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G-protein-coupled receptor kinases

Krzysztof Palczewski and Jeffrey L. Benovic

Rhodopsin kinase and the β-adrenergic receptor kinase (βARK) catalyse the phosphorylation of the activated forms of the G-protein-coupled receptors, rhodopsin and the β_2-adrenergic receptor (β_2AR), respectively. The interaction between receptor and kinase is independent of second messengers and appears to involve a multipoint attachment of kinase and substrate with the specificity being restricted by both the primary amino acid sequence and conformation of the substrate. Kinetic, functional and sequence information reveals that rhodopsin kinase and βARK are closely related, suggesting they may be members of a family of G-protein-coupled receptor kinases.

VISUAL AND CHEMICAL signal transduction share many common features. Two of the best-studied examples are phototransduction in retinal rod cells and hormonal transduction via receptor-activated adenylyl cyclase. Visual transduction in photoreceptor cells results from a cascade of chemical reactions that translate a light signal into a hyperpolarization of the cell plasma membrane. The highly regulated reactions in this cascade are responsible for initiating, amplifying and quenching the light-induced electrical response. Similarly, a wide variety of hormones and neurotransmitters regulate activation of the enzyme adenylyl cyclase. The cascade of events that follows adenylyl cyclase activation includes changes in intracellular cyclic AMP (cAMP) levels, activation of the cAMP-dependent protein kinase and subsequent protein phosphorylation leading to a wide variety of cell-specific changes in protein function. In both signal transduction pathways, three distinct proteins modulate these various reactions via their binding to the surface of stimulated receptors: guanine nucleotide regulatory proteins (G proteins), protein kinases and arrestin proteins that specifically interact with phosphorylated receptors (for reviews see Refs 1–3). Transducin, the retina-specific G protein (also termed G_t), is involved in amplifying the light signal, while rhodopsin kinase and arrestin (also termed the 48 kDa protein or S-antigen) participate in quenching light activation (Fig. 1). Similarly, stimulation of adenylyl cyclase activity in response

to catecholamine activation of the β_2-adrenergic receptor (β_2AR) is mediated by the G protein G_s while the β-adrenergic receptor kinase (βARK) and β-arrestin act in combination to uncouple G protein activation. Dephosphorylation of both receptors appears to be catalysed by general Ser/Thr protein phosphatases, such as protein phosphatase 2A in the case of rhodopsin[4-6].

In this article, we will focus on the quenching mechanisms involved in regulating photolysed rhodopsin and the agonist-activated β_2AR that are mediated by specific receptor kinases. Only brief consideration will be given to the other proteins involved in this quenching process.

Quenching of rhodopsin and β_2AR

The regulation of cellular sensitivity to a wide variety of sensory and hormonal stimuli occurs in diverse signaling systems. This phenomenon, termed desensitization, adaptation, quenching, tolerance or tachyphylaxis, has been studied extensively for the G-protein-coupled receptors rhodopsin and β_2AR[1-3]. The mechanisms for quenching both photo and chemical signal trans-

duction are broadly similar, although photolysed rhodopsin can also be quenched by other non-chemical processes. In both systems, however, receptor phosphorylation provides a rapid mechanism for inactivation. In phototransduction, rhodopsin kinase multi-phosphorylates metarhodopsin II (Ref. 2) on its carboxy-terminal domain[7]. This phosphorylation partially reduces the ability of rhodopsin to interact directly with transducin and significantly increases its ability to interact with the protein arrestin. The binding of arrestin to phosphorylated metarhodopsin II completely blocks further activation of the phosphodiesterase cascade (Fig. 1)[8]. To recycle the system, phosphorylated opsin is regenerated with 11-cis retinal to phosphorylated rhodopsin, and the arrestin is released. Once arrestin is released, the deactivated photo-receptor can be dephosphorylated by protein phosphatase 2A (Refs 4–6) and the regenerated rhodopsin can then interact with transducin when activated by light.

The ability of the β_2AR to respond to catecholamines is also significantly reduced within minutes of activation.

