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Did Primitive Microorganisms Use Nonhem Iron Proteins in Place of NAD/P?

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Abstract. Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are of universal occurrence in living organisms and play a central role in coupling oxidative with reductive reactions. However, the evidence that the origin and early evolution of life occurred at high temperatures (>95°C) is now strong, and at these temperatures some modern metabolites, including both the reduced and oxidized forms of these coenzymes, are unstable. We believe there is good evidence that indicates that in the most primitive organisms nonhem iron proteins carried out many or all of the functions of NAD/P(H). This has important implications for the way in which investigations of archaebacterial metabolism are conducted.

Key words: Archaebacteria — Evolution of metabolism — Nicotinamide coenzymes — Nonhem iron proteins — Redox coenzymes — Thermophiles

Introduction

Of the three kingdoms of living organisms (Woese and Fox 1977a; Fox et al. 1980) (Fig. 1), there is now a significant body of evidence suggesting that the common ancestor of the archaebacteria is likely to be most closely related to the earliest form of life. Woese (1987; Woese et al. 1990; Fischer et al. 1983) and others have argued that the ancestral archaebacterium was an extremely thermophilic anaerobe, dependent on sulphur reduction. Extreme thermophily (optimum growth temperature >70°C) is widespread among the archaebacteria and the most extreme thermophiles of the group also appear to be the most slowly evolving (Woese 1987). Furthermore, extreme thermophily is also most strongly represented toward the root of the archaebacterial tree (Woese et al. 1990). Indeed, the archaebacteria most closely related to the ancestral phenotype of the archaebacteria seem to be those with the highest optimum growth temperatures, over 100°C in some cases. Additional evidence for a high temperature origin of life is available when we consider the eubacterial evolutionary tree. The most deeply rooted branch in this tree is that leading to the thermotogales (Achenbach-Richter et al. 1987) (optimum growth temperatures up to 85°C), the most extremely thermophilic of the eubacteria (Huser et al. 1986; Huber et al. 1986). The next-most-deeply-rooted branch is that leading to the green nonsulphur bacteria containing a number of thermophiles such as *Chloroflexus* and *Thermomicrobium.* The third-most-deeply-rooted group, the deinococci, includes one of the most widely occurring eubacterial extreme thermophiles, *Thermus* (Woese 1987). Thermophiles are thus very strongly represented at the root of the eubacterial phylogenetic tree, and all these groups are also relatively slowly evolving, strongly sug-

Abbreviations: NAD/P(H): Oxidised and reduced forms of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate

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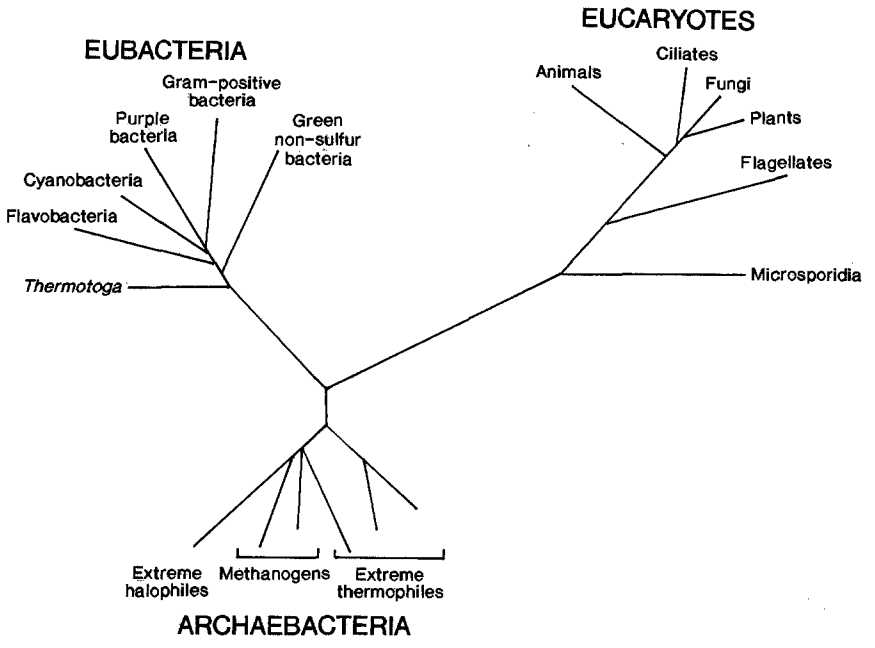

Fig. 1. Universal phylogenetic tree determined from rRNA sequences.

gesting that the eubacteria have also arisen from a thermophilic ancestor (Achenbach-Richter et al. 1987).

