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METALLOPROTEINS

Metalloproteins and the Pyrite-based Origin of Life: A Critical Assessment

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Abstract We critically examine the proposal by Wächtershäuser (Prokaryotes 1:275–283, 2006a, Philos Trans R Soc Lond B Biol Sci 361: 787–1808, 2006b) that putative transition metal binding sites in protein components of the translation machinery of hyperthermophiles provide evidence of a direct relationship with the FeS clusters of pyrite and thus indicate an autotrophic origin of life in volcanic environments. Analysis of completely sequenced cellular genomes of Bacteria, Archaea and Eucarya does not support the suggestion by Wächtershäuser (Prokaryotes 1:275–283, 2006a, Philos Trans R Soc Lond B Biol Sci 361: 787–1808, 2006b) that aminoacyl-tRNA synthetases and ribosomal proteins bear sequence signatures typical of strong covalent metal bonding whose absence in mesophilic species reveals a process of adaptation towards less extreme environments.

Keywords FeS clusters · FeS protein folds · Pyrite-dependent primordial autotrophy · Aminoacyl-tRNA synthetases · Reverse Krebs cycle

Abbreviations

RS	aminoacyl-tRNA synthetase
r-proteins	ribosomal proteins
CoA	coenzyme A

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Introduction

During the past few decades a number of opposing hypothesis on how life first appeared have been suggested. Although the proposal of an heterotrophic origin of life based on the prebiotic synthesis and accumulation of organic compounds is supported by several major lines of evidence (Bada and Lazcano 2009), the antiquity of the reverse Krebs cycle, the basal distribution of hyperthermophilic organisms in some phylogenetic trees, and the catalytic properties of pyrite have been advocated in support of an autotrophic origin of life (Wächtershäuser 1988; Smith and Morowitz 2004; Russell and Hall 1997; Martin and Russell 2003). So far, the most articulate autotrophic hypothesis stems from the work of Wächtershäuser (1988, 1997, 1998, 2006a, b, 2007), who has argued that life started without genetic information with the appearance of a prebiotic autocatalytic reductive tricarboxylic acid (rTCA) cycle (also called the reductive citric acid cycle, or reverse Krebs cycle), that is assumed to be originally based on the formation of the highly insoluble mineral pyrite in sulfur-rich volcanic environments.

Recognition of the key metabolic role of the citric acid cycle led Hartman (1975) to propose an autotrophic origin of life involving clays, transition state metals, disulfides, dithiols, ultraviolet light and CN^-. The primordial chemoautotrophic carbon fixation pathway advocated by Wächtershäuser (1988, 1992, 1997, 2006a, b, 2007) is assumed to be a reductive citric acid cycle, i.e., an Arnon-type cycle, like the one originally described for the photosynthetic green sulfur bacterium *Chlorobium limicola* (Evans et al. 1966). Molecular phylogenetic trees show that this mode of carbon fixation is found in anaerobic Archaea and in the most deeply divergent (eu)Bacteria, which has been interpreted as evidence of its primitive character (Maden 1995). Similar arguments have been applied to other biosynthetic routes, such as the reductive acetyl-CoA or the different versions of the hydroxypropionate/hydroxybutarate pathway found in Archaea (Berg et al. 2010). Wächtershäuser's proposal, which is part of a trend that assumes that genetic material was not essential for the origin of life (Lazcano 2010), has been modified by Russell and Hall (1997) and Martin and Russell (2003), who have argued that primordial life depended on a primitive version of the acetyl-CoA pathway that is assumed to have originated within the FeS-rich mineral boundaries of low-temperature alkaline hydrothermal vents.

As argued by Wächtershäuser (1988, 2007), the formation of pyrrhotite ($Fe_{1-x}S$) and pyrite (FeS_2) provides a continuous input of redox energy that fueled the formation of the first living entities. The FeS/H_2S combination is a strong reducing agent, and has been shown to provide an efficient source of electrons for the reduction of nitrogen and organic compounds under mild and extreme conditions. Although not all proposals of a hydrothermal origin of life favor a high-temperature regime (Martin and Russell 2007), the scenario proposed by Wächtershäuser (1988, 2007) fits well with the environmental conditions found deep-sea hydrothermal vents rich in transition metals and H_2S, CO_2, and CO.

