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Identification of camelid specific residues in mitochondrial ATP synthase subunits

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Abstract ATP synthase is an enzyme involved in oxidative phosphorylation from prokaryotic to eukaryotic cells. In mammals it comprises at least 16 subunits from which the mitochondrial encoded ATP6 and ATP8 are essential. Mitochondrial genes variations have been suggested to allow rapid human and animal adaptation to new climates and dietary conditions (Mishmar et al. 2003). *Camelidae* taxa are uniquely adapted to extremely hot and dry climates of African-Asian territories and to cold and hypoxic environments of the South American Andean region. We sequenced and analyzed ATP6 and ATP8 genes in all camelid species. Based on the available structural data and evolutionary conservation of the deduced proteins we identified features proper of the group. In Old World camels the ATP8, important in the assembly of the F0 complex, showed a number of positively charged residues higher than in the other aligned species. In ATP6 we found the camelid specific substitutions Q47H and I106V that occur in sites highly conserved in other species. We speculate that these changes may have functional importance.

Keywords *Camelidae* · ATP6 and ATP8 · Specific residues · Environment adaptation

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Introduction

Mitochondrial DNA is a double-stranded circular molecule which contains coding genes for 2 rRNAs, 22 tRNAs, and 13 polypeptides. Polypeptides are all subunits of the oxidative phosphorylation (OXPHOS) enzyme complexes. In aerobic organisms, OXPHOS supplies most of the ATP needed for cell metabolism. During this process electrons from NADH or $FADH_2$ are transferred to O_2 by a series of electron carriers which pump protons through the inner mitochondrial membrane generating a proton gradient which drives ATP synthesis by the ATP synthase (complex V).

Complex V consists of two main structural domains: an intrinsic membrane domain (F0) and an extrinsic globular domain (F1), linked together by a central and a peripheral stalk (Walker and Dickson 2006).

The mammalian mitochondrial ATP synthase comprises at least 16 subunit types (Pedersen et al. 2000) from which the mitochondrial encoded ATP6 and ATP8 are essential subunits (Nijtmans et al. 1995). Recent research on human mitochondrial genes has revealed that ATP6 presents the highest amino acid variation, though in other species it is one of the most conserved mitochondrial proteins (Mishmar et al. 2003). On the other hand, De Giorgi et al. (1997) found that ATP8, which is one of the most conserved proteins in humans, is highly variable between distant species. In fact, amino acid similarity between sea urchin and sea star ATP8 peptides was shown to be two-fold higher than that observed between echinoderms and mammals, in spite of the divergence time between the formers (560 My) compared to that between vertebrates and invertebrates (600 My).

Recently, Mishmar et al. (2003) suggested some associations between mitochondrial protein variants and the adaptation of human ancestors to different regions of the

globe. For instance, a dramatic correlation between amino acid substitutions of particular mitochondrial genes and climatic zones has been described. Indeed, ATP6 gene variants that reduce the coupling efficiency of the electrochemical gradient to ATP synthesis would decrease ATP production but increase heat generation, a fact quite advantageous in extremely cold areas. Based on this theory, Mau et al. (2005) analyzed more than 1200 *Homo sapiens* ATP6 sequences and identified 25 variable amino acids probably representing the ATP6 residues specific for human adaptation.

Among Cetartiodactyl species, the *Camelidae* family is currently represented by two Old World camelid species: *Camelus bactrianus* (bactrian) and *Camelus dromedarius* (dromedary), and four South American species: *Lama guanicoe (*guanaco), *Lama glama (*llama), *Lama pacos (*alpaca) and *Vicugna vicugna (*vicuña*)*. *Camelidae* taxa are uniquely adapted to the extremely hot and dry climates of African-Asian territories and to the high altitude cold and hypoxic environment of the South American Andean region. Reports on the mitochondrial COX genes of all *Camelidae* have shown a significant increase of the evolutionary rate with respect to other related *Cetartiodactyl* species. Furthermore, a particular amino acid substitution close to the electron transference site has also been described for the two Old World camels (Di Rocco et al. 2006). These findings and their particular adaptation to such a wide range of environmental conditions make it interesting to focus on those mitochondrial genes encoding the respiratory chain proteins of *Camelidae.*

We report here the study of ATP6 and ATP8 mitochondrial genes and the inferred ATP6 and ATP8 amino acid sequence variations which might be associated to the adaptive evolution of these taxa.