On this basis, it now seems likely that life arose at high temperatures, possibly in marine hydrothermal systems (Corliss et al. 1981; Russell et al. 1989; Holm 1992), and there is good reason to suspect that the earliest organisms (Woese and Fox 1977b) may have evolved and grown at temperatures at or above 100°C. Although proteins can be stable at these temperatures, some low-molecular-weight compounds are not (Daniel 1992; Coolbear et al. 1992). The early development of metabolic pathways may have been somewhat constrained by metabolite stability, but in the case of some key metabolites, including widely used coenzymes such as NAD/P(H), such a constraint could have posed serious problems. Even below 100°C NADH (Walsh et al. 1983) and NADPH (R. Hudson, personal communication) are quite unstable, having half-lives of only a few minutes at neutral pH at 95°C. The rate of degradation is unaffected by the presence of a dehydrogenase or of reducing agents and, while the end products of the breakdown are not known, they cannot be re-reduced by a dehydrogenase and the appropriate cosubstrate (Hudson, personal communication). NMR spectra of NADP solutions show significant changes after 30 min at 90°C (Consalvi, personal communication). Given the fundamental importance of NAD/P(H) in metabolism, and the apparent lack of any stabilizing mechanism, did primitive organisms have a means of circumventing the potential constraints posed by the instability of NAD/P(H)?

Proposal

Both nonhem iron proteins and NAD/P(H) are redox coenzymes. Recent research shows that in some archaebacteria nonhem iron proteins fill the role played by NAD/P(H) in more modern (in the sense of less closely related to the progenote) organisms. We propose that in primitive microorganisms, notably primitive archaebacteria, more of these functions were carried out by nonhem iron proteins, i.e., that nonhem iron proteins partially or fully replaced the use of NAD/P(H). During evolution nonhem iron proteins were largely replaced by NAD/P(H), possibly during the adaption of organisms to lower (<90°C) temperatures.

Evidence

Nonhem iron proteins seem likely to have occurred early in evolution. Hall et al. (1971) have proposed that a ferredoxin may have been among the earliest proteins formed, a view based partly on their simplicity (in anaerobic bacteria), and on the observation that their amino acid composition is dominated by amino acids that are readily synthesized abiogenically and that have been found to occur extraterrestrially. Wachtershauser (1992) also considers nonhem iron proteins to be the earliest of the redox catalysts. Given the important role some authors (Wachtershauser 1988, 1992; Russell et al. 1988, 1989, 1990, 1992; Cairns-Smith et al. 1992) have proposed for iron sulphides in the origin of life, it would not be surprising if iron-sulphur-containing proteins played an important role in primitive metabolism.

The upper temperature limit for protein stability is not known, but a variety of proteins have been found with half-lives of tens of minutes at temperatures above 100°C (Coolbear et al. 1992). Primitive organisms probably grew at ~100°C, and there is no reason to doubt the feasibility of a nonhem iron redox protein stable and

