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ORIGINAL ARTICLE

On an Early Gene for Membrane-Integral Inorganic Pyrophosphatase in the Genome of an Apparently Pre-LUCA Extremophile, the Archaeon *Candidatus Korarchaeum cryptofilum*

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Abstract A gene for membrane-integral inorganic pyrophosphatase (miPPase) was found in the composite genome of the extremophile archaeon *Candidatus Korarchaeum cryptofilum* (CKc). This korarchaeal genome shows unusual partial similarity to both major archaeal phyla Crenarchaeota and Euryarchaeota. Thus this Korarchaeote might have retained features that represent an ancestral archaeal form, existing before the occurrence of the evolutionary bifurcation into Crenarchaeota and Euryarchaeota. In addition, CKc lacks five genes that are common to early genomes at the LUCA border. These two properties independently suggest a pre-LUCA evolutionary position of this extremophile. Our finding of the miPPase gene in the CKc genome points to a role for the enzyme in the energy conversion of this very early archaeon. The structural features of its miPPase indicate that it can pump protons through membranes. An miPPase from the extremophile bacterium *Caldicellulosiruptor saccharolyticus* also has a sequence indicating a proton pump. Recent analysis of the three-dimensional structure of the miPPase from *Vigna radiata* has resulted in the recognition of a strongly acidic substrate (orthophosphate: Pi, pyrophosphate: PPi) binding pocket, containing 11 Asp and one Glu residues. Asp (aspartic acid) is an evolutionarily very early proteinaceous amino acid as compared to the later appearing Glu (glutamic acid). All the Asp residues are conserved in the miPPase of CKc, *V. radiata* and other miPPases. The high proportion of Asp, as compared to Glu, seems to strengthen our argument that biological energy conversion with binding and activities of orthophosphate (Pi) and energy-rich pyrophosphate (PPi) in connection with the origin and early evolution of life may have started with similar or even more primitive acidic peptide funnels and/or pockets.

Keywords Archaeon · Extremophile · Pyrophosphatase · miPPase · Energy · Evolution · Bioinformatics

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Introduction

The genome of the extremophilic archaeon *Candidatus Korarchaeum cryptofilum* (CKc) has been shown by the Karl Stetter team (Elkins et al. 2008) to have a gene composition partly similar to each of two major established archaeal phyla, the Crenarchaeota and the Euryarchaeota. This indicated a very early position of CKc in the archaeal evolutionary tree, apparently ahead of an early evolutionary branching leading to these archaeal phyla. In addition five expected LUCA (Last Universal Common Ancestor) protein genes were not found in the CKc genome. On the other hand, in this genome we have found a gene for a membrane-integral inorganic pyrophosphatase (miPPase). In the genomes of two very early symbiotic organisms, *Ignicoccus hospitalis* and *Nanoarchaeum equitans*, no gene for a miPPase was found.

As a result of work on the evolution of functional classes in the three domains of life, archaea, bacteria and

eukaryotes, the LUCA concept was created (Kyrpides et al. 1999). An original estimate of the genome content of the last common ancestor of Archaea, Bacteria, and Eukaryotes resulted in 324 proteins containing 301 biochemical functions, of which 246 were unique. The universal function set contained mostly genes for energy metabolism or information processing. We assume that CKc is not the only still existing pre-LUCA prokaryote, but no report of any other pre-LUCA organism is known to us.

A possible role of PPi as a first alternative chemical energy carrier to ATP, in biological electron transport coupled phosphorylation, had been found in chromatophores isolated from the photosynthetic bacterium *Rhodospirillum rubrum*. In this system energy from light produced energy-rich PPi from added Pi (Baltscheffsky et al. 1966), and PPi could substitute for ATP in driving various energy requiring reactions (Baltscheffsky 1967). The similarity to ATP and ATP synthase in these respects was further strengthened by the demonstration that also the membrane-integral PPi synthase/PPase is a proton pump (Moyle et al. 1972). The primary structure of this miPPase (Baltscheffsky et al. 1998) is homologous to all known miPPases, but it is still the only one which was shown to efficiently catalyze also the PPi synthase direction. Notably Lipmann, who had established ATP as the universal chemical carrier of useful energy to energy requiring biological reactions (Lipmann 1941), later also assumed that PPi preceded ATP as energy donor in connection with the origin of life (Lipmann 1965).