A wide range of minerals, including Fe and Cu sulfides, have been shown to be catalysts, with Ni and Co being particularly efficient in abiotic carbon fixation reactions under laboratory conditions simulating extreme environments (Huber and Wächtershäuser 1997, 2006; Cody 2004; Cody et al. 2004). It has also been argued that the hypothetical evolutionary relationship between FeS metalloproteins and the FeS clusters found in pyrite and other hydrothermal minerals is one of the outcomes of the pyrite-dependent abiotic synthesis of activated organic compounds (Huber and Wächtershäuser 1997). Here we critically examine the suggestion by Wächtershäuser (2006a, b) that the patterns of sequence signatures present in protein components of the translational machinery of

hyperthermophiles support his proposal of a pyrite-dependent origin of life in volcanic environments rich in transition-metal sulfides.

The iron-sulfur protein fold clusters are versatile prosthetic groups found in a wide variety of proteins present in all known cells. Due to their electron transfer properties, they play a major role in bioenergetic processes, catalysis and regulation, and are involved in iron/sulfur storage, and regulation of gene expression processes involved in the response to oxidative stress and photosynthetic and respiratory processes (Johnson et al. 2005; Meyer 2008; Lill 2009).

The possibility that extant metabolic processes reflect the early recruitment of widely distributed minerals like FeS endowed with catalytic properties was suggested long ago (Lipmann 1965; Eck and Dayhoff 1966; Hall et al. 1971, 1974a,b). The presence of two [4Fe-4S] clusters in ferredoxins (Eck and Dayhoff 1966), and the demonstration that certain denaturalized apoproteins would spontaneously refold by the addition of sulfur and iron ions (Malkin and Rabnowitz 1966) was first interpreted as indicating an enzyme-free process of self-assembly leading to primitive electron-transfer polypeptides.

Wächtershäuser (2006a) has argued that several ancient protein components of the translation machinery bear evidence of the gradual loss by "degeneration rather than by adaptation" of strong S-metal covalent bonds as mesophily evolved. According to this proposal, a number of hyperthermophilic aminoacyl-tRNA synthetases (RSs), i.e., valyl-, isoleucyl-, leucyl-, methionyl-, histidyl-, phenylalanyl- and prolyl-RSs, and of ribosomal proteins (S4, S14, S17, S18, L24, L28, L31, L33, and L36) exhibit patterns of sequence signatures formed by 4n Cys units (n=1 to 6), which are present in protein folds found in zinc-finger families and other strong covalent metal bonding sites Wächtershäuser (2006a, b). The availability of large numbers of completely sequenced cellular genomes from the three major lineages has allowed us to test this scheme. Here we present the results of such an analysis.

Material and Methods

Sequences

Sequences encoding valyl-, isoleucyl-, leucyl-, methionyl-, histidyl-, phenylalanyl- and prolyl-RSs, together with those of the genes of ribosomal proteins L33, S17, L24, S14, L36, S4, L31, L28 and S18 discussed by Wächtershäuser (2006a), were retrieved from the completely sequenced *Escherichia coli* (eco), *Methanocaldococcus jannaschii* (mja), *Homo sapiens* (hsa) and *Saccharomyces cerevisiae* (sce) genomes available in the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa and Goto 2000). The KEGG, BRENDA (Schomburg et al. 2002) and PDB (Berman et al. 2000) databases were searched for bibliographic reports of the chemical interaction of aminoacyl tRNA synthetases with Zn and other transition metal cations.

Homology Searches

In order to detect the presence of any protein signature associated with a strong covalent metal bonding (e.g., Zn) on the RSs and the r-proteins discussed by Wächtershäuser (2006a), their sequences were compared to the Pfam (Finn et al. 2010) database of protein domains, using HAMMER software (http://hmmer.org/). The cut-off value used here was 10.00, in order to avoid false negatives.

In order to identify and retrieve the sequences encoding these RSs and r-proteins, a search was made using BLAST (Karlin and Altschul 1990, 1993) against 86 archaeal, 508 bacterial, and 133 eucaryal complete genomes derived from the KEGG database as of October 2010. Redundancies were avoided by selecting only one genome when several organisms of the same species were available, and only one strain was selected for each species. The cut-off value was E=0.00001. In order to identify the 4n C units (n=1 to 6) signature, the homologous sequences were aligned using ClustalW (Thompson et al. 1994).

