Deleterious interactions among genes cause reductions in fitness of interpopulation hybrids (hybrid breakdown). Identifying genes involved in hybrid breakdown has proven difficult, and few studies have addressed the molecular basis of this widespread phenomenon. Because proper function of the mitochondrial electron transport system (ETS) requires a coadapted set of nuclear and mitochondrial gene products, ETS genes present an attractive system for studying the evolution of coadapted gene complexes within isolated populations and the loss of fitness in interpopulation hybrids. Here we show the effects of single amino acid substitutions in cytochrome c (CYC) on its functional interaction with another ETS protein, cytochrome c oxidase (COX) in the intertidal copepod Tigriopus californicus. The individual and pairwise consequences of three naturally occurring amino acid substitutions in CYC are examined by site-directed mutagenesis and found to differentially effect the rates of CYC oxidation by COX variants from different source populations. In one case, we show that interpopulation hybrid breakdown in COX activity can be attributed to a single naturally occurring amino acid substitution in CYC.

Introduction

When two genetically differentiated populations (or agricultural strains) are crossed, F1 hybrids often display some degree of enhanced performance (hybrid vigor) relative to the parental populations. However, when F1's are intercrossed, the F2's frequently show dramatic declines in performance. This phenomenon of F2 hybrid breakdown has broad implications for diverse areas of biology. For example, in evolutionary biology, hybrid breakdown is viewed as a critical step in the speciation process; in agriculture, the phenomenon underlies the common practice of planting F1 (but not F2) hybrid seed. A model first proposed by Dobzhansky (1937) and Muller (1940) suggests that in isolation, allopatric populations accumulate mutations that are advantageous or neutral in their native genetic background. As a consequence, each independent evolutionary unit will develop functional networks of genes with unique allelic combinations (i.e., coadapted gene complexes). If barriers to gene flow are subsequently removed, mating and recombination will breakup these coadapted gene complexes, bringing together mutations that have not previously been “tested” together. These novel combinations set the stage for deleterious interactions among genes that result in reduced hybrid fitness (Coyne and Orr 1997; Turelli, Barton, and Coyne 2001; Presgraves 2003). Despite wide acceptance of this model, the functional basis for hybrid breakdown at the biochemical or molecular level remains largely unexplored (Rawson and Burton 2002), and few examples of the genes involved have been described (Orr, Masly, and Presgraves 2004; Wu and Ting 2004).

Although not specifically addressed by the Dobzhansky-Muller model, incompatibilities between nuclear and mitochondrial genes may play a significant role in hybrid breakdown due to some unique characteristics of mtDNA. First, mitochondrial genes typically have higher mutation rates than nuclear genes and commonly carry a higher number of slightly deleterious mutations (Sackton, Haney, and Rand 2003). Second, clonal inheritance results in the accumulation of a greater number of fixed differences between isolated populations over time. Finally, mitochondrial genes are all critical subunits of electron transport system (ETS) enzyme complexes where they must interact properly with a suite of nuclear genes (see below).

The intertidal copepod Tigriopus californicus is an excellent laboratory system for investigating the consequences of interpopulation hybridization. Genetic divergence among populations from isolated rocky outcrops is high at both nuclear and mitochondrial gene loci (Burton and Lee 1994; Burton 1998; Edmands 1999, 2001; Edmands and Harrison 2003). Mitochondrial sequence divergence exceeding 18% between populations is common, yet crosses between these populations produce fertile hybrid offspring in the laboratory (Burton 1990). Second generation hybrids exhibit partial hybrid breakdown in a number of fitness-related traits including response to osmotic stress (Burton 1986), developmental time (Burton 1990), hatching number, metamorphosis number, survivorship (Edmands 1999), and cytochrome c oxidase (COX) activity (Edmands and Burton 1999; Rawson and Burton 2002).

