The Journal of Experimental Biology 206, 2911-2922
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doi:10.1242/jeb.00516

Review

Evolution of the coordinate regulation of glycolytic enzyme genes by hypoxia

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Accepted 27 May 2003

Summary

Two billion years of aerobic evolution have resulted in mammalian cells and tissues that are extremely oxygen-dependent. Exposure to oxygen tensions outside the relatively narrow physiological range results in cellular stress and toxicity. Consequently, hypoxia features prominently in many human diseases, particularly those associated with blood and vascular disorders, including all forms of anemia and ischemia. Bioenergetic enzymes have evolved both acute and chronic oxygen sensing mechanisms to buffer changes of oxygen tension; at normal P_{O_2} oxidative phosphorylation is the principal energy supply for eukaryotic cells, but when the P_{O_2} falls below a critical mark metabolic switches turn off mitochondrial electron transport and activate anaerobic glycolysis. Without this switch cells would suffer an immediate energy deficit and death at low P_{O_2}. An intriguing feature of the switching is that the same conditions that regulate energy metabolism also regulate bioenergetic genes, so that enzyme activity and transcription are regulated simultaneously, albeit with different time courses and signaling pathways. In this review we explore the pathways mediating hypoxia-regulated glycolytic enzyme gene expression, focusing on their atavistic traits and evolution.

Key words: hypoxia, anaerobic, glycolysis, gene expression, HIF-1, evolution.

Introduction

Hypoxia is a strong and usually positive regulator of gene expression (D'Angio and Finkelstein, 2000; Prabhakar, 2001; Semenza, 2001). This may be the result of selection pressures operating over millions of years to conserve essential biological functions that were acquired during anaerobic evolution. Life evolved on earth under anaerobic conditions for about 2 billion years, close to one half of the total time period of biological evolution (Barnabas et al., 1982; Papagiannis, 1984). Therefore the fundamental features of biology and genetics, including DNA synthesis, transcription, translation and their regulation, were established under strictly anaerobic conditions, and perhaps as a consequence have an absolute requirement for a reducing environment in order to function (Segerer et al., 1985). Likewise it may be expected that certain biological activities, pathways and regulatory processes function preferentially under conditions of hypoxia. This may be particularly true for anaerobic bioenergetic pathways, including glycolysis, which were established as the first energy generators and ultimately became integrated with oxidative pathways. The suppression of oxidative metabolism under conditions of severe hypoxia reduces oxidative stress, decreases antioxidant levels, and simulates the primordial, reducing environment, even without molecular antioxidants (Webster et al., 2001). At least eight out of the twelve functually distinct glycolytic enzyme genes are coordinately induced by hypoxia in mammalian cells (Webster, 1987; Webster et al., 1990; Webster and Murphy, 1988). The regulation involves contributions from at least four separate molecular pathways, some of which may have been conserved through 4 billion years of evolution, dating back to the origin of life. An intriguing question that will be addressed in this review is whether the hypoxia-mediated gene induction involves activation of ancestral positive-acting factors, repression of oxygen-induced negative factors, or combinations of these. The molecular regulation of glycolysis at the level of enzyme activity has been reviewed extensively elsewhere and will not be addressed here (for reviews, see Hofer, 1996; Hardie, 2000; Romano et al., 1996; Siebers et al., 1998).

Oxygen regulation of glycolysis

Fig. 1A shows the glycolytic pathway, where 12 enzymes catalyze the anaerobic fermentation of glycogen to lactic acid, generating 3 moles of ATP per glucosyl unit. The process is an order of magnitude less efficient than oxidative metabolism, where 32 moles of ATP are generated per 2 or 3 moles of glucose, depending on whether glucose or glycogen is the substrate. The scheme shows the input and output points of the pathway. There are numerous molecular modulators of glycolytic flux, the most famous of which was discovered in 1860 by Louis Pasteur (Pasteur, 1861). Pasteur showed that oxygen inhibits fermentation and that glucose consumption is inversely proportional to the oxygen availability, i.e. that the glycolytic pathway is positively regulated by hypoxia. Pasteur received wide recognition for this stunning observation that became universally known as the 'Pasteur Effect'. In 1987, our laboratory reported the observations shown in Fig. 1B (Webster, 1987). We theorized that since oxygen is a potent and ancient regulator of glycolytic flux, it might also be a regulator of glycolytic enzyme gene expression. We isolated and

cloned rodent cDNAs for six glycolytic enzymes (indicated with asterisks on Fig. 1A), and we used these to measure transcription rates of the genes in muscle cells exposed to hypoxia. Fig. 1B shows a composite of the transcription of six glycolytic enzyme cDNAs compared with that of mitochondrial cytochrome *c*. Chronic hypoxia caused a significant and coordinated activation of transcription of these genes.

Conservation of glycolytic enzyme genes

The 12 mammalian glycolytic enzyme genes are genetically unlinked and dispersed around the genome, mostly on different chromosomes (Webster and Murphy, 1988). These are some of the most ancient and highly conserved proteins and genes known, with strong conservation of both the peptide and DNA sequences even between higher mammals and bacteria (Lonberg and Gilbert, 1985; Peak et al., 1994; Poorman et al., 1984). Fig. 2 shows a Southern blot illustrating the remarkable conservation of pyruvate kinase (PK) and lactate dehydrogenase (LDH) with strong cross-homology of DNA fragments between yeast and human DNA. Glycolytic enzymes were probably among the very first enzyme pathways to appear, allowing primitive organisms to utilize simple carbohydrates as energy stores and to release energy by coupling the breakdown to high-energy phosphates (Fothergill-Gilmore and Michels, 1993; Romano and Conway, 1996). Although structural and functional aspects of glycolytic enzyme genes and proteins have been strongly conserved, it is not clear how gene regulatory mechanisms evolved or how the pathway established a coordinate response of widely dispersed genes to oxygen tension. Fig. 2 also illustrates a second intriguing feature of glycolytic enzyme genes, namely an apparently selective accumulation of pseudogenes in rodents, particularly mouse and rat. This is reflected in the dramatic increase of the number of hybridizing bands in these species, and was first described by Piechaczyk for the GAPDH gene (Piechaczyk et al., 1984). Our results demonstrate increased numbers of pseudogenes of PK and LDH (Fig. 2), as well as GAPDH, aldolase, triosephosphate isomerase, phosphoglycerate kinase and enolase (not shown), and suggest that the effect may be common to the entire pathway of genes. We do not know why or how this occurred.

Precambrian: bacterial glycolytic genes

The chart in Fig. 3 shows sections of time dating back to when life first appeared on earth. This early period is known as the Precambrian and it is divided into Hadean, Archean, Paleoproterozoic, Mesoproterozoic and Neoproterozoic. The oldest fossils include bacteria and other microorganisms that date to about 3.8 billion years ago (BYA). Glycolytic enzymes are evident in the Archean period, 2 BY before the earliest oxygen-requiring species and almost 4 BY before the present pathways (Gebbia et al., 1997; Kelly and Adams, 1994; Peak et al., 1994). Qualitative trends in the amount of global biomass are projected in Fig. 3B. Acquisition of methanogenesis by Archaebacteria probably supported an early expansion of life forms (DeLong et al., 1994; Koch, 1998; O'Callaghan and Conrad, 1992; Papagiannis, 1984; Reeve, 1992), and biomass probably increased significantly before the dip and subsequent massive expansion of the Cambrian period. Natural selection working on the expanding biomass produced increasingly high levels of biological sophistication and diversity within the anaerobic kingdoms. In fact, molecular studies of extant bacterial species such as the Archaebacteria and thermophyllic sulfur bacteria indicate complex patterns of gene expression under anoxia, including the regulation of bioenergetic gene expression by elemental sulfur and phosphorus (Brunner et al., 1998; Fardeau et al., 1996; Friedrich, 1998; Janssen and Morgan, 1992; Kelly and Adams, 1994; Ma et al., 1995; Segerer et al., 1985). There is an intriguing parallel between sulfur regulation of bioenergetic pathways in the Archean era microorganisms and oxygen regulation in eukaryotes. Oxygen replaced sulfur as the terminal electron acceptor of carbohydrate catabolism, and may simultaneously have parasitized some molecular features of the regulation over billions of years.

