Ebony, a Novel Nonribosomal Peptide Synthetase for β-Alanine Conjugation with Biogenic Amines in *Drosophila**

Arnd Richardt‡, Tobias Kemme‡, Stefanie Wagner, Dirk Schwarzer§, Mohamed A. Marahiel§, and Bernhard T. Hovemann¶

From the Fakultät für Chemie, AG Molekulare Zellbiochemie, Ruhr-Universität Bochum, Universitätsstrasse 150, 44780 Bochum, Germany and §Fachbereich Chemie/Biochemie, Philipps-Universität, Hans-Meerwein-Strasse, 35032 Marburg, Germany

Using Ebony protein either expressed in *Escherichia coli* or in Schneider S2 cells, we provide evidence for its substrate specificity and reaction mechanism. Ebony activates β-alanine to aminoacyladenylate by an adenylation domain and covalently attaches it as a thioester to a thiolation domain in a nonribosomal peptide synthetase (NRPS) related mechanism. In a second reaction, biogenic amines act as external nucleophiles on β-alanyl-*S*-pantetheine-Ebony, thereby releasing in a fast reaction the dipeptide (peptidoamine) in a process that is novel in higher eucaryotes. Therefore, we define Ebony as a β-alanyl-biogenic amine synthetase. Insight into the reaction mechanism stems from mutational analysis of an invariant serine that disclosed Ebony as a multienzyme with functional analogy to the starting modules of NRPSs. In light of a putative biogenic amine-deactivating capacity, Ebony function in the nervous system must be reconsidered. We propose that in the *Drosophila* eye Ebony is involved in the transmission process by inactivation of histamine through β-alanyl conjugation.

The *ebony* mutation (1) affects cuticle tanning and sclerotization as well as nervous system functions like vision (2) and behavior (3, 4). Previous biochemical investigations (5, 6) led to the conclusion that Ebony has β-alanyl-dopamine synthetase function. The visual phenotype of *ebony* is reflected by a reduced phototaxis and optomotor response (7, 8). Based on electroretinogram measurements that lack "on" and "off" transients, the visual phenotype has been attributed to altered responses in cells postsynaptic to the terminals of retinula cells R1-R6 of the lamina (2, 9). Histamine, which has been identified as neurotransmitter at these terminals, is released when retinula cells are depolarized (10). Because Ebony is expressed in the lamina glia surrounding the photoreceptor terminals that lack dopamine immunoreactivity, we previously proposed that glial expression reflects histamine conjugation to β-alanine (11). Recent evidence from the elucidation of histamine metabolism in fly heads (12) supported our above-mentioned hypothesis. Throughout fly development, the optic neuropile is not the sole but the major site of *ebony* expression in the nervous system (11, 13). This finding raised the intriguing

possibility that β-alanylation reflects an as yet unknown general pathway for biogenic amine neurotransmitter inactivation. A direct approach and first step to support this hypothesis therefore is to determine the biochemical activity of purified Ebony protein *in vitro*. Along these lines, it is important to consider that the close relationship of Ebony to the family of nonribosomal peptide synthetases (NRPSs)[1] should be reflected by a comparable reaction mechanism of peptide bond formation (Fig. 1).

NRPSs are of modular structure with each module incorporating one amino acid into the final product. Amino acid binding to the module is a rather specific two-step process, which involves selection of the cognate amino acid and activation as aminoacyladenylate in the adenylation domain followed by transfer to a 4′-phosphopantetheinyl (P-pant) group attached to an invariant serine of a neighboring thiolation domain (Fig. 2). A comparison of the Ebony amino acid sequence with that of known NRPSs reveals solely the core motifs of a putative adenylation and thiolation domain, an arrangement that is normally found in initiation modules (Fig. 3). Therefore, only the initial step of the reaction, namely amino acid activation, can follow the thiotemplate mechanism described for NRPSs (Fig. 2). Interestingly, the C-terminal 230 amino acids of the Ebony protein do not share homology with any known NRPSs and might represent a novel type of domain responsible for the selection of dopamine. In this report, we provide evidence by mutational analysis and *in vitro* assays that Ebony exclusively activates one substrate amino acid, β-alanine, in a two-step process hitherto only known from microbial peptide synthetases. However, nucleophilic release of β-alanine is not restricted to dopamine alone but instead Ebony accepts a variety of biogenic amines.

