Molecular and Functional Analyses of Amino Acid Decarboxylases Involved in Cuticle Tanning in Tribolium castaneum

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Aspartate 1-decarboxylase (ADC) and 3,4-dihydroxyphenylalanine decarboxylase (DDC) provide β-alanine and dopamine used in insect cuticle tanning. β-Alanine is conjugated with dopa-mine to yield N-β-alanyldopamine (NBAD), a substrate for the phenol oxidase laccase that catalyzes the synthesis of cuticle protein cross-linking agents and pigment precursors. We identified ADC and DDC genes in the red flour beetle, Tribolium castaneum (Tc), and investigated their functions. TcADC mRNA was most abundant prior to the pupal-adult molt. Injection of dsTcADC led to the accumulation of dopamine, and abnormally dark pigmentation of the adult cuticle. Injection of β-alanine, the expected product of ADC, into dsTcADC-treated pupae rescued the pigmentation phenotype, resulting in normal rust-red color. A similar pattern of catechol content consisting of elevated dopamine and β-alanine was observed in the genetic black mutant of Tribolium, in which levels of TcADC mRNA were drastically reduced. Furthermore, from the T. castaneum black mutant and dsTcADC-injected insects both exhibited similar changes in material properties. Dynamic mechanical analysis of elytral cuticle from beetles with depleted TcADC transcripts revealed diminished cross-linking of cuticular components, further confirming the important role of oxidation products of NBAD as cross-linking agents during cuticle tanning. Injection of dsTcDDC into larvae produced a lethal pupal phenotype, and the resulting grayish pupal cuticle exhibited many small patches of black pigmentation. When dsTcDDC was injected into young pupae, the resulting adults had abnormally dark brown body color, but there was little mortality. Injection of dsTcDDC resulted in more than a 5-fold increase in levels of DOPA, indicating that lack of TcDDC led to accumulation of its substrate, DOPA.

Insect cuticle tanning (sclerotization and pigmentation) is a complex process that involves the oxidative conjugation and cross-linking of cuticle proteins by quinones, which renders the proteins insoluble and also hardens and darkens the exoskeleton (1). The quinones are derived from catecholic metabolites of the amino acid tyrosine, produced by a series of enzymatic modifications, including hydroxylation, decarboxylation, N-acyl transfer, and oxidation (Fig. 1) (2). During tanning, cross-links form between adjacent polypeptide chains, causing progressive hardening, dehydration, and close packing of the polymers. This cross-linking occurs as a result of reactions of quinones and quinone methides derived from N-acylcatecholamines with nucleophilic side chains of structural proteins, leading to changes in mechanical properties and coloration (3). The major catechols used by insects as cuticle tanning agent precursors are dopamine, N-β-alanyldopamine (NBAD), and N-acetyldopamine (NADA) (1).

Different types of quinones and quinone methides produced from catecholic metabolites may contribute to the variety of physical properties of insect cuticle. For example, NADA is predominant in the unpigmented hard larval head capsule of Manduca sexta (tobacco hornworm), whereas the hard pigmented cuticles of the larval mandible and pupa contain primarily NBAD (4, 5). However, both NADA and NBAD are major catechols in the rust-red colored cuticle of the red flour beetle, Tribolium castaneum (6, 7). Previously, we analyzed the levels of catechols and β-alanine in both wild-type (rust-red body color) and black body color mutant strains of Tribolium, and we demonstrated that the black mutant had decreased concentrations of β-alanine and NBAD when compared with levels present in wild-type beetles (6). The black mutation influenced not only cuticle pigmentation but also the puncture resistance of the cuticle, suggesting that both β-alanine and NBAD are critical for beetle cuticle tanning (7). The precise genetic basis for the black and other dark body mutations, however, was not established.

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EU019709, EU019710, and EU030256.

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2 The abbreviations used are: NBAD, N-β-alanyldopamine; DOPA, 3,4-dihydroxyphenylalanine; NADA, N-acetyldopamine; AMD, α-methyl dopa; DA, dopamine; DMA, dynamic mechanical analysis; RNAi, RNA interference; RT, reverse transcription; dsRNA, double-stranded RNA; TcADC, T. castaneum aspartate 1-decarboxylase; TcDDC, T. castaneum DOPA decarboxylase; dsDNA, double-stranded DNA; HPLC, high pressure liquid chromatography.
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Two related decarboxylases, aspartate 1-decarboxylase (ADC, EC 4.1.1.11) and DOPA decarboxylase (DDC, EC 4.1.1.28), are predicted to be involved in the Tribolium cuticle sclerotization and pigmentation pathway, and each is a candidate for the gene that is mutated in black. ADC catalyzes the synthesis of β-alanine by decarboxylation of the α-COOH group of L-aspartic acid. β-Alanine subsequently plays a critical role in cuticle tanning because of its conjugation with dopamine to produce NBAD (6, 8–10). DDC catalyzes the conversion of DOPA to dopamine. To examine more fully the functional roles of ADC and DDC in the synthesis of catechols in the Tribolium cuticle tanning pathway, and to determine whether those enzymes and their products influence the physical properties of the cuticle, we performed RNA interference (RNAi) experiments to suppress the transcript levels of those enzymes and their products influence the physical properties of the cuticle.

The effects of altered catechol and dopamine metabolism on the dynamic mechanical properties of the elytra to determine whether those enzymes and their products influence the physical properties of the cuticle were performed RNA interference (RNAi) experiments to suppress the transcript levels of those enzymes and their products influence the physical properties of the cuticle. These experiments were then followed by measurement of the dynamic mechanical properties of the elytra to determine the effects of altered catechol and β-alanine content on physical properties of the exoskeleton. Such tests can distinguish between cross-linked and uncross-linked high molecular weight compounds, a distinction key to evaluating the roles of enzymes and metabolites in cuticle tanning.

