Evolution of energetic metabolism: the respiration-early hypothesis

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The main energy-transducing metabolic systems originated and diversified very early in the evolution of life. This makes it difficult to unravel the precise steps in the evolution of the proteins involved in these processes. Recent molecular data suggest that homologous proteins of aerobic respiratory chains can be found in Bacteria and Archaea, which points to a common ancestor that possessed these proteins. Other molecular data predict that this ancestor was unlikely to perform oxygenic photosynthesis. This evidence, that aerobic respiration has a single origin and may have evolved before oxygen was released to the atmosphere by photosynthetic organisms, is contrary to the textbook viewpoint.

THERE ARE TWO widely accepted views about aerobic metabolism: first, that it was possible only after oxygen released by photosynthesis became abundant, and second, that it developed independently in diverse evolutionary lines. Both views are commonly accepted in textbooks[1,2], and are backed up by considerable geological data. For example, stromatolites (sedimentary structures thought to have been produced by cyanobacterial communities) have been reported from more than 20 geological locations dating from 2.5–3.5 billion years ago and the oldest known cyanobacterium-like fossils are estimated to be 3.5 billion years old[3]. On the other hand, analyses of the temporal distribution and geochemistry of banded iron formations, paleosols (ancient soils) and red beds (oxidized subaerial deposits) suggest that the transition from a reducing to a stable oxygenic environment occurred later, between 2.3 and 1.8 billion years ago[4]. The commonly accepted interpretation of these data[1,2] is that the photosynthetic apparatus developed first (more than 3.5 billion years ago), leading to a steady increase in the level of free oxygen (culminating about 2 billion years ago) and subsequently making the emergence of aerobic metabolism possible. Distinct evolutionary lineages were already established 2 billion years ago. Therefore, the textbooks suppose that aerobic respiratory chains evolved independently in these different lines.

Molecular data now available allow inferences to be made about the evolution of metabolic processes. We have sequence information for the central bioenergetic enzymes and redox proteins from many aerobic and photosynthetic organisms. The analysis of these sequences within the framework of our current knowledge of bacterial phylogeny leads to a picture that is quite different from that presented in the textbooks, but one that remains compatible with the geological data.

The last common ancestor of Bacteria and Archaea

The phylogenetic analysis of all present-day organisms has revealed the existence of three domains, Bacteria, Archaea and Eucarya[5,6] (also called eubacteria, archaebacteria and eukaryotes, respectively). Aerobic respiration occurs as a primary trait only in Archaea and Bacteria, and chlorophyll-based photosynthesis only in Bacteria. In Eucaria, both metabolic capabilities were secondarily acquired from bacterial endosymbionts[7]. Plant chloroplasts are known to have descended from cyanobacteria and mitochondria from proteobacteria. Therefore, we can limit our discussion on the evolution of energy-transducing systems to the components found in Archaea and Bacteria.

Molecular data support a phylogenetic tree that puts Eucarya and Archaea into the same group, with Bacteria as the earliest branching domain[6,8,9] (Fig. 1). The exact position of Eucarya within the tree does not affect our conclusions because the present discussion only relies upon archaeal and bacterial proteins. The main line in our argument is that every protein that has archaeal and bacterial homologs can be assumed to have been present in their last common ancestor. (This conclusion would be wrong if lateral gene transfers had occurred between the domains during evolution; however, generally these processes can be detected in phylogenetic trees.) If the position of Eucarya in the tree in Fig. 1 is correct, the last common ancestor of Bacteria and Archaea is also the last universal ancestor (Fig. 1).

The biochemical character of the last universal ancestor has been the subject of recent debate[10,11]. Some authors have

Glossary

Last common ancestor	The last common ancestor of any group of species is the most recent species from which all them descend. It is possible to reconstruct characteristics of a last common ancestor by searching homologous features in the descending species.
Last universal ancestor	The last common ancestor of all species that are alive today. Since all living species can be divided into three domains, Archaea, Bacteria and Eucarya, the last universal ancestor can also be defined as the last common ancestor of these three domains. Homologous molecular features in Archaea, Bacteria and Eucarya suggest that the last universal ancestor was a cellular organism with most of the central metabolic activities.
Progenote	A precellular entity with a rudimentary, imprecise linkage between its genotype and phenotype. It might have existed before the last universal ancestor.