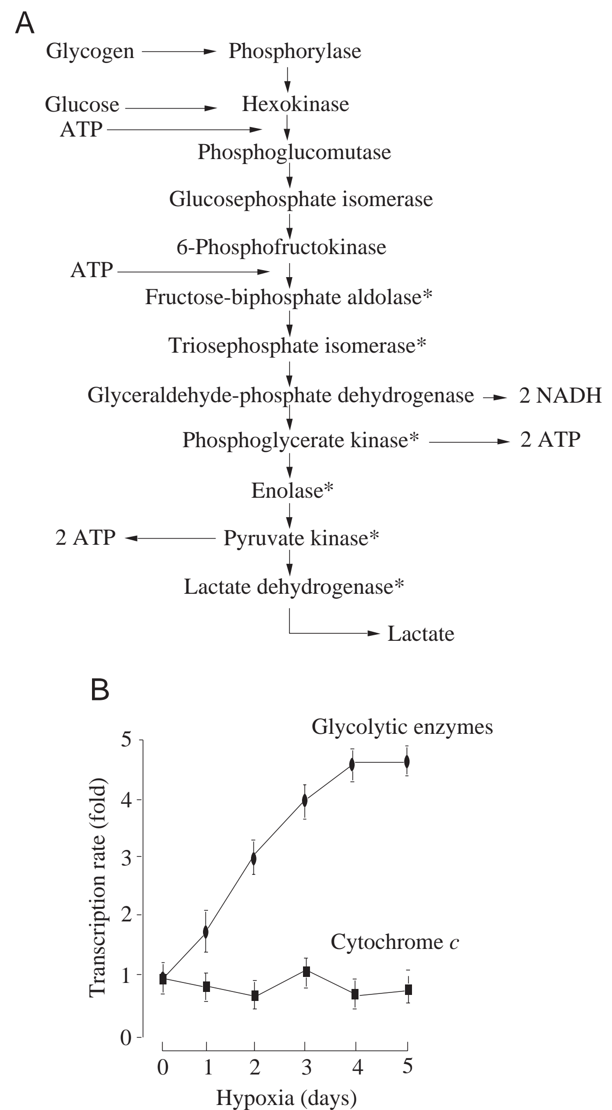


Fig. 1. Composition and regulation of the glycolytic pathway in higher animals. (A) Linear pathway of glycolytic enzymes showing substrate input and sites of ATP utilization and generation. (B) Induction of glycolytic enzyme mRNA levels by hypoxia. Skeletal myocytes were exposed to hypoxia and mRNA transcript levels were measured at the indicated time points by the nuclear run-on technique, described in Webster (1987). The figure is a composite of six glycolytic enzyme gene transcripts using cDNAs to the enzymes indicated by the asterisks in A.

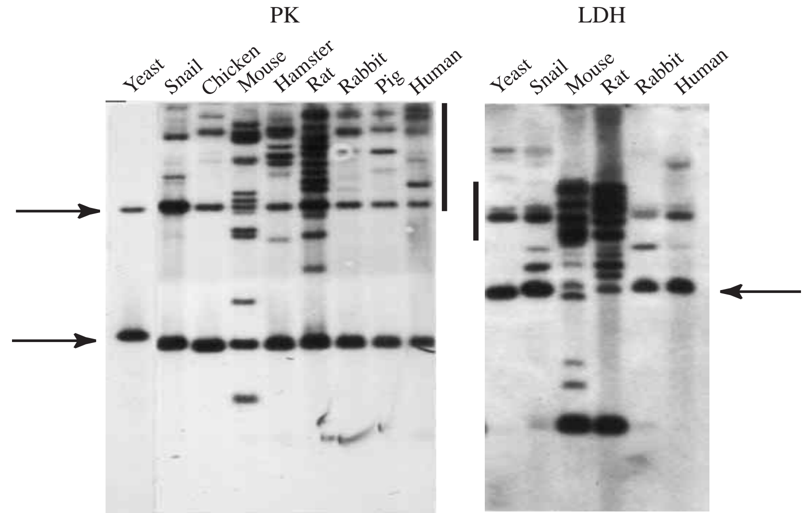
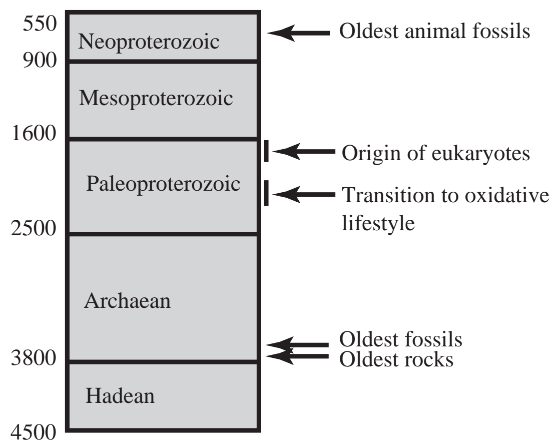


Fig. 2. Southern blots illustrating strong conservation of glycolytic enzyme gene sequences across species. Cells or tissues from the indicated organisms were lysed and genomic DNA was extracted by standard techniques (Webster, 1987; Webster et al., 1990; Lonberg and Gilbert, 1985). DNA was digested with restriction enzyme *Eco*RI, separated on agarose gels and blotted onto nitrocellulose. Membranes were probed with ^{32}P-cDNAs coding for pyruvate kinase (PK) and lactate dehydrogenase (LDH) as described in Webster (1987). Arrows indicate conserved DNA fragments. Note the increased number of hybridization bands for both genes in rodents (blocks indicated by vertical bars) that probably represent increased numbers of pseudogenes in these species (see text).

Numerous aspects of the Archaebacteria and bacterial gene regulatory mechanisms have been conserved and elaborated in higher animals while others, including the bacterial operon, have been largely replaced. The rearrangement of primitive prokaryotic glycolytic enzyme gene operons into unlinked genes on eukaryotic chromosomes requires the parallel segregation, multiplication and/or insertion of regulatory elements with *trans*-acting protein factors to allow the coordinated function of the pathway (Alefounder and Perham, 1989; Barnell et al., 1990; Gebbia et al., 1997).

The Archean period is characterized by what would be an extremely toxic atmosphere for current life forms, with methane, nitrogen and ammonia as the major components (Kasting, 1993; Papagiannis, 1984). Fig. 4A illustrates an Archean coastline 3.5 BYA. The mounds in the foreground are stromatolites, multiple layers of calcified microbial colonies, mostly bacteria and fungi, dating back almost to the beginning of life (Papagiannis, 1984; Reid et al., 2000). These structures form the best record of Archean and the early Proterozoic period, known as the third domain of life (Koch, 1998). Stromatolite fields can still be found in parts of South African and Western Australia. They were common throughout the Precambrian periods until about 1.0 BYA, when herbivorous predators probably featured significantly in their decline. Fig. 4B shows a piece of stroma fossil from the Bitter Springs formation of central Australia, dated at 0.85 BYA. These fossils are known as carbon films, dark compressions in the rock revealing the outlines of ancient species in the forms of spheres, circles, ribbons and leaf-like structures. The diversity represents more than 2 BY of anaerobic evolution generating complex phyla of obligate microbial anaerobes, including Archaebacteria, cyanobacteria and possibly unicellular flagellates. Studies on present day descendants of these microorganisms, in particular the obligate anaerobic hyperthermophilic Archaea, indicate that they have complex systems of bioenergetic pathways (Fardeau et al., 1996; Janssen and Morgan, 1992; Kelly and Adams, 1994; Ma et al., 1995). *Thermoproteus tenax* is an obligate anaerobic hyperthermophile and a descendent of one of the earliest Archea dating back to 3–4 BYA.