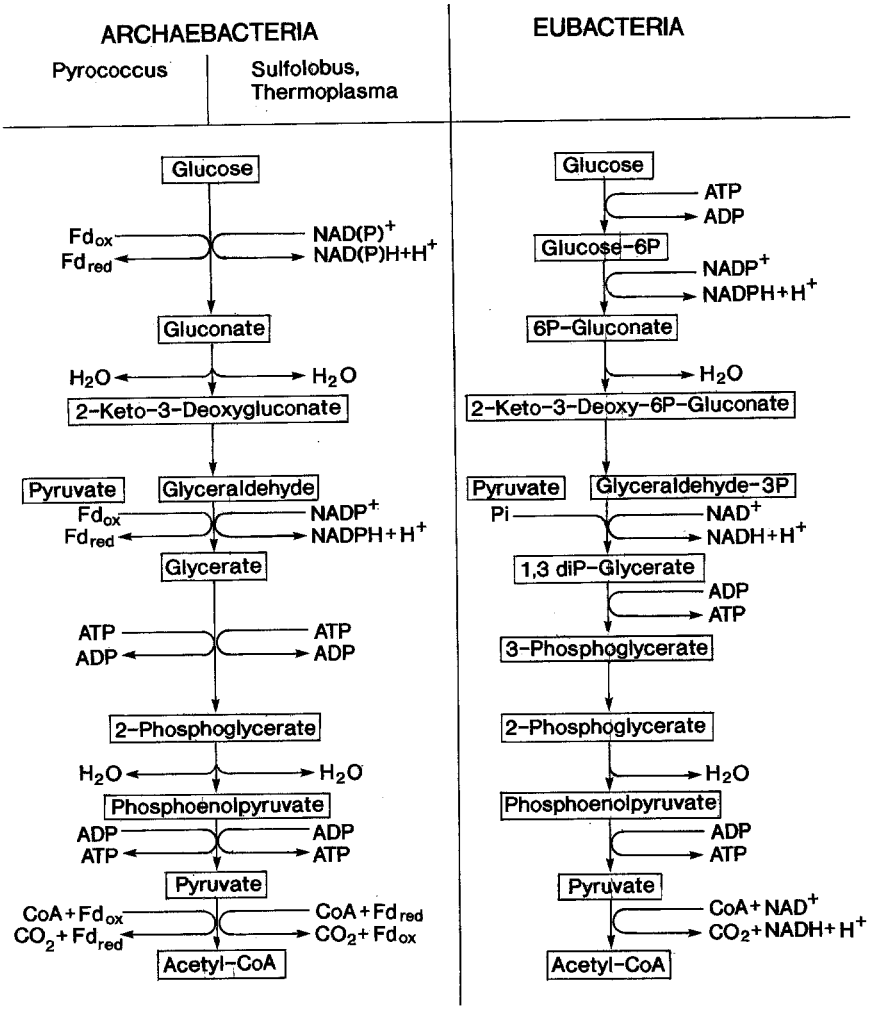

Fig. 2. The Entner-Doudoroff pathways of glucose oxidation in thermophilic archaebacteria and in eubacteria. The coenzymes for primitive archaebacteria (*Pyrococcus* [Makund and Adams 1991]) and less-primitive (Kjems et al. 1992) archaebacteria (*Sulfolobus* [De Rosa et al. 1984] and *Thermoplasma* [Budgen and Danson 1986]) are shown on the same pathway.

functional at 100°C. Indeed, the aldehyde oxidoreductase from the hyperthermophile *Pyrococcus furiosus* belongs in this category (Makund and Adams 1991; Park et al. 1991).

There are a number of enzymes in the central metabolic pathways of thermophilic archaebacteria that use nonhem iron protein for oxidation-reduction reactions, where NAD and NADP are used in their nonarchaebacterial (and less primitive) counterparts (Danson 1988). For example, in eukaryotic and the majority of eubacterial organisms, pyruvate is oxidatively decarboxylated to acetyl-CoA via the pyruvate dehydrogenase multienzyme complex (Perham 1991). This enzyme system uses NAD as the oxidant, with lipoic acid as the protein-bound carrier of both acyl and reducing moieties. A similar oxidative decarboxylation of pyruvate is carried out by the archaebacteria, but the enzyme catalyst is an iron-sulphur pyruvate oxidoreductase, having no dependency on NAD/P (Kerscher and Oesterhelt 1982). The iron-sulphur cluster abstracts the electrons from the oxo-acid and passes them to ferredoxin (Fig. 2). In the hyperthermophile *P. furiosus,* oxidized ferredoxin is regenerated through a hydrogenase with the production of molecular hydrogen (Makund and Adams 1991). The pyruvate oxidoreductase is found in all archaebacterial phenotypes (thermophiles, halophiles, and methanogens) and also in anaerobic eubacteria; therefore, Kerscher and Oesterhelt (1982) have proposed that this enzyme existed before the divergence of the three Kingdoms and that the NAD-linked dehydrogenase complex evolved after the development of oxidative phosphorylation.

