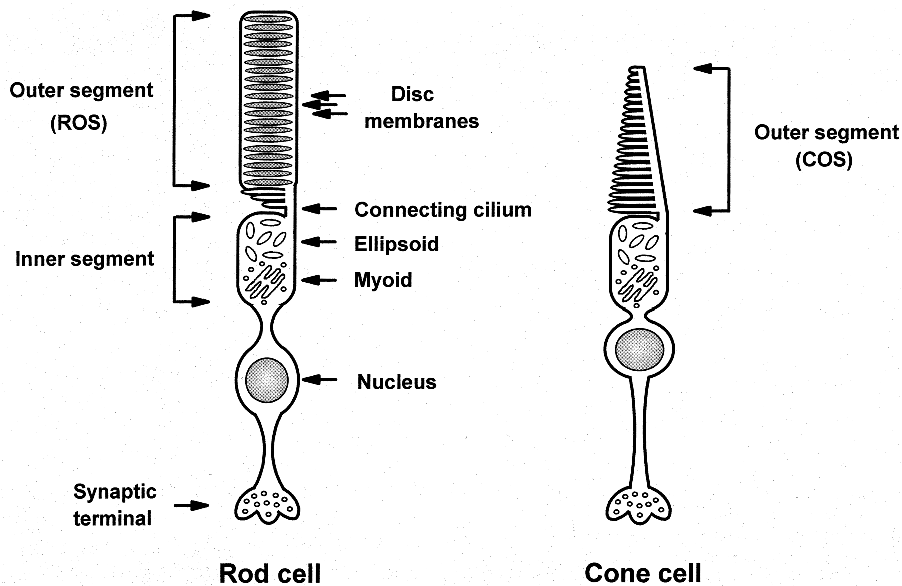

Fig. 1. Diagram of rod and cone photoreceptor cells. Shown here are the two photoreceptor types found in the vertebrate retina and their major cellular subdivisions. ROS, COS, are rod or cone outer segments respectively.

to be a critical modification of proteins involved in signal transduction. Acylation can be important for protein structure or for membrane interactions, and its documented effects include stimulation of protein activity and enhancement of interactions with other signaling proteins. Proteins can be fatty acylated by palmitoylation, which usually occurs on cysteine, and by N-acylation with shorter chain fatty acids, usually myristic acid, which occurs on N-terminal glycines. Many reviews are available on the N-terminal acylation of proteins [1–13]. This review will cover N-terminal acylation reactions in general, but unlike previous reviews, it will additionally focus on the unusual pattern of N-terminal acylation reactions found so far only among signal transduction proteins in vertebrate photoreceptor cells. These retinal proteins are heterogeneous with regard to the types of fatty acids covalently linked to the glycine, in sharp contrast to similar proteins found in other tissues where myristic acid is uniformly the predominant fatty acid. The major goal of this review is to present current knowledge of the N-terminal acylation of photoreceptor proteins and of the pathways leading to the unusual precursor fatty acids and acyl Co-A species along with our own recent findings and hypotheses.

1.1. Photoreceptors of the vertebrate retina

Photoreceptors are highly specialized cells found in the vertebrate retina that generate the initial neuronal response of this organ to light (Fig. 1). They fall into two morphologically distinct cell types, rod and cones [14, 15]. Rods are sensitive to low levels of light and are thus most active at night. Cones are less sensitive to dim light, but provide most of our visual information in daylight conditions, and are responsible for fine visual discrimination and color vision. Both rods and cones are subdivided into common compartments: outer segment, inner segment, nucleus, and synaptic terminal. The synaptic terminal, at the base of each cell (nearest the pupil), is the site of storage and release of the neurotransmitter glutamate. Lying above the nucleus is the inner segment which contains the biosynthetic machinery of the cell. A thin cilium connects the inner segment to the outer segment, the most specialized part of the cell. Rod outer segments (ROS) consist of a stack of 500–2000 flat membrane sacks (disc membranes) encased by a plasma membrane sheath. Only for the most basal rod discs is there a membrane connection between the disc and the plasma membrane. In cones, the outer segment is made up of stacked plasma membrane invaginations, so there is continuity between the disc and plasma membrane. The cytoplasmic surface of the rod disc membranes or cone invaginations is where most proteins involved in phototransduction are found. These membranes are dynamic structures whose integral protein components are completely renewed in vertebrates every ten days [16]. New membranes synthesized in the inner segment are incorporated at the base of the outer segment. Older membranes at the tip of the outer segment are shed and phagocytized by the retinal pigment epithelium. This process is controlled in a circadian manner entrained by light. Rods shed their tips in the morning (at presumed dawn) ([17]) and cones shed their tips in the evening (at presumed sunset) [18]. This renewal process maintains the outer segments at constant length and serves to replace old proteins and lipids in a controlled and orderly manner.

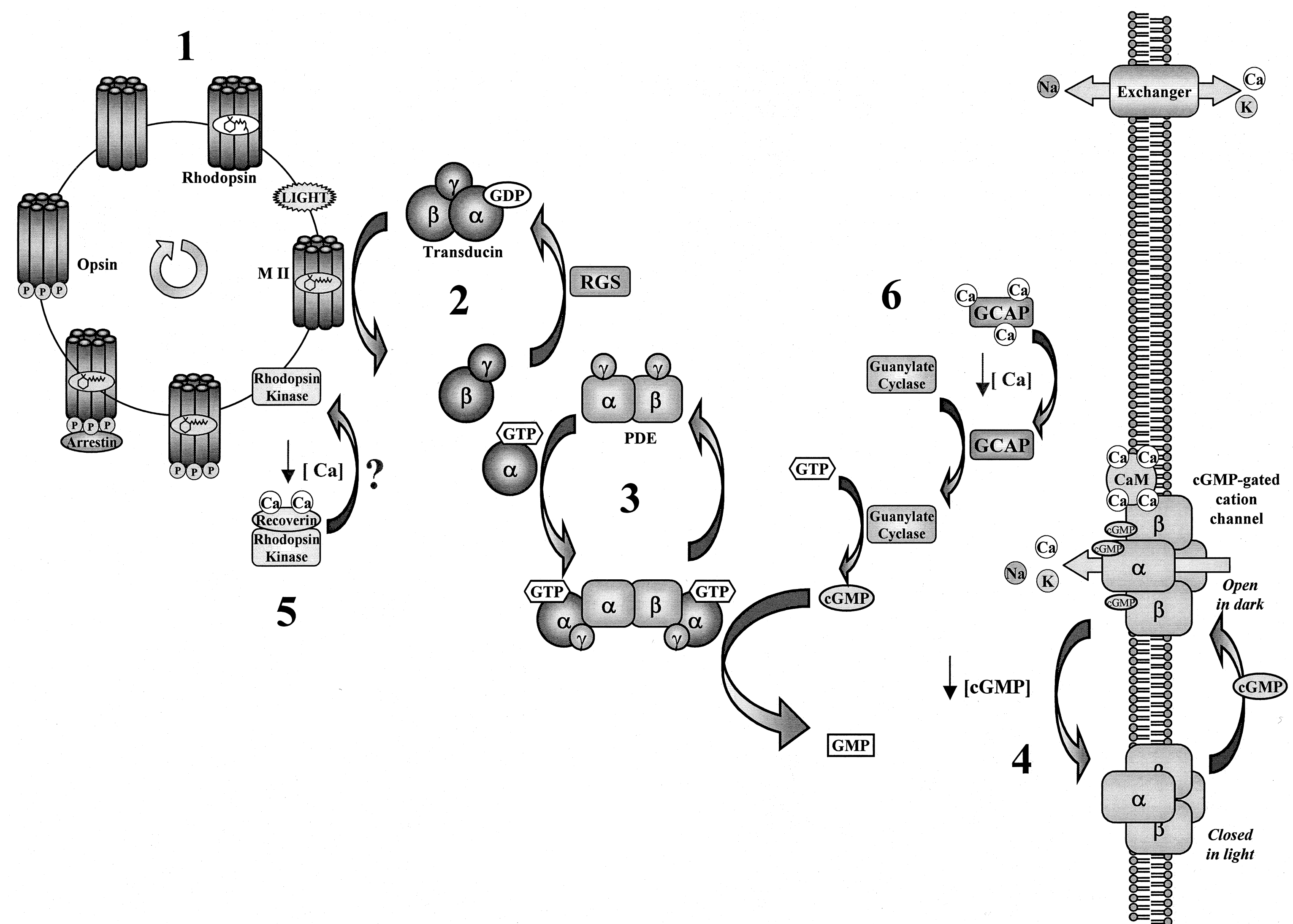

Fig. 2. Diagram of phototransduction cascade. Shown here are both the excitation and recovery pathways that make up the process of phototransduction.

1.2. Phototransduction

The process of phototransduction, in which light absorption by photopigment proteins controls a synaptic signal, is the most thoroughly understood signal transduction process in the retina, and one of the best understood pathways in all of neurobiology. Most of what we know about heterogeneous fatty acylation of retinal proteins derives from studies of the highly specialized set of proteins involved in phototransduction in rod outer segments, which are more biochemically accessible than cones or other retinal neurons. This review focuses on these proteins, beginning with an overview of the major phototransduction proteins and their roles in the signaling pathway (diagrammed in Fig. 2).

1.2.1. Phototransduction proteins

1.2.1.1. Excitatory phase proteins. Four major proteins are involved in the excitation phase of rod phototransduction: rhodopsin, transducin, cGMP phosphodiesterase, and the cGMP-sensitive cation channel. *Rhodopsin* is a seven transmembrane α-helix/G-protein coupled receptor [19–24], that contains a covalently bound 11-cis retinal, which acts as a chromophore [25]. Two adjacent cysteines near the C-terminus of this protein are palmitoylated [26, 27].

Transducin (G_t) is a peripheral heterotrimeric G-protein composed of α, β, and γ-subunits ($G_{t\alpha\beta\gamma}$) [28–30]. The α-subunit is the site of GDP/GTP binding and the βγ-subunits are important for tight coupling of $G_{t\alpha}$ activation to photoexcitation of rhodopsin. As will be discussed (Section 3.2), the N-terminus of the α-subunit is heterogeneously acylated. The C-terminus of the γ-subunit is covalently modified with the isoprenoid farnesyl [31, 32]. $G_{t\alpha}$, like α-subunits of other heterotrimeric G-proteins [33], shuttles on the membrane surface between a transmembrane receptor (rhodopsin) and membrane-bound effectors and GTPase accelerating proteins (see below), so it is not surprising that lipid modifications play an important role in its function.

cGMP-specific phosphodiesterase (PDE), the effector enzyme for transducin, is responsible for rapid hydrolysis of cGMP in response to illumination. It is a peripheral membrane protein composed of an α, β, and two γ-subunits ($PDE_{\alpha\beta\gamma\gamma}$) [28, 29, 34, 35] (reviewed by Wensel [36]). The catalytic domain is composed of the α and β-subunits and the γ-subunits inhibit PDE activity. Like transducin, PDE has lipid modifications: the C-termini of the α and β-subunits are covalently modified with the isoprenoids farnesyl and geranylgeranyl, respectively [37].

The *cGMP-sensitive cation channel* is the sensor of changes in cytoplasmic [cGMP] brought about by light. It is a transmembrane channel protein composed of at least two of each α- and β-subunits which passively transport cations with a fairly low specificity [38–40]. Under physiological conditions, Na^+ is the major current carrier, but the ability to conduct Ca^{2+} is important in phototransduction and recovery as well. The channel is held open by cooperative binding of cGMP, where the affinity for cGMP may be regulated by association of calmodulin with the channel's β-subunits (Fig. 2, Fig. 4) [41, 42].

1.2.1.2. Recovery phase proteins. At least six major proteins are involved in the recovery phase of phototransduction: RGS protein, guanylate cyclase activating protein, guanylate cyclase, recoverin, arrestin, and rhodopsin kinase. All are associated with disc membranes.

RGS protein (*R*egulator of *G*-protein *S*ignaling) is a peripheral membrane protein which acts as a GTPase accelerating protein (GAP), speeding GTP hydrolysis by G-protein α-subunits [43–45]. The RGS protein subtype expressed in rod and cone photoreceptors is RGS9 [46, 47]. While no lipid modifications have been detected or proposed for RGS9, it is very tightly associated with disk membranes.

Guanylate cyclase activating protein (GCAP) is a peripheral membrane protein and a Ca^{2+}-binding protein of the EF hand type [48]. There are two GCAPs known as GCAP-1 and GCAP-2 [49–51]. GCAP-1 is localized to the outer segments of rod and cone photoreceptors, whereas GCAP-2 is found primarily in the inner segments of these cells [52]. As to be discussed (Section 3.2), both GCAP-1 and GCAP-2 are heterogeneously acylated on their N-termini.

Recoverin [53, 54, 48] is a peripheral membrane protein and another EF hand Ca^{2+}-binding protein of the calmodulin superfamily found in photoreceptors. As will be discussed (Section 3.2), like GCAP and transducin ($G_{t\alpha}$), recoverin is modified by heterogeneous N-terminal acylation. Its physiological function is not firmly established, but in vitro recoverin acts as a calcium-sensitive inhibitor of rhodopsin kinase [55–57].

Guanylate cyclase, a single transmembrane segment protein, is found as two types known as RetGC1 and RetGC2 [58–61] and is regulated by interactions with the acylated GCAPs.

Arrestin is a peripheral membrane protein also known as S-antigen and 48 K protein [62–65].