The first glycolytic enzymes in the Archean period probably contributed mainly anabolic, gluconeogenic functions (Conway, 1992; Romano and Conway, 1996; Selig et al., 1997), with catabolic functions being acquired subsequently as kinases appeared to use ATP, ADP or pyrophosphate as phosphate shuttles (Romano and Conway, 1996). There are some unique characteristics of Archean era glycolysis; for example catalysis of reactions by the enzymes



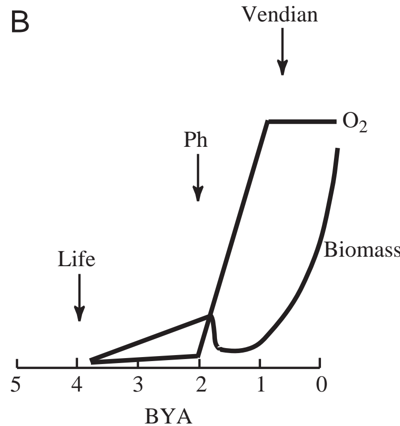


Fig. 3. Milestones in evolution. (A) Paleontological periods of the Precambrian era. (B) Estimates of total earth biomass as a function of time. The graph is only a qualitative representation because it is not possible to establish or extrapolate precise levels of precambrian biomass from paleontological records (Kelly and Adams, 1994; Papagiannis, 1984; DeLong et al., 1994; Koch, 1998; O'Callaghan and Conrad, 1992; Reeve, 1992). BYA, billion years ago; Ph indicates initiation of the major increase in photosynthesis by cyanobacteria.

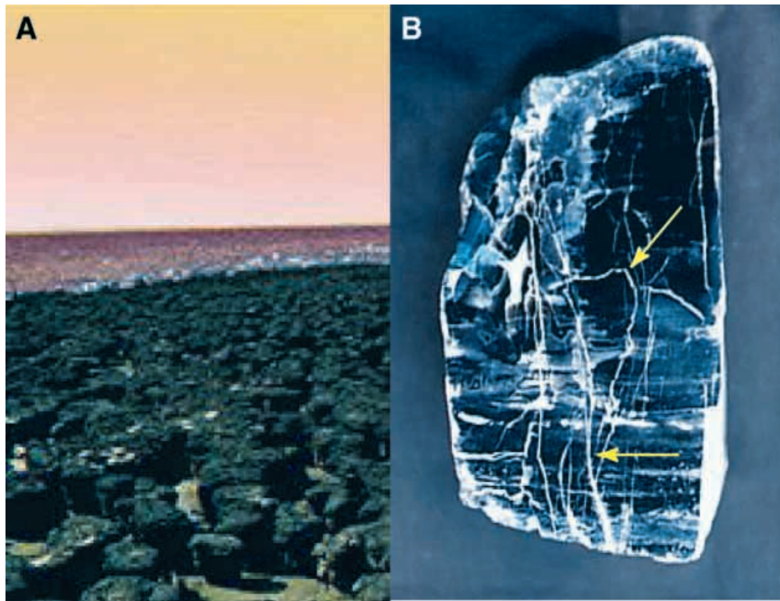


Fig. 4. The Archean Age. (A) Stromatolite field as it may have looked 3.5 BYA (see text for details). (B) Stromatolite fossil; the arrows indicate 'carbon films' where microscopic details reveal microbial fossils dating back to the earliest life forms on earth. (From the University of California at Berkeley Paleontological Museum; with permission).

glucokinase and phosphofructokinase (PFK) in *T. tenax* is dependent on ADP and pyrophosphate as cofactors. This allows these key steps to be functionally reversible, permitting gluconeogenesis as well as glycolysis, a feature not possible in the later bacterial and eukaryotic pathways (Mertens, 1991; Siebers et al., 1998; van der Oost et al., 1998). There is evidence for both divergent and convergent evolution of glycolytic genes, but not divergence from a single multifunctional glycolytic protein or gene cluster (Barnell et al., 1990; Fothergill-Gilmore, 1986; Fothergill-Gilmore and Michels, 1993; Rossman, 1981). Sequence and crystallographic data favor the divergent evolution of for example monophosphoglycerate mutase and diphosphoglycerate mutase, and possibly glyceraldehyde-3-P dehydrogenase and phosphoglycerate kinase from respective common ancestors, but convergence appears to have played a greater role in the development of all of the other 11 enzymes (Fothergill-Gilmore, 1986; Fothergill-Gilmore and Watson, 1989). For example, there is no evidence of a common ancestor for any of the four glycolytic kinases or of the seven enzymes that bind nucleotides, with the exception of those mentioned above. Rather, it seems likely that the pathway resulted from the chance assembly of independently evolving enzymes and genes, probably in association with the co-evolution of other functions and linked pathways.

Substrate regulation by operons in bacteria

Many functionally related bacterial genes are organized into physical operons that are regulated by a master operator element, usually positioned at the 5′ end of the operon, which regulates the transcriptional rate of all genes in the operon (Alefounder and Perham, 1989; Barnell et al., 1990; Hannaert et al., 2000; Liaud et al., 2000; Unkles et al., 1997). Evidence for glycolytic enzyme gene operons include linked pyruvate kinase and PFK genes in *Clostridium acetobutylicum* (Belouski et al., 1998); clustered genes for phosphoglycerate kinase (PGK), triosephosphate isomerase (TPI), phosphoglycerate mutase and enolase in *Baccilus subtilis* (Leyva-Vazquez and Setlow, 1994); linkage of GAPDH, PGK and TPI in *Borrelia megaterium*, *Borrelia bungorferi* and *Borrelia hermsii* (Gebbia et al., 1997; Schlapfer and Zuber, 1992); clustering of fructose 1,6-biphosphate aldolase, 3-phosphoglycerate kinase and GAPDH in *E. coli* (Alefounder and Perham 1989), and clustering of the glucose-6 dehydrogenase, 6-phosphogluconate dehydratase and glucokinase genes with a putative glucose transporter in *Zymomonas mobilis* (Barnell et al., 1990). These glycolytic enzyme gene operons may be regulated independently of each other or globally. In the latter condition the multiple operons behave as a unit, termed a *modulon*, which is coordinately regulated by one or more wide-ranging master regulatory proteins. The best example of modulon regulation is through the cAMP receptor protein (CRP) or catabolite gene activator protein (CAP), which can activate or repress numerous regulons in response to substrate availability (Bledig et al., 1996; Kumari et al., 2000; Luesink et al., 1998; Tobisch et al., 1999). Because substrate fluctuation was the principal selection pressure for evolving Archean microorganisms, modulons became the principal mechanism for the coordinated regulation of all genes involved in carbohydrate metabolism, including glycolytic enzymes. However even in early Moneras there is evidence for fine tuning in the form of functional segregation and preferential targeting of specific genes, in particular those destined to become the 'key regulatory enzymes'. For example, in *E. coli* an operon containing phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase, all 'key enzymes', is selectively regulated through a 5′ cAMP response element that binds the positive factor CcpA. Levels of CcpA in turn are determined by substrate availability (glucose, galactose, fructose) (Luesink et al., 1998; Tobisch et al., 1999). The grouping of PFK and PK is clearly significant because the operon components tend to favor contiguous functions within the glycolytic pathway.

