Rapid electrostatic evolution at the binding site for cytochrome c on cytochrome c oxidase in anthropoid primates

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Cytochrome c (CYC) and the subunits of CYC oxidase (COX) are mitochondrial-functioning proteins that play a central role in aerobic energy production. By catalyzing the transfer of electrons from CYC to oxygen, COX greatly increases the electrochemical gradient used for ATP synthesis. Modeling three-dimensional structural data on CYC and COX reveals that 57 of the >1,500 COX residues can be implicated in binding CYC. Because of the functional importance of the transfer of electrons to oxygen, it might be expected that natural selection would drastically constrain amino acid replacement rates of CYC and COX. Instead, in anthropoid primates, although not in other mammals, CYC and COX show markedly accelerated amino acid replacement rates, with the COX acceleration being much greater at the positions that bind CYC than at those that do not. Specifically, in the anthropoid lineage descending from the last common ancestor of haplorhines (tarsiers and anthropoids) to that of anthropoids (New World monkeys and catarrhines) and that of catarrhines (Old World monkeys and apes, including humans), a minimum of 27 of the 57 COX amino acid residues that bind CYC were replaced, most frequently from electrostatically charged to noncharged residues. Of the COX charge-bearing residues involved in binding CYC, 11 of 22 have been replaced with uncharged residues. CYC residues that interact with COX residues also frequently changed, but only two of the CYC changes altered charge. We suggest that reducing the electrostatic interaction between COX and CYC was part of the adaptive evolution underlying the emergence of anthropoid primates.

Abbreviations: CYC, cytochrome c; COX, cytochrome c oxidase; ES, electrostatically significant; nDNA, nuclear DNA.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY236506, AY585857, AY585861, AY585862, AY585864, and AY918493–AY918495).

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Materials and Methods

DNA Sequences. RNA was prepared from either heart, kidney, lung, or liver tissues, and isolated with either a phenol-chloroform method (28) or with the Qiagen Midi kit. Some RNAs were obtained from the Center for Reproduction of Endangered Species, San Diego. RACE PCR (29) was performed on these RNAs, and the products were either sequenced directly or cloned into the pGEM-T easy vector (Promega) and sequenced. CYC was amplified for sequencing by PCR from genomic DNA. Additional sequences are from previous studies (1, 4, 6, 8, 10–12, 17) and from GenBank (see Tables 3 and 4, which are published as supporting information on the PNAS web site). Alignments of inferred amino acid sequences were performed with CLUSTALX, version 1.81 (30). Insertions relative to the bovine amino acid sequence were removed from the data set to avoid speculation as to the three-dimensional position of amino acids not included in the known crystal structures. COX8L was substituted for COX8H because COX8H is absent in catarrhine primates (11).

Binding-Site Residues. COX residues that form the CYC binding site were extracted from these alignments by two criteria: that they lie on the surface of COX and that an atom of a surface COX residue side chain lies within 10 Å of any CYC atom when CYC is bound to COX (15). Surface residues were determined with the program GETAREA 1.1 (31) by using a radius for water of 1.4 Å. Polar interactions of up to 8.5 Å have been hypothesized in the docking model of CYC on COX (15). We have added 1.5 Å to allow for the possibility of longer range indirect interactions as well as minor conformational changes. Using a more restricted distance between amino acid side chains does not affect the conclusions presented here, although fewer residues would be included.

ES Residue Positions and ES Changes. If a positively charged (Arg or Lys) or negatively charged (Asp or Glu) residue in the binding site resides occurred among the taxa examined, that position was treated as an ES position. Among ES positions, ES changes were amino acid replacements that changed electrostatic state, there being three states: negatively charged, noncharged, and positively charged.

Evolutionary Analyses. Relative rate tests comparing tarsier (a nonanthropoid primate) COX to human or other anthropoid primate COX were performed by using Tajima’s (32) method in MEGA 2.1 (33). Results obtained by using cow, pig, rat, or mouse as the outgroup did not substantially change the results. This analysis excluded COX5B, COX6A, and COX7B, for which the tarsier sequence was unavailable. Placement of amino acid replacements in the evolutionary history of primates was accomplished by using generally accepted phylogenetic relationships among primates (34) and among eutherian mammals (35, 36). By using this phylogeny, the number and location of changes along lineages were estimated in two ways: the most parsimonious solutions and Bayesian solutions as implemented in MRBAYES 2.0.1 (37) using the Equalin amino acid replacement option. This analysis excluded the eight binding site residues of COX6A. For human, these residues differ from the other mammals in the data set by only one non-ES residue. Dates