Rhodopsin kinase is a peripheral membrane protein and a serine/threonine protein kinase [63, 66–69]. The C-terminus of rhodopsin kinase is covalently modified with the isoprenoid farnesyl [70].

1.2.2. Phototransduction mechanism

1.2.2.1. Excitatory phase. Phototransduction is the process whereby photons of light elicit a neuronal signal via a cascade of specific protein-protein interactions (Fig. 2) [71–80]. The excitation phase of phototransduction begins with a single photon of light causing the 11-*cis* retinal chromophore in rhodopsin to undergo isomerization to a *trans* configuration (Fig. 2, 1). This leads to a conformational change in rhodopsin (R*) that allows interaction with $G_{t\alpha\beta\gamma}$-GDP. This binding promotes release of GDP and binding of GTP to $G_{t\alpha}$, thus allowing dissociation of $G_{t\alpha\beta\gamma}$ into $G_{t\alpha}$-GTP and $G_{t\beta\gamma}$ (Fig. 2, 2). $G_{t\alpha}$-GTP then interacts with $PDE_{\alpha\beta\gamma\gamma}$, relieving the inhibitory constraint of the PDE_{γ}-subunits. This interaction generates an active PDE, likely to be found in the form of a $(G_{t\alpha})_2$-$PDE_{\alpha\beta\gamma\gamma}$ complex (Fig. 2, 3) [81].

In the dark, open cGMP-sensitive cation channels on the plasma membrane permit the passage of a dark current, largely due to the influx of Na^{+} ions, which maintains the cell in a depolarized state in the absence of light (Fig. 2, 4). Light-activated PDE hydrolyzes cGMP to GMP and the reduced cytoplasmic cGMP levels allow the cGMP-sensitive cation channels to close. This causes hyperpolarization of the plasma membrane leading to a reduction in glutamate release at the photoreceptor synaptic terminal. The drop in glutamate release is recognized by nearby bipolar cells which subsequently generate a signal that is further processed in the retina and is ultimately transmitted to the visual cortex in the brain.

The signal amplification of the phototransduction cascade is remarkable in that within a few hundred milliseconds in mammalian rods, each rhodopsin photoisomerization can lead to the hydrolysis of 10^5 molecules of cGMP. The subsequent reduction in membrane current amounts

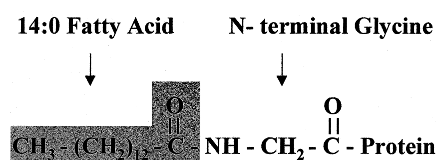

Fig. 3. Chemical structure of N-terminal acylation. The fatty acid myristate (14:0) is amide linked to the N-terminal glycine. In photoreceptors, the acyl group may be 12:0, 14:0, 14:1n-9, or 14:2n-6 linked to glycine.

to blocked entry of about 10^6 Na^+ ion through the plasma membrane channels [82, 83]. This amplification occurs at multiple stages in the transduction pathway. Thus the specificity and timing of protein-protein and protein-membrane interactions are critical, with lipidation likely playing an important role.

1.2.2.2. Recovery phase. Almost as quickly as the excitation phase begins, biochemical events leading to recovery are initiated [84] and, as with excitation, require lipidated proteins. As depicted in Fig. 2, these involve R* (light-excited rhodopsin) inactivation by phosphorylation and binding of the capping protein arrestin (Fig. 2, 5), G-protein and PDE inactivation by RGS9-catalyzed GTP hydrolysis (Fig. 2, 2), and guanylate cyclase activation, all leading to restoration of dark levels of cGMP and reopening of the cation channels. Both R* inactivation and guanylate cyclase activation are stimulated by decreases in cytoplasmic $[Ca^{2+}]$ brought about by the channel closure that also blocks Ca^{2+} entry, while a $Na^+/Ca^{2+}/K^+$ exchanger continues to extrude Ca^{2+} [85]. Ca^{2+} effects are mediated by calcium binding proteins of the calmodulin superfamily, including the N-terminal acylated proteins recoverin which inhibits rhodopsin kinase at high Ca^{2+}; (Fig. 2, 5), and GCAP-1 and GCAP-2 which stimulate guanylate cyclase at low Ca^{2+}; (Fig. 2, 6).

2. General properties of N-terminal acylation

The most common means of covalently attaching fatty acids to a protein are through palmitoylation and N-terminal acylation. Palmitoylation occurs when the 16 carbon saturated fatty acid palmitic is thioester-linked to one or more internal cysteines in a protein. This modification has been demonstrated to be functionally important for many signal transduction proteins, including the photoreceptor protein rhodopsin [86–89, 26, 27, 90–92]. A number of excellent reviews have been written on this subject [93–98, 4, 99–106, 11, 107, 108].

In N-terminal fatty acylation, the fatty acid is attached to the protein through an amide bond to the α-amino group of the N-terminal amino acid, as depicted in the schematic diagram in Fig. 3. In all N-terminally fatty acylated proteins studied in eukaryotes, the N-terminal amino acid is glycine, the essential requirement for which will be discussed in Section 4.4. In tissues other than the retina, the modifying fatty acid is the 14 carbon saturated fatty acid myristic acid (14:0). For this reason, the modification is often referred to as N-terminal myristoylation. Amide linkage of myristate to a protein has also been shown to occur on the ϵ-amino group of internal lysines, as in interleukin 1α and tumor necrosis factor α [109, 110].

Table 1
Known myristoylated proteins (acylated with only 14:0)

	Protein	Reference
Protein kinases	Abl	[121]
	Enterokinase	[122]
	Flagellar creatine kinase	[123]
	Fgr	[124]
	Fyn	[125]
	Hck	[116]
	Lck	[126]
	Lyn	[127]
	c-AMP protein kinase	[128]
	c-GMP protein kinase II	[129]
	Src	[113, 130]
	Syn	[131]
	Vps 15p	[132, 133]
	Yes	[134, 135]
ADP-ribosylation factors	Arf1p, Arf2p	[136–138]
	ARF1	[115]
	ARF2	[139]
	ARF3	[139]
	ARF4	[140]
	ARF5	[141]
	ARF6	[142]
Calcium binding proteins	Cnb1p	[143]
	Calcineurin	[112]
	Hippocalcin	[144]
	Neurocalcin	[145]
Heterotrimeric G-proteins	Gpa1p	[146]
	Giα	[147]
	Goα	[119]
	Gzα	[148]
Miscellaneous	ATCH receptor-associated (43 KD)	[149, 117]
	Cytochrome b5 reductase	[118]
	Erythrocyte, band 4.2	[150]
	Fuctinin	[151]
	Glycoprotein IX	[152]
	Intestine-specific annexin	[153]
	Igloo	[154]
	MARCKS	[156]
	Nitric oxide synthetase	[155]
	Ppz1p, Ppz2p	[157, 158]
	Tapa 1	[159]

However this amidation reaction appears to be enzymatically unrelated to N-terminal myristoylation.

The amide linkage found between the fatty acid and protein in N-terminal myristoylation is chemically stable and can be hydrolyzed only under strongly acidic conditions, such as

Table 2
Relative amounts (%) of fatty acids found on N-termini of photoreceptor proteins

Protein	Animal	12:0	14:0	14:1n-9	14:2n-6
1. GCAP	Bovine	13	7	30	50
2. PKA (C-subunit)	Bovine	7	57	15	21
3. Recoverin	Bovine	ND	10	48	43
4. Recoverin	Human	4	36	21	39
5. Transducin (Gtα)	Bovine	23	5	30	35
6. Transducin (Gtα)	Bovine	18	22	35	25
7. Transducin (Gtα)	Frog	ND	ND	ND	100

Shown here are the fatty acid species found to heterogeneously acylate photoreceptor proteins. Individual protein data is from: 1 [64]; 2, 4, 7 [158]; 3 [53]; 5 [170]; and 6 [171]. Table is adapted from [158], Table 1. **ND = Not detected (< 1%).

treatment with 6M HCl or 2N HCl/83% methanol at 100°C for several hours, which release the N-acyl group as the free fatty acid or fatty acid methyl ester, respectively. N-terminal fatty acids released from proteins in this manner can be identified by high performance liquid chromatography (HPLC) or gas liquid chromatography (GLC) [111–120].

3. Known N-terminally acylated proteins

3.1. Homogeneously acylated proteins (14:0 only)

Many proteins of eukaryotic origin are acylated exclusively with 14:0 (myristoylation) on their N-terminal glycine residues [1–6, 9–13]. A broad representative selection of these proteins is given in Table 1. While only a small fraction of total cellular proteins are myristoylated, this modification is strikingly common among proteins involved in signal transduction. The largest group is the protein kinases of both the serine/threonine and tyrosine kinase types [122, 123, 105, 9, 11, 129]. The ADP ribosylation factor (ARF) subgroup of small G-proteins and EF hand Ca^{2+}-binding proteins represent sizable families of myristoylated proteins [144, 160, 140, 11]. Myristoylation occurs on some G-protein α-subunits (G_i, G_o, and G_z), but not on all G-proteins that have the required glycine (G_{12}, G_{13}, and G_s); others lack a modifiable glycine (G_q) or have not been examined for this modification (G_{gust}, G_{olf}, and G_x) [147, 98, 4, 161, 11, 162, 163, 107, 108]. Other diverse proteins reported to be myristoylated include acetylcholine receptor-associated 43 kDa protein, cytochrome b_5 reductase, fuctinin, Gpa1, glycoprotein IX, intestine-specific annexin, Igloo, nitric oxide synthetase, MARCKS (*m*yristoylated-*a*lanine *r*ich-*C*-*k*inase-*s*ubstrate), PPZ1, and Tapa-1 [157, 154, 164, 151, 152, 11].

Yet another large group of myristoylated proteins important for eukaryotic biology are the viral coat proteins [165–167, 11]. Preventing myristoylation of the pr55gag protein of HIV by mutagenesis was found to block viral assembly [168, 169].

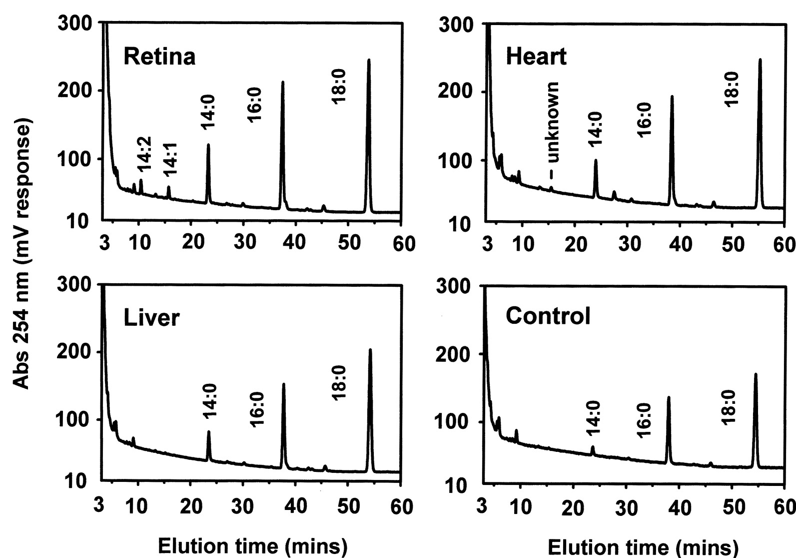

Fig. 4. Acidic methanolysis of total protein from heart, liver, and retina. HPLC profiles for fatty acids released from retina, heart, and liver total protein by acidic methanolysis. Control represents contaminant fatty acids in reagents. Identities of all peaks were based on retention times for standard fatty acids (not shown).

3.2. *Heterogeneously acylated proteins*

Proteins in photoreceptors with N-terminal acylation include $G_{t\alpha}$, GCAP, recoverin, and the catalytic subunit of cAMP dependent protein kinase [*P*rotein *K*inase *A* (PKA)] [53, 158, 170, 171, 51, 120]. Identification of the N-terminal acyl group was made by mass spectroscopic analysis of the isolated N-terminal peptide fragment of these proteins [172]. In contrast to proteins from other tissues, the photoreceptor proteins were found to be heterogeneously modified with 12:0 (12 carbon saturated fatty acid), 14:1n-9, and 14:2n-6, as well as 14:0. The 14:1n-9 and 14:2n-6 fatty acids always have constituent double bonds at the fifth (Δ^5) and fifth and eight (Δ^5,Δ^8) positions from the carboxyl group, respectively. Table 2 lists the percentage of the 12:0, 14:0, 14:1n-9, and 14:2n-6 found on four photoreceptor proteins from several vertebrate species (bovine, frog, and human). Interestingly, the acylated forms of each protein are likely distributed equally among all photoreceptors, since an antibody specific for a 12:0 acylated $G_{t\alpha}$ peptide is immunoreactive across the entire rod outer segment layer of the retina [173].

Tissue specificity of heterogeneous acylation is best exemplified by bovine PKA, which contains only 14:0 in heart and brain, but contains 12:0, 14:0, 14:1n-9, and 14:2n-6 in photoreceptors. [128, 158] The relative amounts of each fatty acid can vary between different proteins within the same species [158]. Frog $G_{t\alpha}$ best exemplifies species acyl chain specificity, since it exclusively carries the 14:2n-6 acyl group, in contrast to its heterogeneous acylation in other species [158]. Possible factors influencing the tissue specificity, species specificity, and the discrepancies between the acyl group percentages for photoreceptor proteins within the same species are discussed below.