Oxygen regulation in prokaryotes

Oxygen exerted massive selection pressures on prokaryotes and engineered cooperativity between energy storing and releasing pathways, including substrate-level phosphorylation and electron transport by dedicated carriers including cytochromes. The oxygen-regulated switching in bacteria (and possibly archaebacteria; Chistoserdova et al., 1988; Iwasaki et al., 1995; Segerer et al., 1985) includes the activation and/or repression of key enzyme genes and operons involved in oxidative metabolism and glycolysis. This includes positive and negative factors regulated by oxygen tension or redox potential and involves contributions from at least three major regulatory pathways. These include the Arc and FNR systems, which regulate gene expression pre-transcriptionally in response to the redox state of the environment, and the CsrA–CsrB system, which differentially regulates the expression of glycogen synthesis, gluconeogenesis and glycolytic genes by conditionally regulating RNA stability. The latter regulation has been recently reviewed and will not be

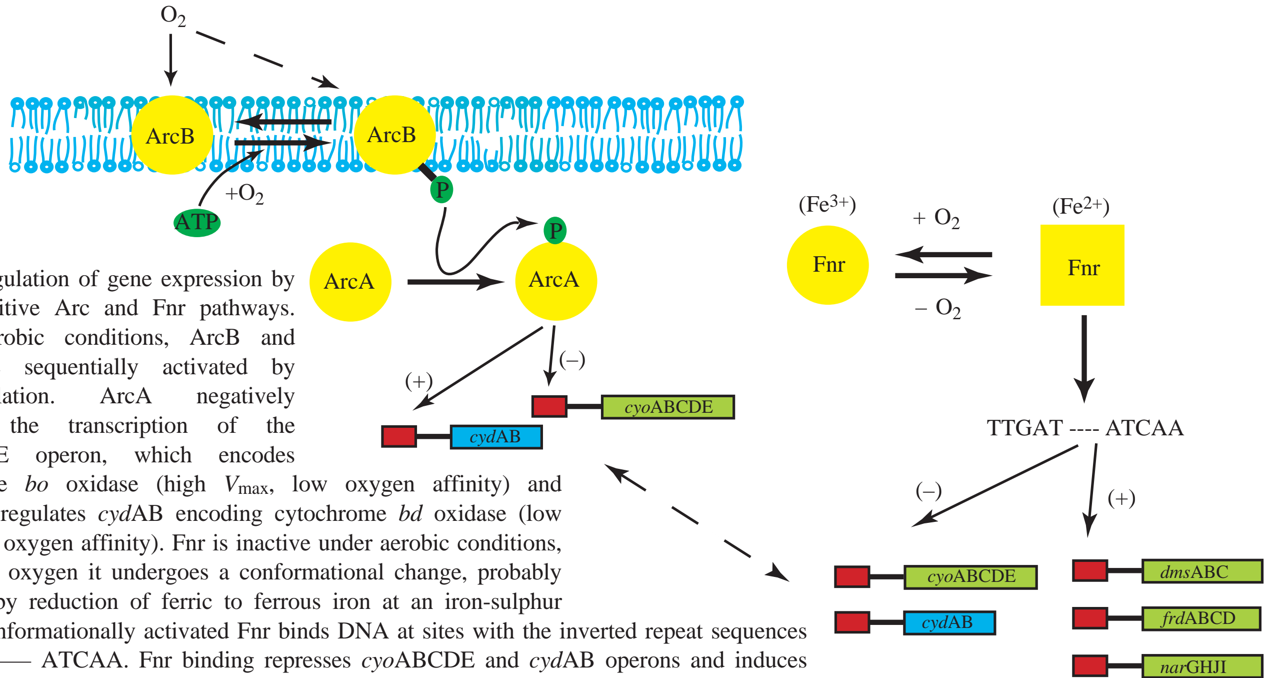


Fig. 5. Regulation of gene expression by redox-sensitive Arc and Fnr pathways. Under aerobic conditions, ArcB and ArcA are sequentially activated by phosphorylation. ArcA negatively regulates the transcription of the *cyo*ABCDE operon, which encodes cytochrome *bo* oxidase (high V_{max}, low oxygen affinity) and positively regulates *cyd*AB encoding cytochrome *bd* oxidase (low V_{max}, high oxygen affinity). Fnr is inactive under aerobic conditions, but at low oxygen it undergoes a conformational change, probably mediated by reduction of ferric to ferrous iron at an iron-sulphur center. Conformationally activated Fnr binds DNA at sites with the inverted repeat sequences TTGAT —— ATCAA. Fnr binding represses *cyo*ABCDE and *cyd*AB operons and induces transcription from the operons *dms*ABC (dimethyl sulfoxide/trimethylamine-*N*-oxide reductase), *frd*ABCD (fumarate reductase), and *nar*GHJI (nitrate reductase). Cross-talk between the two pathways at *cyo*ABCDE and *cyd*AB is indicated by the broken arrow. Under microaerophilic conditions as oxygen becomes limiting, *cyd*AB is optimally active and ArcA may successfully compete Fnr to activate the regulator under these conditions (Bunn and Poyton, 1996).

discussed here (Bunn and Poyton, 1996); we will briefly consider the oxygen-regulated Arc and FNR systems because they may be the precursors of eukaryotic glycolytic enzyme gene regulation by hypoxia.

The Arc system is involved in the repression of aerobic functions under anaerobic conditions. Arc A represses the expression of the succinate dehydrogenase, citric acid cycle and glyoxylate cycle enzyme genes, and activates cytochrome *d* oxidase under hypoxic conditions, thereby shutting off the succinate dehydrogenase–cytochrome oxidase pathway and activating electron transport through the *d*-cytochrome, which has a higher affinity for oxygen (Parkinson and Kofoid, 1992). The mechanism is a classical two-component signal-transducing system, involving a membrane-bound redox sensor and protein kinase (ArcB) and a cytoplasmic regulator (ArcA) with a DNA-binding domain (see Fig. 5). Signals from the electron transport chain (probably the redox state of heme or other iron-containing component) activate ArcB, which transmits the signal to ArcA and initiates the cascade of gene regulation. The FNR system is also involved in the anaerobic activation and repression of a wide variety of metabolic enzymes by a mechanism that parallels that of the CAP system (Chang and Meyerowitz, 1994; Parkinson and Kofoid, 1992). Expression of more than ten enzymes involved in anaerobic energy metabolism, including fumarate reductase and glycerol-3-phosphate dehydrogenase, is induced when the FNR system is activated (Spiro and Guest, 1991). Activation is believed to involve a redox switch within the FNR protein involving cysteine-bound metal ions. A conformational change of the protein creates an active DNA binding site that promotes transcription. The target sequence for activated FNR usually resides about 40 bp upstream of the transcriptional initiation site and includes the consensus sequence nTTGATnnnnATCAAn, which is a typical binding site for dimer-DNA-binding proteins containing helix-turn-helix motifs (Kiley and Reznikoff, 1991). This is significant because it may be the first example of a

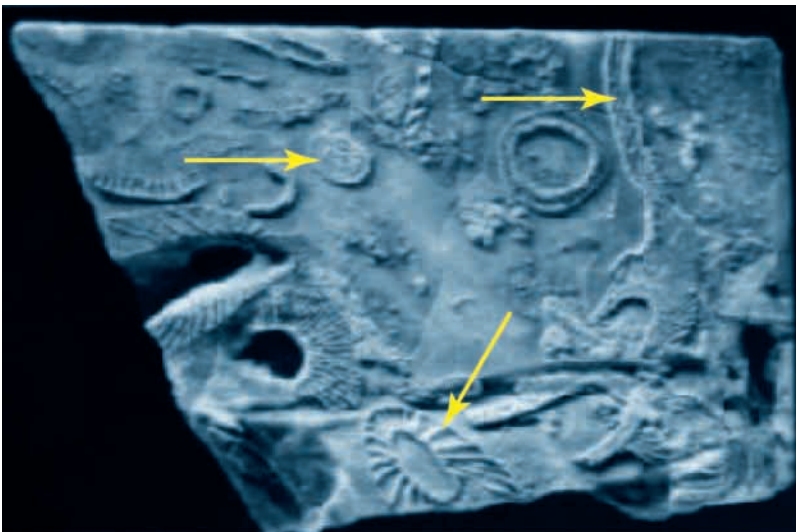


Fig. 6. Example of Vendian flossils from the Ediacara Hills region in Southeast Australia. Arrows indicate microorganisms with amoeboid, hydra and sponge-like features, many with cilia-like structures indicating motility. (From the University of California at Berkeley Paleontological Museum: with permission).

positive-acting transcription factor with helix-turn-helix motifs that is activated by hypoxia and involved in the coordinate regulation of genes that ultimately determine glycolytic functions. Conformational regulation by reversible binding of metals to cysteine sites is reminiscent of the redox responses of zinc finger transcription regulators, the most common regulators of gene expression in eukaryotes (Webster et al., 2001). Redox-dependent conformational changes also contribute to the transcriptional activation of mammalian glycolytic enzyme genes by specific helix-turn-helix factors (see below).