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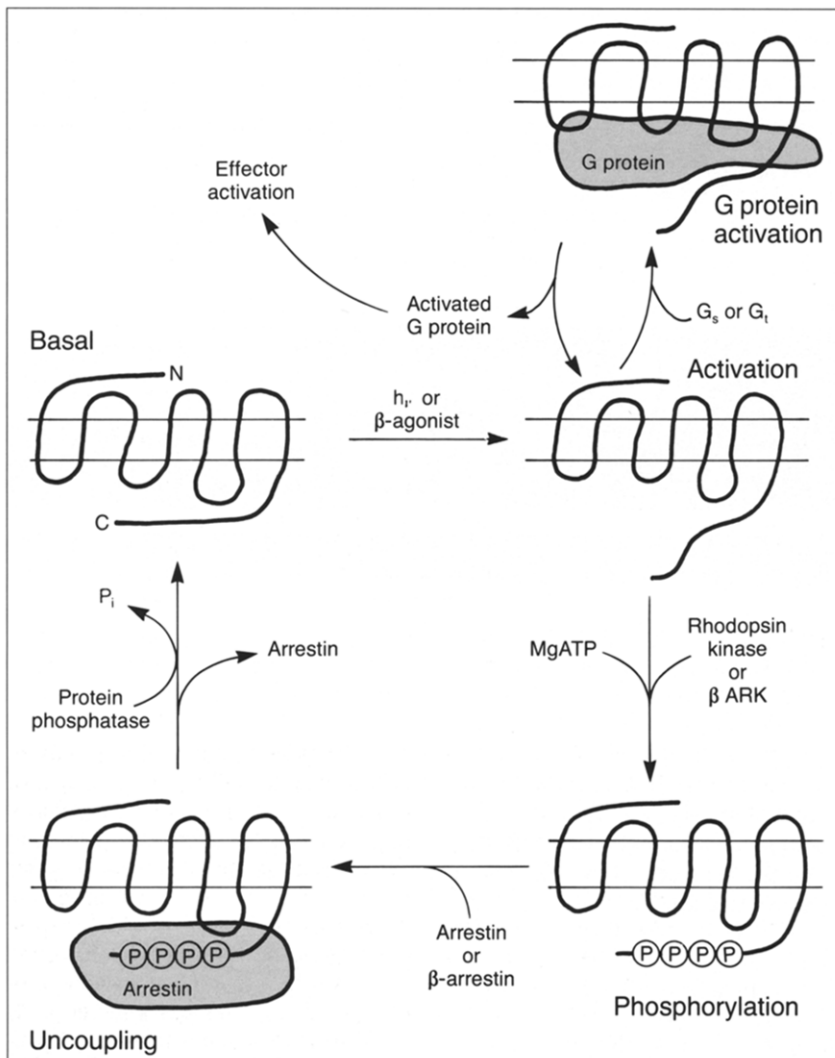
Figure 1

Stimulus-dependent phosphorylation and desensitization of rhodopsin and the β_2AR. Receptor activation, either by light (rhodopsin) or a β-agonist (β_2AR), promotes interaction of the receptor with transducin or G_s, respectively, leading to G protein and effector activation. Receptor activation also promotes phosphorylation of the receptor, which is mediated either by rhodopsin kinase or βARK. Phosphorylation of the receptor appears to uncouple it partially from the G protein, but also promotes its interaction with arrestin (for rhodopsin) or β-arrestin (for β_2AR). This interaction further uncouples the receptor from the G protein. Dephosphorylation of rhodopsin requires regeneration with 11-*cis* retinal, which is followed by arrestin dissociation and dephosphorylation of the receptor by protein phosphatase 2A. The mechanism of β_2AR dephosphorylation remains poorly understood but may involve the sequestration of the receptor into a compartment where dephosphorylation can occur.

Rhodopsin kinase

Rhodopsin kinase is a single subunit polypeptide with a molecular weight of 63 kDa. It is present only in the retina and pineal gland[12,13]. In the rod cells of the retina, the kinase is thought to be either loosely associated with the membrane or present in the cytosol. While at present there is no direct evidence that the kinase is found in cone cells, the photoreceptor found in retinas that contain predominantly cone cells (e.g. lizard) does undergo phosphorylation upon exposure to light[14]. In addition, purified rhodopsin kinase can phosphorylate the cone-specific pigment, iodopsin[15].