EXPERIMENTAL PROCEDURES

Chemicals—3,4-Dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylethylamine (dopamine, DA), the internal standard, 2-methyl-3-(3,4-dihydroxyphenyl)alanine (α-methyl dopa, AMD), and N-acetyldopamine (NADA) were purchased from Sigma. N-β-Alanyldopamine (NBAD) was obtained from the Chemical Synthesis and Drug Supply Program of NIMH (nimh-repository.rti.org). All reagents were of the highest purity available. Other chemicals, unless noted otherwise, were purchased from Fisher.

Insects—T. castaneum (Coleoptera, Tenebrionidae) strain GA-1 (11) and two black body color mutants, black and bChu/bSt, were reared at 30 °C under standard conditions (12). The black mutation is a spontaneous, incompletely recessive, homozygous viable mutation on the third chromosomal linkage group (13). The Charcoal (bChu) and black-of-Scott-Thomson (bSt) mutations are radiation-induced, dominant, homozygous lethal alleles of black (14). The lethality of each of the latter two alleles appears to be due to an independent lesion in an unrelated gene pseudolinked by chromosome rearrangement to a nonlethal lesion in the black gene.

Thus, bChu/bSt forms a balanced lethal system.

Cloning of the T. castaneum Aspartate 1-Decarboxylase (TcADC) and DOPA Decarboxylase (TcDDC) cDNAs—Putative amino acid decarboxylase genes in Tribolium, including TcADC and TcDDC, were identified by searching Beetlebase using Drosophila Dgd2 (CG7811-RA) and the DOPA decarboxylase, epidermal form (CG10697-RB, 15) as queries, respectively. To clone the partial cDNAs of TcADC and TcDDC, the following primers were used: 5′-AGG GAA CGA ACC GCA AGA AC-3′ and 5′-CAA GGC CTT AAA GAT CGC TC-3′ for TcADC, and 5′-GTT GAA TTC TTA ATT TTA CTA GTG TGT CTG CTT C-3′ for TcDDC. 5′- and 3′-rapid amplifications of cDNA ends (Invitrogen) were performed to obtain the full-length cDNA of TcADC. The sequences of TcADC and TcDDC cDNAs have been deposited in GenBank™ with the accession numbers EU019709 and EU019710, respectively. The sequence of TcADC from the black body color mutant was also deposited in GenBank™ with the accession number EU302526.

Gene Expression Analysis by RT-PCR and Real Time PCR—To analyze expression profiles of TcADC and TcDDC genes, total RNA was isolated from whole insects at the pharate pupal to adult stages by using the RNeasy mini kit (Qiagen). cDNA synthesis and RT-PCR were done as described previously (16) using the primer pair 5′-ACA ACG TCT ACT TGA TAG CAG G-3′ and 5′-GTC GTA TTT GGT GTC GTA G-3′ for TcADC, and the primer pair 5′-ATG GAA GCT AAT CAG TTC AAG G-3′ and 5′-TCC AGC ATC ACG ACT TCT AG-3′ for TcDDC. As an internal control, the following pair of primers designed for Tribolium ribosomal protein S6 (rpS6) were used: 5′-AGA TAT ATG GAA GCA TCA TGA AGC-3′ and 5′-CGT GGT
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CTT CTT TGC TCA AAT TG-3’. To analyze the transcript levels of TcADC in the three strains, GA-1 (wild type), black, and b<sup>Ch</sup>/b<sup>ST</sup>, total RNAs were isolated from whole insects 5–6 days after pupation (pharate adults). Real time PCR was performed using SYBR Premix Ex Taq (Takara) and the Mx3000PTM real time PCR System (Stratagene). The following pairs of primers were used: 5′-TGC CAG ATA CAG GGT TAT GCC CGA-3′ and 5′-GAT GCC TTG GAA AGA AGC GCC TTT-3′ for TcADC; 5′-CGT GAG GCT GCC CTT ATT CCA TTT-3′ and 5′-GTC GAG CCA GAT GGT GTC GGA GTC-3′ for TcDDC; and 5′-ACG CAA GTC AGT TAG AGG GTG CAT-3′ and 5′-TCC TGT TCG CCT TTA CGC ACG ATA-3′ for TcrpS6 as an internal control. To analyze the knockdown level of TcADC transcripts, dsRNA for TcADC was injected into penultimate- or last-instar larvae, and total RNA was isolated 5–6 days after pupation (13–14 days post-injection).

Synthesis of dsRNAs and Injection into Insects—dsRNAs for TcADC, TcDDC, and T. castaneum vermillion (Tcv) (17) were prepared according to the protocol described previously using the AmpliScribe T7-Flash transcription kit (Epiceric Technologies) (18). dsTcv was used as a negative control dsRNA in this study. The vermillion gene (v) encodes tryptophan oxygenase (EC 1.13.11.11) that catalyzes the conversion of tryptophan to N-formylkynurenine in the ommochrome pigment pathway and is required for dark eye pigmentation in Tribolium and other insects, but it lacks any apparent role in cuticle formation (19). The length of the dsRNAs for TcADC, TcDDC, and Tcv were 433 bp (nucleotides 1192–1624), 301 bp (nucleotides 1251–1551), and 609 bp (nucleotides 310–918), respectively. dsRNAs for laccase 2 (TcLac2) and tyrosinase (TcTyr) were prepared as described by Arakane et al. (19). For RNAi experiments, 200 ng of the dsRNA were injected into penultimate- and/or last-instar larvae of strains GA-1 (wild type), black, and b<sup>Ch</sup>/b<sup>ST</sup> (20). After injection, larvae were kept at 30 °C for visual monitoring of phenotypes and other analyses.

Phylogenetic Analysis of Decarboxylases—A core region of homologous sequence highly conserved in all decarboxylases, including a putative catalytic region, was aligned with ClustalW software prior to phylogenetic analysis. The MEGA 3.0 program (21) was used to construct the consensus phylogenetic tree using the Unweighted Pair Group Method with Arithmetic Mean. To evaluate the branch strength of the phylogenetic tree, bootstrap analysis of 5,000 replications was performed.