Materials and methods

Genomic DNA was isolated from blood samples from South American camelid (SAC) guanaco, vicuña, llama, and alpaca, and the Old World bactrian and dromedary, as described in Bustamante et al. (2002). An entire fragment spanning ATP6 and ATP8 mitochondrial genes was amplified by PCR polymerase chain reaction using the flanking primers CTGCCTCTATATTATAAGCTCA forward and GTCATTAAAAGGGCTGAGAG reverse, designed on the mitochondrial sequence of guanaco (EU681954). For Old World camels, AGGGCTAGGATT CACTATGT reverse primer was designed on a conserved region of COXIII from all *Camelidae* species (Di Rocco et al. 2006). PCR reactions were carried out in 50 µl solution containing 15 ng genomic DNA, 10X PCR-buffer (10 X buffer 500 mM KCl, 200 mM Tris-HCl pH 8.4), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 U Taq polymerase (Invitrogen, Carlsbad, CA, USA), 0.35 µM of each primer. Cycling profile included an initial denaturation step at 94ºC for 3 min, followed by 35 cycles of 1 min at 94ºC, 1 min at 51ºC, 1 min at 72ºC, and a final 5 min extension at 72ºC.

Afterwards, PCR products were purified using the QIAquick PCR-purification kit (QIAGENE Inc., Valencia, CA, USA) and sequenced on both strands with Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were analyzed in an Automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequences obtained from each species were deposited in the GenBank under the following accession numbers: *Lama pacos*: EU195432; *Lama glama*: EU195433; *Lama guanicoe*: EU195434; *Vicugna vicugna*: EU195435; *Camelus bactrianus*: EU195436; *Camelus dromedarius*: EU195437, for ATP6 and *Lama pacos*: EU195438; *Lama glama*: EU195439; *Lama guanicoe*: EU195440; *Vicugna vicugna*: EU195441; *Camelus bactrianus*: EU195442; *Camelus dromedarius*: EU195443, for ATP8.

Multiple alignments of camelid deduced proteins with ATP6 and ATP8 sequences from other species *Bos taurus* (Cow) V00654; *Balaenoptera musculus* (blue whale) X72204; *Ovis aries* (sheep) NC001941; *Hippopotamus amphibius* (hippopotamus) AJ010957; *Equus caballus* (horse) AY584828; *Sus scrofa* (pig) AF034253; *Felis catus* (cat) U20753; *Ursus arctos* (brown bear) NC003427; *Muntiacus muntjak* (muntjak deer) AY225986; *Canis lupus lupus* (Eurasian wolf) NC009686; *Phoca vitulina* (harbour seal) NC001325; *Pan paniscus* (pygmy chimpanzee) NC001644, *Homo sapiens* (human) AY738947 and *Escherichia Coli* (NP290377) were carried out using Clustal X software (Thompson et al. 1997).

Results

The mitochondrial ATP8 and ATP6 genes from SAC and Old World camelid species were amplified and sequenced.

Alignment of these sequences with those from related cetartiodactyl species allowed us to identify the ATG and TAA triplets as the start and stop codons of both genes, except for dromedary which showed a GTG (Val) start codon in ATP6. Moreover, the translation products deduced from each ATP6 and ATP8 genes were found to contain 226 and 67 amino acids respectively, this latter being one amino acid longer than ATP8 of sheep and cow, and four amino acids longer than the whale protein. Alignment of the deduced protein sequences through the whole *Camelidae* taxa showed 21 polymorphic sites for ATP6 and 16 variable sites for ATP8. Similarity among camelid species for both proteins resulted higher than between camelids and

Table 1 Comparison of ATP6 amino acid sequence between *Camelidae* and other *Cetartiodactyl* species

Species	guan.	llama	alpaca	vicuña	bact.	drom.	pig	sheep	cow	whale
guanaco	-	4	2	1	17	20	31	33	37	50
llama	98.2	-	2	5	14	17	30	32	36	48
alpaca	99.1	99.1	-	3	15	18	29	31	35	49
vicuña	99.6	97.8	98.7	-	18	19	31	33	37	50
bactrian	92.5	93.8	93.4	92.0	-	6	29	29	35	47
dromedary	91.1	92.5	92.0	91.6	97.3	-	31	29	35	48
pig	86.3	86.7	87.2	86.3	87.2	86.3	-	34	39	47
sheep	85.4	85.8	86.3	85.4	87.2	87.2	84.9	-	15	51
cow	83.6	84.1	84.5	83.6	84.5	84.5	82.7	93.4	-	51
whale	77.9	78.8	78.3	77.9	79.2	78.8	79.2	77.4	77.4	-

Number of amino acid differences is shown above the diagonal and percent identity is below it.