Notably, none of the aerobic/anaerobic regulatory systems described above directly regulate glycolytic enzyme genes, although cross talk between the Arc, FNR and CcpA networks causes changes in the transcription rates of glycolytic enzyme operons in response to carbohydrate. The absence of a direct regulation of glycolytic enzyme gene transcription (by substrates, alternative pathways such as sulfur, or oxygen) in the Archean era and subsequently in bacteria would be predicted if such regulation was acquired during the selection and gene shuffling that accompanied the transition to oxidative metabolism. The establishment of direct oxygen regulation of glycolytic enzyme genes may in fact have paralleled mitochondrial symbiosis and the establishment of compartmented energy pathways. Photosynthetic cyanobacteria underwent a major expansion 1.5 BYA, producing >1000 different variants and initiating a rapid increase of atmospheric oxygen (Kasting, 1993; Reid et al., 2000). Atmospheric oxygen during the Archean period was less than 1% of the current level, but by about 1.8 BYA it was 15%, and probably increased to the current level by 0.5 BYA. This accumulated oxygen had a major impact on life. It has been estimated that as much as 99% of the existing anaerobic life forms were extinguished by the toxic byproducts of reactive oxygen (Cannio, 2000). Oxygen allowed the rapid diversification and expansion of survivors because of the increased energy made available from oxidative metabolism. The main expansion occurred within the eukaryotic kingdom, stimulated by the high energy-producing potential of mitochondria. Mitochondria contributed a highly efficient energy production system that was partially insulated from other cellular functions. Metabolic and gene regulatory pathways, including responses to hypoxia, arose in parallel to coordinate mitochondrial and glycolytic function (Webster et al., 1990).

Eukaryotic glycolytic genes

The archeological period known as the ‘Vendian’ is thought to include the earliest species that survived the oxygen explosion (Li et al., 1998; Rasmussen et al., 2002; Seilacher et al., 1998). Organisms within this period bridge the gap between the late Precambrian and early Cambrian periods and represent the ancestors of most if not all eukaryotes. Rich deposits of Vendian fossils have been discovered in three major locations: the Ediacara Hills in Southeast Australia, the Russian Winter coast, and Misty Point in Newfoundland, Canada. Examples of these fossils are shown in Fig. 6. Vendian life forms representing the transition to eukaryotic organisms include sponges, hydra, filamentous algae and fungi. Estimates of the start of the Vendian period vary from about 0.8 to more than 1 BYA. Yeasts belong to the Fungi, and are all facultative anaerobes capable of growth with or without functional mitochondria (Ferguson and von Borstel., 1992).

Studies with the yeast *Saccharomyces cerevisae* illustrate elegantly the powerful selection pressures that are inherent for a dual aerobic/anaerobic lifestyle. The requirement to survive and grow under different oxygen tensions has promoted the establishment of a system for coordinately and simultaneously regulating multiple unlinked genes (Zitomer et al., 1997). To grow anaerobically, *S. cerevisae* requires supplements of sugar, unsaturated fatty acids, sterol and methionine because of oxygen-dependent steps in the biosynthesis of these essential metabolites. Under hypoxic conditions the genes encoding these oxygen-dependent enzymes are coordinately induced so that optimal use can be made of oxygen as it becomes the limiting substrate (Jiang, Y. et al., 2001; Vasconcelles et al., 2001). At least 10 genes are induced when *S. cerevisae* are cultured under hypoxia and there are close parallels between these regulatory pathways and those that regulate glycolytic enzyme and other hypoxia-responsive genes in higher mammals. The *S. cerevisae* OLE1 gene encodes a δ9 fatty acid desaturase that is essential for fatty acid synthesis. OLE1 is induced by hypoxia, transition metals and iron chelators. An element in the OLE1 gene promoter with the sequence ACTCAACAA is responsible for the response to hypoxia. This element named LORE (for low oxygen responsive element) can confer hypoxia inducibility to a heterologous promoter and binds a specific hypoxia-inducible protein. Additional LORE elements have been identified in the promoters of other hypoxia-inducible genes, suggesting a mechanism for a global synchronized response to hypoxia in *S. cerevisae* (see Fig. 7). This is perhaps the earliest evidence of a regulatory system capable of mediating a global response of multiple unlinked genes to changes in oxygen tension. Although the sequence of the LORE does not resemble any identified mammalian LORE (or hypoxia-response element, HRE), the common responses of *S. cerevisae* LOREs and eukaryotic HREs to hypoxia, transition metals and iron chelators suggests related pathways (see below; Jiang, Y. et al., 2001; Vasconcelles et al., 2001).

Genes that are coordinately regulated by LORE or equivalent elements in yeast include SUT1, GPG2, PAU, DNA1 and TIR/SRP1 (Vasconcelles et al., 2001). A second negative regulatory system operates in combination with LOREs to provide an enhanced and inversely coordinated response to selected stimuli (Fig. 7). These two systems probably represent combinations of ancient and newly acquired regulators that have undergone co-adaptation. In the ROX-1p repressor system, hypoxia/anaerobic responsive genes are repressed under aerobic conditions by ROX-1 binding to elements with the consensus sequence CCATTGTTCTC. ROX-1 in turn is regulated by a second factor, HAP1, which binds to sites with the sequence CGG(N6)CGG. HAP1 requires bound heme to activate transcription and when heme levels are high under aerobic growth conditions, HAP1 is active, high levels of ROX-1 are produced and the target genes are repressed. Conversely, when heme levels drop under anaerobic/hypoxic conditions HAP1 becomes inactive, ROX-1 levels decrease and repression of the target genes is relieved. There are clear parallels between the ROX-HAP pathway and the Arc system in bacteria discussed above (reviewed in Bunn and Poyton, 1996).

Neither the LORE nor ROX elements appear to directly regulate glycolytic enzyme genes. This implies that the regulation of these genes by hypoxia was a later acquisition, possibly associated with