Extraction of Catechols—To analyze the levels of catechols such as DOPA, dopamine, NADA, and NBAD, three groups of five pharate adults each were weighed and homogenized in a glass tissue grinder in 0.5 ml of ice-cold 0.1 M perchloric acid containing 1 mM EDTA and 1 mM sodium metabisulfite to minimize oxidation of the catechols. The extracts were subsequently centrifuged at 13,000 × g for 30 min at 4 °C, and the supernatants were used for the analyses.

Catechol Analysis—Extracts were resolved and analyzed using an HP 1050 HPLC system fitted with a programmable HP 1049A electrochemical detector. Twenty μl of each sample was loaded onto a C18 reversed phase column (Gemini, 250 × 4.6 mm, 5 μm) obtained from Phenomenex (Torrance, CA) using an HP autosampler and a sequence program. Catechols were resolved using an isocratic mobile phase consisting of 12% acetonitrile containing 1.4 mM octane sulfonic acid as an ion-pairing agent, 1 mM EDTA, and 1 mM lithium chloride, pH 3.05, at a flow rate of 0.5 ml/min. The catechols were detected using the electrochemical detector with a glassy carbon electrode and the oxidative potential set at +700 mV. DA, DOPA, NADA, AMD, and NBAD were identified by their retention times in comparison with the known standards as follows: DOPA, 6.8; NADA, 11.1; AMD, 11.9; DA, 12.9; and NBAD, 23.5 min. All samples were run in two replicates. The detection limit was ≤0.1 μM, and the recovery was ≥95%. Quantitation was accomplished by comparing peak areas of beetle catechols with those of the corresponding standards of known concentrations and using AMD as an internal standard. Peak areas and retention times were computed using a PC aided by HP ChemStation version revised 08.03 software.

Mechanical Testing of Elytra from Tribolium—At 7–14 days after ecdisis, adult beetles were sacrificed by exposure to −20 °C for 30 min, and then elytra were immediately removed with forceps and mounted onto a custom sample holder with Devcon 1.5-Ton quick-setting epoxy cement. The fast-setting epoxy was allowed to dry for 1 h prior to testing, fixing the samples in place without damage. Sample dimensions were determined via calipers and microscopic measurements using a digital micrometer. A TA Instruments RSAIII dynamic mechanical analyzer was used to perform all of the dynamic mechanical measurements under ambient conditions. The instrument was used to apply a sinusoidally oscillating tension stress on an elytron and to record the strain response. The lag between stress and strain is a function of the elastic (cross-linked) versus the viscous (noncross-linked) response of a sample.

Strain sweeps at 1 Hz (measuring stress as strain amplitude is gradually increased at constant frequency) were used to determine the extent of the linear viscoelastic region of the elytron, the region where an applied strain maintains a linear stress-strain response and where the processes are reversible. This is a necessary prerequisite to frequency sweep tests, as it ensures that the results are strain-independent and that the applied force does not damage a sample during the course of the experiment (22). For all of the elytral types tested, the material properties were found to be strain-independent beyond a strain of 0.2%, and instrument noise was acceptable at this value. Thus, a strain of 0.2% was used for all of the reported frequency sweep experiments.

RESULTS

Characterization of TcADC and TcDDC Genes and Encoded Proteins—Searching Beetlebase with the tblastn program using the sequences of two Drosophila enzymes, Dgad2 (= black, an aspartate decarboxylase) and DOPA decarboxylase (epidermal form), revealed 11 Tribolium genes that encode members of the pyridoxal phosphate-dependent amino acid decarboxylase family, in which all members exhibit a large and highly conserved pyridoxal phosphate-binding motif, Ser-X-X-Lys (Fig. 2 and supplemental Fig. 1). In comparison, the Drosophila melanogaster.
The genome contains only nine members of this gene family. The encoded proteins can be divided into three major clades as follows: with clade A (green in Fig. 2), including Drosophila histidine and tyrosine decarboxylases; clade B (yellow in Fig. 2), including Drosophila DOPA decarboxylase (DmDDC) and the H9251 -methyl DOPA resistance gene (DmAMDR); and the more distantly related clade C (blue in Fig. 2) containing the Drosophila aspartate decarboxylase (DmADC) and glutamate decarboxylase (DmGluDC) proteins. Another D. melanogaster gene, CG1486, and a highly related T. castaneum gene, G05445, encode several long insertions in the conserved domain. The latter two genes were not included in this phylogenetic analysis, but they would define a fourth, more distant clade (data not shown). Putative T. castaneum orthologs can be identified for Drosophila Tyr decarboxylase 2 (G12567), His decarboxylase (G10062), DDC (G13480), GluDC (G09324), and ADC or black (G00038). However, additional T. castaneum genes are represented in the branches that contain TyrDC, DDC, and ADC, indicating that expansions of this gene family have occurred in the red flour beetle. For this study, we have further analyzed the Tribolium genes that are most closely related to DmDDC and DmADC.

We cloned and sequenced multiple full-length cDNAs encoding TcADC from the wild-type GA-1 strain and the black body color mutant to analyze whether any mutations could be detected in the transcript from black individuals. The GA-1 TcADC cDNA sequence was identical to the transcript predicted from the genome except for one nucleotide that did not result in an amino acid substitution. However, it differed from the TcADC cDNA prepared from the black strain at 14 nucleotide positions, which resulted in only four conservative amino acid substitutions (supplemental Fig. 2). However, none of these mutations resulted in an amino acid substitution in the putative decarboxylase catalytic domain, and none created a premature stop codon in the open reading frame. As the wild-type and black body color strains are not closely related, the minor sequence differences in the TcADC transcripts may be due to allelic variation that would not be expected to significantly affect enzyme function. Therefore, the black phenotype is unlikely to be due to production of a nonfunctional ADC. Instead, results presented below demonstrate that TcADC transcript levels are much lower in the black mutants than in the wild-type beetles.