As the energy content of PPi is slightly lower than that of ATP, the inorganic pyrophosphate is sometimes called "poor man's ATP". It has an important role in different stress situations, and in photosynthetic bacteria the miPPase enzyme can be induced by light (López-Marqués et al. 2004). The miPPase bioenergetics field was reviewed in 2007 (Serrano et al. 2007). It has now become of increasing evolutionary actuality, with Na^+-translocation apparently preceding H^+-translocation (Luoto et al. 2011) and PPi possibly preceding ATP (Holm and Baltscheffsky 2011). An excellent review of the known subfamilies of cation-pumping pyrophosphatases has recently appeared (Baykov et al. 2013).

Based on results from genome and three-dimensional structure investigations an analysis of possible pre-LUCA redox enzymes has been presented (Baymann et al. 2003). An early chemiosmotic model for the origin of life (Russell et al. 1994) was later complemented (Russell and Hall 1997) and additionally broadened (Nitschke and Russell 2009). More recently, several papers with focus on early chemiosmosis (Lane et al. 2010) in membrane bioenergetics (Lane and Martin 2010; Schoepp-Cotenet et al. 2013) have been discussed in great detail (Branscomb and Russell 2013). So have also the possible roles of the earliest amino acids and their peptides in the molecular origin and evolution of life (Milner-White and Russell 2011). The most detailed consideration of the possibly early role of PPi in the origin and evolution of life was given by Russell and his co-workers.

A cloud model (Lazcano and Miller 1999) had presented a picture of the relationship between prebiotic chemistry and the emergence of metabolic pathways. The only great similarity between the prebiotic and the metabolic parts in this cloud model was the energetic pathway, where inorganic phosphate compounds make a continuity over the clouded gap between prebiotic chemistry and early metabolism after the origin of life. A possible continuum from prebiosis to biosis with respect to energy and PPi was recently discussed (Holm and Baltscheffsky 2011). A plausible such continuum from volcanic production of polyphosphates had been presented from results with cooling of hot lava, where its compound P_4O_{10} upon cooling gave PPPPi (tetrapolyphosphate), PPPi (tripolyphosphate), PPi and Pi (Yamagata et al. 1991).

A first comparison between different pro- and eukaryotic miPPases showed indications of a partial gene duplication (Baltscheffsky et al. 1999). Today, two duplication events have been traced in miPPase sequences and interpreted as starting from a first 6 transmembrane domain and leading to their well-known 16 transmembrane structure over 4 + 6 or 6 + 4 additions (Au et al. 2006; Hedlund et al. 2006; Kellosalo et al. 2012). We have earlier drawn attention to the high concentration of the first coded four proteinaceous amino acids (Gly, Ala, Val, Asp) and specially the locations of the aspartic acid residues in the loop of the transmembrane 5–loop 5–transmembrane 6 region (Baltscheffsky et al. 2001). A preliminary idea of a proposed early binding site for PPi in this region was also given (Figs. 1, 2 in Baltscheffsky et al. 2001, with 4 Mg^{2+}-ions "borrowed" from soluble PPases).

The novel 3-D structure of a proton-pumping miPPase (Lin et al. 2012) shows 5 Mg^{2+}-ions at the PPi-binding site and, more evolutionary important, a very acidic pocket or funnel-containing 11 Asp and only one Glu residues involved in the binding and reactions of Pi and PPi. The Mg^{2+} ion is a well known link between biological phosphate compounds and acidic groups of polypeptides. The 11/1 excess of Asp over Glu in the phosphate-binding miPPase funnel indicates to us that a first site for the origin and evolution of biological energy transfer with phosphate compounds had emerged before the genetic code had evolved from producing the first four proteinaceous amino acids (Gly, Ala, Val, Asp) to producing the next four. The original PPi-binding polypeptide structure may well have contained only Asp, say at least six, and no Glu residues, stepwise evolving to the present 12 (involving one Glu). How the evolution from phosphate binding over simple

energy transfer to cation pumping, and from a polypeptide with six up to one with 16 transmembrane segments as in the present miPPases is an open question.