Since only PKA from brain and heart was rigorously examined for the absence of heterogeneous N-terminal acylation, studies were undertaken to identify other proteins in non-ocular tissues that might carry this modification [174]. Fatty acid analysis was performed on the total protein precipitates from bovine heart, liver, and retinas by subjecting the proteins to acidic methanolysis (2N HCl/83% MeOH) and separating the released fatty acids by HPLC. Acidic methanolysis of heart and liver total protein released 14:0, but not 12:0, 14:1n-9, or 14:2n-6, as shown by the HPLC profiles in Fig. 4. Retina total protein released 14:0, 14:1n-9, and 14:2n-6 as 20.4%, 4.0%, and 4.3% of the total fatty acids, respectively (Fig. 4). The fatty acid results for total protein from bovine heart, liver, and retina strengthen the idea that heterogeneous N-terminal fatty acylation is a photoreceptor-specific phenomenon, although they do not eliminate the possibility that other retina neuronal cells may also contain heterogeneously N-acylated proteins.

4. N-terminal myristoyl transferases

4.1. Physical characteristics

N-terminal fatty acylation is carried out by myristoyl-CoA:protein *N*-myristoyltransferase (E.C.2.3.1.97), also known as N-terminal myristoyltransferase (NMT) and glycylpeptide N-tetradecanoyltransferase [2, 3, 5, 7, 8, 10, 11, 13]. Studies on NMTs from multiple organisms and tissues have provided valuable information which can be used to generate hypotheses regarding heterogeneous acylation. NMTs have been purified from bovine brain, bovine spleen, murine leukemia cells (L1210), rat brain, rat liver, and *Saccharomyces cerevisiae* [175–181]. Purified NMTs have molecular masses on SDS–PAGE of 50, 55, 60, and 68 kDa for bovine spleen, *S. cerevisiae*, bovine brain, and murine leukemia cell, respectively [175, 176, 178–180]. In its native state, NMT can exist as a monomer, dimer, or hexamer, and formation of these multimers may influence enzymatic activity [176].

NMT cDNAs have been cloned from *Saccharomyces cerevisiae*, *Candida albicans*, *Histoplasma capsulatum*, *Cryptococcus neoformans,* bovine cardiac muscle, human liver, mouse liver, and bovine spleen [182–189]. A single copy gene encodes NMT in *C. albicans*, *C. neoformans*, *H. capsulatum*, and *S. cerevisiae*. The chromosomal locus for *S. cerevisiae* NMT has been mapped to the right arm of XII proximal to the *cdc42* gene [182, 3]. In contrast to lower eukaryotes, mammalian NMTs arise from more than one gene. Southern analysis of the bovine spleen NMT suggests the protein has two distinct genomic copies [187]. In mouse and human liver, the NMT is encoded by two genes to produce NMT–1 and NMT–2 proteins having ~77% similarity [184]. The *C. albicans*, *C. neoformans*, *H. capsulatum* and *S. cerevisiae* NMT genes encode proteins of 52, 55, 59, and 53 kDa, respectively [182, 185, 189]. While the bovine spleen NMT is predicted to be 47 kDa and the expressed bovine heart protein is 50 kDa, the human and mouse NMT–1 and NMT–2 both have estimated molecular weights of 57 kDa, whereas the expressed human NMT–1 form is 49 kDa [190]. NMT is an evolutionarily conserved protein, since human NMT 1, and NMT's from *C. albicans*, *C. neoformans*, and *H. capsulatum* have 44, 55, 50, and 50% amino acid identity to *S. cerevisiae*, respectively [183, 185, 189]. When the mammalian liver NMT–1 and NMT–2 are compared

across species, both are highly similar in human and mouse with > 95% amino acid sequence identity for each inter-species comparison. Thus, the NMT isoforms have undergone little genetic divergence in mammals.

The subcellular localization pattern of bovine heart, bovine spleen, human, and *S. cerevisiae* NMTs is predominantly in the cytosol [191–194, 186]. However, a small fraction of the human and bovine NMT activity is tightly associated with membranes [192–194, 186]. Membrane bound fractions of NMT are also noted for human NMT–1 and NMT–2 when expressed in COS cells [184]. The membrane fraction may represent NMT bound to membrane fused to polyribsomes [193]. Immunohistochemical staining of NMT in human heart using a human NMT–1 polyclonal antibody showed strong immunoreactivity in myocardium muscle fiber [188]. Ultrastructural localization using immunogold labeling and electronmicroscopy showed primarily a cytoplasmic localization with some labeling in mitochondria and myofilaments [188]. These studies would suggest that the photoreceptor NMT is also dispersed throughout the cell within the various cytosolic compartments. However, the highest concentration of NMT would be expected near the rough endoplasmic reticulum where protein synthesis occurs, since N-terminal fatty acylation is described as a co-translational event (Section 4.2). In photoreceptors, the rough endoplasmic is in the inner segment, whereas the fully synthesized phototransduction proteins become localized to the outer segment [195, 196]. Consequently, the NMT should be expected to be primarily an inner segment protein.

4.2. Catalytic mechanism

N-terminal acylation occurs during or soon after translation of the polypeptide, as shown by blockage of the incorporation of myristic acid into muscle cell proteins by the protein synthesis inhibitor puromycin, and by isolation of polyribosomes containing nascent polypeptide chains with 14:0 attached [197]. Inhibition of myristoylation by cycloheximide has been observed for bovine $G_{t\alpha}$, suggesting that the modification is also a co-translational process in photoreceptor cells [120]. For *S. cerevisiae* and mammalian cells, the anticipated events leading to myristoylation of a protein are translation on ribosomes to 30–40 amino acids, removal of the initiator methionine by the enzyme methionine aminopeptidase [198]. and acylation of the N-terminus by NMT [7]. The likely catalytic mechanism for NMT, a sequential ordered Bi-Bi reaction, was proposed based on studies with fatty acid coenzyme A derivatives and peptide analogs [199, 3, 5, 200–202, 10, 203, 11]. The enzymatic reaction requires two substrates, an activated fatty acid in the form of acylated coenzyme A (acyl-CoA) and a peptide with a free N-terminal α-amino group. Acyl-CoA binds first, followed by the peptide's N-terminal glycine to form the activated complex. The fatty acid is transferred from CoA to the peptide N-terminus to form an amide bond. Free CoA is released first, followed by the acyl-peptide. Recent work has provided greater insight into the catalytic region. The crystal structure of *C. albicans* NMT has been resolved to show a novel alpha/beta fold having a symmetry that may possibly result from gene duplication [204]. The crystal structure reveals a long, curved, and relatively uncharged groove containing a deep center pocket which is negatively charged due to its proximity to the C-terminus carboxylate group and to a glutamate that separates the pocket and groove. Mutations effecting binding and activity infer that the groove and pocket are substrate binding sites, with the negative center being the catalytic site. Mutagenic deletion of

the C-terminal domain of bovine spleen NMT eliminates its activity, suggesting that this region contains an essential component of the catalytic site [187]. A highly conserved region within the catalytic domain is EEVEH (289–293) and site directed mutagenesis of His 293, Val 291, and Glu 90 resulted in loss of NMT activity [205]. Similar deletions of the N-terminus did not abolish activity, and one was found to increase activity, suggesting that this domain contains an allosteric regulator of the catalytic site [187]. Interestingly, oxidized free CoA inhibits and reduced free CoA promotes bovine spleen NMT activity, suggesting that an allosteric binding site may be present on the enzyme for CoA [206]. Catalysis does not appear to require a divalent metal cation, in that activity is resistant to strong chelating agents like 1,10-phenanthroline [10]. For the *S. cerevisiae* NMT, a reactive cysteine or serine is not involved, since activity is not sensitive to alkylating agents and phenyl methyl sufonyl fluoride (PMSF), respectively [10]. In contrast, mammalian NMTs might contain an essential cysteine, since reducing agents stimulate and alkylating agents abolish activity of bovine spleen NMT [206]. In support of this possibility, human NMT contains a conserved cysteine which, when mutated, leads to decreased formation of an acyl-peptide product [207]. It is possible that the cysteine sulfhydryl group acts as an acceptor for myristic acid during its transfer from CoA to the peptide's N-terminal α-amino group [207]. In the absence of peptide, *S. cerevisiae* NMT forms a covalent complex with fatty acid that is hydroxylamine resistant, suggesting an intermediate involving amide bonding to the ε-amino group of a lysine occurs in this NMT [201]. NMT contains four highly conserved histidine residues, one of which maybe important to enzyme function since DEPC treatment abolishes the enzymatic activity [206, 208, 10]. In support of this hypothesis, mutation of three of the histidines in human NMT leads to decreased acyl-enzyme intermediate and acyl-peptide formation [207] and mutation of His 293 results in loss of activity [205]. Histidine may be functioning as a proton donor to CoA during formation of the acyl-enzyme intermediate and a proton acceptor from the N-terminal α-amino group during transfer of myristic acid to the protein [207].

Biochemically, N-terminal acylation is considered to be an irreversible modification due to the strong chemical nature of amide bonds. However, bovine spleen has been shown to undergo reverse catalysis to demyristoylate a 14:0-peptide reaction product when levels of free

Table 3
Acyl-CoA specificity of human and *S. cerevisiae* N-terminal myristoyl transferases (NMT). **Relative to 14:0 CoA (1.00)

Human			*S. cerevisiae*				
Acyl-CoA	V_{max}/K_m	Reference	Acyl-CoA	V_{max}/K_m	Specific activity	Kd	Reference
14:0	**1.00**		14:0	**1.00**	**1.00**	**1.00**	
12:0	0.32	[214]	12:0	–	0.44	0.24	[199]
14:1n-9	0.53	[214]	14:1n-9	1.00	–	–	[213]
14:2n-6	–		14:1n-9	0.55	–	–	[214]
			14:1n-9	0.33	–	–	[212]
			14:1n-9	–	1.06	0.77	[199]
			14:2n-6	–	0.05	0.20	[199]

CoA used to produce 14:0 CoA are sufficiently depleted [206]. This may represent a regulatory mechanism, governed by fluctuations in free CoA or acyl-CoA levels, which converts a protein within a cell back to its non-myristoylated state. While rare, demyristoylase activity towards proteins in vivo has been reported. A 68 kDa protein (p68) from *Dictyostelium discoideum* appears to be transiently myristoylated on its N-terminal glycine [209]. *D. discoideum* may not follow the normal biochemistry of myristoylation since N-acylation of p68 is a post-translational rather than a co-translational event. Demyristoylation of the MARCKS protein by an uncharacterized enzyme in bovine brain has been also observed [210]. Apparently, MARCKS must be phosphorylated first by protein kinase C before it is a substrate for the demyristoylation enzyme(s), suggesting that the reaction is a signal transduction coupled process.

4.3. Fatty acyl-CoA substrate specificity

All NMTs utilize only coenzyme A derivatives of fatty acids. Acyl-CoA analogue recognition is mainly determined by the fatty acid and not the CoA portion [202, 10, 211]. Although photoreceptor proteins have 12:0, 14:0, 14:1n-9, and 14:2n-6 on their N-terminus, all NMTs have a very high specificity for 14:0 CoA [2, 3, 5, 10, 11, 13]. While 14:2n-6 CoA has not been tested, 12:0 and 14:1n-9 CoAs are utilized by the human NMT-1 at a 3.1 and 1.9-fold, respectively, lower catalytic efficiency (V_{max}/K_m) than 14:0 CoA [212]. Studies comparing *S. cerevisiae* NMT using 14:1n-9 and 14:0 showed that 14:1n-9 was utilized at a rate either equal to or up to 3.0-fold less than the rate of 14:0 [213, 214, 212]. A study focusing on the specific activity of *S. cerevisiae* NMT for 12:0, 14:1n-9, and 14:2n-6 CoAs compared to 14:0 CoA showed similar activity for 14:1n-9 CoA (106%), but lower activity towards 12:0 and 14:2n-6 CoAs (44 and 5%, respectively) [215]. In this same study, it was shown that the binding affinity (K_d) of the NMT for 12:0, 14:1n-9, and 14:2n-6 CoAs was lower than that for 14:0 CoA (4.1-,

Table 4
N-terminal sequences of N-acylated proteins

Protein	N-terminal Octa-Peptide
Giα	14:0-G-C-T-L-S-A-E-D-
Goα	14:0-G-C-T-L-S-A-E-E-
Gzα	14:0-G-C-R-Q-S-S-E-E-
Src	14:0-G-S-S-K-S-K-P-K-
Fyn	1 4:0-G-C-V-Q-C-K-D-K-
Lck	14:0-G-C-V-C-S-S-N-P
Photoreceptor proteins	
GCAP	F.A.-G-N-I-M-D-G-K-S-
PKA (C-subunit)	F.A.-G-N-A-A-A-A-K-K-
Recoverin	F.A.-G-N-S-K-S-G-A-L-
Transducin (Gtα)	F.A.-G-A-G-A-S-A-E-E-

N-terminal octapeptide sequences for selected proteins acylated with only 14:0 (myristoylated) and heterogeneously acylated photoreceptor proteins. F.A. = 12:0, 14:0, 14:1n-9, and 14:2n-6.

1.3-, and 5.0-fold, respectively). A summary of these studies shown in Table 3 indicates that 12:0, 14:1n-9, and 14:2n-6 CoAs are not superior substrates to 14:0 CoAs.