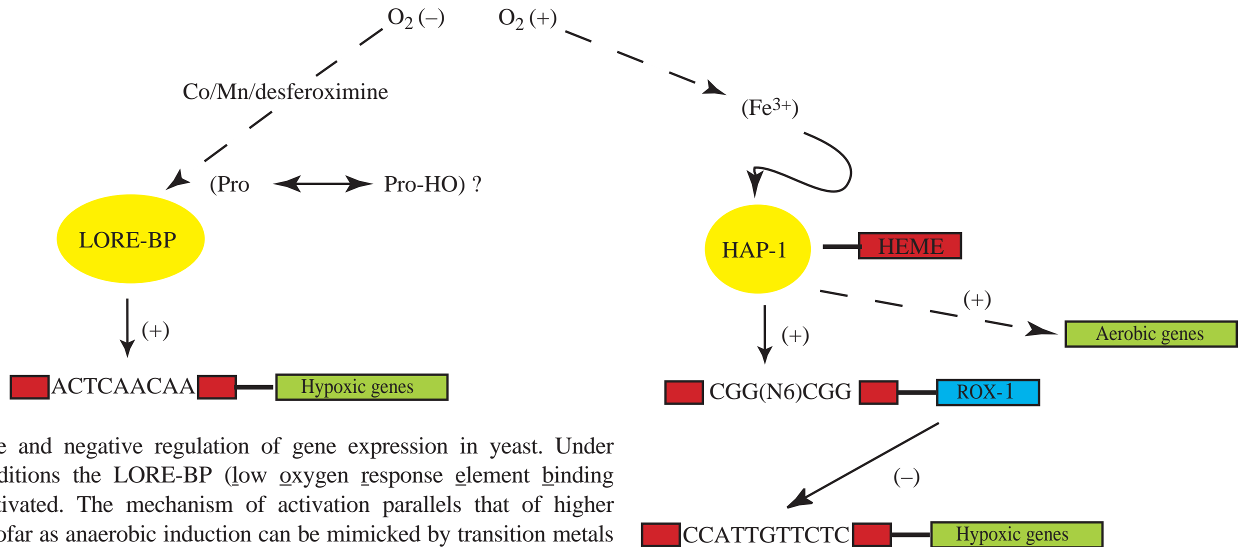


Fig. 7. Positive and negative regulation of gene expression in yeast. Under anaerobic conditions the LORE-BP (low oxygen response element binding protein) is activated. The mechanism of activation parallels that of higher eukaryotes insofar as anaerobic induction can be mimicked by transition metals and desferroximine. The possible involvement of a proline-hydroxyproline active site has not been demonstrated in this system as it has in the HIF-1 pathway (see Fig. 9). LORE-BP is a positive-acting transcription factor that binds and activates hypoxia-response genes with the sequence ACTCAACAA. The HAP1-Rox-1 pathway operates in parallel with the LORE pathway. HAP-1 requires heme as a cofactor and is activated under aerobic conditions when heme levels are high. HAP-1 is a positive transcription factor for multiple aerobic genes (genes required for aerobic metabolism and functions) through binding to the recognition sequence CGG(N6)CGG. The ROX-1 promoter region contains the HAP-1 binding site and is activated by HAP-1. ROX-1 is a repressor that negatively regulates (hypoxic) genes containing the sequence CCATTGTTCTC. Consequently HAP-1 coordinately regulates both aerobic and hypoxic genes.

multicellularity and genome expansion. Glycolytic enzyme genes in *S. cerevisae* are, however, strictly regulated by the carbon source through a mechanism that may be the forerunner of oxygen regulation. The rates of transcription of different glycolytic enzyme genes increases by up to 100-fold when *S. cerevisae* is switched from acetate to fermentative growth on glucose. The regulation involves complex interactions between a number of *cis*-acting promoter elements and *trans*-acting transcription factors that include GCR1, RAP1, ABF1 and GAL11 proteins (Bunn and Poyton, 1996). There is evidence that genes encoding the key glycolytic control enzymes PK and PFK are preferentially regulated (for a review, see Nishi et al., 1995). The regulation of glycolytic enzymes and genes by carbohydrate metabolism in yeast is a powerful illustration of the importance of bioenergetic pathway switching in determining the fitness of an organism. Under anaerobic fermentative conditions glycolytic enzyme proteins can account for >50% of the total yeast protein (Nishi et al., 1995). This level of protein production would be a selective disadvantage for oxidative growth and in fact it is not seen in any other eukaryote. Aerobic adaptation and multicellularity during the Cambrian expansion involved a systematic reduction in the maximum expression levels of glycolytic genes through a decrease of basal promoter strength. Genome expansion for glycolytic enzyme genes also included the acquisition of multiple tissue-specific isoforms as separate genes and the acquisition of mechanisms to simultaneously and coordinately regulate the entire pathway of genes in response to oxygen tension.

Glycolytic genes in multicellular eukaryotes

Multicellular eukaryotes of increasing complexity developed during the Vendian period and expanded rapidly in the Devonian,

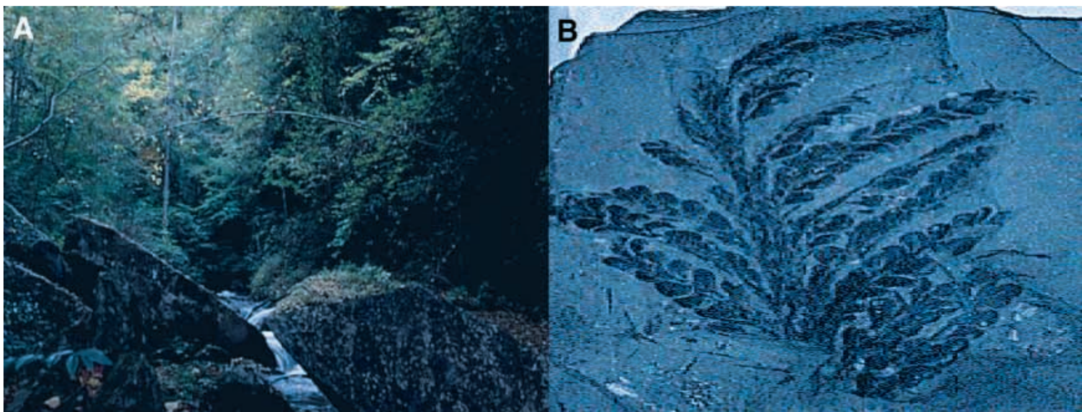


Fig. 8. (A) The Rhynie valley in Scotland. (B) Devonian fossilized fern leaves about 0.8 billion years old. (From the University of California at Berkeley Paleontological Museum; with permission).

Carboniferous, and Permian periods. The Rhynie valley in Scotland is one of the richest sources of Devonian deposits (Fig. 8) with extensive fossil evidence of higher plants dating back 0.8 billion years. Like fungi, higher plant glycolytic enzyme genes have introns, TATA control elements and transcriptional regulatory sites; unlike earlier eukaryotes and prokaryotes, all of the glycolytic enzyme genes are physically separated and scattered seemingly randomly around the genome, mostly on separate chromosomes, posing additional mechanistic requirements for coordinate regulation (Webster and Murphy, 1988).

Analyses of the root system of the monocotyledon *Zea mays* (maize) lead to the first identification of hypoxia-responsive DNA elements in glycolytic enzyme gene promoters. The root systems of many higher plants penetrate deeply into anaerobic waterlogged earth and cells, particularly at the root tips, have adapted to anoxia. Exposure of maize root cells to hypoxia results in the induction of approximately 20 proteins, deemed anaerobic polypeptides (Dennis et al., 1988; Dolferus et al., 1994; Olive et al., 1991). Many of these proteins are enzymes involved in glycolytic or fermentative carbohydrate metabolism and include two alcohol dehydrogenases, glucose phosphate isomerase, aldolase and lactate dehydrogenase. Two anaerobic response elements (ARE) were identified in the proximal promoters of the *aldolase* and *Adh1* genes. The first site contained the consensus sequence TGGTTT and was present in the *aldolase* promoter at –70 bp upstream from the transcription start site, and in the *Adh1* promoter at position –111. The second site contained the consensus GC(G/C)CC and was present at –135 and –120 of the *Adh1* promoter (Olive et al., 1991). Mutation of these elements resulted in the loss of response to hypoxia. Further studies revealed the specific binding of a protein to the GC-rich element and this protein was designated GCBP-1 (GC-rich binding protein-1). This protein has not been fully characterized; its abundance is not changed by hypoxia, it requires accessory proteins and/or post-translational modifications to mediate transcriptional activation by hypoxia, and its binding to the GC site is competed by members of the Sp1 family of zinc finger transcription factors. There may be strong parallels between this regulatory pathway and that described below for the regulation of mammalian muscle-specific pyruvate kinase (PKM) and β-enolase genes (Discher et al., 1998; Webster et al., 2001). These elements represent the earliest examples of hypoxia response elements directly controlling individual glycolytic enzyme genes. They may also provide a clue as to how hypoxia response elements were selected from other stress response pathways of regulation, including temperature and osmolarity, both of which featured significantly as evolutionary selection pressures.