The fact that hybrid breakdown impacts a broad range of traits suggests that its molecular basis may lie in central biochemical pathways with broad physiological consequences. Energy production via the mitochondrial ETS is an attractive candidate system, not only because it meets this criterion but also because extensive genic interactions are required for ETS function (Blier, Dufresne, and Burton 2001; Sackton, Haney, and Rand 2003). For example, COX, which catalyzes the oxidation of cytochrome c (CYC) in the terminal step of the ETS, consists of 3 mitochondrial (COXI, COXII, and COXIII) and 10 nuclear gene products. Rawson and Burton (2002) found that the activity of COX isolated from a Santa Cruz (SC) T. californicus population was significantly higher with CYC from SC than with CYC from a second population (San Diego, SD). Reciprocally, SD COX activity was higher with native SD CYC than with that from SC. These data provide direct evidence of functional coadaptation between CYC and COX within populations. Furthermore, Willett and Burton (2001, 2003) have found that the relative viability of CYC genotypes in interpopulation hybrids can differ dramatically in reciprocal crosses, implicating a direct role for cyto-nuclear interactions on fitness. Interactions between CYC and COX...
could underlie such fitness differences and contribute to hybrid breakdown.

In considering the interactions between CYC and COX within populations and in interpopulation hybrids, we note that COX shows much higher levels of population-specific amino acid substitution than CYC. For example, the COXII subunit alone, encoded in the mitochondrial genome, differs by 14 amino acid substitutions between the SC and SD populations (table 1). COXII is part of the catalytic core of COX, and it has been suggested that interactions between CYC and COXII are important for proper function (Roberts and Pique 1999). In contrast, CYC genotypes from SC and SD differ by only three substitutions (table 1). Therefore, the observed functional coadaptation must be attributed to the three amino acid substitutions in CYC and their interaction with the large number of substitutions in the COX enzyme.

Here we report on the consequence of each CYC amino acid substitution on the observed coadaptive phenotype. Using site-directed mutagenesis, we constructed CYC variants accounting for the three amino acid substitutions and determined their effect on SC and SD COX activity. Each substitution differentially affected the rates of CYC oxidation by COX from different source populations. Furthermore, we surveyed additional natural populations for CYC variants and found a population (Punto Morro, Baja California, Mexico) where one of our constructed CYC mutants occurs naturally. This PM genotype differs from the SD genotype by a single lysine to glutamine (charge changing) amino acid difference. Using population-specific COX assays, we show in this case that CYC/COX type. Using site-directed mutagenesis, we constructed CYC from two natural T. californicus populations (SC and SD) and determined their effect on SC and SD COX activity.

Furthermore, we surveyed additional natural populations for CYC variants and found a population (Punto Morro [PM], Baja California, Mexico) where one of our constructed CYC mutants occurs naturally. This PM genotype differs from the SD genotype by a single lysine to glutamine (charge changing) amino acid difference. Using population-specific COX assays, we show in this case that CYC/COX coadaptation can be attributed to a single naturally occurring amino acid substitution in CYC and its interaction with COX.

Materials and Methods

CYC Cloning and Expression

CYC variants from two natural T. californicus populations (SC and SD) were previously cloned into a CYC expression vector pBTR. This vector facilitates the coexpression of CYC heme lyase which is necessary for the heterologous expression of functional CYC in *Escherichia coli* (Pollock et al. 1998; Rawson and Burton 2002). We removed these CYC/heme lyase constructs from the pBTR vector using restriction enzymes *Nco*I and *Hind*III. After gel purification, the CYC/heme lyase fragment was ligated into the expression vector pET21(d) (Novagen, San Diego, Calif.) prepared in the same manner. After ligation, standard site-directed mutagenesis was used to introduce the single amino acid changes necessary to systematically test the individual and pairwise consequences of the three naturally occurring amino acid differences in SC and SD CYCs (see table 1). All changes were confirmed by sequencing prior to expression. BL21(DE3) cells (Novagen) were transformed with recombinant plasmids, and colonies bearing recombinant plasmids were used to inoculate 3 ml Luria bertani (LB) broth. After growing overnight at 37°C, 1 ml of this culture was used to inoculate 800 ml of Super-Broth with 200 μg/ml ampicillin. Cultures were incubated in an orbital shaker at 35°C and 150 rpm until growth reached an OD<sub>600</sub> of 0.6. At this point, expression was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and cultures were allowed to grow for another 24 h at 35°C.