![Fig. 1. COX residues at the binding site of CYC. (A) The position of the docked CYC (shown in red). (B) Residues that form the binding site for CYC on COX. Green residues are those that have been neutralized in anthopoid primate evolution, yellow residues are those that have changed from neutral to positive, and gray residues are the remainder of residues that form the binding site. (C) Alignment of residues at the binding site. The point of electron transfer (COX2W104) is shown highlighted in orange. Residues 119 and 158, involved in salt bridge formation (15), are shown in boldface.](https://www.pnas.org/content/102/11/6380.short)
of divergence for calculating amino acid replacements over time were taken from generally accepted sources (34–36). CYC coding sequences were aligned and ancestral nodes were reconstructed by maximum likelihood using TrN+F model parameters as chosen by both hierarchical likelihood ratio test and Akaike Information Criterion by MODELTEST (38). Inferred ancestral sequences were then translated to protein.

Complete mitochondrial genomes were used to obtain the mtDNA docking site residues in a range of tetrapods. One representative species was sampled for each available tetrapod order. African lungfish (Protopterus dolloi) and coelacanth (Latimeria chalumnae) were used as outgroups. Accession numbers and taxon names are given in Table 4. A phylogenetic tree representing the ordinal relationships among the species was used to infer amino acid changes during descent of the tetrapods. Phylogenetic relationships were taken from previously published studies (35, 36, 39–42). Inferred amino acid changes were reconstructed with DELTRAN in a maximum parsimony framework by using PAUP* 4.0b10 (43). This same procedure was used to infer amino acid changes in available complete mtDNA genomes for primates.

Results

Definition and Evolution of the Binding Site. We have identified 57 residues of COX that are likely to interact with CYC (Fig. 1). These binding site residues, i.e., the COX residues on which CYC docks, include 33 mtDNA-encoded residues (10 COX1, 20 COX2, and 3 COX3 residues) and 24 nDNA-encoded residues (eight COX6A, nine COX6B, five COX7A, one COX7C, and one COX8 residue). Results of relative rate tests for these residues indicate a statistically significant anthropoid rate acceleration relative to the most closely related nonanthropoid, tarsier (44, 45), for the entire COX monomer, for the 57 residues that are the binding site, and for residues that are not part of the binding site (Table 1). However, binding site residues have sustained the most pronounced rate acceleration, undergoing over four times the number of changes per amino acid than have amino acids that are not part of the binding site (0.43 vs. 0.10 for human vs. tarsier, respectively).

Table 2. Relative rates of change to the residues of COX near CYC when docked

<table>
<thead>
<tr>
<th>Region</th>
<th>Outgroup</th>
<th>Residues</th>
<th>A</th>
<th>B</th>
<th>P</th>
<th>Replacements/Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full monomer</td>
<td>Human</td>
<td>Tarsier</td>
<td>Cow</td>
<td>1,541</td>
<td>164</td>
<td>28</td>
</tr>
<tr>
<td>Nondocking residues</td>
<td>Human</td>
<td>Tarsier</td>
<td>Cow</td>
<td>1,492</td>
<td>143</td>
<td>27</td>
</tr>
<tr>
<td>Docking residues</td>
<td>Human</td>
<td>Tarsier</td>
<td>Cow</td>
<td>49</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OWM</td>
<td>Tarsier</td>
<td>Cow</td>
<td>49</td>
<td>23</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NWM</td>
<td>Tarsier</td>
<td>Cow</td>
<td>49</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Tarsier</td>
<td>Cow</td>
<td>Mouse</td>
<td>49</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Nondocking, ES</td>
<td>Human</td>
<td>Tarsier</td>
<td>Cow</td>
<td>252</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Docking, ES</td>
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<td>Tarsier</td>
<td>Cow</td>
<td>20</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Docking, non-ES</td>
<td>Human</td>
<td>Tarsier</td>
<td>Cow</td>
<td>29</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

A and B refer to lineages being compared relative to the indicated outgroup. OWM, Old World monkeys; NWM, New World monkeys.
Electrostatic Changes. Because binding CYC to COX predominately involves an electrostatic interaction (15), the expectation is that ES residues are functionally important and, thus, under the scrutiny of natural selection but not always the purifying form. We observed that the rate acceleration in anthropoids is most pronounced at ES residue positions. For example, as may be inferred by parsimony from an interspecies alignment of residues at the binding site (Fig. 1C), there are a minimum of 27 changes (31 changes by Bayesian inference) from the earlier eutherians (cetartiodactyl–rodent–primate ancestor) to humans, of which 13 are at 22 ES residue positions (59%), compared with 14 changes at 35 non-ES residue positions (31%).