This presentation is focusing on the derived primary structure of the CKc miPPase and some of its apparent functional, structural, and evolutionary relations with other miPPases. In addition, the great potential similarity of the CKc miPPase substrate-binding site to that unusually Asp-rich acidic funnel-shaped pocket of the corresponding enzyme found in the first detailed 3-D structure of a miPPase (Lin et al. 2012) leads us further into the question (Baltscheffsky et al. 2002, 2004) about the origin of pre-biological and biological binding and use of inorganic phosphate compounds. Was a pyrophosphate engine the first chemical energy conversion source for life? Fuel for this assumed engine may have been abundant in cooling volcanic lava as described above.

Materials and Methods

Amino acid sequences of membrane-bound inorganic pyrophosphatases (miPPases) from *Korarchaeum cryptofilum*, *Caldicellulosiruptor saccharolyticus*, the Thaumarchaeote *Cenarchaeum symbiosum*, *Thermotoga maritima*, *Chlorobium limicola* (two forms), *Vigna radiata*, *Rhodospirillum rubrum*, and *Streptomyces coelicolor* were extracted from the UniProt database (UniProt Consortium 2011). Furthermore, we added amino acid sequences for miPPases with experimentally determined cation specificity (Baykov et al. 2013). Sequences were aligned using Mafft (Katoh and Standley 2013). Dendrograms were calculated using Clustal Omega (Sievers et al. 2011) and illustrated using NJPlot (Perrière and Gouy 1996).

Results

The miPPase sequences were extracted from the UniprotKB database (UniProt Consortium 2011) and multiply aligned using Mafft (Katoh and Standley 2013). Based upon the multiple sequence alignment, a dendrogram was calculated (Fig. 1a). A subset of nine representative miPPases was selected for more detailed investigation (Table 1). A dendrogram of these is shown in Fig. 1b. The dendrogram shows that the very early extremophiles, the archaeon CKc and the bacterium *Caldicellulosiruptor*, a hydrogen-producing Clostridium, form separate branches. Furthermore, the K^+-insensitive proton pumps from *Chlorobium*, *Streptomyces*, and *Rhodospirillum* form one branch, while the K^+-stimulated forms from *Chlorobium*, *Vigna* and *Thermotoga* form another branch together with the *Cenarchaeum* miPPase. According to information in the UniProt database (the HAMAP annotation rule MF_01129), the *Cenarchaeum symbiosum* enzyme is not K^+ stimulated, indicating that K^+ dependence has appeared between the *Cenarchaeum* and *Thermotoga* forms. However, some branch lengths are very short with low bootstrap values (not shown), reflecting that the exact branching order is not unambiguously determined based upon included data. Furthermore, lateral gene transfer cannot be excluded, especially at the early prokaryotic level.

The evidence showing that the genome of CKc contains a gene for a miPPase is shown in Fig. 2a. In the miPPases, a segment of 57 residues has attracted special interest, since it contains three well-conserved sequence motifs (Baltscheffsky et al. 2001). In a sequence comparison of this segment (with addition of two flanking residues at both sides) between *Rhodospirillum rubrum* and *Korarchaeum*, we obtained 85 % identity. The central 35-residue region, with the two Asp-rich nonapeptidyl regions and a high amount of the four earliest proteinaceous amino acids, is identical between the thermophile archaeum CKc and the photosynthetic bacterium *R. rubrum.*

The same pattern to a somewhat lesser extent is also clearly distinguished when all species from the dendrogram in Fig. 1b are compared, as shown in Fig. 2b. From the multiple sequence alignment, it can be seen that only the thermophiles CKc and *C. saccharolyticus* have Q and not R at the second position of the 61-residue segment shown, but uniquely R instead of M (or alternatively P or L) at position 50 of this segment in *R. rubrum* and *S. coelicolor*. *C. saccharolyticus* has been found to use PPi as a central energy carrier (Bielen et al. 2010), which together with the here presented similarities between the two early thermophiles would seem to add support to our hypothesis about a very early evolutionary significance of both PPi and CKc miPPase in biological energy transfer.