EXPERIMENTAL PROCEDURES

Fly Stocks—*Drosophila melanogaster* were cultured on standard corn meal medium. The wild type strain examined was Canton-S. w^{1118}; $In(3R)e^{AFA}$ (14, 15) served as *ebony* null mutant in white background for rescue transformation.

Ebony$_{His-6}$ Production in S2 Cells—The complete *ebony* reading frame was PCR-amplified from the previously described cDNA clone (13) by using a 5′-*Kpn*I primer 5′-**GGTACC**ATGGGTTCGCTGCCA-CAATTGTCG-3′ and a 3′-*Age*I primer 5′-**ACCGGT**TTTGCCCACCTC-CTTCCAATGGAC-3′. The product was cloned via *Kpn*I and *Age*I into the pMT-V5-HisA vector (Invitrogen), yielding pMT-*ebony*-HisA, which codes for the Ebony protein with a tag of six C-terminal histidines attached by a threonine-glycine bridge. A stable cell line was obtained by selection with hygromycin B according to the manufacturer's direc-

[1] The abbreviations used are: NRPS, nonribosomal peptide synthetase; P-pant, phosphopantetheinyl; *e*-cDNA, *ebony*-cDNA; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GABA, γ-aminobutyric acid; WT, wild type; UAS, upstream activating sequence.
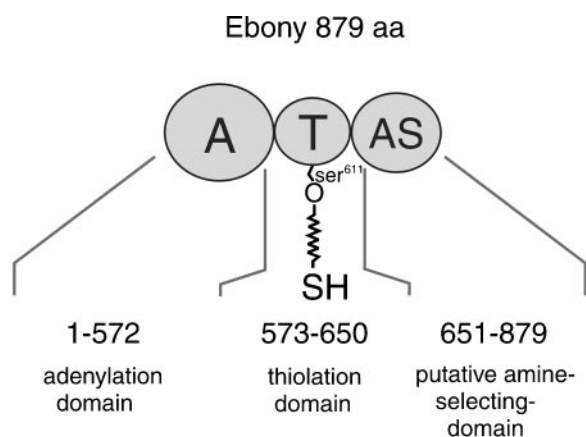
FIG. 1. **Functional domains of Ebony.** The 879 amino acids comprising Ebony protein possesses an NRPS-like domain organization including an adenylation (amino acid 1–572) and a thiolation domain (amino acids 573–650). Both domains can be predicted from structural data of analyzed NRPSs (34, 35). The C-terminal 230 amino acid residues do not share homologies with NRPSs and might represent a new type of domain responsible for selection of biogenic amines for the conjugation with β-alanine.
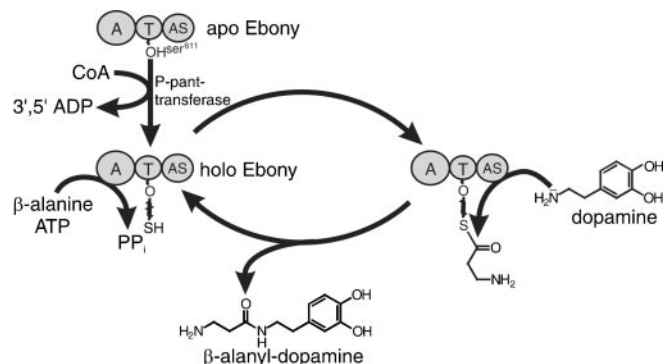
FIG. 2. **Proposed mechanism for Ebony-catalyzed binding of dopamine to β-alanine.** After translation, the apo-Ebony protein needs to be modified with the prosthetic P-pant, a reaction catalyzed by a dedicated P-pant transferase. The A-domain of holo-Ebony activates β-alanine as aminoacyladenylate at the expense of ATP and subsequently transfers it onto the thiol group of the P-pant thiolation domain. In the next step, the amine group of dopamine performs a nucleophilic attack onto the thioester of the Ebony-bound β-alanine that might be catalyzed by the putative amine-selecting domain. This leads to the formation of β-alanyl-dopamine and regenerates holo-Ebony for a new reaction cycle.