Guan. = guanaco; bac.t = bactrian camel; drom. = dromedary

other cetartiodactyls. Within the South American group, identity level of the two proteins was similar, ranging between 97–99%, whereas identity between these and the Old World group was lower for ATP8. Comparison between camelids and other cetartiodactyls also showed less percent identity for ATP8 (Tables 1 and 2).

Characteristics of ATP6 and ATP8 proteins

Alignment of ATP6 camelid inferred proteins with other mammals sequences showed three main fully conserved segments between amino acids 88 to 101, 155 to 170 and at the COOH terminus (Fig. 1). Shorter conserved stretches are also observed along the sequences. On the other hand, amino acid substitutions in the camelid ATP6 protein were found in both variable and conserved residues from other mammals (Fig. 1). Within the latter, a I23V and I106V substitution was detected in the South American group, being the former also present in chimpanzee and the muntjak deer. At 47 position, which is totally conserved in all aligned species, SAC and dromedary showed a Q47H substitution while Q47Y was shown by the bactrian. The I195V replacement is shared by all camelid species and also present in the bear. Moreover, camelids harbor a non polar A residue at 81 position which in the other mammals is occupied by S or T polar amino acid.

Further alignment of camelid ATP6 with the E. coli homologous subunit *a* indicated that H47 and V106 residues from the former are equivalent to E. coli E80 and L155, respectively (Fig. 2). The 80 residue which is negatively charged in E. coli, is replaced by a Q conserved uncharged residue in most mammals and by an H polar charged amino acid in SAC and dromedary. In E. coli this residue is located within one of the two cytoplasmic loops

Table 2 Comparison of ATP8 amino acid sequence between *Camelidae* and other *Cetartiodactyl* species

Species	guan	llama	alpaca	vicuña	bact.	drom.	cow	sheep	pig	whale
guanaco	-	1	1	1	11	12	18	19	19	19
llama	98.5	-	2	2	12	13	18	19	19	18
alpaca	98.5	97	-	2	12	11	19	20	20	20
vicuña	98.5	97	97	-	12	13	19	20	20	20
bactrian	83.6	81.8	81.8	81.8	-	7	23	21	21	18
dromedary	81.8	80.3	83.6	80.3	89.4	-	22	22	21	18
cow	72.7	72.7	71.2	71.2	65.1	66.7	-	7	25	21
sheep	71.2	71.2	69.7	69.7	68.7	66.7	89.4	-	25	19
pig	71.6	71.6	70.1	70.1	68.7	68.7	62.1	62.1	-	21
whale	69.8	71.4	68.2	68.2	71.4	71.4	68.7	71.2	68.7	-

Number of amino acid differences is shown above the diagonal and percent identity is below it.