We also cloned and sequenced a cDNA encoding TcDDC from the GA-1 strain and found the sequence to be identical to the transcript predicted from the genome, except for seven nucleotide differences that resulted in only one amino acid substitution (Met to Lys at position 450). The amino acid sequence identities of TcADC and TcDDC with other insect ADC-like
Table 1
Amino acid identity/(similarity) of insect ADCs

| T. castaneum | TcADC | 60% | 64% | 66% | 68% | 69% |
| AgADC | 50% | 54% | 54% | 54% | 54% |
| TmDDC | 71% | 73% | 74% | 75% | 75% |
| DmDDC | 71% | 74% | 75% | 76% | 76% |
| Tcv | 75% | 77% | 78% | 78% | 79% |
| AgDDC | 70% | 70% | 70% | 71% | 71% |
| TmDDC | 68% | 69% | 70% | 71% | 71% |

Table 2
Amino acid identity/(similarity) of insect DDCs

| T. castaneum | TcDDC | 73% | 74% | 75% | 76% | 77% |
| DmDDC | 72% | 73% | 74% | 75% | 76% |
| AgDDC | 78% | 79% | 80% | 81% | 82% |
| TmDDC | 75% | 76% | 77% | 78% | 79% |
| DmDDC | 75% | 76% | 77% | 78% | 79% |
| AgDDC | 76% | 77% | 78% | 79% | 80% |
| TmDDC | 74% | 75% | 76% | 77% | 78% |

Effect of Injection of Double-stranded RNA for TcADC on Cuticle Pigmentation and Catechol Levels—To examine the function of ADC in vivo, we injected dsRNA (200 ng per insect) for TcADC into penultimate- and last-instar larvae and observed the resulting phenotypes. The level of TcADC transcripts observed after injection of dsRNA for TcADC was less than 10% of that detected in controls, indicating that a substantial knockdown of TcADC expression had occurred (Fig. 4B). Insects injected with dsTcADC displayed darkened body pigmentation when compared with controls (Fig. 4A). Pupa-specific cuticular structures such as the gill traps were darker than those of control insects (data not shown). This effect, however, was most striking in adults, which were black rather than the rust-red color of un.injected beetles or controls injected with a different dsRNA. Wild-type adults treated with dsTcADC had the same body color as the black mutant strains.

Previously, we demonstrated that injection of β-alanine into the black body color mutant restored the rust-red pigmentation of the adult cuticle (6). We conducted a similar experiment to determine whether β-alanine injection could also rescue the black body color phenotype produced by injection of dsRNA for TcADC, restoring the normal rust-red pigmentation. As shown in Fig. 5, a successful rescue of black adult pigmentation was obtained after injection of β-alanine, the expected product of ADC, into dsTcADC-treated pharate adults.

Levels of catechols in dsTcADC-treated insects were compared with those in controls (dsTcv-treated wild-type insects) and the two black mutant strains (Fig. 6). After TcADC knockdown NBAD content of pharate adults was decreased nearly 10-fold when compared with controls (Fig. 6A). The black mutant strains also had significantly lower NBAD content than wild-type controls as well as higher levels of dopamine (Fig. 6B). These data are consistent with the hypothesis that the black body color mutant has a defect(s) in the gene encoding ADC and that the black phenotype is a result of an abnormally low expression of ADC, which leads to a deficiency in β-alanine required for the synthesis of NBAD.

Effect of Injection of Double-stranded RNAs for Two Phenol Oxidases, TcLac2 and TcTyr, on Cuticle Pigmentation of the Black Mutant—We previously demonstrated that laccase 2 is responsible for cuticle tanning in wild-type T. castaneum, in which NADA, NBAD, and dopamine are the predominant catechols available as laccase substrates (19). To test whether either or both of the phenol oxidases, laccase and tyrosinase, catalyze cuticle tanning in the black body color mutant that has a reduced NBAD content, dsRNA (200 ng per insect) for TcLac2 or the two TcTyr genes was injected into last-instar larvae or pharate pupae of the b^{Chr}/b^{ST} mutant. Tanning of cuticle was almost completely prevented by injection of dsTcLac2, whereas injection of either dsTcTyr had no effect (Fig. 7). These results are consistent with those reported from previous TcLac2 and TcTyr RNAi experiments using the wild-type strain GA-1 (19) and support the following hypotheses: 1) laccase and not tyrosinase catalyzes both sclerotization and pigmentation of Tribolium larval,
pupal, and adult cuticles, and 2) black rather than rust-red pigmentation is due to an increased content of dopamine-quinone (and dopamine melanin) produced via oxidation of DA by laccase 2 along with a concomitant decrease in the NBAD quinone content.

**Effect of Injection of Double-stranded RNA for TcDDC on Cuticle Pigmentation and Catechol Levels**—As dopamine is required as a precursor for synthesis of NBAD and NADA, the predominant catechols utilized for cuticle tanning, we predicted that reduced TcDDC expression would result in aberrant sclerotization and pigmentation. We analyzed the function(s) of TcDDC by injection of dsRNA corresponding to a unique region of this gene. A lethal pupal phenotype was observed after injection of dsTcDDC into late larvae (Fig. 8). The resulting pupal cuticle attained a grayish color and exhibited many small black patches with a bilateral symmetry, after which all of the pupae died.

To evaluate the effect of down-regulation of TcDDC transcripts on adult cuticle tanning, dsTcDDC was injected into 1–2-day-old pupae. Pupae treated with dsTcDDC developed normally and molted to the adult stage, but tanning of the adult cuticle was delayed by several hours with the cuticle eventually becoming slightly darker than the normal rust-red color (Fig. 9). Levels of DOPA were more than 5-fold higher in dsTc-ADC-treated insects (1500 pmol/mg) relative to dsTcv-treated control insects (200 pmol/mg). However, an unexpected result was that injection of dsTcDDC did not result in any decrease in the levels of dopamine, NBAD, or NADA (data not shown).