tions. Ebony$_{His-6}$ expression was induced by treatment with 0.5 mM CuSO$_4$ for 24 h. Cells were lysed, and the protein was affinity-purified on nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Ebony$_{His-6}$ Production in E. coli BL21(pREP4-gsp)—Ebony cDNA was PCR-amplified and cloned in the *Bam*HI site of pQE60 (Qiagen). *E. coli* BL21(pREP4-*gsp*) cells were transformed with the construct. Expression was induced from cells in early log phase with 0.1–0.4 mM isopropyl-1-thio-β-D-galactopyranoside overnight at 30 °C. The bacterial cell pellet was lysed in 50 mM Hepes, 300 mM NaCl, pH 8.0, with three rounds of French press treatment (1000 p.s.i.). From the cell lysate, Ebony was purified by affinity chromatography on nickel-nitrilotriacetic acid-agarose. Pooled enzyme fractions were dialyzed against 50 mM Na$_3$PO$_4$, 100 mM NaCl, pH 7.0. Protein concentration was determined according to Bradford (16).

ATP-pyrophosphate Exchange Assay—The ATP-pyrophosphate exchange assay was carried out according to Stachelhaus *et al.* (17) with modifications. In a final volume of 100 μl, the reaction mixture contained 500 nM affinity-purified enzyme, 1 mM amino acid, 50 mM sodium phosphate buffer, pH 7.0, 25 mM MgCl$_2$, 1.0 mM ATP, 50 μM *tetra*-sodium pyrophosphate, and 0.5 μCi (30 Ci/mmol) of *tetra*-[^{32}P]sodium pyrophosphate (PerkinElmer Life Sciences). After incubation at 37 °C for 15 min, the reaction was quenched by the addition of cold stop mixture containing 0.1 M sodium pyrophosphate, 0.56 M perchloric acid,

and 1.2% (w/v) activated charcoal (Norit A, Sigma). The charcoal was pelleted by centrifugation, washed twice with 1 ml of water, and resuspended in 0.5 ml of water. After addition of 5 ml of scintillation fluid (Rotiscint Eco Plus, Roth, Karlsruhe, Germany), the charcoal-bound radioactivity was determined by liquid scintillation counting.

Thioester Formation (Loading Assay)—100 μl of reaction mixture contained 500 nM enzyme, 1 mM ATP, and 1–3 μCi of [^{3}H]β-alanine (50 Ci/mmol) and 25 mM MgCl$_2$ in 50 mM sodium phosphate buffer, pH 7.0. After a 15-min incubation at 37 °C, the reaction was stopped by the addition of 15 μl of 2.5% (w/v) bovine serum albumen and 800 μl of chilled 10% (w/v) trichloroacetic acid. After 30 min on ice, the precipitate was pelleted by centrifugation and washed twice with 1 ml of chilled 10% trichloroacetic acid. The pellet was dissolved in 200 μl of formic acid. Incorporated radioactivity was counted in 5 ml of scintillation fluid.
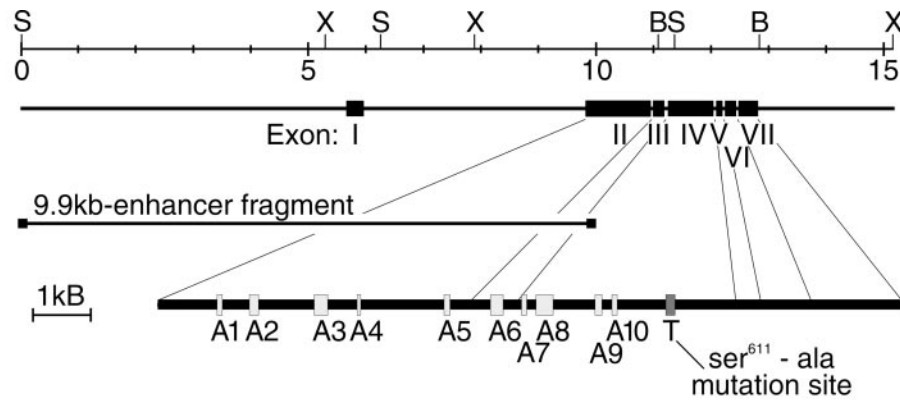
Peptide Bond Formation (Product Assay)—To ensure extensive loading of Ebony with its ^{3}H-labeled cognate amino acid, a preincubation was carried out for 15 min under loading assay conditions. The condensation reaction was initiated by the addition of the respective amino group containing reaction partner in a final concentration of 1 mM. At time points 0 (before addition of the second substrate), 10 s, 30 s, 1 min, and 5 min, aliquots were taken for trichloroacetic acid precipitation and liquid scintillation counting as described above.