Gua. = guanaco; bact. = bactrian camel; drom. = dromedary

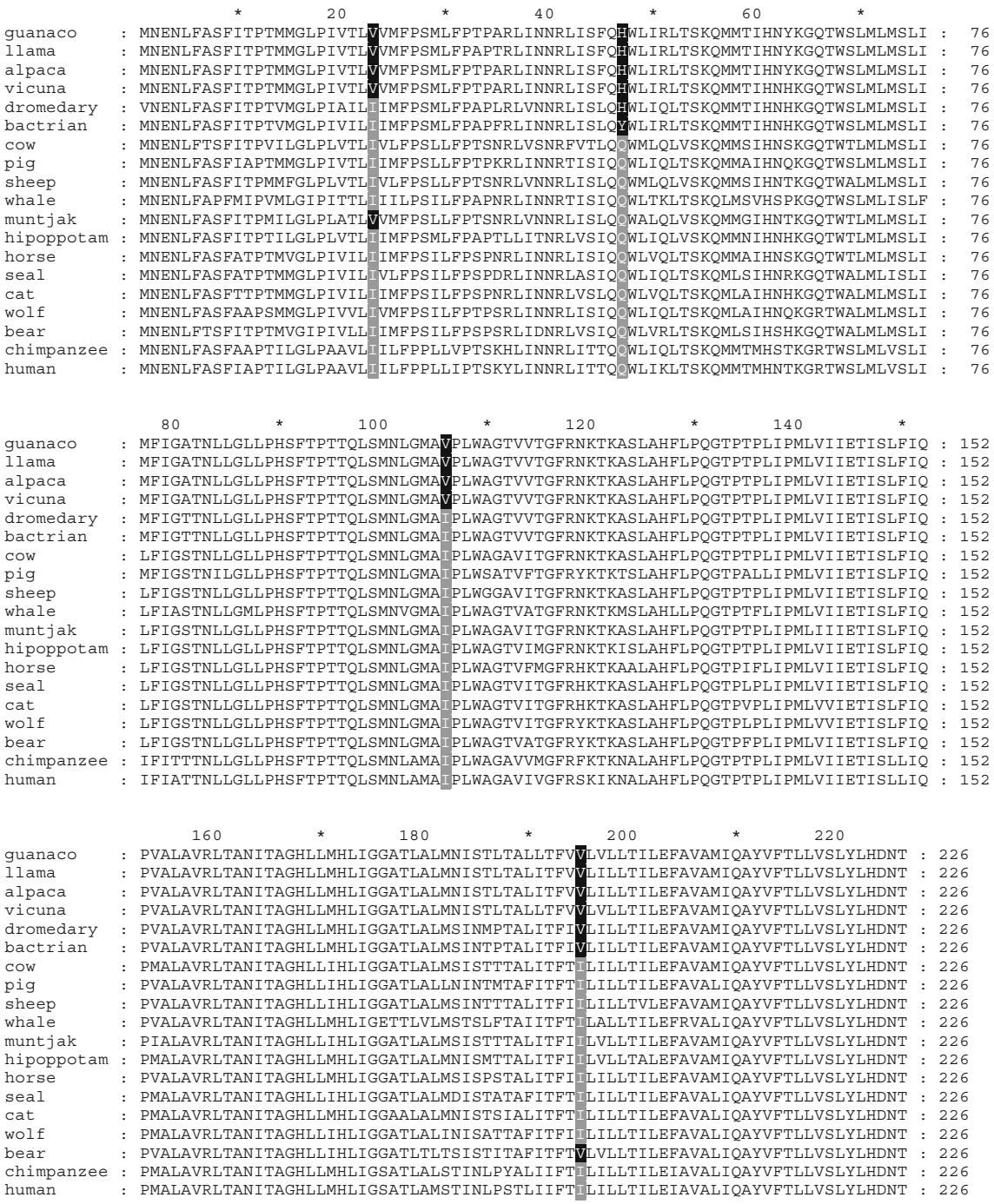

Fig. 1 Alignment of ATP6 subunit from different species. Camelid amino acid substitutions in conserved sites are highlighted in black

of the ATP6 protein (Deckers-Hebestreit et al. 2000). Non polar characteristics at the 106 site are conserved from mammals to bacteria, being in SAC occupied by V. This residue is located within the transmembrane α-helix 2, as predicted by the homology modeling described by Sgarbi et al. (2006).

As for ATP8, we observed only 20 conserved residues in all aligned species. Among these, the N termini motif MPQL(X4)W has been reported to be evolutionarily conserved among mammals (De Giorgi et al. 1997). Several lysine residues concentrated towards the C-terminus of the sequence were also present in camelids. Bactrian and dromedary camel protein showed between 40–49 positions a number of positively charged amino acids higher than in any other of the aligned mammals. In fact, camels exhibit four lysines and one arginine within this segment, meaning additional charges over the number registered for the rest of the aligned species (Fig. 3).

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                    80         *       100         *       120         *       140
guanaco    : ---RLISFQHWLIRLTSKQMMTIHNYKGQTWSLMLMSLIMFIGATNLLGLLPHSFT-------------- :  93
llama      : ---RLISFQHWLIRLTSKQMMTIHNYKGQTWSLMLMSLIMFIGATNLLGLLPHSFT-------------- :  93
alpaca     : ---RLISFQHWLIRLTSKQMMTIHNYKGQTWSLMLMSLIMFIGATNLLGLLPHSFT-------------- :  93
vicuna     : ---RLISFQHWLIRLTSKQMMTIHNHKGQTWSLMLMSLIMFIGATNLLGLLPHSFT-------------- :  93
dromedary  : ---RLISLQHWLIQLTSKQMMTIHNHKGQTWSLMLMSLIMFIGTTNLLGLLPHSFT-------------- :  93
bactrian   : ---RLISLQYWLIRLTSKQMMTIHNHKGQTWSLMLMSLIMFIGTTNLLGLLPHSFT-------------- :  93
E.coli     : VPGKFQTAIHLVIGFVNGSVKDMYHGKSKLIAPLALTIFVWVFLMNLMDLLPIDLLPYIAEHVLGLPALR : 140