Among the membrane-bound inorganic pyrophosphatases, there are a few sequence motifs of special interest for pump specificity (Luoto et al. 2011). In Fig. 3, the three motifs starting at positions 168, 231, and 465, respectively (*R. rubrum* numbering) are shown for the nine selected miPPases. Sequence similarities are greater within K^+-dependent and K^+-independent groups, respectively, in agreement with a recent review (Baykov et al. 2013).

Figure 4 shows our attempt to visualize a current evolutionary picture for miPPases from the origin of life, over LUCA to the present time. The separate branches of CKc and *C. saccharolyticus* are taken into account, however, assuming an extremely early separation from a common origin. The separate evolution between *C. saccharolyticus* (Cs) on one side and *R. rubrum* (Rr) and *S. coelicolor* (Sc) on the other is shown as different lines, corresponding to the initial branching in Fig. 1b. The picture also includes the in 2013 published (Na^+,H^+) pump, at both physiological ionic concentration (Luoto et al. 2013b) and H^+ ion

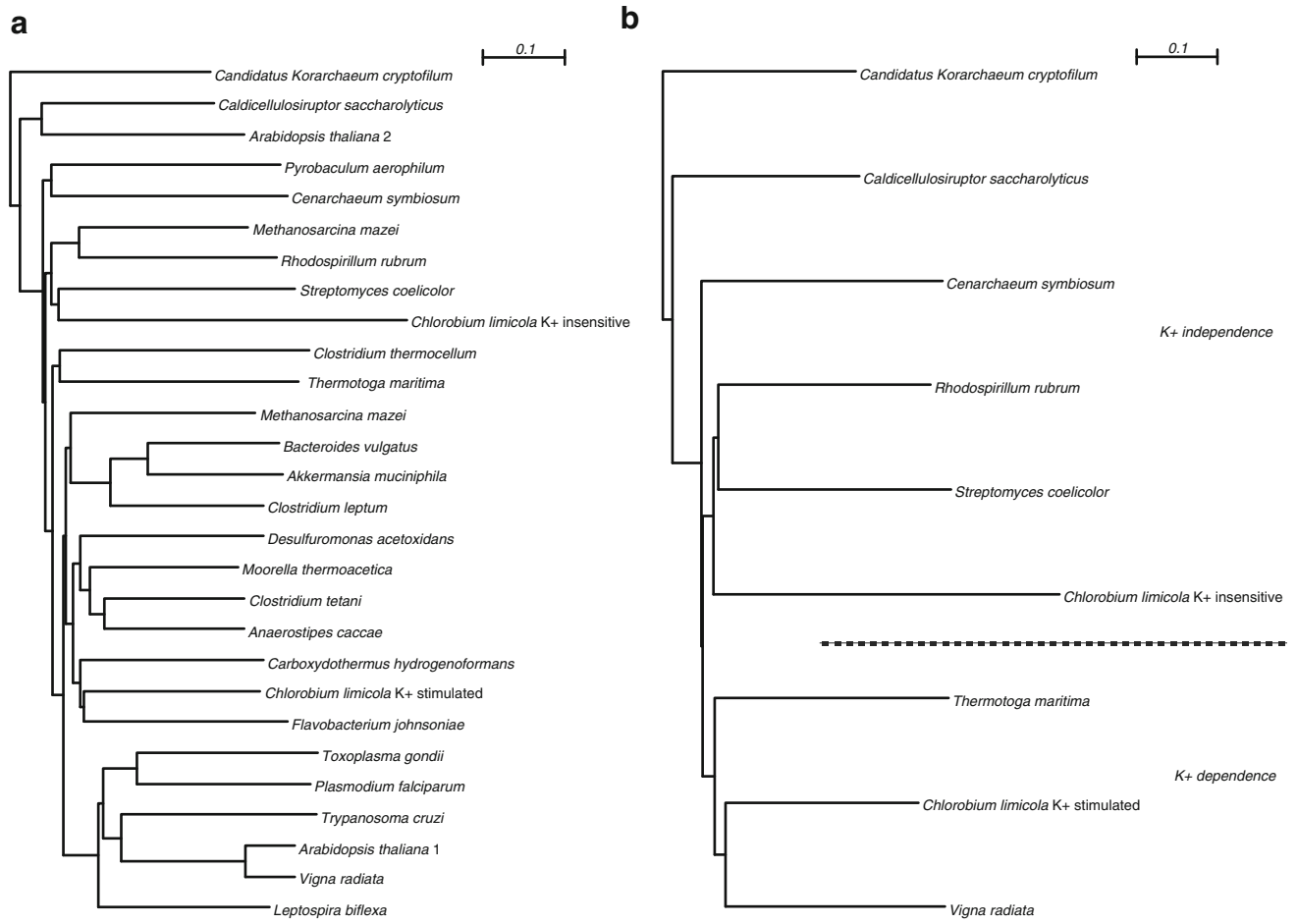

Fig. 1 Dendrograms of miPPases. The dendrograms are based upon multiple sequence alignments calculated using Mafft (Katoh and Standley 2013). In **a**, all sequences investigated are included, while in **b** only the nine selected representative miPPases. The *dashed line* separates the K^+-independent forms from the K^+-dependent ones. The *scale bars* represent 10 % difference in sequence identity