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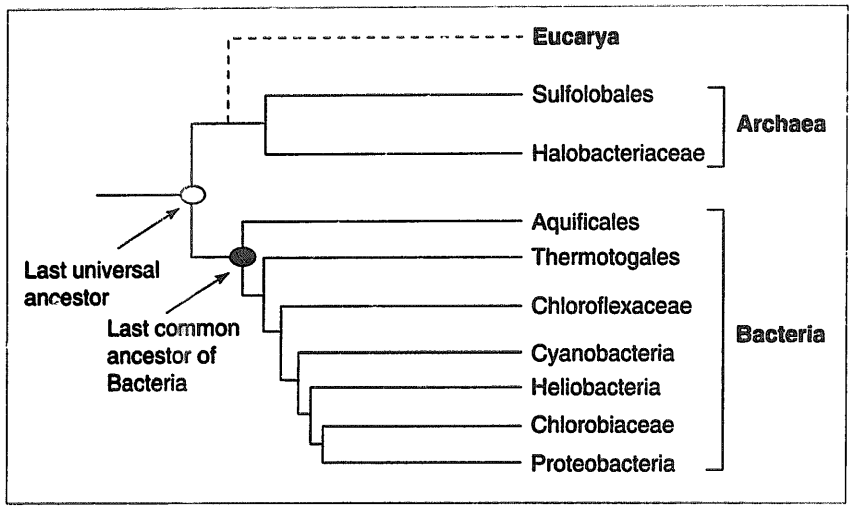

Figure 1

Phylogeny of the taxa discussed in the text, adapted from Ref. 9. The cyanobacterial evolutionary line is drawn in green. Branch lengths are not drawn to scale.

suggested that it was a precellular entity (or progenote[5]) and lacked a cell membrane. However, homologs of membrane proteins such as cytochrome oxidase, cytochrome *b* and ATP synthase[12,13] are inferred to exist in the last universal ancestor. Oxidative phosphorylation, which uses these enzymes to produce ATP from a proton gradient across a membrane, must have been present in the last universal ancestor and so a cell membrane in this ancestral organism was an absolute requirement. An intact cellular membrane would, in turn, limit the fluid exchange of genes between descending lineages.

The aerobic respiratory chain of the last universal ancestor

The following redox proteins are known to be present in aerobic respiratory chains of both Archaea and Bacteria, and therefore can be assigned to the last universal ancestor: cytochrome oxidase subunits I and II, cytochrome *b*, Rieske iron–sulphur protein, blue copper protein, 2Fe–2S and 4Fe–4S ferredoxins, and the iron–sulphur subunit of succinate dehydrogenase. To prepare this list of proteins, we first made an extensive screening of the EMBL database[14] and detected a total of 19 archaeal redox proteins known to participate in electron transfer reactions. Numerous sequences were obtained for their bacterial homologs (Table I).

Phylogenetic trees have been constructed for cytochrome oxidase subunits, cytochrome *b*, Rieske iron–sulphur protein, blue copper protein and 4Fe–4S ferredoxin[15–17]. They are all consistent with phylogenetic expectations and so there is no need to consider lateral gene transfer between Bacteria and Archaea. The phylogenetic tree for the iron–sulphur subunit of succinate dehydrogenases and for homologous fumarate reductases (not shown) shows evidence of a gene duplication, since both succinate dehydrogenase and fumarate reductase are present in *Escherichia coli*, and the archaeal sequence is placed in the expected position on the tree. Phylogenetic trees for 2Fe–2S ferredoxins (not shown) suggest a larger number of gene duplications within Bacteria. So far, homologous archaeal proteins have only been found in *Halobacterium*. Thus, the possibility of lateral transfer remains but, in the absence of any clear evidence for horizontal gene transfer, we assign 2Fe–2S ferredoxin to the last universal ancestor.