Hypoxia, dehydration and hypothermia induce the *Adh* gene in the roots of the dicotyledon, *Arabidopsis thaliana*. The promoter contains a single GT/GC motif, which has a similar sequence to the monocotyledon *Zea mays* GC site described above, except that the GT motif is in reverse orientation (Dolferus et al., 1994). However, the *Arabidopsis Adh* promoter contains a second motif with the sequence CCACGTGC. The core sequence of this motif, ACGTG, is the binding site for the major hypoxia regulatory binding protein in mammalian cells called hypoxia inducible factor-1α (HIF-1α). Interestingly this motif appears to be required for *Adh* gene induction by hypothermia, dehydration and UV light, but not hypoxia, whereas the hypoxia response is determined by the GT/GC sequence. The reversal of the use of these elements in mammalian genes seems remarkable, although as discussed below, both HIF-1 and GC elements may contribute to the hypoxia response of glycolytic enzyme genes in mammals.

Regulation of glycolytic enzyme genes in fish, insects and mammals

The regulation of glycolytic enzyme genes by hypoxia in insects, fish, reptiles, birds and mammals and possibly all mobile multicellular species is multifactorial, with clear origins in the prokaryotic and fungal regulatory systems (Bacon et al., 1998; Bruick and McKnight, 2001; Hochachka and Lutz, 2001; Jiang, H. et al., 2001; Soitamo et al., 2001). Animal glycolytic enzyme genes are regulated both coordinately and individually by hypoxia-responsive transcription factors including hypoxia-inducible factor-1α (HIF-1α), SP-1 family factors, AP-1 and possibly metal response elements (MREs) (Discher et al., 1998; Hochachka and Lutz, 2001; Murphy et al., 1999; Webster et al., 2000). HIF-1α is probably the main component and is largely responsible for coordinating the induction. The core consensus sequence for HIF-1α binding is ACGT, and active HIF-1α binding sites have been reported in at least eight glycolytic enzyme genes, usually in the proximal promoter regions (Riddle et al., 2000). Glycolytic enzyme genes with HIF-1 sites include PFK, aldolase, pyruvate kinase, PGK, enolase, LDH, hexokinase and GAPDH (Firth et al., 1994; Semenza et al., 1996). Active HIF-1 binding sites are present in these genes at the following positions: mouse PFKL, first intron, +336/+361; human PGK1, promoter, –309/–290, and 5′ untranslated region, +31/+11; human ENO1, promoter, –585/–610; human ALDA, promoter, –204/–180, and first intron, +125/+150; mouse LDHA, promoter, –75/–50. Although other regulatory elements may be involved, the HIF-1 pathway appears to be sufficient to account for the observed induction of these genes by hypoxia. It is not yet clear whether HIF-1 contributes to the regulation of the other glycolytic enzyme genes, glycogen phosphorylase, phosphoglucomutase, phosphoglucose isomerase or triosephosphate isomerase. Glycogen phosphorylase is induced by hypoxia in tissues from turtles to humans (Mehrani and Storey, 1995; Parolin et al., 2000) and our laboratory has show that TPI is induced coordinately by hypoxia with the other glycolytic enzymes in cultured muscle cells (Webster, 1987). It seems probable that the full complement of glycolytic enzyme genes is induced at some level by hypoxia.

The HIF-1 pathway has been described in insects and fish but not in plants or fungi, and it is possible that the pathway developed in the Silurian period about 500 MYA when highly mobile sea and land species were evolving. The sequence ACGTC is essential (although not sufficient) for gene activation by HIF-1 and, as discussed above, the same sequence is required for the hypothermia, dehydration and the UV response of *Arabidopsis* genes involved in carbohydrate metabolism. It seems likely that this recognition sequence and the protein that binds it are related in plants and animals, and this may provide the link between gene regulation in hypoxic root tips and the HIF-1 pathway of insects, fish, birds and mammals.

Role of HIF-1α

Fig. 9 shows the essential features of gene regulation by HIF-1α. The pathway allows for a rapidly reversible activation of genes in response to hypoxia because the HIF-1α gene is constitutively active and regulation is at the level of protein stability. Under aerobic conditions HIF-1α is targeted by the ubiquitin degradation system through a redox modulated hydroxyproline residue, which appears to regulate the conformation of the protein (Bruick and McKnight, 2001; Jaakkola et al., 2001). This results in rapid degradation such that HIF-1α protein is undetectable in most aerobic cells and tissues but accumulates within minutes when the cells are exposed to hypoxia. The development of a rapid and coordinated response to changes of oxygen tension may have been a significant force in the early Cambrian period, providing a selective advantage to cells and organisms that could tolerate aerobic and hypoxic environments and shuttle rapidly between the two. Our experiments show that switching of muscle cells from aerobic to hypoxic growth conditions results in an approximately 12-fold increase of glucose consumption (and lactic acid production) and net 3- to 5-fold increase of glycolytic enzyme proteins (Webster et al., 1993, 1994, 1999). To maintain equilibrium, glucose transport and acid efflux must be correspondingly increased. The glucose transporter GLUT-1 is also positively regulated through HIF-1α, introducing an elegant coordination of glucose utilization and uptake. Hypoxia-regulation of proton translocation genes has not been reported but seems likely. The coordination of glycolytic enzyme activity with glucose and acid regulation may have been an early Silurian adaptation that paralleled multicellularity and increased mobility.

HIF-1 appears to be the only transcription factor that is dedicated specifically to the regulation of gene expression by hypoxia. However, it is not the only factor involved in the response. It may be significant that the ‘TGGTTT’ and G/C elements that mediate the response of *Arabidopsis* to hypoxia resemble the binding sites for the transcription factors activator protein-1 (Ap-1; TGATTC) and the Sp-1 family (GGGCCC), both of which contribute to the regulation of glycolytic enzyme genes by hypoxia. The Ap-1 proteins, c-Fos and c-Jun, are induced by hypoxia in neuronal cells, cancer cells and cardiac myocytes (Webster et al., 1993), and Ap-1 binding has been shown to be required for the induction of tyrosine hydroxylase as well as the endothelin-1 gene by HIF-1α (Hu et al., 1998; Millhorn et al., 1997; Yamashita, 2001). Both factors are present in most glycolytic enzyme gene promoters, often in multiple copies. Sp1 has an important role in the regulation of the muscle-specific glycolytic enzyme genes encoding pyruvate kinase (PKM) and β-enolase (Discher et al., 1998). The gene promoters of the latter genes do not have consensus HIF-1α binding sites but they are induced by hypoxia (Discher et al., 1998). Regulation in this case appears to correlate with the differential binding of Sp1 and Sp3 to common sites (Fig. 9). The differential regulation of Sp1 and Sp3 has been shown to regulate other GC-dependent promoters by interacting with multiple classes of related factors (Birnbaum et al., 1995; Hagen et al., 1994; Kumar and Butler, 1997; Luca et al., 1996; Majello et al., 1997). Therefore Ap-1 and Sp1 can operate in concert or separately with HIF-1α to fine-tune and coordinate the responses of glycolytic enzyme genes to hypoxia in higher animals and humans.