Table 1 Comparison of miPPase residues discriminating between different enzyme types (Hedlund et al. 2006)

Enzyme	Position							Assignment
Type 1 vs Type 2	197 LM vs FI	253 IV vs CS	266 EG vs VA	507 A vs K	510 G vs TA	521 VT vs AG	720 I vs PV	
Candidatus Korarchaeum cryptofilum	F2	C2	A2	K2	A2	A2	V2	Type 2
Caldicellulosiruptor saccharolyticus	F2	C2	A2	K2	T2	A2	V2	Type 2
Rhodospirillum rubrum	F2	C2	V2	K2	T2	G2	P2	Type 2
Streptomyces coelicolor	L1	C2	V2	K2	T2	A2	P2	Type 2
Chlorobium limicola K^+ insensitive	I2	S2	V2	K2	A2	G2	P2	Type 2
Cenarchaeum symbiosum	I2	A?	V2	K2	T2	A2	P2	Type 2
Thermotoga maritima	L1	V1	G1	A1	G1	A2	I1	Type 1
Chlorobium limicola K^+ stimulated	L1	V1	G1	A1	G1	T1	I1	Type 1
Vigna radiata	L1	I1	E1	A1	G1	V1	I1	Type 1

Type 1 is K^+ dependent and Type 2 is K^+ independent. For each of the seven discriminating positions, the occurring residue types together with type assignment [1 or 2, or unclear (?)] are shown. In all cases, a clear type 1 or type 2 assignment can be made. Positional numbers according to the *Streptomyces coelicolor* sequence (Hedlund et al. 2006)

a

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                          |---------------------------------|
R. rubrum         ARLGGGIFTKCADVGADLVGKVEAGIPEDDPRNPAVIADNVGDNVGDCAGMAADLFETYAV
K. cryptofilum    AQLGGGIYTKAADVGADLVGKVEAGIPEDDPRNPAVIADNVGDNVGDCAGRGADLFESISA
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b