Table 1

<table>
<thead>
<tr>
<th>CYC AA Residue</th>
<th>SC</th>
<th>SD</th>
<th>PM</th>
<th>Variable COXII AA Residues&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>CYC Variant</td>
<td>43</td>
<td>47</td>
<td>48</td>
<td></td>
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<tr>
<td>SC</td>
<td>Gln</td>
<td>Phe</td>
<td>Asn</td>
<td>SCVLSDMLVSLIVGAFPLMNIVLSMVLMVTPQMR</td>
</tr>
<tr>
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<td>Lys</td>
<td>Tyr</td>
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<td>Phe</td>
<td>Ser</td>
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<tr>
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<td>Tyr</td>
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<tr>
<td>SD</td>
<td>Lys</td>
<td>Tyr</td>
<td>Ser</td>
<td>SD</td>
</tr>
</tbody>
</table>

<sup>a</sup> All variants except SC and SD were constructed using site-directed mutagenesis. SC, SD, and K43Q variants are found in natural populations.

<sup>b</sup> SC = Santa Cruz, California, USA (36°57' N, 122°03' W); SD = San Diego, California, USA (32°45' N, 117°15' W); and PM = Punta Morro, Baja California, Mexico (31°52' N, 116°40' W).

<sup>c</sup> Polymorphic acidic residues are indicated in bold.

CJC Purification

Cells from 1.6 liters of culture for each CYC variant were pelleted and resuspended in 150 ml of 100 mM Na<sub>2</sub>PO<sub>4</sub> buffer (pH 7.1) with 0.5 g lysozyme and crystals of DNase and RNase. Suspensions were incubated at 4°C, with mixing, for 24 h. Cellular debris was pelleted, and the supernatant was removed and stored at 4°C. This process was repeated two to three times until the pellet was no longer pink in color. The supernatant from each round of digestion was combined and passed through an Ultrafree-15 Centrifugal filter (Millipore) three times, each time resuspending in 0.05 M potassium phosphate buffer (PB) at pH 7.3. After the final pass through the filter, each sample was resuspended in 3 ml PB. CYC from each sample was then reduced with 0.5 mg ascorbic acid. Ascorbate was removed using a PD-10 desalting column (Amersham Biosciences, Piscataway, N.J.) equilibrated with PB.
COX Activity Assay

Homogenates prepared from 30 adult females were used as a source of population-specific COX. Animals were homogenized in 600 μl of PB with 0.05% Tween 20 and then centrifuged at 5000 × g for 5 min; pellets were discarded. Activity of COX for each population was measured with each CYC variant at both 18 and 25°C and replicated four times. Reaction rates were determined by monitoring the oxidation of CYC at 550 nm. Reactions were performed by adding 25 μl of the homogenate to 400 μl of CYC (20 μM) and monitored for 2 min. Reaction rates were calculated using the best linear fit to the 2-min trajectories. Rates were normalized to protein concentration of homogenates determined by standard bicinchoninic acid method (Pierce) using bovine serum albumin as a standard.

Results and Discussion

Using site-directed mutagenesis, we constructed CYC variants and tested each of the three amino acid substitutions for their effect on activity of SC and SD COXs (table 1). COX activity assays demonstrate significant functional coadaptation between CYC and COX within populations (Rawson and Burton 2002) (fig. 1). In vitro assays of population-specific COX activity with each of our constructed CYC variants indicate that each amino acid change has a COX-dependent functional consequence (fig. 1). In addition, assays run at 18 and 25°C show a significant effect of temperature on COX activity. Three-way analysis of variance (ANOVA) indicates that the main effects of COX, CYC, and temperature are all significant, as are all the two-way interactions ($P \leq 0.01$).