Figure 2 shows the position (membrane-bound, peripheral or cytoplasmic) of the redox proteins listed in Table I within the cell of the last universal ancestor. ATP synthase is also shown, as sequences of this protein complex are known from both Archaea and Bacteria[12,13]. This ancestral cell probably employed the chemiosmotic mechanism for energy conversion, as it contained the necessary molecular apparatus including membrane-bound proton pumps (cytochrome oxidase and cytochrome *b* complexes), as well as ATP synthase.

Cytochrome oxidase, the key enzyme in aerobic metabolism, catalyses the reduction of oxygen to water and acts as a redox-linked proton pump[18]. The databases contain 18 homologous sequences of subunit I (the main catalytic subunit) from Bacteria and three from Archaea (Table I). Cytochrome oxidases have been sequenced from Sulfolobales and Halobacteriaceae, two deeply divergent evolutionary lines within Archaea. Thus, it is unlikely that archaeal cytochrome oxidases have been acquired through recent horizontal gene transfers. We have previously conducted a phylogenetic analysis of the cytochrome oxidase superfamily[16] and concluded that two gene duplications had already happened before Bacteria and Archaea diverged. This means that three different cytochrome oxidases were present in the last universal ancestor (Fig. 3). Sequences of two of them, which we call the SoxB- and SoxM-type cytochrome oxidases, are known from Archaea and Bacteria (Table I), and can be clearly assigned to the last universal ancestor. The third type, the FixN-type cytochrome oxidases, is an outgroup to the two other types, which indicates that it might also have existed in the last universal ancestor. FixN oxidases have not yet been found in Archaea (and therefore we do not represent this type in the ancestral cell in Fig. 2), but the phylogenetic tree suggests that they could be present in aerobic archaeal species. On the basis of a similar phylogenetic analysis[16,17], we can assign two different homologs of subunit II of cytochrome oxidase to the last universal ancestor, one associated with the SoxB-type and the other with the SoxM-type subunit I.

The other components of the respiratory chain in the last universal ancestor included cytochrome *b*, blue copper protein and four different types of proteins with iron–sulphur centres: Rieske protein, 2Fe–2S ferredoxin, 4Fe–4S ferredoxin and one subunit of succinate dehydrogenase. Ferredoxins might have been involved in redox reactions by feeding electrons to the respiratory chain. Other redox proteins, such as NADH dehydrogenase or cytochrome *c*, were probably also present in the last universal ancestor, although they have not yet been found in archaeal genomes. We conclude that the respiratory chain of the last universal ancestor was elaborate and probably used oxygen as the terminal electron acceptor.

Aerobic respiration has a single origin

Aerobic metabolism occurs in Bacteria, Archaea and Eucarya. Although most bacterial species are

anaerobic, many are facultative or obligate aerobes. The majority of archaeal species live in extreme environments that are often highly anaerobic. There are, however, several cases of aerobic archaea such as *Halobacterium*, *Thermoplasma*, *Sulfolobus* and *Pyrobaculum*[5]. Most of the known eukaryotes carry out aerobic metabolism within their mitochondria. All aerobic organisms contain oxidases of the cytochrome oxidase superfamily, but some members of the proteobacteria (*E. coli* and *Acetobacter vinelandii*) can also use the unrelated cytochrome *bd* complex as a respiratory terminal oxidase[19].

Thus, in contrast with previous views[1,2], and with the exception of the cytochrome *bd* complex, we conclude that aerobic respiration has a single origin. This notion has been masked by the lack of a close phylogenetic relationship among present-day aerobic organisms. This phylogenetic diversity is due to the loss of the ancestral respiratory system in many evolutionary lines of Archaea and Bacteria, so that it presently exists in unrelated taxa. Early eukaryotes probably acquired aerobic respiration when the atmospheric levels of oxygen increased, by means of a bacterial endosymbiont that gave rise to mitochondria[7].