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                          |---------------------------------|
K. cryptofilum    AQLGGGIYTKAADVGADLVGKVEAGIPEDDPRNPAVIADNVGDNVGDCAGRGADLFESISA
C. saccharol.     AQLGGGIYTKAADVGADLVGKVEAGIPEDDPRNPAVVADLVGDNVGDCAGRGADLFESTAA
R. rubrum         ARLGGGIFTKCADVGADLVGKVEAGIPEDDPRNPAVIADNVGDNVGDCAGMAADLFETYAV
S. coelicolor     MRVGGGIFTKAADVGADLVGKVEQGIPEDDPRNAATIADNVGDNVGDCAGMAADLFESYAV
C. lim. K+ins.    LRIAGGIFTKIADIGSDL-MKIVFKIKEDDARNPGVIADCTGDNAGDSVGPTADGFETYGV
C. symbiosum      IRIGGGIFTKAADMGADLVGKVEANIPEDDPRNPATIADNVGDNVGDAAGMGSDVYESYIV
T. maritima       DRVGGGVYTKAADMAADLVGKTELNLPEDDPRNPATIADNVGDNVGDVAGLGADLLESFVG
C. lim. K+stim.   ARVGGGIYTKAADVGADLAGKVYEGIPEDDPRNPATIADNVGDNVGDVAGMGADLFESYVG
V. radiata        GRVGGGIYTKAADVGADLVGKVERNIPEDDPRNPAVIADNVGDNVGDIAGMGSDLFGSYAE
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Fig. 2 **a** Multiple sequence alignment of miPPases. Alignment of the 57 residue conserved region (Hedlund et al. 2006), plus two flanking residues at both sides, of miPPases between *Rhodospirillum rubrum* and *Korarchaeum.* Identical residues are marked with *gray shading*. The first cysteine residue in the *R. rubrum* sequence has also been reported as glycine. **b** Multiple sequence alignment of the miPPases from Fig. 1. Residues in *gray* indicate identity with the *R. rubrum* sequence. Remarkable identity exists between the inner core of 35 residues, as is evident from Fig. 3. The 100% identity is here restricted to CKc and the photosynthetic bacterium *R. rubrum*. *C. saccharolyticus* and *V. radiata* show two differences and the five other compared sequences are somewhat more different

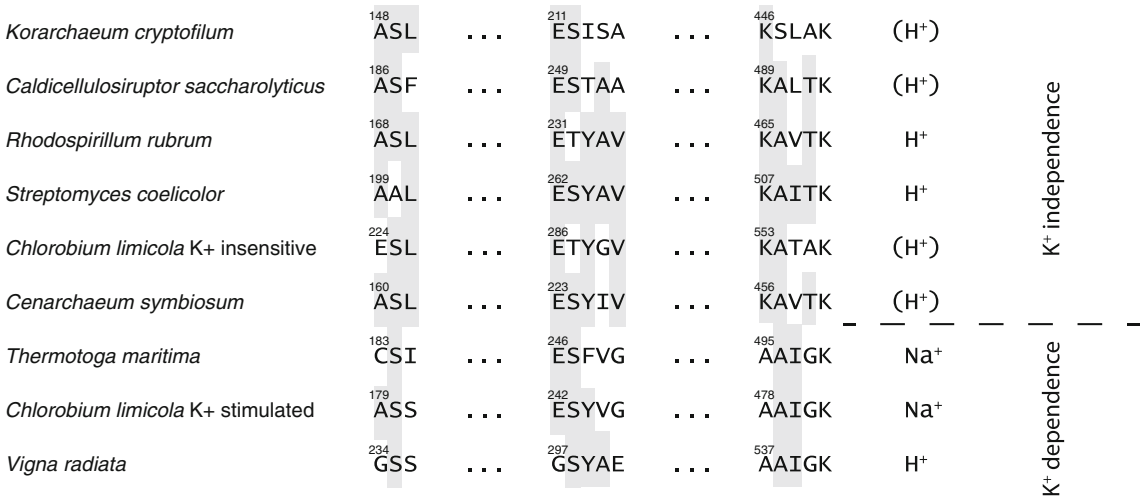

Fig. 3 Sequence motifs in the nine miPPases. The most frequent residue type at each position is shown against *gray background*. Positional numbers are given above the first residue of each motif. H^+ or Na^+ specificities are shown to the right where data from ion transport measurements are indicated without *parentheses* (Luoto et al. 2011)