Summary and Conclusions

The principal stages in the evolution of glycolytic enzyme gene regulation by hypoxia are shown in Table 1. It is not clear precisely how or when coordinate regulation was acquired by higher animals, but there are several interesting features that may represent developmental milestones. The *S. cerevisae OLE1* gene, encoding a rate-limiting enzyme for fatty acid biosynthesis, is one of several genes that are coordinately regulated by hypoxia through common LORE regulatory sites in fungi. The LORE site has properties that strongly parallel the HIF-1α pathway, including

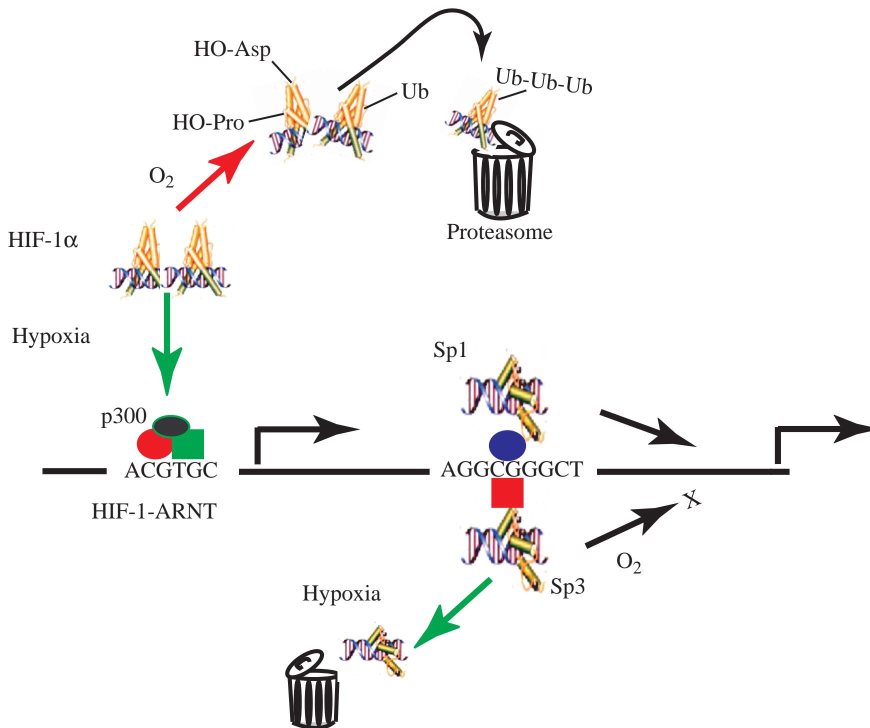


Fig. 9. Regulation of mammalian glycolytic enzyme genes by the HIF-1α and Sp1 family transcription factors. A proline residue at position 564 on the HIF-1α protein (illustrated as a helix-turn-helix structure) is hydroxylated at physiological oxygen tension, causing a conformational change and rendering the protein susceptible to ubiquitination (Ub). Ubiquitinated protein is rapidly degraded by the proteosome. Under hypoxia, this pathway is blocked; HIF-1α accumulates, dimerizes the aryl hydrocarbon nuclear transporter (ARNT), translocates to the nucleus, and activates responsive genes including glycolytic enzymes by binding to sequences containing the consensus ACGT site. In a second redox-regulated step, p300 can only be recruited to activate the HIF-1α complex when the Asp-851 residue is not hydroxylated. In a parallel hypoxia-regulated pathway, Sp1 and Sp3 (zinc fingers) compete for binding to GC-rich DNA sequences; Sp1 is a positive transcriptional activator while Sp3 can repress transcription. Hypoxia favors degradation of Sp3 (by an unknown mechanism), promoting the full inducing activity of Sp1.

Table 1. *Milestones in the acquisition of coordinate regulation of glycolytic enzyme genes by hypoxia*

BYA	Period	Organism	Regulator	Element	Response
3.5	Archea[1]	Therm/S-Bacteria	Carbohydrate Sulfur/Phosphate	cre/ccpA ?	Global Global
1.0	Vendian[2]	Fungi/Yeast	Carbohydrate Hypoxia/Anoxia Hypoxia (Repression)	GCR1 LORE: ACTCAACAA ROX-1: CCATTGTTCTC HAP1: CGGN6CGG	Key enzymes Key enzymes
0.6	Devonian[3]	Higher plants	Hypoxia/anoxia (Other stress)	HREs TGGTTT GC(G/C)CC ACGTG	Key enzymes
0.4	Silurian[4]	Fish/Insects	Hypoxia	HRE/HIF-1α ACGTG	Coordinate
0	Cambrian	Animals/Mammals	Hypoxia	HRE/HIF-1/Ap-1/Sp1 ACGTG TGATTC GC(G/C)GC	Coordinate

BYA is billion years ago.

[1]Strictly anaerobic organisms in the Archean period developed sophisticated pathways for substrate regulation of bioenergetic enzyme gene expression, including regulation by sulfur and phosphorus.

[2]The Vendian organisms are representative of the transition to oxidative metabolism and include the first examples of oxygen-regulated gene switching.

[3]The Devonian period includes the first example of anaerobic response elements in glycolytic enzyme genes, including regulation by the sequence element ACGT that binds factor HIF-1α in higher animals (but not plants).

[4]Contemporary pathways that coordinate glycolytic enzyme gene expression in response to hypoxia probably originated in the Silurian period, coincident with multicellularity and metabolic compartmentation.

activation by transition metals and desferrioxamine, suggesting a similar pathway of regulation. Therefore LOREs may be the forerunners of animal hypoxia response elements, although LORE-regulated glycolytic enzymes were not reported in fungi. A second pathway with close parallels to hypoxia and glycolysis is the stress response in plants. Common sequence elements regulating alcohol dehydrogenases, glucose phosphate isomerase, aldolase and lactate dehydrogenase have been described in plants. In *Arabidopsis thaliana*, two elements determine the response to osmotic, hypothermic or hypoxic stress. One of these elements is a GC-rich sequence with similarity to the binding sites of the Sp1-family factors, and the second is an element with a sequence identical to the mammalian HIF-1α binding site. Both of these elements play important roles in the responses of glycolytic enzyme genes to hypoxia in higher animals. It seems probable therefore that coordinated regulation of glycolytic enzyme genes by HIF-1, Sp-1 and AP-1 has its roots in the early Vendian and Devonian periods and obtained its current arrangement by genetic reorganization during the Silurian period, possibly in parallel with increased mobility and multicellularity.

The ability to modulate glycolytic enzyme gene expression in response to oxygen tension probably conveyed significant selective advantages to life forms at many stages of evolution. The advantages of such a switch are evident from the waterlogged root tips of plants to hibernating mammals, exercising ‘glycolytic’ skeletal muscles in all higher animals, and ischemic skeletal and cardiac muscles in humans (Hochachka and Lutz, 2001; Mehrani and Storey, 1995; McClelland, et al., 1998; Vogt et al., 2002; Webster et al., 2000). Hypoxia is a much more frequent condition than is generally realized. The HIF-1 pathway is activated at oxygen tensions less than about 40 mmHg (5% O_2) (Iyer et al., 1998; Semenza, 2001). Whereas the P_{O_2} in the atmosphere is about 150 mmHg, the normal P_{O_2} of most tissues is in the range 50–70 mmHg (Hochachka, 1999). Therefore small changes in the supply and demand for oxygen can tilt the balance to promote the activation of hypoxia-dependent genes. Both cardiac and skeletal muscles can survive extended periods of hypoxia, during which time glycolytic enzyme genes become fully induced (Webster and Murphy, 1988; Webster et al., 1990). When maximally activated, the levels of glycolytic enzymes in muscle can reach almost 20% of total soluble protein (Webster, 1987; Webster and Murphy, 1988). Continuous production of this level of protein may be of negative selective value under aerobic conditions when oxidative phosphorylation produces more than 95% of cellular ATP, but it may be essential for the survival of cells and tissues subjected to chronic or repetitive hypoxia.

Dedication

This review is dedicated to the memory of Dr Peter W. Hochachka (1937–2002), an inspired scientist whose contributions to zoology, physiology and the study of adaptations to hypoxia in higher organisms is unsurpassed. Peter, a close friend and much-admired colleague was also the inspiration behind this review.

This work was supported by National Institutes of Health grants HL44578, HL69812, HL07294 and award # BM034 from the Chiles Endowment Biomedical Research Program of the Florida Department of Health. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the views of the Biomedical Research Program or the Florida Department

of Health. I also acknowledge the University of California at Berkeley, Museum of Plaeontology for their generous permission to use their materials for Figs 4, 6 and 8.

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