A further example of the use of nonhem iron proteins in place of NAD/P(H) is provided by the pathway of glucose catabolism in *P. furiosus.* It has been proposed (Makund and Adams 1991) that this hyperthermophilic archaebacterium oxidizes glucose to pyruvate via a non-phosphorylated Entner-Doudoroff pathway in which the redox reactions are catalyzed by ferredoxin-linked oxidoreductases (Fig. 2). In the less-primitive thermophilic archaebacteria, *Sulfolobus* and *Thermoplasma* (Kjems et al. 1992), glucose is also catabolised via this nonphosphorylated pathway, but the equivalent redox enzymes (glucose dehydrogenase and glyceraldehyde dehydrogenase) are NAD/P-linked enzymes (De Rosa et al. 1984; Budgen and Danson 1986) (Fig. 2). Interestingly, in those eubacteria that possess an Entner-Doudoroff path-

way, the redox enzymes are also NAD/P-linked but the pathway comprises phosphorylated intermediates (Fig. 2).

A nonhem iron protein from *P. furiosus* has been shown to transfer electrons to a metal electrode without the need for a mediator (Park et al. 1991). It seems likely that this is due to a reduced distance between the electrode and the redox center of the enzyme, implying a relatively exposed site for the iron-sulphur cluster. This exposure should facilitate the exchange of electrons between different nonhem iron proteins to have enabled them to function as a "redox currency" in the same way as NAD/P(H) couples do in modern organisms. It might also confer on such nonhem iron proteins an enhanced ability to carry out coenzyme-type redox reactions in solution, e.g., the transfer of electrons to a separate hydrogenase.

In any event, the wider redox potential range over which nonhem iron proteins act compared with NAD/P(H) may have favored their early use as redox coenzymes.

Some of the properties of the NAD/P(H)-linked dehydrogenases in archaebacterial enzymes may be interpreted as being due to less well-developed enzyme-coenzyme interactions than in the eubacterial and eukaryotic equivalents. The dehydrogenases from thermophilic archaebacteria can often use both NAD and NADP, and in some cases with equal efficiency, whereas most dehydrogenases from eubacteria and eukaryotes are specific for one or the other (Perham 1991). Examples of dual-cofactor specificity in thermophilic archaebacteria include isocitrate dehydrogenase from *Sulfolobus acidocaldarius* (Danson and Wood 1984), malate dehydrogenase from *S. acidocaldarius, Thermoplasma acidophilum* (Grossebuter et al. 1986), and *Methanothermus fervidus* (Honka et al. 1990), glyceraldehyde dehydrogenase from *M. fervidus* (Fabry and Hensel 1987) and *Pyrococcus woesei* (Zwickl et al. 1990), and glutamate dehydrogenase from *P. furiosus* (Robb et al. 1992) and *S. solfataricus* (Consalvi et al. 1991).

Finally, there are some data (Makund and Adams 1991) to suggest that free NAD/P(H) levels are lower in thermophilic archaebacteria, although it is not clear whether this reflects lower total intracellular NAD/P(H) or whether these are more tightly bound to dehydrogenases.

Predictions

If our suggestion that the use of nonhem iron proteins as redox coenzymes preceded the use of NAD/P(H) is correct, then there will be fewer NAD/P-linked enzymes and more nonhem iron protein-coupled enzymes in primitive archaebacteria than in more modern organisms. This possibility should be borne in mind when assaying for dehydrogenases in the archaebacteria; that is, the absence of an NAD/P-dependent activity may not imply that the dehydrogenase in question is absent, but rather that the nicotinamide coenzymes are not the redox agents. The pathway of glucose catabolism in *P. furiosus* is one such example (Makund and Adams 1991). To detect dehydrogenases in primitive organisms, monitoring the change of levels of substrate or product in relatively undiluted cell extracts, using an external oxidant or reductant and in the presence and absence of an iron chelator, should supplement the monitoring of the redox change of NAD/P(H).

Although nonhem iron proteins from "modern" organisms transfer electrons between proteins, those from "primitive" archaebacteria may show properties that especially fit them to this role. We take the ability of a *P. furiosus* nonhem iron protein to transfer electrons directly to a metal electrode (Park et al. 1991) to be an example of this.

The structures of the dual-specific NAD/P-linked dehydrogenases in primitive archaebacteria will be less well adapted in terms of their protein-coenzyme interaction than the equivalent enzymes in nonarchaebacterial species.