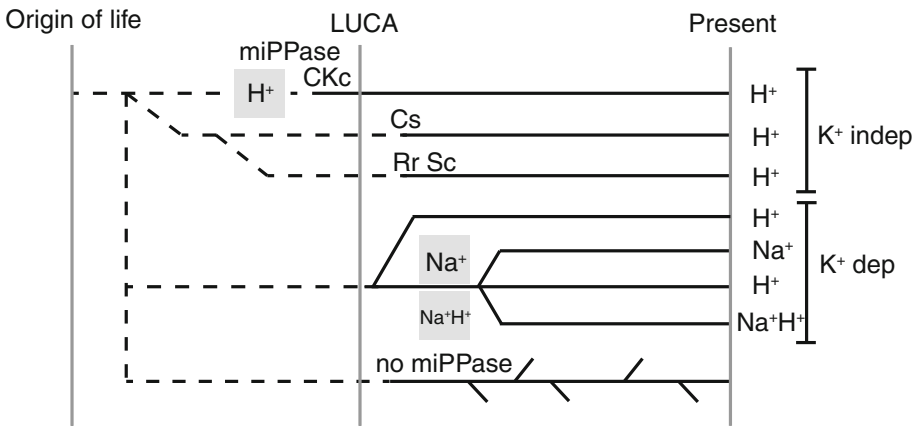

Fig. 4 A current picture of miPPase evolution. The *lines* indicate evolution from the origin of life via LUCA to the present. *Dashed lines* show plausible evolutionary links between different miPPases. The only assumed pre-LUCA form is that of CKc. *Gray boxes* indicate proton (H^+), sodium ion (Na^+), and sodium ion + proton (Na^+, H^+) pump specificity

pumping at low Na^+ concentrations (Luoto et al. 2013a). In the figure, the three alternatives of K^+-dependent forms are schematically shown as single lines, although multiple occurrences likely exist. The Na^+ pump seems to have evolved to H^+ pump multiple times (Luoto et al. 2011). The Na^+,H^+-PPases appear to have evolved independently (Luoto et al. 2013a), so we place them "neutrally" below Na^+ in the picture, until further information appears. The bottom line represents all the organisms which manage without miPPases.

Our current provisional indication that very early in evolution (starting before LUCA!) a H^+ pump may have preceded the Na^+ pump is in contrast to data from a somewhat later evolutionary phase (Mulkidjanian et al. 2008). It should soon become further elaborated after the very recent demonstration of still another variety of miPPases (Luoto et al. 2013a). The connection of this early H^+ pump with a current very detailed MiPPase evolutionary scheme (Baykov et al. 2013) is at present suggested to be located somewhere on the K^+-independent H^+-PPases side of the line between the Na^+-PPases and the K^+-independent H^+-PPases.

Discussion

The miPPase found in the CKc genome provides new information about very early energy-linked transport of ions across membranes. The fact that this miPPase shows the structural characteristics of a proton pump reopens the question of primacy for H^+ or Na^+ ions in early biological chemiosmotic energy transfer. The question of evolutionary primacy for PPi- or ATP-driven chemiosmosis may also be considered, as the plausible function of both in an apparently pre-LUCA organism is now established.

Central phosphate binding parts of the CKc miPPase primary structure is shown in Fig. 2b to be very similar to those of a proton pumping PPase of the extremophile bacterium *Caldicellulosiruptor saccharolyticus* (Bielen et al. 2010) and also to those of the other known proton pumping PPases. In particular, the CKc and the *C. saccharolyticus* enzymes show strong similarity to that miPPase subfamily which does not require K^+ ion for H^+-translocation (Luoto et al. 2011). Comparing those with the homologous PPases from the sub-families pumping Na^+ ion (Malinen et al. 2007), or both Na^+ ion and H^+ ion (Luoto et al. 2013b), increases the difference only slightly, reflecting the different specificities. Of particular significance seems the similarity in the Pi and PPi substrate-binding site with respect to the sequence placement of its 12 acidic residues (11 Asp and only 1 Glu). They provide an unusually acidic environment in the funnel-shaped Pi- and PPi-binding site in the first published 3-D structure of an miPPase (Lin et al. 2012). The high Asp content may well reflect an original-binding site for phosphate (perhaps with no Glu), as Asp in contrast to Glu is one of the four very early coded proteinaceous amino acids.