Table I. Proteins of respiratory chains sequenced in Archaea and Bacteria[a]

Protein	Archaea[b]	Bacteria[c]
Cytochrome oxidase (I)		
SoxM-type	*Halobacterium halobium* *Sulfolobus acidocaldarius*	17
SoxB-type	*Sulfolobus acidocaldarius*	1
Cytochrome oxidase (II)		
SoxM-type	*Sulfolobus acidocaldarius*	14
SoxB-type	*Sulfolobus acidocaldarius*[d]	1
Cytochrome *b*	*Sulfolobus acidocaldarius*[e]	9
Rieske protein	*Sulfolobus acidocaldarius*	9
Blue copper protein	*Natronobacterium pharaonis* *Sulfolobus acidocaldarius*	28
2Fe–2S ferredoxin	*Halobacterium halobium* *Halobacterium* sp.	24
4Fe–4S ferredoxin	*Methanosarcina barkeri* *Methanococcus thermolithotrophicus* *Thermoplasma acidophium* *Thermococcus litoralis* *Pyrococcus furiosus* *Sulfolobus acidocaldarius*	49
Succinate dehydrogenase[f] (Fe–S subunit)	*Thermoplasma acidophilum*	5

[a]Entry names for all sequences are available upon request.
[b]Species from which the protein sequences have been reported.
[c]Number of different protein sequences.
[d]The SoxB-type subunit II of cytochrome oxidase does not have a copper centre, which was probably lost during evolution.
[e]Two cytochromes *b* have been sequenced from *Sulfolobus acidocaldarius*.
[f]Sequences of the homologous fumarate reductase from Bacteria are included.

Denitrification processes were present in the last universal ancestor

We and others[20,21] have previously shown that the bacterial NO reductase, a key enzyme in denitrification, is homologous to cytochrome oxidases. In the phylogenetic tree, NO reductase is an outgroup of all cytochrome oxidases and so NO reductase was most probably present in the last universal ancestor. No archaeal sequences of this enzyme are yet known, but one can predict that denitrifying archaeal species (which exist in Halobacteriaceae[22]) possess a homologous protein. We have postulated[20] an evolutionary pathway in which the FixN-type cytochrome oxidases evolved from NO reductase. The reactions catalysed by NO reductase

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$$

and cytochrome oxidase

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

are similar. Since NO was easily generated in the early atmosphere[23], the binuclear Fe–Fe catalytic centre[21] that reduces NO is probably more ancient

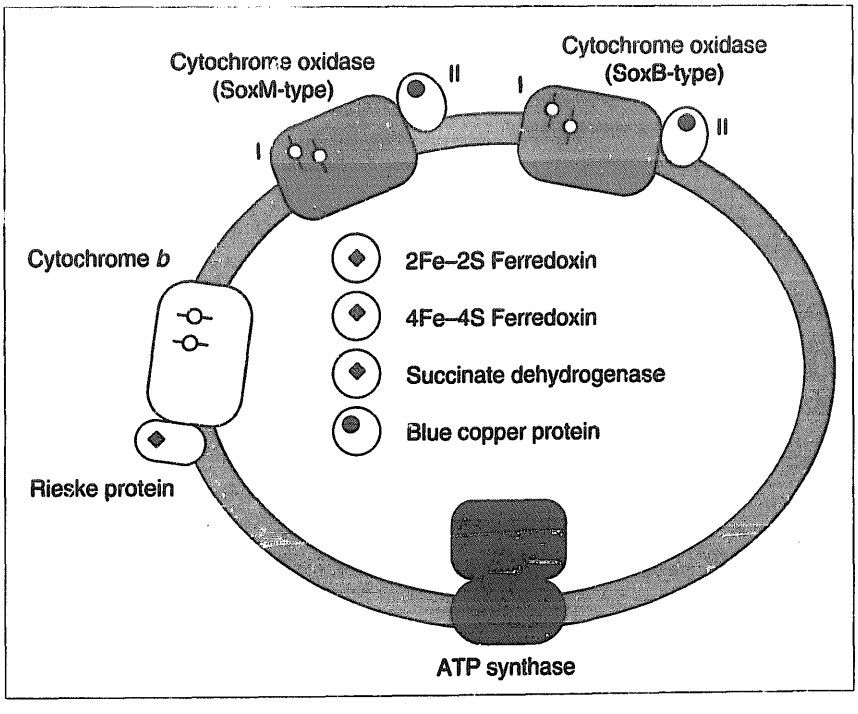

Figure 2
Redox proteins involved in the respiratory chain in the cell of the last universal ancestor. These proteins are present in both Archaea and Bacteria (see Table I) and can therefore be assigned to the last universal ancestor. Redox groups in these proteins are -○- (heme), ◆ (iron–sulphur centre) and ● (copper centre). Subunit I of cytochrome oxidases is coloured orange. The ATP synthase is also shown.