**Effect of Reduced TcADC Expression on Mechanical Properties of Elytral Cuticle**—The tanning process involves not only pigment formation but also reactions of oxidized catechols that modify or cross-link cuticular proteins, modulating cuticular physical properties such as the elastic modulus, a measure of material stiffness. We used dynamic mechanical analysis (DMA) to test whether an altered catechol content resulting from reduced TcADC transcripts affects the physical properties of the cuticle in elytra from dsTcADC-treated beetles and black mutant beetles when compared with dsTcv-injected control insects. DMA provides insight into the internal molecular structure of a material, in addition to direct measurement of fundamental material mechanical proper-

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**FIGURE 3. Expression profiles of TcADC and TcDDC mRNAs during development. A, total RNA was extracted from whole beetles (n = 5) at late developmental stages (pharate pupa to young adult). PP, pharate pupae; P1, 0–1-day-old pupae; P2, 1–2-day-old pupae; P3, 2–3-day-old pupae; P4, 3–4-day-old pupae; P5, 4–5-day-old pupae; A1, 0-day adults; A2, 5–6-day-old adults. B, expression levels of TcADC gene in the wild-type and two black body color mutants. cDNA templates for RT-PCR and real-time PCR were prepared from total RNA isolated from whole beetles (n = 5, duplicate for wild-type and b Chr/b ST, and triplicate for BI) at pharate adult stage corresponding to P5. Wt, wild type; b, black body color mutant; b Chr/b ST, balanced lethal black body color mutant. The numbers in parentheses represent PCR cycles. RT-PCR and real-time PCR of Tribolium ribosomal protein 6 (rpS6) transcript with the same cDNA template served as an internal control. For real-time PCR, expression levels for TcADC and TcDDC are presented relative to the wild-type level or the levels of expression in pharate pupae (PP). qPCR, quantitative PCR.
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FIGURE 4. Effect of dsRNA for TcADC on larval, pupal, and adult cuticle tanning. A, dsRNA (200 ng per insect) for TcADC (dsTcADC) or Tcv (dsTcv) were injected into penultimate instar or last instar larvae (n = 60, triplicate of 20 insects each). Left panel, 1-day-old last instar larvae; middle panel, 5-day-old pupae (pharate adults); right panel, 4-day-old adults. B, knockdown level of TcADC by RNAi. cDNAs for RT-PCR and real time PCR were prepared from total RNA isolated from whole beetles (n = 3) at 5 days (10-day post-injection). The numbers in parentheses represent PCR cycles. RT-PCR and real time PCR of Tribolium ribosomal protein 6 (rpS6) transcript with the same cDNA template served as an internal control. For real time PCR, expression levels of TcADC in dsTcADC-treated insects are presented relative to the levels in dsTcv control. qPCR, quantitative PCR.

FIGURE 5. Effect of injection of β-alanine on adult cuticle pigmentation of dsTcADC-treated Tribolium pupae. dsRNAs for TcADC (dsTcADC) or Tcver (dsTcv) (200 ng per insect) were injected into 1-day-old pupae (1st injection), and then β-alanine (0.4 μmol per insect) or buffer were injected into 5-day-old pupae (2nd injection) (n = 40 for each treatment, duplicate of 20 insects each). Rescue of the black body adult phenotype produced by injection of dsADC was observed by injection of β-alanine in 100% of the resulting adults.

ties, unlike more conventional mechanical measurements such as puncture resistance tests. Furthermore, because DMA does not require a material to be tested to failure, irreproducibility in sample preparation, mounting, and testing is minimized.

We performed frequency sweep experiments on tanned elytra freshly harvested prior to each test. Frequency sweep experiments on an elytron yield its complex modulus as function of the imposed oscillation stress frequency (ω). The complex modulus can be resolved into the elastic modulus (designated E’ and the viscous modulus (designated E”). The dependence of such moduli on frequency for polymeric materials in general is a function of the relaxation modes available to the constitutive polymer chains (23). The elastic modulus (E’) for an entangled, but uncross-linked, polymeric material is expected to increase with frequency, whereas E” is expected to become frequency-independent for a well cross-linked material at frequencies below ~100 Hz (24, 25). Experimentally, the elastic modulus of a lightly cross-linked biopolymer is typically observed to have a weak power-law frequency dependence E’(ω) ~ ω^{0.1-0.3}. As the extent of cross-linking increases, the frequency exponent falls toward zero, exhibiting frequency independence (26, 27).

The E’ frequency dependence of the dsTcADC knockdown sample and the black mutant are remarkably similar to each other and significantly different from the control wild-type insects injected with dsTcv (Fig. 10 and Table 3). An important, statistically confirmed similarity of the former two samples is indicated by the power law exponent (Table 3). Significantly, these exponents are more than 50% higher for the black and dsTcADC-treated elytra relative to that of the dsTcv-injected control elytra. As noted earlier, the significantly higher E’ power law dependence of cuticle with reduced ADC levels suggests that the black and dsTcADC knockdown elytra are similar in having a less effectively cross-linked structure than dsTcv-injected control elytra. This interpretation of the differences between the cuticle sets at the molecular level is further corroborated by the frequency dependence of the E”/E’ ratio (Fig. 11). The ratio of the E” and the E’ (equivalent to tan δ, where δ is the phase angle between the applied stress and the material strain) has an inverse power dependence on frequency, because viscous effects become relatively more important at low frequencies. Entangled, but uncross-linked, polymer chains are able to flow past one another at these longer time scales. Therefore, the ratio of E”/E’ is a function of the molecular interconnectivity, whether entangled or cross-linked. Uncross-linked materials exhibit relatively high viscous dampening and hence a high E”/E’ ratio. Cross-linking reduces the magnitude of the E”/E’ ratio as well as its inverse dependence on frequency (23). Elytra from the dsADC knockdown insects and the black color mutant had a greater E”/E’ ratio and stronger inverse frequency dependence than elytra from the dsTcv knockdown insects. This behavior demonstrates a higher damping ability or viscous energy dissipation in elytra from ADC-deficient insects, consistent with the interpretation suggested by the data in Fig. 10 that the ADC-deficient insects have less effectively cross-linked cuticle than dsTcv-injected insects. Overall, these data are consistent with the hypothesis that a product of TcADC mediates cross-linking and that the black mutant is deficient in ADC.
DISCUSSION