A number of kinetic parameters have been determined for rhodopsin kinase purified from bovine retinas (Table I). The kinase utilizes freshly bleached rhodopsin either in native membranes or in detergent solution, suggesting that phospholipids are not required for its activity[16]. Unlike the cAMP-, cGMP-, Ca^{2+}/calmodulin- and Ca^{2+}/phospholipid-dependent protein kinases, rhodopsin kinase is not regulated by second messengers[16,17]. However, preliminary data suggest that a rod-specific calcium-binding protein might play a role in regulating rhodopsin kinase (K. Palczewski, pers. commun.).

The substrate specificity of rhodopsin kinase has been investigated by using rhodopsin from different species, modified rhodopsin, related receptors and peptides derived from phosphorylable fragments of rhodopsin and unrelated proteins and peptides[15,16,18–20]. While rhodopsin kinase can phosphorylate rhodopsin from different species[16], cone pigment (iodopsin)[15] and the β_2AR (Ref. 21), these receptors have to be in their activated state to serve as substrates. While these substrates have a very similar overall topology, many have only modest sequence homology. This suggests that rhodopsin kinase may require a multipoint attachment to the surface of the properly folded receptor. The poor phosphorylation of short synthetic peptides by rhodopsin kinase further supports this view.

We and others have recently shown that rhodopsin kinase is activated upon binding the cytoplasmic loops of photolysed rhodopsin[19,22]. While the carboxy-terminal region of rhodopsin (which contains the sites of phosphorylation) does not appear to be a critical binding site, the cytoplasmic loop connecting transmembrane helices V and

Several mechanisms appear to regulate β_2AR responsiveness. There are rapid mechanisms involving receptor phosphorylation and slower events that involve increased receptor degradation and decreased receptor synthesis (for review see Ref. 3). Additional effects may include direct changes in G protein and effector activity. Together, these events blunt the effects of a given agonist and effectively stop signal transduction. As with rhodopsin, rapid desensitization of the β_2AR is achieved by phosphorylation of the receptor.

Phosphorylation of the β_2AR by the cAMP-dependent protein kinase directly uncouples the receptor from G protein interaction[9]. In contrast, phosphorylation by βARK promotes interaction of the receptor with β-arrestin, a homolog of retinal arrestin, which binds to the phosphorylated receptor, thereby preventing further activation of G_s[10,11] (Fig. 1). Presumably, the recycling of phosphorylated β_2AR will also involve dephosphorylation by an as yet unidentified protein phosphatase.

VI is[22]. This binding may trigger a conformational change in the kinase that improves catalysis of the phosphotransfer. The catalytic site of the kinase may thus be brought into closer proximity to the carboxy-terminal phosphorylation sites on rhodopsin, thus facilitating the phosphotransfer. It may be that such a loose association is necessary to enable the kinase to phosphorylate multiple sites on the carboxy-terminal tail of photolysed rhodopsin. This mechanism of catalysis distinguishes rhodopsin kinase from other extensively investigated protein kinases such as the cAMP-dependent protein kinase and casein kinase II where the recognition and catalytic sites are closely coupled[23].

The nucleotide-binding domain of rhodopsin kinase has also been extensively studied in an effort to search for selective and potent inhibitors of rhodopsin kinase as well as to elucidate the substrate specificity of the enzyme. Rhodopsin kinase was found to preferentially use Mg^{2+}-complexed ATP over GTP as the phosphate donor and bind nucleotides (or nucleosides) in an anti-configuration[24]. However, the nucleotide polyphosphate chain appears to play only a minor role in enzyme recognition of the nucleotide. In addition, sangivamycin, an ATP analog, was found to be a very potent and selective inhibitor of rhodopsin kinase (Table I).

An additional important feature of rhodopsin kinase is its ability to autophosphorylate. Kelleher and Johnson noted that autophosphorylation does not change the overall kinetic properties of rhodopsin kinase[20]. However, we have found that autophosphorylation changes the affinity of rhodopsin kinase for its substrate, especially for partially phosphorylated rhodopsin[25]. Thus, autophosphorylation could play a role in the dissociation of kinase from its substrate.