Concluding Remarks

Clearly, further data are required to test these possibilities, especially needed is an increased knowledge of the metabolic pathways in the hyperthermophilic archaebacteria and of detailed 3D structures of their NAD/P-linked dehydrogenase and nonhem iron protein-linked oxidoreductase enyzmes. Furthermore, it will be particularly interesting to investigate, at the primary sequence level, any evolutionary relationship within the archaebacteria between an NAD/P-linked dehydrogenase and a nonhem iron-linked oxidoreductase catalyzing the oxidation of the same substrate (e.g., glucose dehydrogenase [De Rosa et al. 1984; Budgen and Danson 1986] and glucose oxidoreductase [Makund and Adams 1991]).

It is tempting to speculate that some of the metabolic pathways observed in modern organisms and not in some primitive archaebacteria—for example, the phosphorylated Entner-Doudoroff pathway, may have developed only when NAD/P(H) became available. The removal of the limitations imposed by the use of a high-molecular-weight redox coenzyme, such as steric hindrance and relatively low diffusion rates compared with NAD/P(H), may have been a factor. However, it is not clear to us how this effect may be distinguished from other reasons for the development of alternative metabolic pathways.

Finally, it is of course clear that NAD/P(H) is not the only metabolite whose instability may have posed constraints for primitive organisms growing >90°C. However, as we have indicated above, we believe that in the case of NAD/P(H) there is good evidence how this might have been overcome. It will be interesting to see whether, as our knowledge of more primitive and extreme ther-

mophilic metabolism grows, we can see similar evidence in respect to other unstable metabolites.

Note Added in Proof

Kengen et al. [(1994) J Biol Chem 269:17537–17541] and Schafer et al. [(1994) FEMS Microbiol Lett 121:107–114] have recently presented evidence for glucose catabolism by an Embden-Meyerhof pathway in *P. furiosus*. Kengen et al. (1994) found that the glyceraldehyde 3-phosphate dehydrogenase would accept either NADP or benzyl viologen (and therefore probably ferredoxin) as electron acceptor, providing evidence for the predictions made above. The discovery of ADP–kinases in the same pathway may, given the relatively higher stability of ADP, be another example of how the instability of a low molecular weight metabolite (ATP) can be overcome in extreme thermophiles.

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References

Achenbach-Richter L, Gupta R, Stetter KO, Woese CR (1987) Were the original eubacteria thermophiles? Syst Appl Microbiol 9:34–39

Budgen N, Danson MJ (1986) Metabolism of glucose via a modified Entner-Doudoroff pathway in the thermophilic archaebacterium *Thermoplasma acidophilum*. FEBS Lett 196:207–210

Cairns-Smith AG, Hall AJ, Russell MJ (1992) Mineral theories of the origin of life and an iron sulphide example. Orig Life Evol Biosph 22:161–180

Consalvi V, Chiaraluce R, Politi L, Gambacorta A, De Rosa M, Scandurra R (1991) Glutamate dehydrogenase from the thermoacidophilic archaebacterium. *Sulfolobus solfataricus*. Eur J Biochem 196:459–467

Coolbear T, Daniel RM, Morgan HW (1992) The enzymes from extreme thermophiles. Adv Biochem Bioeng Biotechnol 45:57–98

Corliss JB, Baross JA, Hoffman SE (1981) An hypothesis concerning the relationship between submarine hot springs and the origin of life. Ocean. Acta No. SP:59–69

Daniel RM (1992) Modern life at high temperatures. Orig Life Evol Biosph 22:33–42

Danson MJ (1988) Archaebacteria: the comparative enzymology of their central metabolic pathways. Adv Microbiol Physiol 29:165–231

Danson MJ, Wood PA (1984) Isocitrate dehydrogenase of the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. FEBS Lett 172:289–293

De Rosa M, Gambacorta A, Nicolaus B, Giardina P, Poerio E, Buonocore V (1984) Glucose metabolism in the extremely thermoacidophilic archaebacterium *Sulfolobus solfataricus*. Biochem J 224: 407–414

Fabry S, Hensel R (1987) Purification and characterisation of the D-glyceraldehyde-3-phosphate dehydrogenase from the thermophilic archaebacterium *Methanothermus fervidus*. Eur J Biochem 165: 147–155