The current model for the NMT acyl-CoA binding pocket is based on fatty acid analogue binding studies and suggests the depth is exactly 14 carbons with a single bend occurring near the fifth carbon of the fatty acid [216–218, 213, 10]. Appropriate chain length and a *cis* double bond at the fifth position explains why 14:1n-9 approaches 14:0 as a substrate. Inappropriate length and presence of a second double bond explains why 12:0 and 14:2n-6, respectively, are poor substrates compared to 14:0 and 14:1n-9. Overall, based on the acyl-CoA specificity studies for the human and *S. cerevisiae* NMT, one would expect the following relationship for utilization of each acyl-group by the photoreceptor NMT, if it shows the same specificity: 14:0 ≥ 14:1n-9 > 12:0 > 14:2n-6. However, this relationship is inconsistent with the acylation

Table 5
Functional consequence of N-terminal myristoylation (modification with only 14:0)

Protein	Functional effect	Reference
Increased protein interactions		
ARF1	GTP/GDP exchange factor	[226]
Fyn	Receptor/Substrate	[227, 228]
Giα	Giβγ, Adenylylcyclase	[229, 230]
Goα	Goβγ	[231, 232]
Gsα	Gsβγ, Adenylylcyclase	[233]
Lck	Receptor/Substrate	[234]
Protein kinase C	Substrate	[235]
Src	Receptor/Substrate	[225]
Nitric oxide synthetase	Increased catalytic activity	[236]
Calcineurin	Increased structural stability	[237]
Lck	Increased structural stability	[234]
c-AMP protein kinase	Increased structural stability	[238]
c-GMP protein kinase 11	Increased structural stability	[129]
Abl	Increased membrane binding	[239]
ARF1	Increased membrane binding	[226]
ARF5	Increased membrane binding	[141]
ARF6	Increased membrane binding	[142]
Cytochrome b5 reductase	Increased membrane binding	[240]
Flagellar creatine kinase	Increased membrane binding	[123, 241]
Fyn	Increased membrane binding	
Giα	Increased membrane binding	[242]
Goα	Increased membrane binding	[148]
Gzα	Increased membrane binding	[243, 244]
Hck	Increased membrane binding	[245]
Hippocalcin	Increased membrane binding	[144]
Lck	Increased membrane binding	[234]
MARCKS	Increased membrane binding	[246]
Nuerocalcin	Increased membrane binding	[145]
Nitric oxide synthetase	Increased membrane binding	[247, 248]
c-GMP protein kinase II	Increased membrane binding	[129]
Src	Increased membrane binding	[225]

pattern of photoreceptor proteins, suggesting that NMT substrate specificity does not establish the pattern of heterogeneous acylation seen in photoreceptors, unless the retinal NMT has strikingly different properties from those of NMTs studied thus far.

4.4. Peptide substrate specificity

There is an absolute requirement that the peptide acceptor has a N-terminal glycine with a free α-amino group [219, 13]. The nitrogen groups within the side chains of arginine (guanidinium), asparagine (amide), glutamine (amide), and lysine (ε-amino) are not acylated by NMT. The photoreceptor proteins GCAP, PKA, recoverin, and $G_{t\alpha}$ all have the requisite N-terminal glycine and are suitable candidates for fatty acid modification by the NMT. Studies on *S. cerevisae* NMT show the next seven amino acids also influence activity [2, 3, 5, 10, 11, 13]. Only uncharged amino acids are allowed at position two [13]. Positions three and four have a variable effect, although neutral amino acids are most commonly found in these positions [13]. While most amino acids are tolerated at position five, a serine residue greatly increases enzyme activity [13]. Position six is permissive for most amino acids other than proline, which eliminates activity [13]. Finally, positions seven and eight show greatest activity with basic amino acids, while neutral and especially acidic amino acids diminish activity [13].

Importantly, while the N-terminal octapeptide sequence has a great influence on myristoylation, specificity may reside in more distant portions of the primary structure as well. For example, an N-terminal peptide from $G_{t\alpha}$ was not a substrate for *S. cerevisiae* NMT [220], whereas $G_{t\alpha}$ protein is myristoylated when co-expressed with *S. cerevisiae* NMT in *E. coli* [221, 120], unpublished observations), as well as in cultured mammalian cells [148], reticulocyte lysates [222, 221] and retina [170, 172, 120].

As shown in Table 4, examination of the N-terminal octapeptide sequences of CGAP, $G_{t\alpha}$, PKA, and recoverin reveals attributes that lower NMT activity [158]. GCAP has a charged amino acid (lysine) at position four, lacks a serine at position five, and has a neutral residue (serine) at position eight. $G_{t\alpha}$ has two acidic residues (glutamate, glutamate) at position seven and eight. PKA lacks a serine at position five and recoverin has two neutral amino acids (alanine and leucine) at positions seven and eight. However, N-termini of proteins in photoreceptors do not deviate dramatically from the myristoylation consensus sequence pattern, which suggests that heterogeneous acylation versus myristoylation is not based on properties of the peptide. The case of PKA, which is heterogeneously acylated only in the retina [158], demonstrates clearly that tissue-specific mechanisms rather than protein sequence provide the key determinants for heterogeneous acylation. This conclusion is further strengthened by comparing the acylation patterns for bovine and frog $G_{t\alpha}$. All four fatty acids are present in substantial proportions on bovine $G_{t\alpha}$, whereas the frog protein is predominately modified by 14:2n-6, [158] with only trace amounts of 14:0 and 14:1n-9 present [114], despite the observation that the two species share 100% sequence identity within the first eight amino acids [158, 223]. Consequently, it appears the species specificity of N-terminal acylation is not established by peptide sequence. It must be considered, however, that amino acids beyond the first eight may also influence NMT activity. Presumably $G_{s\alpha}$ is not myristoylated because it has an asparagine instead of serine in position six. However, substitution of the asparagine with serine does not produce myristoylation of $G_{s\alpha}$ in COS cells [161].

5. Functional significance of N-terminal acylation

5.1. Function of homogeneous N-terminal acylation (14:0 only) of proteins

Studies on the functional effects of N-terminal acylation have mostly involved proteins in tissues other than photoreceptors that are modified only with 14:0. Identified functional effects include the enhancement of membrane binding, protein/protein interactions, catalytic activity, or structural stability [4, 5, 224, 6, 161, 7, 140, 225, 105, 11, 162, 107, 108]. Table 5 provides a partial list of proteins that demonstrate functional effects of myristoylation. Membrane association by 14:0 occurs through the hydrophobicity of this fatty acid, where its ΔG of binding is ~8 kcal/mol [105]. Consequently, the 14:0 moiety anchors the protein to the membrane by energetically favoring insertion into the lipid bilayer over remaining in aqueous solution. The dissociation constant (K_d) for bilayer insertion is 100 μM, which implies that 14:0 is not a stable membrane anchor [105]. It has been shown that the unitary free energy for binding an acylated peptide to phospholipid vesicles increases 0.8 kcal/mol per each carbon in the acyl chain of the attached fatty acid [249]. Consequently, membrane attachment is often assisted by having other lipid modifications present. Addition of a palmitoyl group would add ~10 kcal/mol to the binding energy. The presence of both 14:0 and 16:0 on a protein therefore represents ~18 kcal/mol in binding energy to lipid membranes. Proteins that carry dual 14:0 and 16:0 modifications are G-protein α-subunits ($G_{i\alpha}$, $G_{o\alpha}$, and $G_{z\alpha}$) and protein tyrosine kinases (fyn, hck, lck, and lyn) [105]. None of the N-terminally acylated photoreceptor proteins carries palmitoylatable cysteines within their N-terminal octapeptide sequences (Table 4) and are unlikely candidates for dual anchoring by myristoylation and palmitoylation. However, tight binding to other polypeptides with additional lipid groups can provide sufficient lipophilicity for membrane anchoring. $G_{t\alpha}$ finds such a partner in $G_{t\beta\gamma}$, which bears a farnesyl group on the γ-moiety, and in PDE, which has two isoprenoids (farnesyl and geranylgeranyl) on its catalytic subunits [31, 37].

Myristoylated protein membrane binding is also aided in proteins containing a N-terminal region rich in basic residues which bind acidic phospholipid head groups via electrostatic interactions [250]. The tyrosine kinase Src has six basic residues near the N-terminus of the protein that increase the affinity for phospholipid vesicles 2500-fold over modification with 14:0 alone [251]. Other myristoylated proteins shown to have increased membrane binding by basic amino acids within their N-terminal regions are ARF and MARCKS [250]. Interestingly, lysine residues are found within the N-terminal octapeptide sequences of GCAP, recoverin, and PKA (one, one, and two lysines, respectively) (Table 4). In contrast, $G_{t\alpha}$ lacks arginine or lysine residues near the amino terminus and contains two negatively charged glutamate residues, which may electrostatically repel the negatively charged membrane surface. Similarly, GCAP has a single negatively charged aspartate, which could cancel the positive effect of the single lysine. In addition to electrostatics, competing sites for 14:0 binding within the myristoylated protein are also important for determining membrane affinities. For example, in low calcium PKA and recoverin display negligible membrane affinity because the alkyl chains of their fatty acids are sequestered in hydrophobic pockets within the protein [252, 253], whereas in high calcium the fatty acids become available for membrane insertion [252].

Protein/protein interactions and catalytic activity are also enhanced by 14:0 membrane anchoring which may allow the protein to make faster contact with its membrane bound effector protein(s) through lateral diffusion, as opposed to a spatially less favorable search when free in solution. Membrane attachment may also orient a protein and its effector in the proper position for interaction. Alternatively, 14:0 itself could participate by binding to a hydrophobic domain on the effector protein, although evidence for such an interaction is still lacking. Enhancement of protein/protein interactions by 14:0 modification has been shown to result in catalytic activity changes such as increased GTP hydrolysis by ARF and G-proteins, decreased or increased cAMP production by adenylyl cyclase ($G_{i\alpha}$ and $G_{s\alpha}$ interactions, respectively), or increased protein phosphorylation by protein kinases [229, 231, 226, 230, 227, 232, 225, 105, 228, 233, 235]. All of these altered effects may occur partly as secondary consequences of interactions with membranes or other proteins. The 14:0 modification is able to increase catalytic activity by means other than through protein/protein interactions. Nitric oxide synthetase shows increased rate of NO synthesis when attached to membranes by 14:0 [236], perhaps by facilitating release of its NO product, since NO is more soluble in lipid than in water.

Enhancement of structural stability by 14:0 has been shown for calcineurin, cGMP-dependent protein kinase type II, PKA, and the protein-tyrosine kinase p56lck [237, 234, 129, 238]. The 14:0 group tethers the N-terminus to a hydrophobic domain on the protein, as observed for calcineurin, PKA, and calcium-free recoverin [252, 254, 253], and these enhanced intramolecular interactions may help preserve the functional structure of the protein or decrease susceptibility to degradation.

5.2. Function of heterogeneous N-terminal acylation of photoreceptor proteins

Hydrophobicity is the most important property to consider in evaluating the functional influence of 12:0, 14:0, 14:1n-9, and 14:2n-6 fatty acids. Since reduction of chain length by one

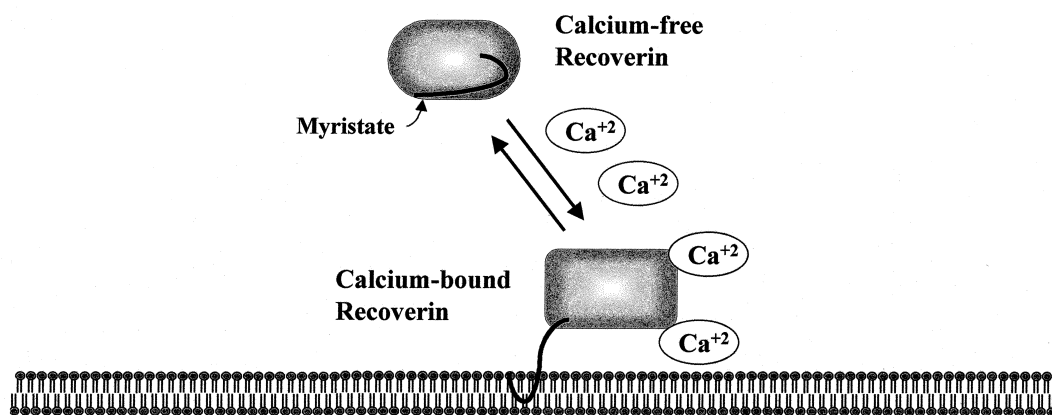

Fig. 5. Functional significance of recoverin myristoylation. The "calcium myristoyl switch" model indicates that calcium bound recoverin releases a sequestered myristoyl group to become membrane bound. While uncertain in vivo, this can lead to inhibition of rhodopsin kinase activity, under high Ca^{+2} concentrations in vitro.

methylene carbon unit or insertion of a double bond results in the transfer energy loss of 0.8 kcal/mol [249], the ΔG for transfer from cytosol to membrane for 12:0, 14:1n-9, and 14:2n-6 would be ~6.4 (verified experimentally [249]), 7.2, and 6.4 kcal/mol, respectively. Additionally, a methylene carbon unit would be expected to represent a slightly higher energy than a double bond. Consequently, the order of hydrophobicity or membrane binding affinity is as follows: 14:0 > 14:1n-9 > 14:2n-6 ≈ 12:0 [217]. Membrane affinity information is important for interpretation of functional studies involving these fatty acids.