In contrast to the strong dominance of early Asp over later Glu in the evolutionary very early type acidic phosphate-binding site, Glu occupies important positions for the apparently later emerging ion pumping of the miPPases. It has already been found at two different positions in transmembrane helix 5 and also in transmembrane helix 6. The evolutionary aspect may be considered in connection with the increase from 6 over 10 or 12–16 transmembrane segments of the miPPase enzyme. Also, studies of the evolution of a designed retro-aldolase (Giger et al. 2013) led to the discovery of complete active site remodeling. This was interpreted to indicate such internal competition between alternative reaction sites in early enzyme evolution. The Glu relocations could well be exemplifying this prediction. It has been described that switching between Na^+ and H^+ specificities requires only subtle changes in the transporter structure (Luoto et al. 2013b).

Recently published 3-D properties of two different PPi-driven ion pump sub-families (Lin et al. 2012; Kellosalo et al. 2012), and the results with a third subfamily (Luoto et al. 2013b) pumping both H^+ and Na^+ ions, when compared with the amino acid sequence of CKc miPPase have given important novel information on structure–function connections. This includes the acidic PPi-binding site (with early Asp in great excess of Glu). It may indicate the early existence of an even more primitive (with only Asp and no Glu) and original pyrophosphate engine for providing what might have become the necessary energy transfer for the origin and early evolution of life.

An early emerging group of H^+-PPase is uniquely independent of K^+ ions. Their ion conductance channel connecting the PPi hydrolysis site to the other side of the membrane contains a putative gate formed by Asp–Lys for the K^+-independent H^+-PPase pump in more modest contrast to Asp-Lys-Glu for the K^+-dependent Na^+-PPase pumps. This suggests an evolution of very early Asp-rich H^+-PPases to later K^+-dependent Na^+-PPases with a Glu-containing gate. So we visualize here a pre-LUCA origin and early evolution of PPi-driven H^+-ion pumping, followed by an added gate Glu residue and K^+ requirement.

Membrane-integral PPases have been found in archaeae, bacteria, protists, and plants. The different subclasses of miPPases are remarkably similar to each other and according to present knowledge very different from all other proteins. Comparison of miPPases and PPi as energy donors with the corresponding ATP synthase and ATP as energy donor gives immediate attention to the relative simplicity of both enzyme and substrate of PPi and miPPase. The miPPase enzyme is a comparatively simple

duplex structure of a single subunit, whereas the well-known ATP synthase is a multi-subunit complex of usually eight different subunits. The greater complexity of ATP gives it the possibility to, for example, adenylate. This advantage in cellular metabolism is one obvious explanation for an early takeover of ATP metabolism from PPi metabolism. On the other hand, a reason for the continued, wide spread biological use of PPi as energy donor in many kinds of organisms would appear to be the lower content of free energy per mol (roughly 2/3) in PPi than the 35 kJ/mol in ATP. This difference has allowed PPi to operate successfully in, for example, different crisis situations. In this connection, it is tempting to ask the question, if the remnant PPi from adenylation reactions of ATP, really always only serves in a pulling hydrolysis reaction, with its content of free energy "lost" as heat.

We like to consider in some detail the possibility that an early takeover might have happened, by ATP, from an original PPi-based energy metabolism in connection with the origin and early evolution of life. Some very small similarity has been indicated with respect to high very early amino acid content of the active sites of both PPi and ATP synthesis (Hedlund et al. 2006). But any evolutionary path from miPPase to ATP synthase cannot be suggested at the present time. In contrast, any solution of this problem may well be expected to become a paradigm shift in this field. For the seekers of an answer to this fundamental evolutionary question, an achievement would be what Joshua Lederberg called an "anastrophic shift" (anastrophe is a new antonym to catastrophe) (Baltscheffsky 1997; Beskow and Baltscheffsky 2012).

Another anastrophic shift in the same area would be the in vitro construction of a Pi- and PPi-binding site, with activity, for example based on the acidic peptide funnel or pocket recently presented with the first detailed 3D picture of a H^+-pumping miPPase (Lin et al. 2012). Current progress in making various models with enzyme-like activities provide a background for hope that such expectations may become realized in a not too distant future (Farid et al. 2013).

Conflict of interest The authors declare that they have no conflict of interest.

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