Results

The compilation of the metallic cofactors required by RSs using the KEGG database is shown in Table 1. With the exception of potassium, the available reports indicate that Mg^{2+}, Mn^{2+} and Zn^{2+}, together with other divalent metallic cations (Ca^{2+}, Co^{2+}) appear to be almost universal requirements for the aminoacyl-tRNA synthetases discussed here.

We have also studied the distribution of the 4nCys (n=1 to 6) signatures in the RSs discussed by Wächtershäuser (2006a) using multiple gene alignments of homologous sequences derived from fully sequenced cellular genomes available in public databases as of October 2010. In contrast to what Wächsterhäuser has suggested, the 4nCys motif is found indistinctly in mesophilic, thermophilic and hyperthermophilic species, i.e., there is no evidence of a correlation of the presence of this motif with the organisms' lifestyles. For instance, the 4nCys box is present only in the archaeal thermophilic and hyperthermophilic methionyl- and phenylalanyl-RSs, but is found in all bacterial methionyl-RSs, regardless of the organisms' growth temperature. Although cysteinyl-sulfur is the most frequently observed ligand of Fe-S clusters, histidyl residues are also known to replace cysteine (Meyer 2008). We therefore searched for cysteine-histidine variants of the 4nCys signatures, such as 3Cys-1His and 2Cys-2His. These modified motifs are present in all bacterial phenylalanyl-RSs, regardless of the optimal environmental temperature of the organisms (Table 2).

Simple 4Cys (i.e., Cys2Cys2) Zn-finger domains are a well-characterized trait of many ribosomal proteins (Dresios et al. 2005). The presence of superkingdom-specific rubredoxin-like zinc fingers in the L24, L31 and S14 ribosomal proteins shown in Table 2 is consistent with the wide phylogenetic distribution of this motif. However, like the RSs discussed above, there is no evidence of a correlation between their presence in r-proteins and the organism's lifestyle (Tables 2). For instance, the archaeal S14 ribosomal protein, together with the bacterial L31 and L36 r-proteins exhibit the 4nCys signature, but the

Table 1 Metallic cofactors required for the different aminoacyl-tRNA synthetases analyzed here. Data was derived from the KEGG, BRENDA and PDB databases

Aminoacyl tRNA synthetase	Metallic Cofactor(s)
Valyl-RS	Mg2+ Mn2+ Zn2+ Ca2+ Co2+
Isoleucyl-RS	Mg2+ Zn2+
Leucyl-RS	Mg2+ Mn2+ Ca2+ Co2+ Fe2+ Al2+ Ba2+ K+ Sn2+
Methionyl-RS	Mg2+ Mn2+ Zn2+ Ca2+ Co2+
Histidyl-RS	Mg2+
Phenylalanyl-RS	Mg2+ Mn2+ Zn2+ Fe2+ Cd2+
Prolyl- RS	Mg2+ Mn2+ Zn2+

Table 2 The optimal growth temperatures are indicated in fourth, fifth and sixth columns. The void boxes in the E rows of the third and fourth columns are due to the absence of any known hyperthermophilic eukaryotes. The void boxes in the prokaryotic B and A rows indicate that no 4nCys or His-containing zinc fingers have been detected. (**a**) 4nCys zinc finger motifs and His-containing zinc finger-like signatures in aminoacyl-tRNA synthetases in the genomes of Archaea (A), Bacteria (B) and Eucarya (E) discussed in this work. The 4nCys and His-containing motifs of phenylalanyl-tRNA synthetases α and β heterodimer subunits are also shown; **(b)** 4nCys and His-containing zinc finger-like boxes identified in the sequences of small (S) and large (L) ribosomal subunit proteins. Paralog pairs for the archaeal S14, S17 (A1, A2) and L24 (A1, A2) ribosomal proteins, and for the bacterial L31 (B1, B2) and L36 r-proteins are shown in the second column. See text for discussion