5.2.1. Recoverin

Recoverin has been the most extensively investigated photoreceptor protein in terms of N-terminal acyl group function. Recoverin contains four EF hand Ca^{2+} binding motifs, but only two are active (EF2 and EF3) [255]. The protein binds ROS membranes in vitro only in the presence of Ca^{2+} (> 10 μM). Myristoylation effects on membrane binding have been studied using myristoylated and non-acylated forms of recombinant recoverin. Only the myristoylated recombinant recoverin shows Ca^{2+}-induced binding to membranes [256, 257]. A "Ca^{2+}-myristoyl switch" model has been purposed for the recoverin membrane binding (Fig. 5), in which Ca^{2+}-free recoverin has 14:0 sequestered in a hydrophobic pocket. Upon Ca^{2+} binding, 14:0 is exposed and inserted into the lipid bilayer [252, 256, 257]. Several lines of evidence support this model. First, the myristoylated N-terminus of recoverin becomes sensitive to tryptic digestion when Ca^{2+} is bound to the protein [256]. Second, fluorescence spectroscopic analysis shows solvent exposure of a hydrophobic region on recoverin accompanies Ca^{2+} binding [258], and the myristoylated form, unlike non-acylated recoverin, has a decrease in the polar environment of tryptophan residues [258, 259], indicating that 14:0 becomes sequestered within the protein in the absence of Ca^{2+}. Third, 2D-NMR shows the acyl chain of 14:0 on Ca^{2+}-free recoverin is in contact with the rings of aromatic amino acids [258]. Likewise, the NMR determined structural model of Ca^{2+}-free recoverin shows the 14:0 is extended into a pocket lined with conserved hydrophobic amino acids [252, 260]. An α-helix covers the mouth of the pocket which is connected to a Ca^{2+}-binding EF hand structure (EF 3). Consequently, the fifth α-helix may act as a "lid" for release of 14:0 after its displacement by Ca^{2+} binding.

The Ca^{2+}-induced membrane attachment led to an investigation to determine if acylation affects the ability of recoverin to bind Ca^{2+}. Flow dialysis, fluorescence spectroscopy, and NMR were used to analyze the interaction between the N-terminal acyl group and the two Ca^{2+} binding sites of recombinant recoverin in the absence of membranes. [255] Non-acylated recoverin bound Ca^{2+} biphasically, showing different affinities at sites EF2 and EF3 (K_d = 6.9 and 0.11 μM, respectively). In contrast, myristoylated recoverin showed cooperative Ca^{2+} binding, with a lower combined affinity than that of each site alone (K_d = 17 μM). The cooperativity in Ca^{2+} binding may be explained by a concerted allosteric model where recoverin is converted from a T (taut) to a R (relaxed) conformational state with 14:0 being either tightly sequestered or loosely exposed [255].

Rhodopsin kinase has been proposed to be the downstream effector of recoverin and is inhibited by recoverin in the presence of Ca^{2+} [261]. Effects of Ca^{2+} and myristoylation on interactions with rhodopsin kinase (RK) provide a convenient assay for their effects on recoverin's ability to interact with membrane-associated proteins. In two studies [55, 262], myrisotylation was found to enhance interactions with RK, and in these, as well as in a

third [263], to introduce cooperativity to the dependence of the inhibition on Ca^{2+}. Another study [264], however, failed to observe these effects. In general, the effects are consistent with the "Ca^{2+}-myristoyl switch" model.

The effect of heterogeneous acylation on recoverin has been investigated using native protein separated into the 14:0, 14:1n-9, and 12:0/14:2n-6 forms by HPLC [265]. The three forms show similar inhibition for rhodopsin kinase with increasing Ca^{2+} concentration (K_i = 0.6, 0.6, and 0.5 μM, respectively), suggesting that cooperativity for Ca^{2+} binding is not altered by the type of acyl group present. At Ca^{2+} concentrations > 1 μM, the three forms show somewhat different inhibition potencies, and the order of inhibition follows the increasing hydrophobicity of the fatty acids with 14:0 > 14:1n-9 > 14:2n-6 ≈ 12:0. Consequently, the effect of heterogeneous fatty acylation may be to decrease recoverin activity through weakened membrane binding (Fig. 5). In a recent study using truncated salamander rods to assay recoverin effects on photoresponse prolongation [266], fatty acylation was found to enhance potency 12-fold over non-acylated recoverin; however, no significant differences were observed when 12:0, 14:0 and 14:2n-6 forms of recoverin were compared to one another.

5.2.2. Transducin ($G_{t\alpha}$)

Studies have been carried out to determine the effect of N-terminal acylation on $G_{t\alpha}$ function, using native acylated protein and the non-acylated recombinant protein produced in baculovirus with an N-terminal Gly → Ala substitution [267]. Non-acylated $G_{t\alpha}$-GDP does not bind to micelles or vesicles, suggesting that the N-terminal fatty acid serves as a membrane anchor. Native $G_{t\alpha}$-GDP shows weak binding, consistent with the low binding energy (ΔG) expected for modification with a N-terminal acyl-group (Section 5.2). When the $G_{t\beta\gamma}$ subunit is introduced into the system, the binding of native $G_{t\alpha}$-GDP is greatly enhanced; this does not occur for non-acylated recombinant $G_{t\alpha}$-GDP. $G_{t\beta\gamma}$ carries a farnesyl lipid modification on the γ-subunit (Section 1.2.1.1), which acts as an additional membrane anchor. Consequently, $G_{t\beta\gamma}$ may enhance $G_{t\alpha}$-GDP membrane binding through hydrophobic interactions between the N-terminal acyl and the farnesyl groups [267]. Interestingly, when native $G_{t\alpha}$ binds GTP or a non-hydrolyzable analogue, it is a highly soluble protein. The acyl group may be folded onto the surface of the protein or sequestered in a hydrophobic pocket, as suggested by a study using a $G_{t\alpha}$ antibody whose epitope requires the acyl group to have close proximity to internal amino acids [268].

Two studies explored the effects of myristoylation on $G_{t\alpha}$ function using recombinant protein expressed by in vitro translation [222, 221]. Altered electrophoretic mobility of myristoylated wild-type $G_{t\alpha}$ was used to confirm that the protein is quantitatively myristoylated when translated in the presence of reticulocyte lysate [221], and in both studies, non-myristoylated protein was generated by synthesis of a protein with the N-terminal glycine mutated to alanine. Both studies showed that myristoylation enhanced binding of $G_{t\alpha}$ to rhodopsin, presumably through enhancement of interactions with membrane lipids. However, interactions with $G_{t\beta\gamma}$ in the presence of R* (Section 1.2.2.1), and with nucleotides, were not greatly affected by myristoylation.

The effect of heterogeneous acylation on the function of $G_{t\alpha}$ has also been examined by comparing the ability of $G_{t\alpha}$ nonapeptides containing 12:0, 14:0, 14:1n-9, and 14:2n-6 to inhibit GTPase activity and the pertussis toxin-catalyzed ADP ribosylation of $G_{t\alpha}$ [170, 173]. Since

both processes require binding of $G_{t\alpha}$ to $G_{t\beta\gamma}$, the peptide inhibition is presumed to represent the potency of acyl-peptides in inhibiting interactions between $G_{t\alpha}$ and $G_{t\beta\gamma}$. The 14:0 peptide is a stronger inhibitor of GTPase activity than the 12:0 peptide ($K_i = 210$ and 740 μM, respectively). For ADP ribosylation, the peptide inhibition order is: 14:0 > 14:1n-9 > 12:0 ≥ 14:2n-6 ($K_i \approx 350$, 800, 1050, and 1075 μM, respectively). The inhibition is not a measure of micelle formation by the acyl peptides, since the effect was the same in the absence and presence of detergent. The order of peptide inhibition follows the predicted hydrophobicity of the fatty acids, 14:0 > 14:1n-9 > 14:2n-6 ≈ 12:0 [217] (Section 5.2).

Additionally, using sequential washes of increasing GTP concentration, it has been shown that 14:0 acylated $G_{t\alpha}$ is the least efficiently solublized form of transducin from bovine photoreceptor membranes [269]. Consistent with the order of fatty acid hydrophobicity, 12:0 and 14:2n-6 acylated $G_{t\alpha}$ showed maximal elution at the lowest concentration of GTP (1 μM); 14:1n-9 $G_{t\alpha}$, while also efficiently released at this concentration, showed a slight increase (~1.2 times) up to 10 μM GTP. Furthermore, the 14:0 acylated $G_{t\alpha}$ was maximally released only under the highest GTP concentration (400 μM) with a sharp increase (~3.3 times) from the lowest GTP concentration (1 μM). The solubility effect apparently is not dependent on affinity of the fatty acids for the lipid bilayer because the four acylated forms of $G_{t\alpha}$ showed no difference in elution when the non-hydrolyzable GTP analogue GTPγS was used [269]. Since the association of $G_{t\alpha}$ with $G_{t\beta\gamma}$ requires $G_{t\alpha}$ to be in the GDP bound form, this suggests that the observed solubility effect results from the affinity of the fatty acid towards the $G_{t\beta\gamma}$ subunit, as noted for the previously mentioned studies involving acylated $G_{t\alpha}$ peptides [170, 173]. Consequently, the effect of heterogeneous fatty acylation may be to decrease affinity of $G_{t\alpha}$ for $G_{t\beta\gamma}$, which would promote increased rate of phototransduction by faster production of GTP bound $G_{t\alpha}$. Alternatively, the more hydrophobic fatty acids may enhance interactions with the GTPase accelerator, RGS9, a tightly membrane-bound protein [46, 47].

5.2.3. Guanylate cyclase activating protein (GCAP)

Currently no studies have been carried out to address specifically the effect of the individual N-terminal acyl-groups (12:0, 14:0, 14:1n-9, and 14:2n-6) on the function of the two forms of GCAP, although the function of heterogeneous acylation has been indirectly examined using native and either myristoylated or non-acylated recombinant proteins [270–272]. Taken together these studies suggest that the acylated N-termini of the GCAPs are important for their structure and function, but that Ca^{2+} binding does not induce a Ca^{2+}/myristoyl switch like the one observed for recoverin. A particular contrast with recoverin was seen with respect to membrane binding, which appears to require neither Ca^{2+} binding nor N-terminal acylation for GCAP-1. Although lack of Ca^{2+} stimulates membrane binding as well as guanylate cyclase activation for GCAP-2 [271], again it is not by means of a Ca^{2+}-myristoyl switch, as described for recoverin (Section 5.2.1). Apparently, membrane binding of GCAP-2 is determined by Ca^{2+} induced changes in the protein itself, perhaps by exposure of a patch of hydrophobic amino acids [271]. The acyl group of GCAP-2 may provide structural stability by anchoring the N-terminus to the surface of the protein [271]. Additionally, the acyl group may be part of a critical interaction domain with guanylate cyclase, as appears also to be the case for GCAP-1.

5.2.4. Protein kinase A (PKA)

Although there are no studies relating N-terminal acylation to functional aspects of the catalytic subunit of PKA within photoreceptors, the protein has been examined for the effect of myristoylation in NIH 3T3 cells using acylated and non-acylated recombinant proteins [273, 238]. Non-acylated PKA shows no decrease in ability to phosphorylate peptides, couple to its regulatory (R) subunit, change cell morphology, stimulate steroid production, activate CRE (cAMP responsive element) transcription factors, or undergo nuclear transport [273]. However, it has been shown that the myristoylated form of PKA is resistant to dephosphorylation and concomitant loss of enzymatic activity, in comparison to the non-myristoylated catalytic subunit, which can be dephosphorylated by a cellular phosphatase and phosphatase 2A [274]. Additionally, thermostablity studies show non-acylated PKA has a lower denaturation temperature than the myristoylated form ($T_{1/2} = 55$ and 58°C, respectively) [238]. This suggests that one function of the acyl group is to provide structural stability by anchoring the N-terminus to the surface of the protein. Indeed, the myristoyl group of PKA has been shown to be deeply buried in a pocket of hydrophobic amino acids [253]. Additionally, cross-linking studies using non-acylated and myristoylated PKA peptide suggest that there is a myristoyl specific PKA receptor (24 kDa) in liver cells [275].

6. Paths toward heterogeneous N-terminal acylation

6.1. Occurrence of 12:0, 14:0, 14:1n-9, and 14:2n-6

The fatty acids 12:0, 14:0, 14:1n-9, and 14:2n-6 found on photoreceptor proteins are known by the generic names of lauric, myristic, physeteric, and goshuyic acids. Appropriate IUPAC

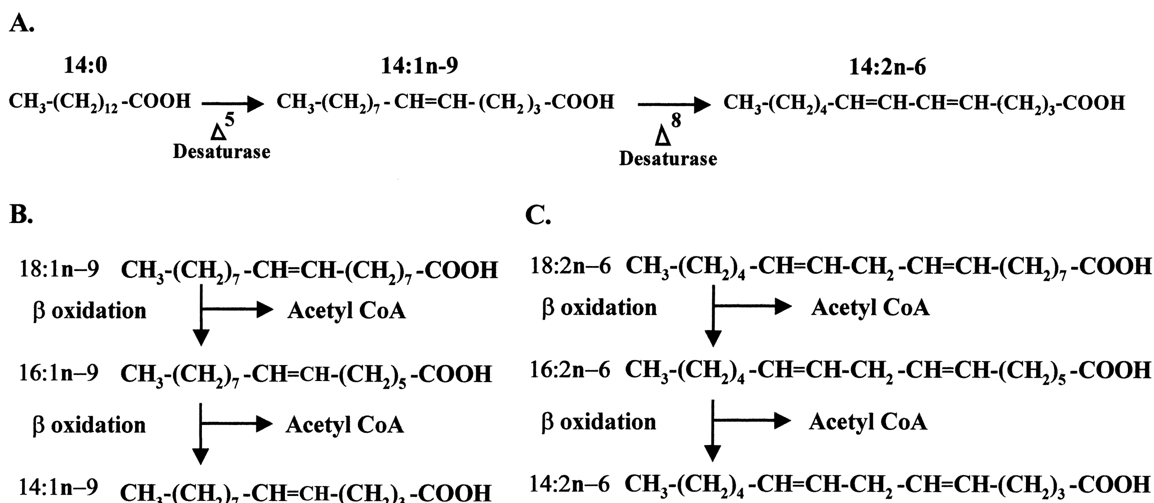

Fig. 6. Hypothetical pathways for production of 14:1n-9 and 14:2n-6. A. Desaturation of 14:0 to generate 14:1n-9 and 14:2n-6. B and C. Production of 14:1n-9 and 14:2n-6 by β-oxidation of 18:1n-9 and 18:2n-6, respectively.