Of these 13 ES changes, 11 were from charged to neutral, thus reducing the number of charged residues at the CYC binding site. Only two ES changes were from neutral to charged (Table 2). This level of ES change to COX residues near the CYC binding site is unique in vertebrates examined to date. For example, the amino acid sequences from cow, pig, rat, mouse, and tarsier do not have ES differences. The ES changes in anthropoids appear to reduce electrostatic interaction between CYC and COX: The seven changes from negatively charged to neutral residues are located on the mitochondrially encoded subunits that face the positively charged residues of docked CYC, whereas the four changes from positively charged residues to neutral are to nuclear-encoded residues near the negatively charged pole of docked CYC. Overall, ES changes are over six times more common at the binding site than in other regions of COX (0.65 vs. 0.10 on the human lineage) (Table 1), showing that the high rate of ES change at the CYC binding site does not arise from an overall high rate of ES change to COX.

The public availability of a large comparative body of mitochondrial genome sequence data (Table 4) allows us to further demonstrate that ES changes rarely occur among mammals and other tetrapods (Fig. 2). Note that the negatively charged surface of the CYC-binding site of COX in most orders of amphibians and mammals has remained at the net electrostatic charge of $-\varepsilon_{\text{H}}$. However, in the catarrhine primates (Fig. 3), this net charge was reduced to $-\varepsilon$. Except for birds, which show a limited charge reduction in the mitochondrially coded COX residues that bind CYC, no other clade of tetrapods as represented by the 45 species examined in 45 orders (Fig. 2) show such a dramatic reduction of
This pattern of accelerated rates followed by decelerated rates is descendent lineages, have been maintained by purifying selection. Ancestral lineages were advantageous and positively selected and, in stem catarrhine lineages but has been slow in more recent lineages.

Residues at the CYC binding site is increased in stem anthropoid and stem catarrhine lineages. Moreover, 12 of the replacements were at positions that are within 10 Å of a COX ES position when CYC is docked (Table 5; see also Table 6, which is published as supporting information on the PNAS web site). Strikingly, unlike the COX changes, in most instances no change charge is found in CYC. The exceptions are a D to A replacement at CYC-50 on the catarrhine lineage, nearest to a gain of a positive charge (H to R) in COX (Table 2), and a G to E replacement at CYC-89 on the catarrhine lineage, near an S to T change in COX (Table 5; see also Table 6, which is published as supporting information on the PNAS web site).

Evolutionary Analysis. Amino acid replacements in the CYC-binding site of COX have occurred predominantly on three lineages (Fig. 4): the stem lineage of anthropoids (platyrhines and catarhines), the stem lineage of catarhines (Old World monkeys and apes, including humans), and in platyrhines (New World monkeys). However, the ES changes occurred only in the stem anthropoids and stem catarhines. In the lineage encompassing the stem anthropoids and the stem catarhines there were, according to the Bayesian analysis, 29 changes from the most recent common ancestor of tarsier and anthropoids (~58 million years ago) to the most recent common ancestor of Old World monkeys and apes (~25 million years ago), whereas zero and four changes have occurred to the ape and Old World monkey lineages, respectively.

The observation that the amino acid replacement rate of COX residues at the CYC binding site is increased in stem anthropoid and stem catarhine lineages but has been slow in more recent lineages (Fig. 4) is consistent with the hypothesis that the changes in the ancestral lineages were advantageous and positively selected and, in descendent lineages, have been maintained by purifying selection. This pattern of accelerated rates followed by decelerated rates is inconsistent with the alternate hypothesis that the amino acid replacement rate increase is due to a relaxation of functional constraints.

Discussion

In cross-species experiments, Osheroff et al. (23) found overly tight binding of human CYC on bovine COX and greatly reduced steady-state reaction kinetics. They found less tight binding and much higher steady-state reaction kinetics when either horse CYC or slow loris CYC docked on bovine COX. In this study, we have conducted and is shown in Figs. 5 and 6, which are published as supporting information on the PNAS web site.

Fig. 3. Evolution of the 33 mitochondrially encoded COX residues that putatively bind CYC in primates. (Left) Phylogenetic relationships among different species of primates (33). (Center) Number of amino acid replacements in the mtDNA-encoded portion of COX that binds CYC. Bars indicate the total number of DELTRAN-inferred amino acid changes on each terminal lineage from the ancestral crown primate to the terminal species; the number of inferred ES changes in the total number of changes are indicated by black fill in the bar graph. (Right) Docking site total charge. The vertical line represents the inferred total charge of the crown primate ancestor; filled ovals are the inferred total charge of the indicated primate species.