	Superkingdom	Hyperthermophiles 80°C or greater	Thermophiles Between 60 and 80°C	Mesophiles 60°C or less
(**a**) Aminoacyl tRNA Synthetase				
Valyl-RS	A	4n Cys	4n Cys	4n Cys
	B	4n Cys	4n Cys	4n Cys
	E	–	–	3C-H/2C-2H
Isoleucyl-RS	A	4n Cys	4n Cys	4n Cys
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4n Cys/3C-H
Leucyl-RS	A	4n Cys	4n Cys	4n Cys
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4n Cys/3C-H
Methionyl-RS	A	4n Cys	4n Cys	4n Cys
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4n Cys/3C-H
Histidyl-RS	A	–	–	–
	B	4n Cys	4n Cys	4n Cys
	E	–	–	3C-H/2C-2H
Phenylalanyl-RS α	A	–	–	3C-H/2C-2H
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4H
Phenylalanyl-RS β	A	2C-2H	–	–
	B	–	–	–
	E	–	–	3C-H/2C-2H
Prolyl- RS	A	4n Cys	4n Cys	4n Cys
	B	3C-H/2C-2H	4H	2C-2H
	E	–	–	4n Cys/2C-2H
(b) Ribosomal Protein				
S4	A1	4H	4H	4H
	A2	4H	C-3H/4H	C-3H/4H
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4n Cys
S14	A	4n Cys	4n Cys	4n Cys
	B	–	–	–
	E	–	–	–
S17	A1	4n Cys	4n Cys	4n Cys
	A2	–	–	–

Table 2 (continued)

	Superkingdom	Hyperthermophiles 80°C or greater	Thermophiles Between 60 and 80°C	Mesophiles 60°C or less
	B	3C-H	–	2C-2H
	E	–	–	–
S18	B	3C-H	3C-H	3C-H
	E	–	–	–
L24	A1	–	–	–
	A2	4n Cys	4n Cys	4n Cys
	B	4n Cys	4n Cys	4n Cys
	E	–	–	4n Cys
L28	B	–	–	C-3H
L31	A	–	–	–
	B1	4n Cys	4n Cys	4n Cys
	B2	–	–	4n Cys
L33	B	–	–	–
L36	B1	3C-H	3C-H	3C-H
	B2	–	–	–

sequence is absent in the archaeal L31 and in one of the bacterial L36 r-protein paralogs included in our sample.

Our search for cysteine-histidine-containing variants of the 4nCys-Zn finger motifs in r-proteins (Dresios et al. 2005) shows that with the exception of the S14, L24 and L31 ribosomal proteins, histidine-containing Zn finger-like variants are present in the S4, S17, S18, L24, L28, L31, L33, and L36 ribosomal proteins of all genomes analyzed here. We found no evidence of the presence of a 4Cys box or its variants in the paralogous copies of the archaeal S17 and L24 ribosomal proteins, nor in hyperthermophilic and thermophilic bacterial L31 paralogs (Table 2).

Discussion

Iron-sulfur clusters are highly ubiquitous prosthetic groups that play a key role in many ancestral fundamental biochemical processes. However, analysis of sets of highly conserved sequences that may have been part of the gene complement of the last common ancestor (Harris et al. 2003; Delaye et al. 2005) shows that they are surprisingly rare among enzymes involved in nucleotide metabolism, as well as among those involved in polymerization, translation, transcription, and RNA processing and degradation. The few cases of FeS protein fold-enzymes associated with nucleic acid synthesis and processing include (a) the (eu)bacterial endonuclease III, which is involved in DNA repair (Cunningham et al. 1989); (b) the *Sulfolobus solfataricus* DNA-dependent RNA polymerase, where the Fe-S cluster is removed from the catalytic site and probably plays a structural role (Hirata and Murakami 2009); and (c) the eukaryotic and archaeal DNA primase endowed with a [4Fe-4S] cluster that appears to have a regulatory role (Weiner et al. 2007; Klinge et al. 2007). A FeS protein fold has also been identified in *Thermotoga maritima* tryptophanyl-tRNA synthase, but no functional role has been assigned to it (Han et al. 2010).

Wächtershäuser (2006a) has hypothesized that protein components of the hyperthermophilic translation machinery exhibit 4nCys patterns whose absence in a number of mesophilic RSs (valyl-, isoleucyl-, leucyl-, methionyl-, phenylalanyl-, histidyl, and prolyl-aminoacyl tRNA synthetases) and ribosomal proteins (S4, S14, S17, S18, L24, L28, L31, L33, and L36) is an outcome of the gradual adaptation of ancestral life forms from hot volcanic environments to low temperature conditions. The structural similarities between the rubredoxin Fe(Cys)4 active site and the Zn-containing folds of zinc-finger proteins (Dauter et al. 1996; Meyer 2008) support the feasibility of such transitions. Cysteine-histidine boxes in RSs are known to bind to zinc, cobalt, and other metallic cations (Miller et al. 1991; Rees 2002). However, the 4nCys boxes and their histidine-containing variants are equally distributed among hyper/thermophilic and mesophilic Archaea and Bacteria (Table 2). As a whole, the results of the searches summarized in Tables 2 demonstrate that detailed analysis of a statistically significant sample of completely sequenced cell genomes do not support Wächtershäuser's (2006a) proposal.