Sequence Analysis and Expression of TcADC and TcDDC Transcripts during Development—Enzymes that catalyze the decarboxylation of two amino acids, aspartic acid and DOPA, are hypothesized to participate in insect cuticle tanning metabolism. One, ADC, catalyzes the decarboxylation of aspartic acid to form L-alanine; and the other, DDC, uses DOPA as its substrate to produce dopamine. Dopamine is subsequently N-acylated with L-alanine to yield NBAD, one of the major catechols used for cuticle tanning. After searching the Tribolium genome data base for ADC-like genes, we identified two genes, including an apparent ortholog of the Drosophila ADC known as black or Dgad2 (28), which we call TcADC, as well as a gene encoding a related protein, G10581. We cloned a full-length TcADC cDNA with an open reading frame encoding a protein of 540 amino acid residues. The Tribolium genome also encodes an apparent ortholog of D. melanogaster glutamate decarboxylase-1 (G09324), as well as another protein (G14177) that groups with the Asp and Glu decarboxylases in a phylogenetic analysis of protein sequences and is very similar to D. melanogaster protein CG5618, whose function has not yet been determined. It is conceivable that the proteins in this group, in

FIGURE 6. Catechol content of T. castaneum pharate adults. GA-1 wild-type larvae or pupae were injected with dsRNA for TcADC or Tcv (tryptophan oxygenase) as a control. When these insects became pharate adults, catechols were extracted and analyzed by HPLC with electrochemical detection (A). Catechols of untreated prepupae from two black body color mutants (black and b^{Chr}/b^{ST}) were also analyzed (B). Bars indicate mean ± S.D. (n = 3). Values significantly different from the dsTcv controls (p < 0.05) are marked with an asterisk (determined by analysis of variance and Newman-Keuls multiple comparison test).

FIGURE 7. Effect of dsRNA for TcLac2 or TcTyr on larval, pupal, and adult cuticle tanning of b^{Chr}/b^{ST} black body color mutant. dsRNA (200 ng per insect) for TcLac2 or TcTyr was injected into late larvae or pharate pupae of b^{Chr}/b^{ST} black body color strain. Little or no cuticle tanning was observed after injection of dsLac2 at each developmental stage (A), whereas dsTyr had no effect on cuticle tanning (B). dsRNA for Tcv was injected as a control.

FIGURE 8. Lethal pupal phenotype produced by injection of dsRNA for TcDDC into late larvae. dsRNA (200 ng per insect) for TcDDC was injected into late larvae. The resulting pupal cuticle turned a grayish color and exhibited many minute black patches. The pupa died after 3–5 days. dsRNA for Tc vermilion (dsTcv) was injected as a control. A, whole body; B, head; C, dorsal view; D, side view; E, urogomphi.

FIGURE 9. Adult phenotypes produced by injection of dsRNA for TcDDC. A, dsRNA (200 ng per insect) for TcDDC was injected into 1–2-day-old pupae (D, right pictures of each panel). The adult body color turned dark rust-red but not black after injection of dsDDC. dsRNA for vermilion (dsTcv) was injected as a control (V, left pictures of each panel). B, knockdown level of TcDDC by RNAi. cDNAs for RT-PCR and real time PCR were prepared from total RNA isolated from whole beetles (n = 3) at 5 days (3–4 day post-injection). The numbers in parentheses represent PCR cycles. RT-PCR and real time PCR of Tribolium ribosomal protein 6 (rpS6) transcript with the same cDNA template served as an internal control. For real time PCR, expression levels of TcDDC in dsTcDDC treated insects are presented relative to the levels in dsTcv control. qPCR, quantitative PCR.

data base for ADC-like genes, we identified two genes, including an apparent ortholog of the Drosophila ADC known as black or Dgad2 (28), which we call TcADC, as well as a gene encoding a related protein, G10581. We cloned a full-length TcADC cDNA with an open reading frame encoding a protein of 540 amino acid residues. The Tribolium genome also encodes an apparent ortholog of D. melanogaster glutamate decarboxylase-1 (G09324), as well as another protein (G14177) that groups with the Asp and Glu decarboxylases in a phylogenetic analysis of protein sequences and is very similar to D. melanogaster protein CG5618, whose function has not yet been determined. It is conceivable that the proteins in this group, in
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![Graph showing the relationship between elastic modulus (E) and frequency (rad/s) for different samples: black, dsTcv, dsTcADC.](image)

**FIGURE 10.** Dependence of the E’ of Tribolium elytral cuticle upon frequency of sinusoidally oscillating stress. Frequency sweeps of a single elytron were performed at 0.2% strain. Each curve is an average of four trials. The lesser frequency dependence displayed by elytra from dsTcv-injected control insects is indicative of a more effectively cross-linked structure than elytra from black or dsTcADC-injected insects.

**TABLE 3**

Physical properties of Tribolium elytral cuticle determined by dynamic mechanical analysis

| Sample      | E’ at 10 rad/s (GPa) | n² | E’/E” at 10 rad/s |
|-------------|----------------------|----|------------------|
| Black       | 3.5 ± 1.5            | 0.054 ± 0.008 | 0.11 ± 0.02     |
| dsTcADC     | 3.2 ± 1.5            | 0.060 ± 0.009 | 0.12 ± 0.02     |
| dsTcv       | 2.3 ± 1.0            | 0.034 ± 0.004 | 0.07 ± 0.01     |

*Exponent of power law frequency dependence is E”.

**FIGURE 11.** Dependence of the ratio of the E’ to the E” of Tribolium elytral cuticle upon frequency of sinusoidally oscillating stress. Frequency sweeps of a single elytron were performed at 0.2% strain. Each curve is an average of four trials. The lesser frequency dependence displayed by elytra from dsTcv-injected control insects is indicative of a more effectively cross-linked structure than elytra from black or dsTcADC-injected insects.

addition to TcADC, may have some degree of aspartate decarboxylating activity.