                   *       160         *       180         *       200         *
guanaco    : --PTTQLSMNLGMAVPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
llama      : --PTTQLSMNLGMAVPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
alpaca     : --PTTQLSMNLGMAVPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
vicuna     : --PTTQLSMNLGMAVPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
dromedary  : --PTTQLSMNLGMAIPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
bactrian   : --PTTQLSMNLGMAIPLWAGTVVTGFRNKTKASLAHFLPQG--TPTPLIPMLVIIETISLFIQPVALAVR : 159
E.coli     : VVPSADVNVTLSMALGVFILILFYSIKMKGIGGFTKELTLQPFNHWAFIPVNLILEGVSLLSKPVSLGLR : 210
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Fig. 2 Alignment of partial amino acid sequence of camelid ATP6 with *E. Coli*. Residues 80 and 155 of *E.coli* and their equivalent camelid amino acids are highlighted in grey

Discussion

Knowledge of the structure and assembly of the ATP synthase subunits has grown largely in recent times (Abrahams et al. 1994; Boyer 1999; Walker and Dickson 2006; Strauss et al. 2008). Although these processes are not fully understood, the close similarity between the structures shown by prokaryotes, lower eukaryotes, and mammalian cells suggests an analogous assembly mechanism for at least the F_1 domain (Houštěk et al. 2006). The F_0 domain shows additional difficulties because of the increased evolutionary complexity of its structure from bacteria to humans. Therefore, the sequencing and alignment of new species with sequences available at databases have provided valuable information on the structure and function of these protein subunits (Hong and Pedersen 2004).

The potential role of the respiratory chain mitochondrial proteins in species adaptation to different environmental and dietary conditions through evolution has been recently discussed (Mishmar et al. 2003; Ruiz-Pesini et al. 2004; Xu et al. 2005)

In the search of particular features which may correlate the outstanding adaptation of Camelidae to extreme climates and high altitude hypoxia, we provide here the complete sequence of ATP6 and ATP8 mitochondrial genes from both New and Old World camelid species, and the alignment of the deduced protein sequences with those from other mammals.

In agreement with the close relationship between SAC and camels previously demonstrated (Randi et al. 1996; Montgelard et al. 1997), we found that ATP6 and ATP8 percent identity among camelids is higher than that with other cetartiodactyl species.

Recent studies on human mitochondrial DNA have revealed that ATP6 is highly variable among individuals from Artic and Subartic zones (Mishmar et al. 2003).This fact has been associated with the human adaptation to extremely cold habitats. Further assays in human ATP6 identified 25 variable amino acids which were well conserved in other