β-adrenergic receptor kinase

βARK was initially identified in a crude cytosolic fraction of S49 lymphoma cells as an activity that specifically phosphorylated the agonist-occupied form of the β_2-adrenergic receptor[26]. This activity appeared to be ubiquitous as it was present in all tissues examined, albeit to varying extents[27]. The purified kinase appears to be composed of a single subunit polypeptide of 80 kDa. Its ability to phosphorylate the agonist-occupied β_2AR is independent of second messengers such as cAMP,

Table I. Kinetic and molecular properties of rhodopsin kinase and βARK

Parameter		Rhodopsin kinase	βARK
Localization		Retina, pineal gland	Ubiquitous Brain, spleen > heart, lung > kidney
Molecular weight		63 000	79 600
Substrate	good	Metarhodopsin II	β_2AR, α_2AR, M2-muscarinic
	poor	β_2-adrenergic receptor Neutral and acidic peptides	Metarhodopsin II Acidic peptides
V_{max}		700 nmol P_i $min^{-1}mg^{-1}$	80 nmol P_i $min^{-1}mg^{-1}$
K_m for rhodopsin		4 μM	6 μM
β_2AR		ND	0.25 μM
ATP		4 μM	35 μM
GTP		>1 mM	ND
peptides		>1 mM	>1 mM
Optimum pH		6.5–8.0	6.0–7.5
Metal ion requirements		$Mg^{2+} > Mn^{2+}$ Extra Mg^{2+}	$Mg^{2+} > Mn^{2+}$
Activators		Spermine, spermidine, polycation	No known activator
Inhibitors		Sangivamycin, polyanions	Polyanions

ND, not determined.

cGMP and Ca^{2+}. The kinetic constants of the phosphorylation reaction (K_m = 0.25 μM for β_2AR, K_m = 35 μM for ATP, V_{max} = 80 nmol phosphate min^{-1} mg^{-1}) suggest a high-affinity interaction between kinase and receptor (Table I). Further evidence for high-affinity binding of the kinase to its native substrate comes from peptide phosphorylation studies that have demonstrated that synthetic peptides derived from the carboxy-terminal domain of the hamster β_2AR serve as substrates for βARK[28]. However, there is a marked difference between the kinetics of the peptide phosphorylation and those of the receptor. In particular, the K_m values for peptide phosphorylation by βARK range from 1–5 mM, ~10 000-fold different to those for the intact receptor. Additional studies have demonstrated that several peptides derived from intracellular domains of the β_2AR (non-substrate) can inhibit βARK phosphorylation of the β_2AR[28]. These studies suggest that βARK may in fact interact with multiple domains on the intracellular surface of the receptor, similar to the interaction of rhodopsin kinase and rhodopsin. The βARK phosphorylation sites on the β_2AR, however, appear to be predominantly localized to the carboxyl terminus[29].

Several lines of evidence suggest that βARK may play a role in the phosphorylation and regulation of multiple G-protein-coupled receptors. It has been demonstrated that stimulation of DDT1

MF-2 hamster smooth muscle cells or S49 lymphoma cells with a β-agonist leads to the translocation of ~80% of the βARK activity from the cytosol to the plasma membrane[30]. This suggests that an agonist-induced translocation of βARK may represent an initial step in homologous desensitization. Moreover, this translocation phenomenon also provides a test to assess whether βARK might be involved in regulating other G-protein-coupled receptors. In S49 cells, prostaglandin E1 (which stimulates adenylyl cyclase) and somatostatin (which inhibits adenylyl cyclase) also induce the translocation of βARK to the plasma membrane, suggesting that βARK may have a general role in regulating receptor activity[30,31]. Additional studies have directly demonstrated that βARK can phosphorylate multiple G-protein-coupled receptors in vitro. The platelet α_2-adrenergic receptor serves as an excellent substrate for βARK, with phosphorylation kinetics similar to that for the β_2AR as the substrate[32]. Moreover, the α_2-receptor phosphorylation was totally agonist-dependent and could be blocked by co-incubation with α_2-antagonists. The chick-heart muscarinic cholinergic receptor, which inhibits adenylyl cyclase, also serves as an excellent substrate for βARK. Again, the phosphorylation was totally agonist-dependent and a stoichiometry of ~4 mol phosphate^{-1} mol receptor^{-1} was measured[33]. While these studies suggest that βARK or a related kinase may be

involved in regulating other G-protein-coupled receptors, the physiological and functional significance of these results remains to be established.