Fischer F, Zillig W, Stetter KO, Schneiber G (1983) Chemolithoautotrophic metabolism of anaerobic extremely thermophilic archaebacteria. Nature 301:511–513

Fox GE, Stackebrandt E, Hespell RB, Gibson J, Maniloff J, Dyer TA, Wolfe RS, Bolch WE, Tannen RS, Mogrum LJ, Zablen LB, Blackmore R, Gupta R, Bonen L, Lenris BJ, Stahl DA, Leuhusen KR, Chen KN, Woese CR (1980) The phylogeny of prokaryotes. Science 209:457–463

Grossebüter W, Hartl T, Görisch H, Stezowski JJ (1986) Purification and properties of malate dehydrogenase from the thermoacidophilic archaebacterium *Thermoplasma acidophilum*. Biol Chem Hoppe Seyler 367:457

Hall DO, Cammack R, Rao KK (1971) Role for ferrodoxins in the origin of life and biological evolution. Nature 233:136–138

Holm N (ed) (1992) Marine hydrothermal systems and the origin of life. Orig Life Evol Biosph 22:1–242

Honka E, Fabry S, Niermann T, Palm P, Hensel R (1990) Properties and primary structure of the L-malate dehydrogenase from the extremely thermophilic archaebacterium *Methanothermus fervidus*. Eur J Biochem 188:623–632

Huber R, Langworthy TA, Konig K, Thomm M, Woese CR, Sletyr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144:324–333

Huser BA, Patel BKC, Daniel RM, Morgan HW (1986) Isolation and characterisation of a novel extremely thermophilic anaerobic chemoorganotrophic eubacterium. FEMS Microbiol Lett 37:121–127

Kerscher L, Oesterhelt D (1982) Pyruvate:ferridoxin oxidoreductase—new findings on an ancient enzyme. Trends Biochem Sci 7:371–374

Kjems J, Larsen N, Dalgaard JZ, Garrett RA, Stetter KO (1992) Phylogenetic relationships among the hyperthermophilic Archaea determined from partial 23S rRNA gene sequences. System Appl Microbiol 15:203–208

Makund S, Adams MWW (1991) The novel tungsten-iron-sulphur protein of the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. J Biol Chem 266:14208–14216

Park J-B, Fan C, Hoffman BM, Adams MWW (1991) Potentiometric and electron nuclear double resonance properties of the two spin forms of the (4 Fe-4S)$^{+}$ cluster in the novel ferredoxin from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 266:19351–19356

Perham RN (1991) Domains, motifs, and linkers in 2-oxo acid dehydrogenase multienzyme complexes. A paradigm in the design of a multifunctional protein. Biochemistry 30:8501–8512

Robb FT, Park J-B, Adams MWW (1992) Characterisation of an extremely thermophilic glutamate dehydrogenase. Biochim Biophys Acta 1120:267–272

Russell MJ, Hall AJ, Cairns-Smith AG, Braterman PS (1988) Submarine hot springs and the origin of life. Nature 336:117

Russell MJ, Hall AJ, Turner D (1989) *In vitro* growth of iron sulphide chimneys: possible culture chambers for origin of life experiments. Terra Nova 1:238–241

Russell MJ, Hall AJ, Gize AP (1990) Pyrite and the origin of life. Nature 344:387

Russell MJ, Daniel RM (1992) Emergence of life via catalytic hydrothermal colloidal iron sulphide membranes. Ann Geophys 10:C506

Wachtershauser G (1988) Pyrite formation, the first energy source for life: a hypothesis. System Appl Microbiol 10:207–210

Wachtershauser G (1992) Ground works for an evolutionary biochemistry: The iron-sulphur world. Biophys Mol Biol 58:85–202

Walsh KAJ, Daniel RM, Morgan HW (1983) A soluble NADH dehydrogenase from *Thermus aquaticus* strain T351. Biochem J 209: 427–433

Woese CR (1987) Bacterial evolution. Microbiol Rev 51:221–271

Woese CR, Fox GE (1977a) Phylogenetic structure of the procaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090

Woese CR, Fox GE (1977b) The concept of cellular evolution. J Mol Evol 10:1–6

Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci USA 87:4576–4579

Zwickl P, Fabry S, Bogedain C, Haas A, Hensel R (1990) Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*. J Bacteriol 172:4329–4338