We also identified an apparent ortholog of *D. melanogaster* *DDC*, which we denoted as *TcDDC*, and we cloned its full-length cDNA. The deduced amino acid sequence (475 amino acids) exhibited high sequence similarity to other insect DDCs as well as to *Drosophila* epidermal DDC. The DmDDC gene has two alternatively spliced transcripts, one that is expressed in the epidermis and the other in the central nervous system (15). However, to our knowledge there is no evidence of tissue-specific expression of alternatively spliced DDC transcripts in two other species, *Manduca sexta* and *Aedes aegypti* (29, 30). The *Tribolium* genome contains two other genes with high sequence similarity to *TcDDC*, G13401 and G13402. It is feasible that they may also encode proteins with DOPA decarboxylating activity, as may be the case for a related gene in *Drosophila* known as the α-methyl dopa resistance gene (31, 32).

Developmental expression profiles of *TcADC*, G10581, *TcDDC*, G13401, and G13402 were analyzed by RT-PCR, and the transcript levels of *TcADC* and *TcDDC* were further analyzed by real time PCR. The relatively low levels of *TcDDC* transcripts observed during pupal development may be due to some expression in the central nervous system as well as in the epidermis. Transcripts of both *TcADC* and *TcDDC*, however, were dramatically increased at the pharate adult stage (Fig. 3A), when *Tribolium* initiates adult cuticle tanning metabolism (Fig. 4A, middle panel). These data support the hypothesis that *TcDDC* provides dopamine and *TcADC* provides β-alanine for subsequent production of NBAD, which serves as a precursor for the quinone-derived cuticle tanning agent, ultimately resulting in the rust-red body coloration of the cuticle of the beetle. The other candidate genes encoding DDC-like proteins (G13401 and G13402) and ADC-like proteins (G10851) were expressed little or not at all at the pharate adult stage, suggesting that they lack a major role in providing metabolic precursors for adult cuticle tanning.

**Is the Body Color Phenotype of the Tribolium Black Mutant Because of a Mutation in *TcADC*?**—NBAD and NADA are the major catechols in the adult cuticle of the wild-type strain and are associated with the rust-red body color, whereas black pigmentation is associated with lower levels of these catechols and β-alanine, as well as an abnormally high level of dopamine (6) (Fig. 1). ADC and NBAD synthase are key enzymes for producing NBAD from the two precursors dopamine and β-alanine (Fig. 1), and the reduction of β-alanine content in the black body color mutant (6) suggested that it might have a mutation in the gene encoding ADC. The *TcADC* gene maps to linkage group LG3 at position 42.6, which is very near the locus of the black body color mutant. To analyze whether the black mutation occurs in the coding region of the *ADC* gene, we cloned and sequenced a full-length cDNA of *TcADC* from the black body color mutant and from the wild-type strain (supplemental Fig. 2). There was no reading frameshift, premature stop codon, or amino acid substitution in the open reading frame encoding the putative catalytic domain (28) of ADC from the black mutant. It is quite possible that *TcADC* in the black body color mutant is enzymatically active but has been down-regulated. We subsequently analyzed the transcript levels of *TcADC* in the black mutant as well as in a second black body color mutant strain, *b*C/ *b*ST, and compared them to wild-type levels (Fig. 3B). The transcript levels of *TcADC* in these black mutant strains were indeed significantly lower than that of the wild-type strain. Real time PCR confirmed that the mRNA level of *TcADC* in the black mutant was more than 2 orders of magnitude less than that in wild-type insects, and the transcript was undetectable in the *b*C/hr/*b*ST strain. These results indicate that the black body

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3 Y. Arakane and R. W. Beeman, unpublished observations.
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mutations lead to severely diminished TcADC mRNA levels, which limit synthesis of β-alanine used for NBAD production and lead to accumulation of dopamine. Rescue of the black mutant by injection of β-alanine into newly eclosed adults (6) supports this interpretation. The observation that even the $b^{CHW}/b^{ST}$ mutant contained some NBAD (Fig. 6) suggests that alternative decarboxylating enzymes, perhaps those with primary specificity for other substrates such as glutamate, might help to compensate for a lack of TcADC and catalyze the synthesis of some β-alanine from aspartic acid.

Injection of dsRNA for TcADC resulted in a black-pigmented adult phenotype similar to that of the black mutant strain (Fig. 4A). Furthermore, these insects had a nearly 90% reduction in NBAD levels when compared with control insects, together with a higher level of dopamine (Fig. 6). Also β-alanine injection could also rescue the rust-red body pigmentation of the black phenotype produced by injection of dsRNA for TcADC (Fig. 5). All of these results support the hypothesis that the Tribolium black mutants have a mutation(s) that causes an abnormally low expression of ADC. The lack of ADC prevents synthesis of β-alanine used for NBAD production and thus allows for the buildup of abnormally high levels of dopamine, which is used for dopamine eumelanin pigment synthesis (black body coloration) and dopamine quinone-mediated protein cross-linking (Fig. 1). Similarly, knockdown of TcADC mRNA in Tribolium results in a deficiency of β-alanine and high levels of dopamine, with the latter used instead of NBAD for tanning of the exoskeleton.

Laccase 2 Is Involved in Dopamine/DOPA Eumelanin Pigmentation Pathway—We were interested in determining whether the same or different phenol oxidases influence the cuticle tanning pathway in wild-type and mutant strains of Tribolium. Previously, we demonstrated that two isoforms of laccase 2 catalyze beetle cuticle tanning (19). To determine whether TcLac2 also affects these processes in the black body color mutant of Tribolium, dsRNA for TcLac2 was injected into $b^{CHW}/b^{ST}$ late-stage larvae and pharate pupae. dsRNA for TcLac2 prevented larval, pupal, and adult cuticle tanning of the black mutant. The albino phenotypes obtained were similar to those of TcLac2 RNAi in the wild-type strain of Tribolium. In contrast, dsRNA for TcTyr1/Tyr2 had no effect on cuticle tanning in either strain (Fig. 7B). This result supports the hypothesis that, in addition to cuticle sclerotization, laccase 2, but not tyrosinase, catalyzes the DOPA/dopamine melanization (pigmentation) pathway in Tribolium (Fig. 1).