The primary sequence of βARK has recently been deduced from a cDNA clone encoding this enzyme[34]. The βARK cDNA encodes a protein of 689 amino acids (79.7 kDa) with a central kinase catalytic domain (239 amino acids) flanked by large amino- (197 amino acids) and carboxy- (253 amino acids) terminal domains. The catalytic domain shares the highest homology with the S6 kinase and the yeast SCH9 gene product, with which it bears ~40% sequence identity, the cAMP dependent kinase (33.1%) and protein kinase C (33.7%). The amino- and carboxy-terminal domains share no significant homology with any other sequenced proteins and their function is currently unknown, however, our initial mutagenesis studies suggest that the amino-terminal domain may play a role in substrate binding (G. Kong and J. L. Benovic, unpublished). Northern blot analysis suggests that βARK mRNA is localized predominantly in tissues that have a high degree of sympathetic innervation. Interestingly, there is little correlation between βARK mRNA levels and the levels of 'βARK' activity actually measured in various tissues[27,34]. The finding of low βARK mRNA levels in some tissues that have high 'βARK' activity suggests the presence of βARK-related enzymes in these tissues.

Evidence for other G-protein-coupled receptor kinases

Rhodopsin kinase and βARK share a number of properties. They can both specifically phosphorylate the activated form of a G-protein-coupled receptor which, to a certain extent, is analogous to the ability of G proteins to recognize receptors. Both kinases are involved in a high-affinity multipoint interaction with their receptor. In addition, both kinases phosphorylate peptides rich in acidic residues, albeit with a very low affinity[35]. The specificity of these enzymes also overlaps, with βARK weakly phosphorylating metarhodopsin II and rhodopsin kinase weakly phosphorylating the agonist-occupied β₂AR (Ref. 21). Moreover, recent evidence demonstrates that rhodopsin kinase and βARK also share significant sequence homology. The amino acid identity between rhodopsin kinase and βARK is 44.2% in the catalytic domain, 22.7% in the amino-terminal region and

27% in the carboxy-terminal domain[36].

Is there any evidence for other G-protein-coupled receptor kinases? To date, no related kinases have been purified; however, Haga and Haga recently identified an activity that phosphorylates the agonist-occupied M1 and M2 muscarinic acetylcholine receptors[37]. Whether this enzyme is βARK or a related kinase remains to be determined. Several lines of evidence also suggest a role for agonist-dependent receptor phosphorylation in lower eukaryotes. In Dictyostelium, a stimulus-dependent phosphorylation of the cAMP receptor, which activates adenylyl cyclase through a G protein, plays a role in regulating chemotaxis[38]. In Saccharomyces cerevisiae, the α-mating factor receptor, which regulates an effector via interaction with a G protein, also undergoes an agonist-dependent phosphorylation which accompanies desensitization[39]. While the kinases in Dictyostelium and Saccharomyces have not yet been isolated, there is good evidence that they play a role in regulating G-protein-coupled receptors.

Perhaps the strongest evidence for the presence of additional G-protein-coupled receptor kinases comes from the cloning of βARK. Southern blot analysis of bovine genomic DNA reveals the presence of multiple hybridizing bands when probed with a catalytic domain fragment of the βARK cDNA[34]. These studies were done under conditions where one band would be expected for the βARK gene itself. Thus, the additional hybridizing species may represent other genes which the βARK cDNA probe is recognizing. Further demonstration of other βARK-related kinases comes from low stringency hybridization studies. When the catalytic domain fragment of the βARK cDNA was used to screen a bovine brain cDNA library, a second class of cDNA clones was isolated. This second cDNA encodes a protein of 688 amino acids (79.6 kDa) which bears a striking similarity to βARK[40]. The catalytic domain of this βARK-related kinase has 95% amino acid identity, while the amino- and carboxy-terminal domains have 81% and 78% identity, respectively, with βARK. The substrate specificity of this related kinase is currently under investigation.

Stimulus-dependent protein phosphorylation may well serve as a general mechanism for regulating many, if not all, G-protein-coupled receptors. The general nature of this phenomenon raises numerous questions about the

number and diversity of the kinases involved, their substrate specificity, distribution and biological roles. Addressing these questions will serve as a major focus of future research in this area.

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BOOK REVIEWS

Sing O for oxygen?

Metazoan Life Without Oxygen

edited by **Christopher Bryant**, Chapman and Hall, 1990. £37.50 (xii + 291 pages) ISBN 0 412 33360 0

The past quarter of a century has been an exciting time for anaerobic metabolism. A bewildering and impressive array of pathways across the animal kingdom have been unravelled, new enzymes have been discovered and both basic and conceptual advances have been made towards understanding the regulation, energetics, utilization and evolution of such pathways. Any book that encompasses all this and puts the understanding into an ecological and evolutionary context is to be welcomed. To some extent *Metazoan Life Without Oxygen* succeeds in doing just this.