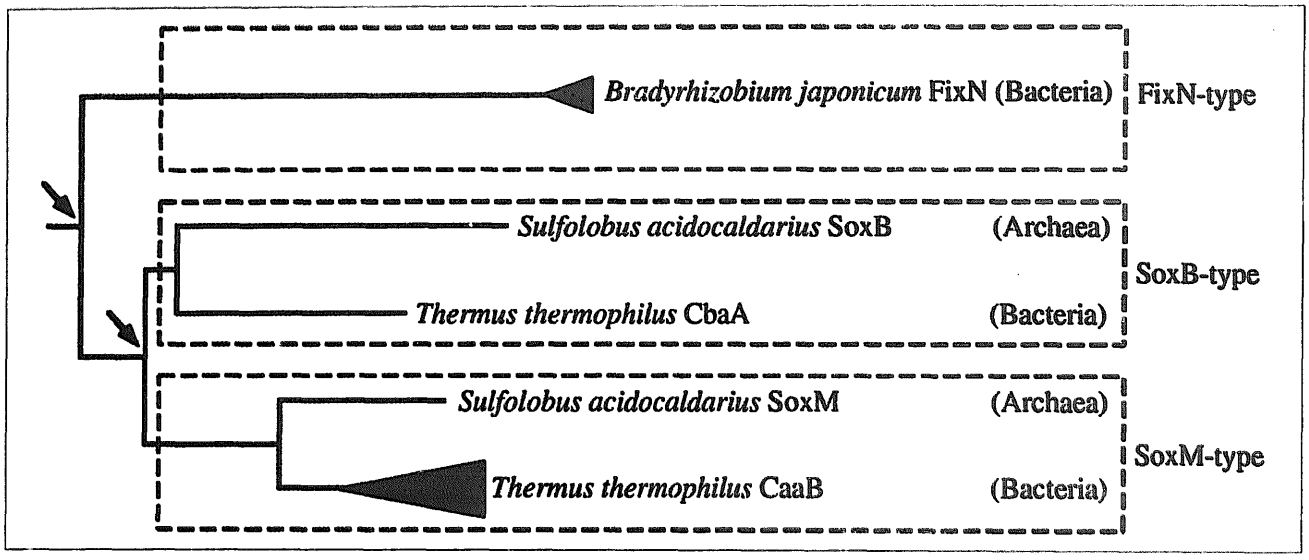

Figure 3

Phylogenetic tree of a set of cytochrome oxidase subunits I (Ref. 16). Arrows point to the gene duplications that happened before the divergence of the last universal ancestor into Bacteria and Archaea. These gene duplications gave rise to three main groups of cytochrome oxidases, which are boxed in different colours. The SoxB-type has been sequenced from Archaea and Bacteria (only one sequence from each domain is available). The SoxM-type is also present in Archaea (two sequences, one from *Sulfolobus acidocaldarius* and one from *Halobacterium halobium*) and in Bacteria (17 sequences, represented as a triangle). It follows that both types were present in the last universal ancestor. The FixN-type has been sequenced only from Bacteria (five sequences, represented as a triangle), but its position in the tree, branching before the divergence of the two other types of cytochrome oxidase, predicts that it was also present in the last universal ancestor. A phylogenetic tree containing all available sequences from Archaea and Bacteria can be found in Ref. 16. More cytochrome oxidase sequences from archaeal species would be necessary to corroborate the main groups of the tree.

than the O_2-reducing Fe–Cu active site. In other words, aerobic respiration may have evolved from denitrification.