Product Analysis—To obtain sufficient reaction product for mass spectroscopic analysis, the reaction mixture was scaled up 10-fold. 0.5 mM enzyme, 1 mM β-alanine, 1 mM biogenic amine, 25 mM MgCl$_2$, and 1 mM ATP in 50 mM sodium phosphate buffer, pH 7.0, in a final volume of 1 ml were incubated for 2 h at 37 °C. To analyze the products, the reaction was stopped by the addition of butanol/chloroform (4:1, v/v). After transfer to a fresh tube, the solvent was removed under vacuum. The pellet was resolved in 1 ml of methanol, and the samples were centrifuged for 15 min to pellet-insoluble components. Afterward the samples were directly injected into an electrospray ionization quadrupole time-of-flight spectrometer (Qstar Pulsar I, Applied Biosystems, Foster City, CA).

RESULTS

Evidence for a Thioesterification Step According to NRPSs—To investigate whether Ebony uses a NRPS-related activation process, Ser[611] of Ebony, the counterpart of an invariant Ser in the thiolation domain of NRPSs, was mutated to Ala. If Ser[611] holds the key function of P-pant cofactor binding, Ser[611]-Ala mutation should abolish Ebony activity completely as previously shown in mutational analysis of surfactin synthetase (18). Dependence on functional activity of Ser[611] *versus* Ala[611] can easily be investigated in *Drosophila in vivo* by cuticle color of transgenic flies. Expression of a transgenic unmutated *ebony*-cDNA (*e*-cDNA) copy should suffice to restore wild type function in an *ebony* mutant background, whereas transgenic Ser[611]-Ala-mutated *e*-cDNA should have no effect. To perform this test, we generated two types of *Drosophila* transformant lines in *w^{1118}*; *In(3R)e^{AFA}* mutant background. An *ebony*-enhancer-*gal*4 construct was transformed to provide the Gal4-driving element at the sites of cuticular Ebony expression. In addition, transformant lines were generated that contained either a mutant or a wild type UAS-*e*-cDNA construct for Gal4-driven expression. In accordance with previous successful *ebony* rescue experiments, we selected a genomic DNA fragment as *ebony*-specific enhancer-driving element that starts with the upstream *Sal*I site ~5.6 kb upstream of the *ebony* transcriptional start and extends just into exon 2 (Fig. 3) (13). *Ebony*-specific cuticle activity of Gal4 in the respective transformant line was verified in a cross with a UAS-*lacZ* line. Faithful β-galactosidase activity as revealed by X-gal conversion to the blue dye 5-bromo-4-chloro indigo at sites of cuticular Ebony expression confirmed the tissue-specific driving capacity (Fig. 4A).

To obtain Gal4-dependent Ebony expression, *e*-cDNA was PCR-amplified and cloned into pUAST using previously isolated cDNA clones (13, 19). A second pUAST-cDNA construct was prepared where a mutation in the *ebony*-reading frame was introduced by PCR amplification changing codon 611 from