The book comprises 14 chapters with prominent researchers in the field covering the anaerobic metabolism of most of the major animal phyla. These chapters are preceeded by others on the nature of oxygen and the early evolution of metazoan life. Others explore the interstitial meiofauna, adaptations to hydrogen sulphide and the exploitation of anaerobiosis by ruminants.

Having sung the praises of the book, one is still left with a feeling of an opportunity not quite fulfilled. Several problems are apparent; the most prominent is the absence of an in-depth chapter that brings together and builds on the findings of the individual chapters. Thus the opportunity to identify common mechanisms and problems of anaerobic metabolism, maximize the phylogenetic/evolutionary perspective and put new ideas of general relevance across the phyla is lost. For example, Chapter 9 ponders on the source of amino groups for alanine formation during anaerobiosis in polychaetes, while Chapter 10 presents evidence on this aspect in molluscs. Low versus high output modes of anaerobic metabolism

are discussed, but considerations of phylogenetic differences, a theme developed in published reviews, is absent. The opine dehydrogenase story is diluted across the chapters, and evolutionary aspects on enzymes and some pathways are minimal. A final overview chapter is included, which mainly gives a useful historical perspective and identifies key ways to future progress. However, as the editor notes, 'the final chapter might also (have been) the first'.

A second problem is an apparent (or real) dichotomy of thought on the selective pressures driving the evolution of anaerobic metabolism. At the beginning of the book, the impression that the pathways evolved to avoid the toxic effects of oxygen radicals is given, whereas the rest of the book follows the much more likely line that it was to exploit/survive situations of oxygen limitation. This difference is reflected in the fact that after an early discourse on antioxidant defenses, they are not mentioned again after Chapter 4. A third, but less important deficiency, is the absence of information on the Echinodermata, Cnidaria and other phyla, which merit inclusion both from a basic and an evolutionary standpoint.

The beginning of the book is stimulating, but somewhat demanding on the reader, as he/she is successively required to cope with chapters on chemistry, biochemistry, geology and palaeontology, as the rise of dioxygen and metazoan life is developed. Indeed, following the early encouragement (or warning!) to 'sing O for oxygen', by Chapter 4 the reader begins to wonder if the book is entitled *Metazoan Life With Oxygen*. Highlights include comments on the original roles of cytochrome oxidase and the early need for DNA repair mechanisms, a reinterpretation of the Nursall/Berkner–Marshall hypotheses, and the possibility of a long Precambrian history to metazoan life. Chapter 5 is an excellent discourse on fumarate reductase (the key to many anaerobic pathways) and its relationship with succinate dehydrogenase and the citric

acid (Krebs) cycle (which evolved from which?). Adaptations to high sulphide environments are enthusiastically pursued in Chapter 6, the requirement for both anaerobic pathways and protective mechanisms against sulphide being emphasized. Chapters 8 to 11 provide comprehensive and very readable reviews on anaerobic metabolism in parasitic helminths, annelids, molluscs and arthropods, including evolutionary aspects and the distinction between functional and environmental anaerobiosis. In Chapter 11, a number of unspecified references are omitted because the experimental conditions employed are considered '[un]related to normal physiological processes'. Chapter 12 discusses interesting concepts of defense against hypoxia in vertebrates, but does not provide detailed or systematic information on anaerobic metabolism in this phylum. The chapters on meiofauna and ruminants are interesting but mainly biological.

The book is admirably conceived and the editor appears to have encouraged a personal approach to the writing, to the point where, unwittingly or otherwise, scientific differences of opinion are revealed; for example the term 'facultative anaerobe', which is used widely by some but considered a misnomer by others. It was three years in the making, which is illustrated by the fact that most references are dated 1987 or earlier, β-alanopine dehydrogenase is not mentioned and the role of the hydroxyl radical (currently being measured *in vivo*) is considered 'a matter of debate'. More could have been made of oxyradical generation in non-mammals (an area of enormous interest and future potential). Overall, it is a good book for undergraduates and researchers alike, but with more rigour and discipline could have been even better. However, it certainly makes a valuable contribution to the subject of anaerobic metabolism.

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