Models of CYC/COX interactions predict that the dipole moment or surface charge potential is critical for proper docking of CYC (Maneg et al. 2004). Electrostatic forces between lysine residues in CYC and acidic residues in COXII are also thought to be important (Roberts and Pique 1999; Zhen et al. 1999; Drosou, Malatesta, and Ludwig 2002). In addition to the lysine to glutamine change at residue 43 of CYC, the SC COXII genotype has two fewer acidic residues than that of SD (table 1). Consistent with these predictions, we observed a notable genotype-dependent pattern with respect to the function of SC COX and the presence or absence of lysine at CYC residue 43. In five out of six cases (3/3 at 18°C and 2/3 at 25°C), the presence of this lysine (the SD wild-type condition) significantly reduces SC COX activity relative to the SC wild type; its absence (the SC wild type condition) either fully recovers SC wild-type activity or significantly increases SC COX activity relative to the SD CYC/SC COX combination (fig. 1). In the 18°C experiments, this single substitution can completely explain the differences in SC COX activity with SC versus SD CYC (fig. 2). Interestingly, the presence or absence of lysine did not have a consistent effect on SD COX activity.
The large temperature effect on COX activity observed here has been noted previously (Rawson and Burton 2002). This result is largely a consequence of increased activity of SC COX at 25°C with most CYC variants. SC COX activity increased by an average of 47% (excluding K43Q CYC variant where there was decreased activity) when the temperature was increased from 18 to 25°C. SD COX activity showed little change between temperatures. In contrast to SC COX, SD COX showed no consistent pattern of an increase or decrease in activity when the experimental temperature was changed from 18 to 25°C (activity moderately increased with five CYC variants [average change 11.2%] and slightly decreased with three CYC variants [average change ~6.3%]). Willett and Burton (2003) reported significant temperature effects on CYC genotype viabilities in experimental crosses between SC and another southern California population Abalone Cove (AB). They found selection against the AB CYC genotype in hybrids at a constant 16°C but not at a temperature regime cycling between 16 and 25°C. This result was independent of cytoplasmic background suggesting involvement of complex epistatic interactions. Our results are consistent with those of Willett and Burton (2003) suggesting that selection for a more “SC type” COX at 25°C (i.e., positive epistasis among SC COX subunits) may likely contribute to the observed pattern.

Because we observed a large effect of individual amino acids on COX activity, we extended a previous survey of T. californicus populations to obtain additional natural CYC variants (Rawson, Brazeau, and Burton 2000). Copepods obtained from PM, Baja California, Mexico (31°52' N, 116°40' W) possessed a CYC variant identical to the experimental CYC mutant K43Q that is differing from SD by the single change found to be most important in the SC/SD comparison. Our finding of multiple T. californicus populations possessing charge differences in CYC is itself remarkable. In contrast, there are almost no charge differences in CYC among divergent lineages of primates (Schmidt et al. 2005).

Interestingly, PM COXII also possesses one less acidic residue than SD COXII (table 1). Results of intraspecific CYC/COX activity assays were again consistent with functional breakdown in the interpopulation assay (fig. 3). Three-way ANOVA again shows the main effects of COX, CYC, and temperature, and all two-way interactions are significant ($P < 0.001$). The activity of PM COX was consistently higher with PM CYC than with SD CYC at both 18 and 25°C ($P = 0.002$ [18°C], $P < 0.001$ [25°C], t-test). SD COX activity was significantly higher with SD CYC than with PM CYC regardless of temperature ($P = 0.013$ [18°C], $P = 0.023$ [25°C], t-test). The significant two-way interaction (ANOVA, $P < 0.001$) and the consistently higher activities of within-population CYC/COX pairs strongly suggest that the observed intrapopulation CYC/COX coadaptation can be attributed to this single amino acid difference between PM and SD CYCs and its functional interaction with COX.

There are a number of studies that have considered the coevolution of ETS proteins within species. These studies typically include sequence analysis to identify correlated rate changes among lineages (Jobson et al. 2004; Schmidt et al. 2005) or viability assays of experimental hybrids (Willett and Burton 2001; Sackton, Haney, and Rand 2003; Willett and Burton 2003). Studies investigating the functional consequences of ETS coevolution have involved highly divergent taxa (Osheroff et al. 1983). Our results add to numerous studies of CYC/COX coevolution within T. californicus populations and make a first attempt at
determining the functional consequences of individual CYC mutations and their implications for the evolutionary dynamics of isolated populations.

Our study was designed to determine which of the three amino acid differences between SD and SC CYCs are required for coadaptation with SD or SC COXs. Results suggest that the K43Q substitution accounts for a large portion of the CYC/COX coadaptation in SC, a result that is consistent with hypotheses concerning charge potential and electrostatic interactions between CYC and COX. In contrast, although SD COX activity is not independent of the CYC source, none of the individual or pairwise amino acid changes explained the decreased activity with SC CYC. Here we suggest that all three amino acid differences between SD and SC CYCs are required for coadaptation with SD or SC COXs. Results on the functional coadaptation at the molecular level can be isolated to a single amino acid change.

activity. Again, intrapopulation COX/CYC combinations resulted in higher COX activity than interpopulation combinations. To our knowledge, this is the first case in natural populations, where the functional basis for functional coadaptation at the molecular level can be isolated to a single amino acid change.

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**Literature Cited**


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