TcDDC Is Required for Pupal Development—A lethal pupal phenotype was observed after injection of dsTcDDC into late-instar larvae (Fig. 8). The pupal cuticle turned a grayish color and exhibited many black-colored patches. This pattern of coloration may be the result of a buildup of high levels of DOPA, some of which are oxidized to DOPA eumelanin in small localized areas only. DOPA cannot serve as a precursor for quinone tanning in most of the pupal cuticle. Huang et al. (33) reported some behavioral modifications and high mortality of Armigeres subalbatus after silencing of its DDC transcript using the double subgenomic Sindbis system, and DDC mutants of Drosophila have neurological phenotypes (15) because of the role of dopamine as a neurotransmitter. We do not know whether tissue-specific alternative splicing of TcDDC mRNA occurs in Tribolium, but it is likely that injection of dsTcDDC suppressed TcDDC transcript levels in the central nervous system also because this tissue is also susceptible to RNAi in Tribolium.3 The lethal pupal phenotype may be due to a down-regulation of transcripts of TcDDC in both the epidermis and central nervous system, adversely affecting both pupal cuticle formation and neurotransmission.

DOPA Is a Poor Substrate for Laccase 2—To study the function(s) of TcDDC in adult cuticle pigmentation and sclerotization in Tribolium, dsRNA for TcDDC was injected into 1–2-day-old pupae, and the adults generated were observed. Pupae treated with dsTcDDC developed normally. However, the initial adult cuticle pigmentation and sclerotization was substantially delayed (see insects in 0 and 1 d panels in Fig. 9). Also, the timing and rate of cuticle tanning of black body-colored beetles were similar to those of wild-type insects, suggesting that dopamine in black body insects is oxidized by laccase 2 and used for dopamine eumelanin synthesis when it accumulated during tanning metabolism. The level of DOPA increased about 5-fold in pharate adults after injection of dsRNA for TcDDC into 1–2-day-old pupae. However, unlike dopamine, DOPA does not appear to be well utilized for DOPA eumelanin or DOPA quinone synthesis, perhaps because of the substrate preference of laccase 2. Thomas et al. (34) reported that the specific activity of Manduca laccase solubilized from pharate pupal integument when DOPA was utilized as the substrate was about 20-fold less than when NBAD was used. Also DOPA is a poor substrate for recombinant M. sexta laccase produced using a baculovirus-insect cell line protein expression system.3 Interestingly, the body color of TcDDC-knockdown adults was darker than that of dsTcv-knockdown control beetles, but it was not as dark as any of the black body color mutants or TcADC-knockdown beetles (Fig. 9). Because DOPA eumelanin and dopamine eumelanin exhibit a similar black pigmentation, this result supports the hypothesis that little DOPA eumelanin accumulates in the Tribolium cuticle tanning pathway relative to NBAD- or NADA-mediated pigmentation and dopamine eumelanin, probably because DOPA is a poor substrate for laccase 2.

Mechanical Properties of Elytra from Black Body Color Mutants and dsTcADC-treated T. castaneum—A change in the frequency dependence of the E’ cuticle as well as that of the E’/E” ratio was observed in response to varying ADC expression. Natural levels of ADC expression present in dsTcv-knockdown control insects imparted the cuticle with an elastic modulus that is relatively frequency-independent, indicating effective cross-linking between cuticular components. In contrast, black and dsTcADC-treated beetles with reduced ADC levels produced a cuticle that had notably higher frequency dependence. This finding is consistent with the interpretation that a reduced ADC level results in an overabundance of melamin-containing pigments at the expense of cuticular protein cross-linking through NBAD, which requires ADC for production of β-alanine. Our observation that the black and dsTcADC-knockdown insects have a less cross-linked cuticle is consistent with the hypothesis that the black mutant is deficient in ADC, which in turn biases the cuticle reaction pathway to...
produce a dopamine eumelanin pigment at the expense of NBAD quinone-mediated cross-linking. This conclusion is further supported by the significantly higher E'/E' ratio found in the dsTcADC-injected insects and the black color mutant. The higher ratio suggests a greater dampening ability of such cuticle, consistent with a less cross-linked material. Cuticle from dsTcv-injected control insects exhibited a lower E'/E' ratio that is less dependent on stress frequency, further suggesting that the wild-type cuticle must possess a greater number of cross-links.

We have shown that tanning of red flour beetle cuticle is regulated in part by the synthesis of β-alanine, dopamine, NBAD, and NADA and also the subsequent oxidation of these catechols by laccase 2. NBAD and NADA are available for production of reddish-brown cuticle, and if β-alanine is unavailable for NBAD synthesis, dopamine, but not DOPA, can be utilized for black cuticle formation. High levels of dopamine in dark body and TcADC-knockdown insects occur because of a lack of β-alanine for synthesis of NBAD. TcDDC knockdown results in high levels of DOPA and either pupae that cannot tan their cuticles and die or adults with a darker than normal rust red-colored cuticle, which nonetheless still survive.

We have also demonstrated that the mechanical performance of the cuticle can be altered by tampering with the synthesis of specific tanning agents and enzymes using specific dsRNAs or mutants. Specifically, cuticle component cross-linking as indicated by modulus frequency dependence appears to depend on β-alanine and NBAD availability during cuticle formation. This observation further implicates NBAD as an important metabolite for cuticle tanning in T. castaneum. Finally, this work also demonstrates the potential of dynamic mechanical analysis to quantify physical differences in phenotype and to help assign a molecular interpretation of these differences. It may be possible to change the mechanical properties of cuticle in desirable ways by changing the ratios of the components needed for cuticle tanning.

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