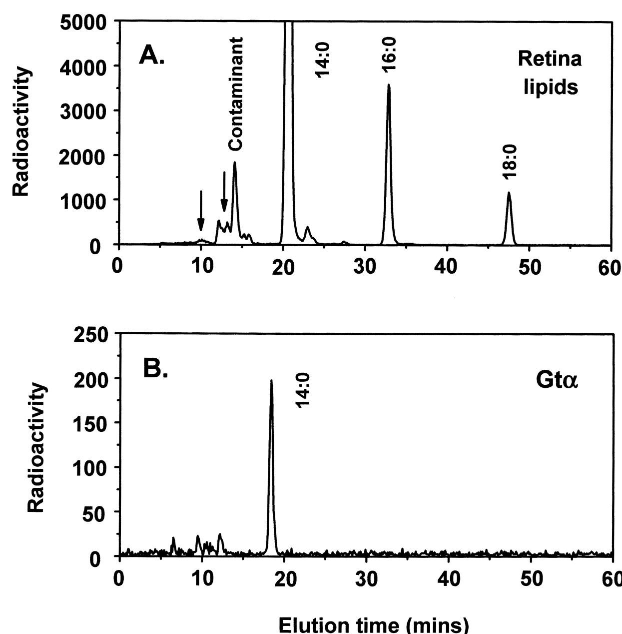

Fig. 7. Total lipid and $G_{t\alpha}$ fatty acids from frog retinas incubated with [9, 10-^{3}H]-14:0. A. HPLC elution profile of fatty acid radioactivity from frog retinas incubated with 1 mCi [^{3}H]-14:0. Arrows mark retention times for 14:1n-9 and 14:2n-6. B. Fatty acid radioactivity for $G_{t\alpha}$ isolated from the frog retinas incubated with [^{3}H]-14:0. Fatty acids were released from the protein by acidic methanolysis.

(International Union of Pure and Applied Chemistry) names for these same fatty acids are dodecanoic, tetradecanoic, *Z*-5-tetradecaenoic, and *Z*-5, *Z*-8-tetradecadienoic acids. Further information on these fatty acids can be accessed in the *Chemical Abstracts Collective Index* (published by the American Chemical Society; Columbus, OH) under the registry numbers 143-07-7, 544-63-8, 544-66-1, and 39039-37-7 for 12:0, 14:0, 14:1n-9, and 14:2n-6, respectively. While 12:0 and 14:0 are readily available from commercial sources, 14:1n-9 and 14:2n-6 currently are not. Routes for the organic synthesis of unsaturated fatty acids have been described which lead directly or indirectly to the production of 14:1n-9 and 14:2n-6 [276–281].

Animal tissues, including the photoreceptors, contain 12:0 and 14:0 (~1 and 3%, respectively) [282, 283]. However, 14:1n-9 and 14:2n-6 are quite rare ($<0.1\%$) and have only been found in abundance in marine mammals (14:1n-9) and the Asian plant *Evodia rutaecarpa* (14:2n-6) [284, 283]. In light of the rarity of these two fatty acids in both plant and animal tissues, it is reasonable to assume they are not being derived from dietary input for photoreceptor protein modification. Consequently, biosynthetic routes must be present in photoreceptors for producing sufficient amounts of 14:1n-9 and 14:2n-6 to modify the substantial protein pools represented by $G_{t\alpha}$, GCAP, and recoverin, which together comprise more than 10% of the total rod outer segment proteins [29, 285].

6.2. Biosynthesis of 14:1n-9 and 14:2n-6

6.2.1. Desaturation of 14:0

Biosynthesis of 12:0, 14:0, and other saturated fatty acids is actively carried out in animal cells by fatty acid synthase, which uses acetyl-CoA as the basic building block [3, 5, 10, 11, 286, 287]. It is conceivable the 14:1n-9 and 14:2n-6 could arise from desaturase enzymes acting on endogenous 14:0 in photoreceptors (~3%). As shown in Fig. 6a, 14:0 would be first desaturated between carbons 5 and 6 to form 14:1n-9 (Δ^5-desaturase), which would then undergo additional desaturation between carbons 8 and 9 (Δ^8-desaturase) to form 14:2n-6. It is known that the n-5 isomer of 14:1 can be made de novo, by a Δ^9-desaturase [288, 289]. However, previously described Δ^5-desaturases prefer long chain

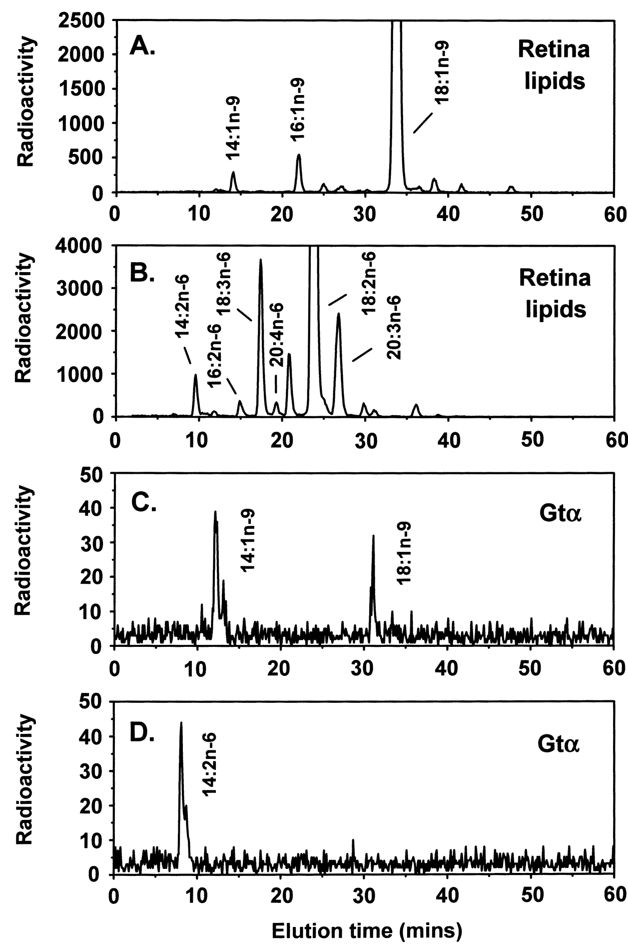

Fig. 8. Total lipid and $G_{t\alpha}$ fatty acids from frog retinas incubated with [9, 10-^{3}H]-18:1n-9 or [9, 10, 12, 13-^{3}H]-18:2n-6. A. HPLC elution profile of fatty acid radioactivity from frog retinas incubated with 1 mCi [^{3}H]-18:1n-9 (A) or 120 μCi [^{3}H]-18:2n-6 (B). C and D. Fatty acid radioactivity for $G_{t\alpha}$ isolated from the [^{3}H]-18:1n-9 (C) or [^{3}H]-18:2n-6 (D) incubated frog retinas. Fatty acids were released from the protein by acidic methanolysis.

polyunsaturated fatty acids as substrates and Δ^8-desaturases have only been found in a few cell types, such as testicular Sertoli and brain glioma cells [290–292].

We investigated the possibility that 14:1n-9 and 14:2n-6 can be generated through desaturation of 14:0 by in vitro incubation of frog retinas with [^{3}H]14:0 as the precursor fatty acid [114]. HPLC of the fatty acids from the total retina lipids, shown in Fig. 7a, revealed only chain elongation to [^{3}H]16:0 (~11%) and [^{3}H]18:0 (~7%). There was no evidence for the formation of [^{3}H]14:2n-6, whose anticipated position is indicated by an arrow (~10 min) in Fig. 7a, arising through desaturation of the [^{3}H]14:0. A peak near the anticipated retention time of [^{3}H]14:1n-9, as indicated by the other arrow (~13 min) in Fig. 7a was observed. However, this peak was also present as a contaminant in the commercial stock of [^{3}H]14:0 and therefore it is uncertain if this represents the 14:1n-9 metabolic product of 14:0. To examine incorporation of original precursor [^{3}H]14:0 or its metabolic products into photoreceptor proteins, $G_{t\alpha}$ was purified from these retinas and subjected to acidic methanolysis (2N HCl/83% methanol) to release any N-terminally linked fatty acids. As shown in Fig. 7b, HPLC of these fatty acids showed the protein released 14:0 along with some minor peaks of uncertain identity. There was no indication of a significant amount of [^{3}H]14:1n-9 or [^{3}H]14:2n-6 associated with the $G_{t\alpha}$ sample. Overall these results demonstrate that 14:0 is not a substrate for desaturase enzymes to produce 14:1n-9 or 14:2n-6 in the retina. They do indicate that a small amount of 14:0 below the detection limit of mass spectroscopy can be found on frog $G_{t\alpha}$. [158]

6.2.2. Retroconversion of longer chain unsaturated fatty acids

A more plausible route for forming 14:1n-9 and 14:2n-6 might be by retroconversion of longer chain fatty acids through partial β-oxidation. As shown in Fig. 6b and c, 18:1n-9 (oleic acid) or 18:2n-6 (linoleic acid) might be converted by this route to 14:1n-9 and 14:2n-6. Both 18:1n-9 and 18:2n-6 are suitable precursor candidates because they are abundant in biological tissues. The 18:1n-9, a non-essential fatty acid produced by desaturation of 18:0 (Δ^9 desaturase), is present in vertebrate photoreceptors at ~8% [293]. While 18:2n-6 is found in vertebrate photoreceptors in small amounts ($<1\%$), this essential fatty acid is present in plasma and retinal pigment epithelial cells (~34 and 6%, respectively). [294] It has been previously hypothesized that 14:2n-6 is generated from 18:2n-6 within the photoreceptor cell by partial β-oxidation [295, 296]. Such partial β-oxidation is characteristic of peroxisomal metabolism, in contrast to mitochondrial β-oxidation which favors complete degradation to acetyl-CoA [297, 210, 298]. Retroconversion pathways of this kind have been demonstrated to convert 13-hydroxy-9,11-octadecadienoic acid (13-OH, 18:2n-6) to 9-hydroxy-5,7-tetradecadienoic acid (13-OH, 14:2n-6) in lymphocytes and 18:2n-6 to 14:2n-6 in rat liver peroxisomes [299, 300]. It has also been demonstrated that rat liver peroxisomes can retroconvert 20:4n-6 to 14:2n-6, with passage through 18:3n-6 and 16:3n-6 intermediates [301, 302]. In the retina, a similar retroconversion pathway was found to convert 22:5n-3 to 20:5n-3 [296]. Finally, biosynthesis of docosahexaenoic acid (22:6n-3), an essential component of neuronal cells including retina photoreceptors (30–40% of total fatty acids), requires the peroxisomal based retroconversion of 24:6n-3 to 22:6n-3 [303, 304].

Since it is possible that 14:1n-9 and 14:2n-6 might be produced in the retina during the partial β-oxidation (retroconversion) of long chain polyunsaturated fatty acids, we performed

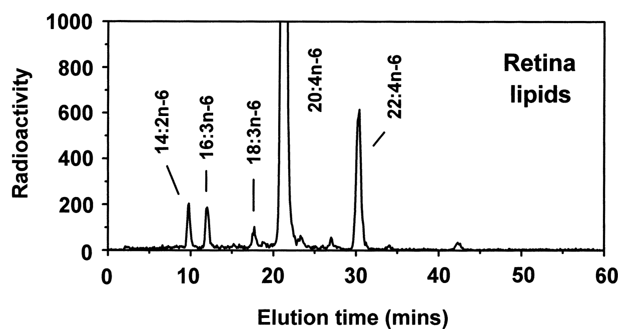

Fig. 9. Total lipid fatty acids from frog retinas incubated with [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]-20:4n-6. HPLC elution profile of fatty acid radioactivity from frog retinas incubated with 25 μCi [^{3}H]-20:4n-6.

in vitro incubations of frog retinas using [^{3}H]18:1n-9 and [^{3}H]18:2n-6, the precursor fatty acids predicted to lead to the formation of 14:1n-9 and 14:2n-6 [114]. As shown in Fig. 8, HPLC of the fatty acids from retina total lipids confirmed that the [^{3}H]18:1n-9 was retroconverted to [^{3}H]14:1n-9 (~2%) and [^{3}H]16:1n-9 (~4%) (Fig. 8a). The [^{3}H]18:2n-6 was retroconverted to [^{3}H]14:2n-6 (~2%) and [^{3}H]16:2n-6 (~1%), along with desaturation and elongation to [^{3}H]18:3n-6 (~8%), [^{3}H]20:3n-6 (~3%) and [^{3}H]20:4n-6 (~7%) (Fig. 8b). Since 18:1n-9 was only retroconverted to 14:1n-9 and 16:1n-9, this result suggests a restricted utilization of this fatty acid in the retina for only retroconversion reactions. While 18:2n-6 was retroconverted to 14:2n-6 and 16:2n-6, it also underwent extensive elongation and desaturation, following the steps towards 20:4n-6 described for liver [305]. These results suggest that 18:2n-6 has multiple functional roles in the retina.