On the basis of this link, a different scenario for the evolution of aerobic respiration can be proposed. The cytochrome oxidase of the last universal ancestor might have used NO rather than O_2 as electron acceptor, and the change in the active site of NO reductase caused by the change in substrate could have happened independently in Archaea and Bacteria. The rise of atmospheric oxygen caused by the photosynthetic activity of evolving cyanobacteria must have created a remarkably strong selective pressure on organisms in both domains. Adaptations to use the new, chemically superior, electron acceptor might have taken place, with similar molecular solutions creating the oxygen-reducing active site. This would mean that the appearance of aerobic respiration was polyphyletic. However, the predicted presence of three different types of cytochrome oxidases in the last universal ancestor (Fig. 3) complicates this hypothesis, since it is unlikely that all three should have changed their active sites independently in Bacteria and in Archaea. The determination of the detailed mechanism of proton translocation coupled to oxygen reduction should clarify this situation. We predict that these steps will prove to be the same in cytochrome oxidases of both domains if the solution for this coupling mechanism was found only once.

The electron transfer chain in the last universal ancestor (see Fig. 2) was probably very adaptable. It had the capacity for aerobic respiration using two or three different terminal oxidases, and it could also generate energy by denitrification (if NO reductase in denitrifying Archaea prove to be homologous to those in bacteria). During evolution, many metabolic systems were probably lost in diverse evolutionary lines in response to adaptation to different niches, leading to a haphazard distribution of metabolic characteristics among present-day organisms. However, the remaining systems could be controlled much better than those in the ancestral, non-specialized organisms[24].

Was the last universal ancestor photosynthetic?

Photosynthetic reaction centres are integral membrane proteins known only in bacterial species. Photosystem I is present in Heliobacteria and Chlorobiaceae, whereas photosystem II occurs in Proteobacteria and Chloroflexaceae[25,26] (Fig. 1). Oxygen-releasing photosynthesis occurs only in Cyanobacteria, which have both types of photosystem. No photosynthetic reaction centres have been found in Archaea and so it cannot be assumed that they were present in the last universal ancestor. Bacteriorhodopsin is a completely different archaeal photosynthetic system, based on the retinal photocycle, and is found exclusively in Halobacteriaceae.

Two hypotheses about the evolution of photosynthesis in Bacteria have been proposed. (1) In the fusion model[25], the last common ancestor of Bacteria (Fig. 1) had only one primitive reaction centre. Later, two evolutionary lines, one with a photosystem I and the other with a photosystem II reaction centre, fused to produce the ancestor of cyanobacteria. (2) In the selective loss model[26], the last common ancestor of Bacteria contained both types of reaction centres. Several bacterial lines lost either one or the other, or both. Only cyanobacteria maintained both reaction centres and added a water-splitting enzyme to photosystem II that could extract electrons from water with the consequent production of oxygen, a system that has been retained by all cyanobacteria.

It has also been shown that bacteriochlorophyll, the pigment used by most bacteria, evolved earlier than chlorophyll[27], which is only used by cyanobacteria. Therefore, the last common ancestor of Bacteria probably had

bacteriochlorophyll. The current distribution among Bacteria of photosynthetic reaction centres and the water-splitting system does not allow the assumption that the last common ancestor of Bacteria could perform oxygenic photosynthesis, as this trait only evolved in cyanobacteria. It follows that the last universal ancestor, a more ancient organism, could not perform oxygenic photosynthesis.

Respiration before oxygenic photosynthesis: the supply of oxygen

We have argued that aerobic respiration was present in the last universal ancestor (Fig. 2). However, it is unlikely that oxygenic photosynthesis was present either in the last universal ancestor or in the last common ancestor of Bacteria (see Fig. 1). It arose only later, in the cyanobacterial evolutionary line. Therefore, molecular evidence shows that aerobic respiration evolved before oxygenic photosynthesis or, in other words, cytochrome oxidase (the enzyme that consumes oxygen) appeared before the water-splitting system (the enzyme that produces oxygen). The existence of very old fossils assigned to ancient photosynthetic cyanobacteria[3] does not affect this conclusion: it is the order of appearance of these metabolic traits, rather than the actual date of appearance, that is relevant here. But was respiration possible before the large increase of oxygen in the atmosphere caused by oxygenic photosynthesis?