It is of course possible that during prebiotic times catalytic FeS in pyrite and other sulfur-bearing minerals interacted with other chemical species, including amino acids and small peptides of abiotic origin. While this may have contributed to enhance the synthesis of organic compounds, such chemical complexes would not have been inherited prior to the emergence of an encoding mechanism. On the other hand, four of the central reactions involved in protein biosynthesis, including peptide-bond formation, are catalyzed by ribozymes, which suggest that they first evolved in an RNA world (Kumar and Yarus 2001). This implies that ribosomal proteins must have emerged after the appearance of catalytic rRNA. Not all the ribosomal proteins are equally ancient (Fox 2010), and the distribution of their zinc-finger sequences exhibits no correlation with their relative age nor appears to support Wächtershäuser's proposal (2006a, b). Of the list discussed by Wächtershäuser (2006a) only L24, which ranks among the oldest proteins (Fox 2010), is endowed with a zinc-finger domain (Laity et al. 2001, and Table 2). The absence of zinc-fingers boxes in the bacterial L33 ribosomal protein, which appears to be a late addition to the ribosome (Fox 2010), fits well with Wächtershäuser's proposal. However, the bacterial L36 proteins are also endowed with zinc fingers (Urlaub et al. 1995). It is unlikely that such complex distribution reflects a non-homogenous process of Zn-finger loss along evolutionary time, and cannot be used to support Wächtershäuser (2006a,b) proposal of an autotrophic hot origin of life.

Conclusions

The recognition that pyrite formation can catalyze the production of molecular hydrogen, the fixation of carbon and nitrogen, and reduce a few organic molecules supports the possibility that Hadean hydrothermal vent systems may have served as primordial chemical reactors where organic synthesis took place fueled by the input of redox energy of iron sulfide minerals. Experiments using the FeS/H_2S combination are also compatible with a more general, modified model of the primitive soup in which pyrite formation is recognized as an important source of electrons for the reduction of organic compounds, i.e., pyrite-dependent abiotic synthesis may have contributed to the formation of the primitive soup (Bada and Lazcano 2002, 2009).

Regardless of their unique catalytic and structural properties, protein-associated FeS clusters do not provide by themselves phylogenetic information (cf. Zuckerkandl and Pauling 1965). However, an evolutionary analysis of the distribution of the different types

of transition-metal clusters, the enzymes to which they are associated, and the type of folds that host them provides important insights on the early evolution of redox reactions.

Wächtershäuser (2006a) has argued that the sequence signatures with 4n Cys units found in hyperthermophilic RSs and r-proteins and their absence in their mesophilic counterparts, provide evidence of the gradual loss of strong S-metal covalent bonds as mesophily evolved. The results presented here, which are based on a detailed analysis of statistically significant numbers of sequences homologous to the proteins discussed by Wächtershäuser (2006a) do not confirm such possibility. In other words, the distribution of transition-metal bonding motifs in protein components of the translation apparatus cannot be used to argue for an origin of life under high temperature sulfur-rich volcanic environments.

The lines of evidence supporting the possibility that ribonucleotide cofactors that provide reducing power are vestiges of the RNA world (Jadhav and Yarus 2002) implies that a ribozyme-dependent biosphere predated the emergence of iron-sulfur protein folds, i. e., that the ubiquity of biological Fe/S complexes does not constitute by itself an indication of a pyrite-dependent origin of life. The discovery of self-acylating ribozymes that produce acetyl-CoA and butyryl-CoA as RNA-bounded products suggest that CoA-SH nucleophilicity and redox SH groups may have appeared in an RNA world (Jadhav and Yarus 2002). This is consistent with the proposal that once protein biosynthesis had evolved, natural selection favored the incorporation of cysteine in primitive proteins because of its ability to form RNA-recognizing zinc-fingers, to bind to Fe/S clusters and to dimerize and covalently link to form disulfide bonds that play a key role in maintaining functional three-dimensionally folded structures (Parker et al. 2010).

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