FIG. 3. **Ebony gene organization and NRPS sequence homology.** In 15 kb of genomic *ebony* DNA, *Sal*I (*S*), *Xho*I (*X*), and *Bam*HI (*B*) restriction sites are shown. *Filled boxes* indicate the seven *ebony* gene exons. The *lower bar* delineates the genomic DNA cloned in front of *gal4* to facilitate *ebony*-like cuticle expression in an *ebony-gal4* transformant line. In the *blow up*, a bar comprising the *ebony* reading frame and the sites of homology to NRPS consensus core sequences A1–A10 and T (29) are indicated. The degree of amino acid conservation in Ebony as compared with the NRPS consensus sequence is listed below.
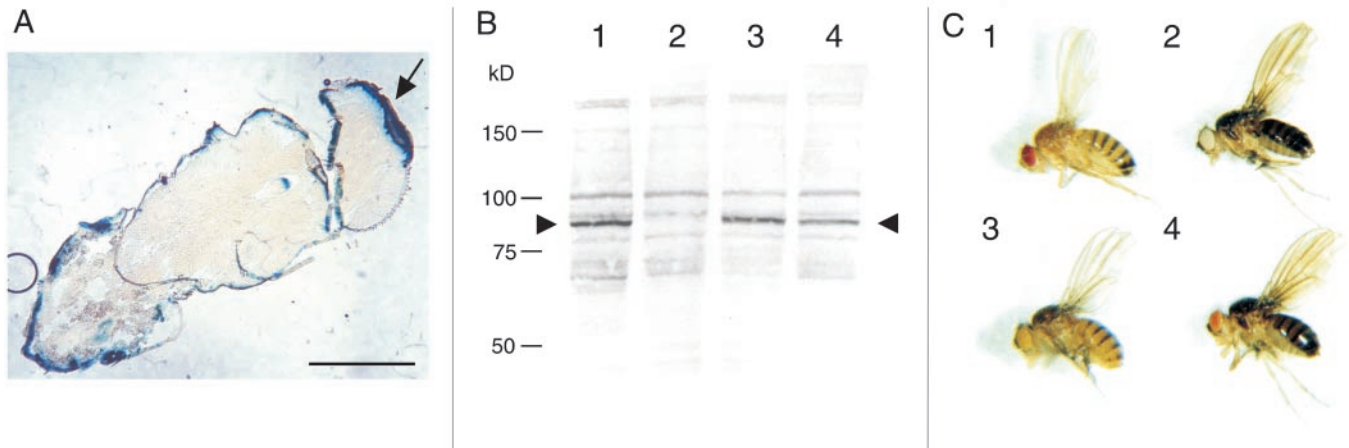
Motif	Core-Sequence		Motif	NRPS	Ebony
	NRPS	Ebony	A6	GELxIxGxG(VL)ARGYL	GEIFASGLNLAAGYV
A1	L(TS)YxEL	LLVAET	A7	Y(RK)TGDL	YRTGDY
A2	LKAGxAY(VL)P(LI)D	WKAGGAYLPID	A8	GRxDxQVKIRGxRIELGEIE	GRTDSQVKIRGHRVDLSEVE
A3	LAYxxYTSG(ST)TGxPKG	IAIVLYTSGSTGVPKG	A9	LPxYM(IV)P	LADYMTP
A4	FDxS	FVDS	A10	NGK(VL)DR	NGKVDR
A5	NxYGPTE	NFYGSTE	T	DxFFxLGG(HD)S(LI)	SNFYELGGNSL

FIG. 4. **Expression and activity of *e*-cDNA as compared with *e*-cDNASer^{611}-Ala.** *A*, confirmation of Gal4 expression in the cuticle (*arrow*) according to the ebony enhancer in a cross with a *UAS-lacZ* transformant line by X-gal staining. The scale bar corresponds to 500 μm. *B*, Ebony immunostaining in a Western blot of a SDS-PAGE containing head protein extracts of WT-Canton-S (*lane 1*), w^{1118}; $In(3R)e^{AFA}$ (*lane 2*), w^{1118}; *p[w, e-gal4]/p[w, UAS-e-cDNA]*; $In(3R)e^{AFA}$ (*lane 3*), and w^{1118}; *p[w, e-gal4]/p[w, UAS-e-cDNA*Ser^{611}-Ala*]*; $In(3R)e^{AFA}$ (*lane 4*) head extracts. *Arrowheads* point toward the Ebony band that appears at 90 kDa instead of the calculated molecular mass of 98 kDa. *C*, cuticle phenotype of transformants expressing a wild type copy of *e*-cDNA (*3*), a Ser^{611}-Ala mutant copy of *e*-cDNA (*4*) as compared with WT-Canton-S (*1*), and null mutant w^{1118}; $In(3R)e^{AFA}$ (*2*) flies.