Fig. 4. Rates of amino acid replacement by lineage, showing the increase in the amino acid replacement rate in ancestral (i.e., stem) anthropoid primates, followed by a decrease in the amino acid replacement rate in descendent anthropoid primate lineages. (A) Vertical bars depict the number of amino acid replacement per amino acid residue per billion years (BY). Gray bars depict the rates for the 57 binding site residues, and the rates for the 1,578 nonbinding site residues are shown in white. (B) Changes to COX at the CYC-binding site as determined by Bayesian analysis. The numbers of inferred changes to the binding site residues are shown above the branches (ES changes are shown in parentheses), and the numbers of nonbinding site changes are shown below the branches.

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mammalian COX than to catarrhine COX and that the nonan-
thropoids lack the extensive ES changes found in catarrhines. Never-
thless, changes in reaction kinetics do not appear to result 
excursively from ES changes to the CYC-binding site of COX. The 
activity of these COX with rhesus monkey COX (23) is only about half 
the activity of human CYC with rhesus monkey COX (23), yet the 
electrostatic interactions in both must be similar. These data further 
suggest that nonelectrostatic changes to anthropoid CYC also affect 
the reaction kinetics between CYC and COX.

As noted, for the extensive charge neutralizations found at the 
COX/CYC interaction site, the reduction in electrostatic attraction 
is carried out almost wholly by COX residues. Chemically signifi-
cantly changes to CYC residues, such as charge alterations, may 
be constrained because CYC has the known additional roles of inter-
acting with the mitochondrial bc1 complex and with apoplastic protease activating factor 1. Despite these constraints, apparently 
evolving changes to CYC are seen and appear to compensate for 
COX charge changes in a more subtle way. Examination of the 
COX/CYC interacting surfaces suggests that at least some of the 
paired substitutions increase hydrophobic contacts near the site of 
electron transfer (for example V→I in CYC-11 paired with 
D→YF in COX2-127; Table 2). Although the effects of altering 
the hydrophobic surface will need to be addressed experimentally, 
they are likely to affect CYC’s on rate and off rate and the rate of

Are changes to the CYC-binding site of COX the only accel-
tered change in the ancestry of anthropid primates? Results of the 
relative rate tests (Table 1) indicate that there is a statistically 
significant rate acceleration in the nonbinding site residues of COX 
during anthropid evolution as well. Additional changes to other 
regions of COX are not surprising, because the primary function of 
the nuclear-encoded subunits is thought to be regulatory in nature 
(46, 47). If so, changes in the regulatory scheme would be called for 
to accommodate a significant shift in enzyme kinetics.

We suggest that the accelerated amino acid replacement rates of 
COX and CYC in the stem anthropid and stem catarrhine lineages

were coadaptive and part of the organismal evolution out of which 
emerged a constellation of new phenotypic features. Two corre-
lated features linked to the mitochondrial electron transport chain 
are enhanced life spans and greatly enlarged brains. Considerable 
evidence (48, 49) has aging and diseases of old age associated with 
damage from mitochondrial-generated radical species, i.e., delaying 
the onset of excessive damage should extend life spans. A possibility 
worth exploring is that an effect of the marked changes in the 
electron transport chain proteins of anthropoids was to reduce the 
radical flux which accompanies oxidative energy production. Brain 
tissues have potentially the greatest stake in changes to energetically 
important proteins. Although the human brain accounts for only 
≈2% of the adult human body weight, it utilizes ≈16% of the total 
oxogen consumed (50) and as much as 65% during fetal develop-
ment (51). Most of the energy demand in a neuron is oxidative (52). 
Moreover, many deleterious mutations in mitochondrial genes are 
associated with neuropathological conditions (53). Thus, we suggest 
that there is a nonfortuitous association between molecular changes 
in aerobic energy metabolism proteins, such as COX and CYC, and 
organisal changes, such as brain enlargements in anthropoids. In 
fact, the same charge reduction tendency is possible to see in birds 
(52), a group with remarkably higher metabolic demands than 
other mammals. We realize that this hypothesis is not amenable to direct 
experimental testing. However, indirect phylogenetic tests are 
possible because evolution of enlarged brains occurred separately 
in a number of clades but not in their sister clades. Thus, it is possible 
to explore in each such pair of closely related clades whether the 
proteins important for brain function evolved faster in the clades 
showing the most encephalization.

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