To determine if the [^{3}H]14:1n-9 and [^{3}H]14:2n-6 produced in these incubations were incorporated into photoreceptor proteins, $G_{t\alpha}$ was isolated from the incubated frog retinas and subjected to acidic methanolysis to release N-terminally linked fatty acids [114]. HPLC of methanolysis products showed release of [^{3}H]14:1n-9 and [^{3}H]18:1n-9 from the [^{3}H]18:1n-9 incubation (Fig. 8c) and [^{3}H]14:2n-6 from the [^{3}H]18:2n-6 incubation (Fig. 8d). Positive identification of both products was made by catalytic hydrogenation and rechromatography which yielded the predicted saturated fatty acid. The presence of [^{3}H]14:1n-9 and [^{3}H]14:2n-6 in $G_{t\alpha}$ shows that these fatty acids were used for N-terminal acylation by the NMT. The fatty acids of the retina lipids and isolated $G_{t\alpha}$ confirm the hypothesis that 14:1n-9 and 14:2n-6 are formed by retroconversion reactions within the retina from longer chain unsaturated fatty acids of the n-9 and n-6 families. The 14:1n-9 and 14:2n-6 produced in this manner are accessible by the photoreceptor NMT for modification of proteins. The incorporation of [^{3}H]14:2n-6 into frog $G_{t\alpha}$ is consistent the protein being ~100% modified with 14:2n-6 [158]. The observed incorporation of [^{3}H]14:0 and [^{3}H]14:1n-9 into frog $G_{t\alpha}$ indicates a small population of 14:0 and 14:1n-9 modified protein is also present. The level of 14:0 and 14:1n-9 in frog $G_{t\alpha}$ is likely below the detection limits of the mass spectroscopy based analysis methods used by previous investigators to characterize the acyl group on this protein [158, 172].

As further proof of the original hypothesis, we investigated whether another fatty acid of the n-6 family could also be retroconverted to 14:2n-6 [114]. Apart from 18:2n-6, 20:4n-6 has the most promising potential for retroconversion because it has already been shown to be

converted to 14:2n-6 in the liver. [301, 302] In vitro incubations of frog retinas with [^{3}H]20:4n-6 revealed production of [^{3}H]14:2n-6 (~2%) along with the anticipated [^{3}H]16:3n-6 (~2%) and [^{3}H]18:3n-6 (~0.5%) intermediates (Fig. 9) [302]. These results demonstrate that the frog retina can retroconvert 20:4n-6 to 14:2n-6, as was observed for 18:2n-6. Since the retina has higher levels of 20:4n-6 (~5%) than 18:2n-6 (<1%), 20:4n-6 is the more likely endogenous precursor of 14:2n-6 for N-terminal acylation reactions.

6.2.3. Specificity of fatty acid retroconversions

N-terminal acylated retinal proteins do not contain 14:3n-3, even though n-3 fatty acids are the most abundant polyunsaturates in the vertebrate retina [282, 158]. This observation suggests that fatty acids of the n-3 family are not retroconverted in the retina to 14:3n-3 or that the specificity of the photoreceptor NMT for 14:3n-3 is extremely low. We incubated frog retinas in vitro with [^{3}H]18:3n-3 and found that they did not produce detectable amounts of 16:3n-3 or 14:3n-3 retroconversion products, although elongation and desaturation products were formed [114].

Consistent with this observation, 14:3n-3 was not incorporated into $G_{t\alpha}$. The fatty acid retroconversions seen in the in vitro incubations of frog retinas with n-9 and n-6 fatty acids (Section 6.2.2) have the limited chain shortening characteristics associated with peroxisomal β-oxidation, as shown by the lack of production of 12:1n-9 and 12:2n-6 or other short chain fatty acids [297, 306, 298]. Retina peroxisomes may prove to be more vigorous or less stringent in retroconverting n-9 and n-6 fatty acids compared to those from other tissues, thus accounting for the tissue specificity of heterogeneous N-terminal acylation. Such differences would account for the absence of 14:1n-9 or 14:2n-6 on N-acylated liver proteins such as cytochrome b_5 reductase, even though liver peroxisomes carry out fatty acid retroconversions of n-6 fatty acids to produce 14:2n-6 [299, 302, 118].

6.3. Differences in fatty acyl-CoA pool of tissues

The retina can generate the 14:1n-9 and 14:2n-6 required for N-terminal acylation of photoreceptor proteins through retroconversion of longer chain unsaturated fatty acids (Section 6.2.2). The formation of 12:0 and 14:0 is more direct, through the fatty acid synthase complex (Section 6.2.1). This suggests that all four of these fatty acids are available to the NMT and that the newly synthesized fatty acids would be in the required acyl-CoA form [307, 308]. Since NMT has the highest preference for 14:0 CoA (Section 4.3), it is difficult to understand why 12:0, 14:1n-9, and 14:2n-6 are incorporated into photoreceptor proteins at equal or greater levels compared to 14:0. One possible explanation is that the amounts of available 12:0, 14:1n-9, and 14:2n-6 CoAs are higher in the retina than that of 14:0, which would allow these fatty acids to successfully compete for the NMT. If true, based on the acyl-CoA activities of both human and *S. cerevisiae* NMTs (Section 4.3), equal utilization in comparison to 14:0 CoA requires the cellular concentration of 12:0, 14:1n-9, and 14:2n-6 CoAs to be 2.3–3.1, 1.0–3.0, and 20.0 times the level of 14:0 CoA.

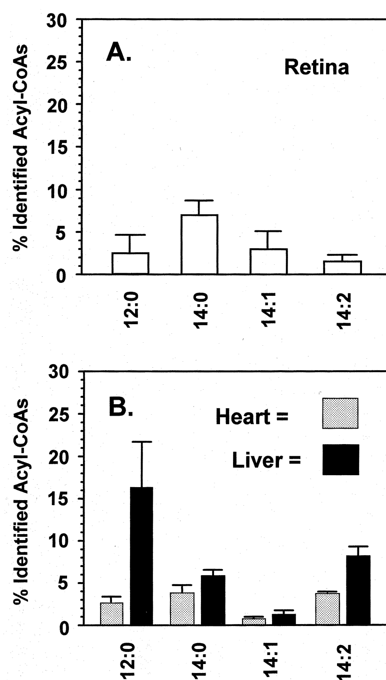

Fig. 10. 12:0, 14:0, 14:1n-9, and 14:2n-6 CoA content in the acyl-CoA pool of bovine retinas, heart, and liver. A and B. Bar graphs summarizing the relative percentages for 12:0, 14:0, 14:1n-9, and 14:2n-6 CoAs found in HPLC profiles for bovine retinas (n = 12), heart (n = 2), and liver (n = 2).

6.4. Acyl-CoA composition of non-retina tissues

According to the hypothesis just stated, non-ocular tissues would have lower levels of 12:0, 14:1n-9, and 14:2n-6 CoAs than 14:0 CoA and therefore have proteins modified with only 14:0. Of all tissues examined, only rat and pig heart, rat liver, and rat skeletal muscle were reported to contain 12:0, 14:0, and 14:1 CoAs [309, 310], and no reports of 14:2 CoA have been made. Levels of 12:0 CoA in pig heart (4.1%), 12:0 and 14:1 CoA in rat liver (2.4 and 4.7%), and 12:0 and 14:1 CoA in skeletal muscle (2.1 and 2.1%) were below that of 14:0 CoA (5.8, 7.1, and 4.4%, respectively). In contrast, 14:1 CoA in pig heart (14.3%) and 12:0 and 14:1 CoA in rat heart (6.5 and 13.6%) were higher than that of 14:0 CoA (7.1 and 5.2%). The results for pig and rat heart are noteworthy, since protein kinase A is exclusively modified on the N-terminus with 14:0 in heart (Section 2), although there apparently exists enough of 12:0 and 14:1n-9 CoA in this tissue to compete with 14:0 CoA for N-terminal fatty acylation of proteins [128, 158].

6.4.1. Acyl-CoA composition of the retina

We addressed whether the acyl-CoA pool of the retina has an unique enrichment of 12:0, 14:1n-9, and 14:2n-6 CoAs over 14:0 CoA by performing HPLC separation of the total acyl-CoAs from bovine retina [174]. Since the acyl-CoA composition of other bovine tissues was not published, bovine heart and liver were also subjected to the same analysis for comparison.

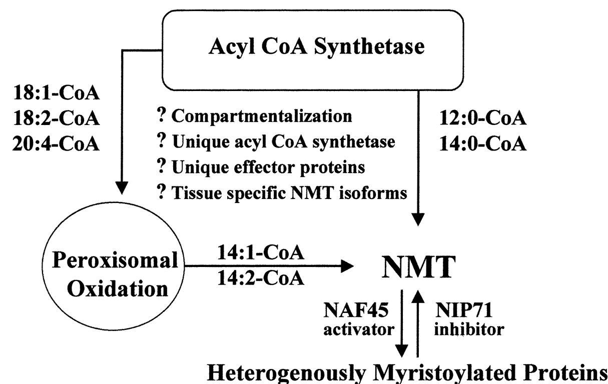

Fig. 11. Possible models for regulation of heterogeneous N-terminal acylation. Other tissues may lack retina specific regulatory proteins, NMT isotypes, or unique peroxisome or acyl-CoA synthetase activities. The NMT may be subcellularly compartmentalized with 12:0, 14:1n-9, and 14:2n-6 CoAs, apart from the pool of available 14:0 CoA.

The percentages of 12:0, 14:0, 14:1n-9, and 14:2n-6 found with in the total acyl-CoA pool of retinas, heart, and liver are shown in Fig. 10. Of the total identifiable acyl-CoAs, retina contains amounts of 12:0, 14:0, 14:1n-9, and 14:2n-6 CoAs at 2.7, 7.0, 2.9, and 1.6%, respectively (Fig. 10a). Likewise, levels of 12:0, 14:0, 14:1n-9, and 14:2n-6 CoAs in heart and liver are 2.6, 3.8, 0.7, and 3.7% and 16.1, 5.9, 1.2, and 8.1%, respectively (Fig. 10b). Overall, there is no indication in heart and retina that 12:0, 14:1n-9, and 14:2n-6 CoAs exceed 14:0 CoA by a substantial amount. Liver levels of 12:0 and 14:2n-6 CoAs were slightly higher than that of 14:0 CoA, while 14:1n-9 CoA was somewhat lower. Like heart and retina, however, in liver there also is no dramatic enrichment of 12:0, 14:1n-9, and 14:2n-6 CoAs over 14:0 CoA.

While the amounts of 12:0, 14:1n-9, and 14:2n-6 CoAs in heart are consistent with the acylation of heart proteins with only 14:0, the levels of these CoAs in the retina are not enough to cause the heterogeneous acylation pattern seen for photoreceptor proteins. Likewise, the relative amounts of 12:0 and 14:2n-6 CoAs in liver suggests that these fatty acids should be found on liver proteins in addition to 14:0, yet this is not the case [174, 118]. Consequently, it is very unlikely that the tissue specificity of heterogeneous N-terminal fatty acylation is determined by differences in the acyl-CoA composition of tissues or the acyl-CoA specificity of the NMT. This strongly suggests alternative models must be considered for regulation of the fatty acid specificity of N-terminal acylation reactions in photoreceptors and other tissues.

7. Alternative models for control of heterogeneous N-terminal acylation

In light of the acyl-CoA composition showing that the retina does not have an enriched pool of 12:0, 14:1n-9, and 14:2n-6 CoAs compared to other tissues [174], alternative models must be considered for regulation of N-terminal fatty acylation in photoreceptors. Some of these alternative models, depicted in Fig. 11, include regulation of the NMT activity through hormonal factors, peroxisomal β-oxidation, acyl-CoA synthetase activity, subcellular

compartmentalization, NMT accessory proteins, NMT isozymes, and proteolytic cleavage. It is likely that one or more of these mechanisms is at work in photoreceptors.

7.1. Hormonal factors

Photoreceptors have morphological and biochemical responses to growth factors [311]. Although acyl chain specificity was not evaluated in response to these factors, it has been shown that streptozotocin induced diabetes in rat results in a 2-fold increase in liver NMT activity, compared to control animals [312], making insulin a possible effector of NMT activity. Consequently, heterogeneous N-terminal acylation could be established by the unique hormonal properties of this tissue which might modulate the activity of the NMT, making it more selective for 12:0, 14:1n-9, and 14:2n-6 over 14:0. For example, in other tissues, glucosamine, γ-interferon, and lipopolysaccharide (LPS) are small molecules that can regulate NMT activity. Glucosamine is a noncompetitive inhibitor (K_i = 0.8 mM) of NMT activity in human H9 lymphocytes and rat liver cells by an unknown mechanism [313]. Lipopolysaccharide and γ-interferon stimulate protein myristoylation in macrophages [13]. For LPS, one of the proteins appears to be antigenically similar to the MARCKS protein, which has also been shown to be a substrate for phosphorylation. Consequently, activation of protein myristoylation in macrophages may be related to up regulation of phosphorylation by hormonal signals. Consistent with this, HL-60 promyelocytic cells show increased protein myristoylation when stimulated with phorbol esters, which are known activators of the protein kinase C signal transduction pathway in many tissues including the retina [314].