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                   *       20          *       40          *       60
guanaco     : MPQLDTSTWFITILSMLMTLFILFQLKLSKHIYYSTPEPKFSKTHKQNTPWETKWTKIYLPLLLPQQ- : 67
llama       : MPQLDTSTWFITILSMLMTLFILFQLKLSKHIYYPTPEPKFSKTHKQNTPWETKWTKIYLPLLLPQQ- : 67
alpaca      : MPQLDTSTWFITILSMLMTLFILFQLKLSKHIYYSTPEPKFSKTHKQNTPWETKWTKIYLPLLLPRQ- : 67
vicuña      : MPQLDTSTWFITILSMLMTLFILFQLKLSKHIYYSTPEPKFSKTHNQNTPWETKWTKIYLPLLLPQQ- : 67
dromedary   : MPQLDTSTWFITILSMLVTLFVLFQLKISKHIYPSDPSPKSNKTRKQKAPWETKWTKIYLPLLLPLQ- : 67
bactrian    : MPQLDTSTWFITILSMLMTLFVLFQLKISKHMYLSAPSPKSNKTRKQKTPWETKWTKIYLPLSLPQQ- : 67
cow         : MPQLDTSTWLTMILSMFLTLFIIFQLKVSKHNFYHNPELTPTKMLKQNTPWETKWTKIYLPLLLPL-- : 66
pig         : MPQLDTSTWFITITSMIMTLFILFQLKISNYSYPASPESIELKTQKHSTPWEMKWTKIYLPLLLPPR- : 67
sheep       : MPQLDTSTWLTMILSMFLVLFIIFQLKISKHNFYHNPELMTTKTPKQNTPWETKWTKIYLPLSLPL-- : 66
whale       : MPQLDTSTWLLTILSMLLTLFVLFQLKISKHSYSPNPKLVPTKTQKQQTPWNITWTKIYLPLL----- : 63
muntjak     : MPQLDTSTWLIMIMSMFLVLFIIFQLKISKHNFYFNPETLPTKAQKQNTPWETKWTKIYLPLLLPQ-- : 66
hipoppotam  : MPQLDTSTWFTTILSMFLTLFIIFQLKISKHTYHPNPETTLPMTQKQPTPWETKWTKIYSPLSLPLQS : 68
horse       : MPQLDTSTWFINIVSMILTLFIVFQLKISKHSYPTHPEVKTTKMTKHSAPWESKWTKIYSPLSLPQQ- : 67
seal        : MPQLDTSTWLIMILSMILTLFITFQLKVSKHYFPTNPEPKHTPLLKNSAPWEEKWTKIYSPLSLPLQ- : 67
cat         : MPQLDTSTWSITIMSMIMTLFIVFQLKISKYLYPSNPEPKSMTTLKQFNPWEKKWTKIYSPLSLPQQ- : 67
wolf        : MPQLDTSTWFIMIFSMFLTLFILFQLKISNHYYPENPMTKSAKIAGQHNPWENKWTKIYSLLSLPPQ- : 67
bear        : MPQLDTSTWSITILSMALTLFIALQLKVSKYKYPETPEPKSLSSLKKLVPWEEKWTKIYLPLLSPQR- : 67
orangutan   : MPQLNTTTWLTVITPTLLALFLITQLKLLNSHLHPPTPPKFTKTKPHAKPWELKWTKIYSPHSLPPQS : 68
chimpanzee  : MPQLNTAVWPTTITPMLLTLFLITQLKMLNSNYHLPPSPKPMKMKNYNKPWEPKWTKICSLHSLPPQS : 68
human       : MPQLNTTVWPTMITPMLLTLFLITQLKMLNTNYHLPPSPKPMKMKNYNKPWEPKWTKICSLHSLPPQS : 68
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Fig. 3 Alignment of ATP8 subunit from different species. Camelid amino acid substitutions in conserved sites are highlighted in black

primates (Mau et al. 2005), suggesting that these residues were specific for human adaptation. Interestingly, our search for ATP6 showed that among these residues, Q47 and I106 were also replaced in camelids.

Since there is no available high-resolution structure of the F_0 sector in mammals and its functional information is scarce, knowledge on these aspects is based on studies on bacterial or yeast systems (Vik and Antonio 1994; Stock et al. 1999). Bacterial subunit *a* and the corresponding eukaryote subunit ATP6 are believed to have two functions: to rotate the ring of *c* subunits and conduct the protons to the opposite side of the membrane (Sgarbi et al. 2006). Residues in E. coli, which are equivalent to mammal conserved 106 and 47 amino acids, are located in the transmembrane helix 2 of the ATP6 and in a cytoplasmic loop. The replacement in the transmembrane helix is conservative since it involves a change of L to V from E. coli to camelids. The second substitution occurs in a site equivalent to *E. coli* 80 glutamic residue of the cytoplasmic loop. This amino acid, highly conserved among bacteria and chloroplasts, has been shown to become deleterious when substituted by a positively charged Lys, probably by producing assembly defects (Vik and Ishmukhametov 2005). Although the cytoplasmic loop is not involved in proton translocation, it probably makes contact with one or both *b* subunits. If these residues were equivalent in bacteria and mammals as we inferred from the alignment, the presence of a positively charged H in the camelid protein might have some effect too.

We did not identify specific residues of SAC within ATP8, though we observed particular features of eventual functional importance in dromedary and bactrian. The C-terminus in these species contains a number of positively charged amino acids higher than in the C-terminus of other species. Studies on *Saccharomyces cerevisiae* mutants showed that the C-terminal positively charged region of subunit 8 is required for the efficient assembly of the F0 sector. The presence of this positively charged amino acid region in many different systems also supports their role in the assembly of the complex (Grasso et al. 1991).

Further studies contributing to the knowledge of the interactions, structure, and function of ATP6 and ATP8 mitochondrial subunits in mammals will be necessary to understand the role of any of these proteins in camelid adaptation to desert and hypoxic environments.

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