It is known that traces of oxygen existed in the early atmosphere, resulting from photolysis of water. It is also believed that 'oases' of high oxygen concentration occurred on the ocean surface[4,23]. The cells of the last universal ancestor living in these restricted environments might have been able to carry out aerobic respiration. In fact, a high oxygen concentration is not required for aerobic respiration, as some cytochrome oxidases of present-day organisms work under a very low oxygen pressure. For example, this is the case for the FixN-type cytochrome oxidases of endosymbiotic proteobacteria living inside root nodules, where the oxygen concentration has to be extremely low to allow nitrogen fixation[28].

Concluding remarks

Our conclusions (see Fig. 4) are based on current knowledge of respiratory chains and reaction centres, homology between redox proteins as deduced from the sequences in databases, and analysis of their distribution among present-day organisms. This molecular evidence leads to a 'respiration-early hypothesis' that can be summarized as follows.

(1) Denitrification (NO reductase) is the probable origin of aerobic respiration.

(2) Aerobic respiration (cytochrome oxidase) arose only once and was already present in the last universal ancestor. Therefore, aerobic respiration in present-day organisms has a single origin. The branched and elaborate respiratory chain of the last universal ancestor had several redox protein components. Traces of oxygen in the early biosphere could have been used for respiration by the last universal ancestor.

(3) Oxygenic photosynthesis (involving a water-splitting enzyme) developed in a single evolutionary line, the cyanobacteria, after the origin of aerobic respiration.

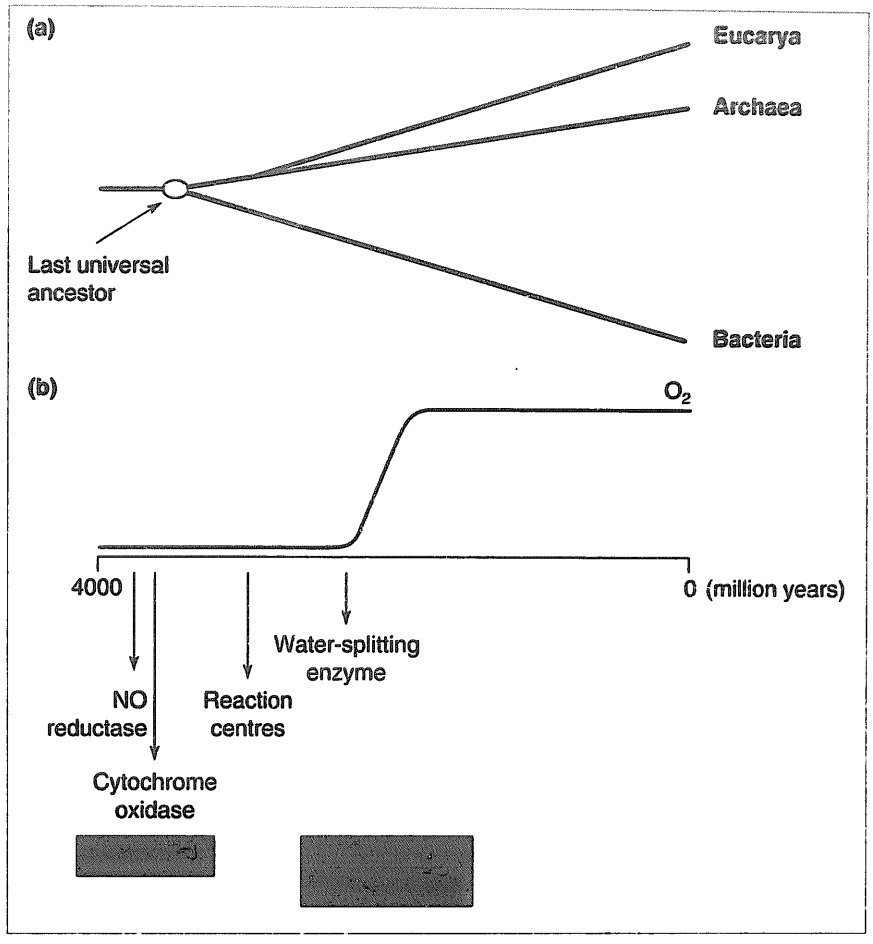

Figure 4

(a) Phylogenetic tree of Archaea, Bacteria and Eucarya, with **(b)** a curve showing the rise of atmospheric oxygen caused by oxygenic photosynthesis, and relative time positions where proteins of respiratory and photosynthetic chains appeared.