7.2. Peroxisomal β-oxidation

The retina synthesizes 14:1n-9 and 14:2n-6 by retroconversion of 18:1n-9 and 18:2n-6 or 20:4n-6, respectively, and 14:1n-9 and 14:2n-6 are used to modify photoreceptor proteins. One previously discussed hypothesis (Section 4.2) is that retina peroxisomes might be more active in producing 14:1n-9 and 14:2n-6 than other tissues. Photoreceptors do contain peroxisomes which are morphologically distinct in shape from those in liver and kidney, although they appear to contain the same enzymes involved in the β-oxidative cascade [315–317]. It has been shown in a *S. cerevisiae* mutant strain with lowered NMT affinity for 14:0 (nmt1–181) phenotypic (myristic acid auxotrophy) reversion can be achieved by supplementation with excess 18:1n-9 under conditions where saturated fatty acid synthesis is suppressed [5]. Since it is known that the retina can synthesize 14:1n-9 from retroconversion of 18:1n-9 and this 14:1n-9 is utilized by the NMT [114], it is possible that the rescue of the nmt1–181 mutant with 18:1n-9 also comes from similar production of 14:1n-9 and incorporation into proteins. These findings suggest that the nmt–181 mutant may be a suitable in vivo model for studying aspects of heterogeneous N-terminal fatty acylation.

7.3. Acyl-CoA synthetase activity

The coupling of long chain fatty acids to CoA is carried out in cells by acyl-CoA synthetases [308]. It is possible that the retina acyl-CoA synthetase is more efficient at

generating the required 18:1n-9, 18:2n-6, and 20:4n-6 CoAs for ultimate peroxisomal production of 14:1n-9 and 14:2n-6. Multiple isozymes of acyl-CoA synthetase with distinctly different fatty acid specificity are known to exist, so it is possible that the photoreceptor contains unique acyl-CoA synthetases that are involved directly or indirectly in providing the NMT with 12:0, 14:1n-9, and 14:2n-6 CoAs over 14:0 CoA.

Acyl-CoA synthetases exist which have a high preference towards utilization of 20:4n-6 over other fatty acids and are termed arachidonic specific acyl-CoA synthetases (AACS). AACS have been enzymatically characterized from numerous tissues including bovine, frog, human, and rat retinas [318, 319]. The AACS is localized to the microsomal fraction of the retina, and in human retinas appears to have higher activity than that of human brain and liver (4.5 and 5.6 times, respectively) [320, 318, 319, 321]. This may suggest that the requirement for 20:4n-6 CoA is higher in the retina than other tissues, such as in the utilization for production of 14:2n-6 to modify photoreceptor proteins.

Long chain fatty acid specific acyl-CoA synthetases (LACS) exist in three distinct isoforms, as shown by cloning [322–328]. The three types of LACS have distinct but very broad fatty acid specificity, utilizing both saturated and unsaturated fatty acids from C12 to C22 in length [323, 324, 326]. LACS has been characterized enzymatically from the microsomal fraction of bovine, frog, human, and rat retinas [321]. The enzyme shows higher activity with saturated fatty acids, in comparison to long chain polyunsaturated fatty acids. Microsomes from human erythroleukemia cells and murine T-lymphocytes contain a LACS with high specificity for short chain saturated fatty acids [212]. Both enzymes have a preference for 14:0, but also utilize 12:0 and 16:0 (14:0 > 12:0 > 16:0). In erythroleukemia cells, the catalytic efficiency (V_{max}/K_m) of 14:0 is 5.5-fold greater than that of 12:0. This type of LACS may be the primary producer of 12:0 and 14:0 CoAs for use by N-terminal myristoyltransferases (NMT). Interestingly, the erythroleukemia cell LACS was shown to utilize 14:1n-9 with a slightly higher activity rate (120%) than 14:0. The photoreceptor may contain a similar LACS for formation of 12:0, 14:0, 14:1n-9, and 14:2n-6 CoAs.

7.4. Subcellular compartmentalization

While NMTs are predominately cytosolic proteins, the retina NMT could be compartmentalized where it has greater access to 12:0, 14:1n-9, and 14:2n-6 CoAs than to 14:0 CoA [191–194]. To address this question, we incubated frog retinas with [^{14}C]14:2n-6 and $G_{t\alpha}$ was purified and subjected to acidic methanolysis [114]. No [^{14}C]14:2n-6 was released from the protein, although significant amounts were elongated to [^{14}C]16:2n-6 (~3%) and [^{14}C]18:2n-6 (~4%) in the retina total lipids (HPLC profiles not shown). These results suggest that exogenous [^{14}C]14:2n-6 may not be available to the retina NMT through a direct cytoplasmic route. Consequently, the NMT may only have access to 14:1n-9 and 14:2n-6 produced by retroconversion. If this reaction and co-translational modification of proteins occur in cellular regions of close proximity, local concentrations of 14:1n-9 and 14:2n-6 CoA could be quite high relative to 14:0 CoA and lead to the heterogeneous N-acylation of photoreceptor proteins.

7.5. NMT accessory proteins

Regulation of NMT activity has been shown to occur through direct interaction with specific protein factors. A protein known as NIP_{71} (*71* kDa, *N*MT *i*nhibitor *p*rotein) has been co-purified with the bovine brain NMT [329, 330, 8], and shown to a potent inhibitor (K_i = 34 nM) of the NMT, preventing the binding of both the 14:0 CoA and peptide substrates. Inhibition follows a sigmoidal curve, suggesting existence of an on/off switch for NMT regulating activity in the brain. Bovine brain NIP_{71} has also been shown to have an equivalent inhibitory action against recombinant human NMT (K_i = 31 nM), suggesting that NMT inhibition by NIP_{71} is a common regulatory mechanism in mammals [190]. A 50 kDa protein purified from bovine heart was described as being representative of myristate specific binding/ transport protein and not an NMT, since the binding of 14:0 was not inhibited by NIP_{71} [331]. However, the protein was able to acylate a PKA decapeptide, in the presence of 14:0 CoA alone with similar activity to bovine spleen and human NMTs. It is likely some heart NMT is present in the protein preparations and these findings show the inhibitory action of NIP_{71} differs with tissue specific NMT isoforms, serving as an additional regulatory mechanism of myristoylation.

Another protein, NAF_{45} (*45* kDa, *N*MT activating *f*actor), isolated from bovine brain stimulates NMT activity 3–4 fold by increasing the rate and extent of product formation [332]. Activation of NMT by NAF_{45} is highly cooperative (Hill coefficient = 5.5), suggesting multiple NAF_{45} monomers can bind to the NMT. NAF_{45} only partially competes against the inhibitory action of NIP_{71}. A regulatory mechanism would have NIP_{71} associated with the NMT to form an inactive complex with NMT activity being progressively restored by the cooperative binding of NAF_{45} [332]. It has not yet been demonstrated if the interaction of NIP_{71} and NAF_{45} with the NMT leads to changes in the acyl-CoA specificity of the protein.

7.6. NMT isozymes

The NMT purified from bovine brain appears to exist in several molecular weight isoforms of 60, 57, 53, 49, and 47 kDa [176, 178, 8], and immunoreactivity suggests a 84 kDa form is also present in brain [186]. Purified bovine brain and spleen [179] NMTs differ in mass by up to 10 kDa. Additionally, immunoreactivity shows bovine pancreas, kidney, liver, spleen, and heart to contain a single 49 kDa form, while lung, skeletal muscle, and brain contain additional 58, 58, and 50 and 84 kDa forms [187]. Northern analysis shows that a single 1.7 kb transcript exists in all of these bovine tissues [187]. Although no evidence of alternative splicing for human NMT mRNA by Northern analysis has been found in heart, gut, kidney, liver, and placenta cell lines, where an identical 2 kb transcript is noted [183], recent work has identified a NMT splice variant in human HEK 293 and HeLa cells and in lymphocytes [178] using 5′ RACE–PCR. The alternatively spliced NMT generated a protein of 48 kDa and the 5′ extended mRNA produced a 63 kDa protein. The 5′ extended mRNA contained a poly-lysine targeting signal, as was previously found in NMT from human leukemia and HeLa cell lines [333]. These studies indicate that alternative splicing may account for the variable protein molecular weight seen in many studies, as well as the presence or absence of a targeting signal which may allow the enzyme to be found in the cytosol or localized to a specific intracellular

site. It is possible that the observed NMT isoforms are generated in bovine tissues by post translational modification, proteolytic cleavage, or by alternative splicing. The specific effect of proteolytic cleavage on NMT activity will be discussed later (Section 7.7).

As mentioned before (Section 4.1), two distinct forms of human and mouse NMTs have been cloned, NMT–1 and NMT–2 [184]. When expressed in COS cells, the human NMT–2 is found as a single 65 kDa protein. However, the expressed NMT–1 is apparently post-translationally processed to produce isoforms of 68, 56, 55, and 49 kDa, similar to those found in bovine brain [184]. The human NMT–1 and NMT–2 isoforms show different rates of 14:0 incorporation into a PKA octapeptide, with the NMT–1 acylation rate being 1.4 times higher than that of NMT–2. This may suggest that the fatty acid as well as the peptide specificity of the two proteins differ. However, the two proteins show the same rate of acylation with a Src decapeptide. Consequently, these studies and others provide little evidence for NMT isozymes with drastically altered acyl-CoA specificity.

7.7. Proteolytic cleavage

As previously described (Section 7.6), bovine brain contains different molecular weight forms of a NMT, which may arise from proteolytic processing of the protein. Remarkably, brain has been shown to have 3.3 to 5000 times the total NMT activity, compared to the other tissues, despite the level of NMT mRNA expression in brain being the lowest [187]. However, the multiple isoforms of bovine brain NMT show no change in activity with decreasing molecular weight [176], making it uncertain if size modification of brain NMT is a regulatory mechanism leading to changes in activity.

Recent studies have shown that heart tissue containing high levels of myristoylated PKA have low NMT activity, although Western blots indicate NMT is abundant in heart tissue [188]. These results indicate regulation of NMT activity occurs in cardiac muscle. The cardiac NMT was cloned and analysis of its amino acid sequence indicated eight regions rich in proline, serine, threonine, and glutamic acid known as PEST regions [188]. These domains are thought to be recognized by Ca^{+2} dependent cysteine proteases called calpains and serve to target proteins for rapid proteolytic degradation [334]. Calpain and its endogenous inhibitor, calpastatin, are expressed in a variety of tissues [335, 336], and were used to study heart NMT activity in vitro [188]. Calpain reduced NMT enzyme activity and Western blot immunoreactivity in a time dependent manner and this effect could be reversed by calpastatin. Because calpain and calpastatin are thought to be localized primarily in the cytoplasm [337], and NMTs are also cytoplasmic enzymes, it is possible that the levels of cytoplasmic proteases and their inhibitors regulate NMT activity by either maintaining or reducing NMT levels.

8. Conclusions and medical implications for heterogeneous N-terminal acylation

N-terminal acylation of a photoreceptor protein with 12:0, 14:1n-9, and 14:2n-6 in general likely provides lower affinity membrane anchors than modification with 14:0 alone (Section 5.1). Functionally, this lessened hydrophobicity may decrease the strength of protein/protein interactions for the photoreceptor proteins, as suggested by results with $G_{t\alpha}$ and recover in

(Section 5.2.1 and Section 5.2.2). Modulation of the strength of these interactions may be important for fine-tuning the kinetics and sensititivity of the photocascade.

Since the 14:1n-9 and 14:2n-6 are generated in the retina primarily through retroconversion of long chain unsaturated fatty acids, these pathways could play a major role in both normal visual function and retinal disease states. Congenital defects such as adrenomyelnoneuropathy, neonatal adrenoleukodystrophy, infantile Refsum's disease, and Zellweger syndrome are known afflictions where peroxisomal β-oxidation is impaired [338–341]. The general phenotype of these diseases is deterioration of the nervous system including the retina. These symptoms may arise in part from impairment of the biosynthesis of docosahexaenoic acid (22:6n-3), an essential component of neuronal cells including retina photoreceptors, which requires the peroxisomal retroconversion of 24:6n-3 to 22:6n-3 [303, 304]. Because N-terminal fatty acylation with 14:1n-9 and 14:2n-6 may be required for proper function of phototransduction proteins, impairment of 14:1n-9 or 14:2n-6 production may also have a deleterious effect on normal visual function. It is known that many types of retinal degeneration involve lipidated phototransduction proteins, such as rhodopsin (autosomal dominant and autosomal recessive retinitis pigmentosa) and the β-subunit of cGMP phosphodiesterase (PDE_{β}) (autosomal recessive retinitis pigmentosa) [342–344]. A specific example of defective protein lipidation in retinal degeneration is choroideremia, where a mutation in geranylgeranyl transferase, the enzyme that isoprenylates PDE_{β} and G proteins of the rab family, has been described [345]. Therefore, genes encoding proteins involved in the pathways required for heterogeneous fatty acylation of retina protein warrant further consideration as retinal degeneration candidates.

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