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Understanding, improving and using green fluorescent proteins

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Green fluorescent proteins (GFPs) are presently attracting tremendous interest as the first general method to create strong visible fluorescence by purely molecular biological means. So far, they have been used as reporters of gene expression, tracers of cell lineage, and as fusion tags to monitor protein localization within living cells. However, the GFP originally cloned from the jellyfish *Aequorea victoria* has several nonoptimal properties including low brightness, a significant delay between protein synthesis and fluorescence development, and complex photoisomerization. Fortunately, the protein can be re-engineered by mutagenesis to ameliorate these deficiencies and shift the excitation and emission wavelengths, creating different colors and new applications.

THE TWO BEST-CHARACTERIZED green fluorescent proteins (GFPs) are from marine invertebrates: a Pacific Northwest jellyfish, *Aequorea victoria*, and a sea pansy from the Georgia coastline, *Renilla reniformis*[1]. Other members of this coelenterate sub-phylum Cnidaria contain fluorescent proteins which remain to be characterized[2,3]. *Aequorea* and *Renilla* GFPs each transmute blue chemiluminescence from a distinct primary photoprotein into green fluorescence. The first written report of such bioluminescence was from Pliny the Elder in the first century AD, who observed the bright glow of certain jellyfish present in the Bay of Naples[4]. His early development of glowing slime that could be scraped from these organisms and used to make various articles luminescent was abruptly terminated by the eruption of Vesuvius in AD79. More recently, biochemical characterization of the GFPs in the labs of Blinks, Cormier, Hastings, Johnson and Shimomura, Prendergast and Ward began in the 1960s and culminated in the cloning of a cDNA (*gfp10*) for *Aequorea* GFP by Prasher *et al.*[5] Chalfie *et al.*[6] then triggered an enormous upsurge of interest in GFP by showing that expression of the cloned gene produces fluorescent protein in a variety of cell types, as also reported by Inouye and Tsuji[7].

As yet there is no definitive explanation as to why these organisms evolved bioluminescence or fluorescent proteins[3]. GFPs probably serve not just to shift the output color from blue to green, because that color change might be achieved more simply by mutating the primary photoprotein. Perhaps GFPs evolved mainly to boost the overall quantum efficiency of emission. The chemiluminescence efficiencies of primary photoproteins are relatively low, but if their excited state energy can be efficiently transferred to a GFP that, in turn, emits with high efficiency, then the organism produces more light for a given energy cost (Refs 1, 8; W. W. Ward, M. W. Cutler and D. F. Davis, pers. commun.).

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Biochemical properties

Aequorea GFP is a protein of 238 amino acid residues. Its biggest absorbance peak is at 395 nm with a smaller peak at 475 nm. The amplitudes of these peaks (i.e. extinction coefficients) have been estimated as 21–30 and 7–15 $mM^{-1}\,cm^{-1}$, respectively (Ref. 8, W. Ward, pers. commun.). Excitation at 395 nm yields an emission maximum at 508 nm. The quantum yield, or probability of re-emitting a photon once the molecule has been excited, is 0.72–0.85 (Ref. 8; E. Kurian, J. Rudzki-Small and F. Prendergast, pers. commun.), and the excited state lifetime is 3.25 ns (Ref. 10). To put these numbers in perspective, the well-known dye fluorescein has an extinction coefficient of 80 $mM^{-1}\,cm^{-1}$ at 490 nm and a quantum yield of 0.91. Because fluorescence brightness is proportional to the product of these numbers, wild-type *Aequorea* GFP excited with fluorescein filters is about an order of magnitude less bright than the same number of molecules of free fluorescein. Switching the excitation to 395 nm does not help because such wavelengths cause rapid photoisomerization (see below) and also excite more background autofluorescence.

The mature purified protein is highly stable, remaining fluorescent up to 65°C, pH11, 1% sodium dodecyl sulphate (SDS) or 6 M guanidinium chloride, and