UCG (Ser) to GCG (Ala). Transformation into w^{1118}; $In(3R)e^{AFA}$ flies resulted in lines harboring the wild type *e*-cDNA construct as well as in lines with the Ser^{611}-Ala-mutated *e*-cDNA construct. Subsequently, the *ebony* enhancer-Gal4 line was crossed with these transformants to express the integrated *e*-cDNA-constructs. Before evaluating the functionality of the expressed Ebony proteins, we had to confirm that each of the transformed cDNA copies was faithfully translated. Immunochemical detection of Ebony was performed with an antiserum raised against the C-terminal portion of the protein (11). As shown in Fig. 4B, protein preparations from fly heads of wild

type Canton-S (*lane 1*), the recipient line w^{1118}; $In(3R)e^{AFA}$ (*lane 2*) and the two cDNA-expressing lines, w^{1118}; *p[w, e-gal4]/p[w, UAS-e-cDNA] $In(3R)e^{AFA}$* (*lane 3*) and w^{1118}; *p[w, e-gal4]/p[w, UAS-e-cDNASer^{611}-Ala]*; $In(3R)e^{AFA}$* (*lane 4*), revealed that Ebony was clearly detectable in all but the w^{1118}; $In(3R)e^{AFA}$ mutant recipient line (Fig. 4B, *lane 2*). After confirmation of Ebony protein expression, we monitored the competence for $In(3R)e^{AFA}$ mutant cuticle color rescue (Fig. 4C). Induction of wild type Ebony in an *ebony-gal4/UAS-e-cDNA* cross gave rise to a clear rescue of the mutant phenotype as revealed by reversion of the dark cuticle color in Fig. 4C, *fly 3*. Flies

carrying the Ser[611]-Ala-mutated *e*-cDNA constructs showed a cuticle color identical to that of the *ebony* mutant parental line (Fig. 4*C*, *fly 4*). We note that the Ser[611]-Ala mutant Ebony protein was not competent in reverting the *In(3R)e*[AFA] mutant phenotype. Because the Ser[611]-Ala mutation is located within the conserved thiolation motif T (Fig. 3), this result is a strong hint to amino acid binding of Ebony by thioester formation.

Expression of Active Ebony Requires P-pant Transfer—The aforementioned genetic evidence has to be confirmed by biochemical determination of Ebony activity. If Ebony indeed binds its amino acid substrate by the two-step process that is known from NRPSs, one has to assume the necessity of an enzyme activation step involving the transfer of a P-pant moiety to Ser[611] (Fig. 2). To ensure production of activated Ebony holoenzyme, we had two options of expressing *ebony* cDNA. We used Schneider S2 cells (20) anticipating that the homologous expression system might provide the necessary activation activity *per se* and in sufficient strength. *Ebony*-cDNA was cloned into pMT-V5-HisA, Schneider S2 cells were transformed with this construct, and a stably transformed cell line was selected. Cell lysis and subsequent affinity purification of the C-terminally His$_6$-tagged protein resulted in a nearly pure protein according to SDS-PAGE. Even though assays on amino acid selection and activation as aminoacyladenylate revealed full activity for one amino acid (see below), loading onto the 4′-P-pant of Ebony was incomplete (Fig. 2). To find an explanation for the low level of activity, we first asked whether S2 cell-expressed Ebony was already substantially modified with the P-pant cofactor. The unspecific phosphopantetheinyltransferase from *Bacillus subtilis*, Sfp (21), was therefore used for additional *in vitro* conversion of potentially unphosphopantetheinylated S2 cell-expressed Ebony into the holoform. When [³H]coenzyme A (CoA) was added as substrate for Ebony phosphopantheteinylation, a significant amount of label was incorporated into Ebony (data not shown). This result indicated that [³H]P-pant had been transferred to Ebony by P-pant transferase activity *in vitro*. Both the thioesterification capacity and the P-pant acceptance of partially activated Ebony corroborated our genetic evidence.

Amino Acid Activation and Binding by Ebony—To obtain highly activated enzyme *in vivo*, we transformed wild type and, as negative control, Ser[611]-Ala mutant *ebony* cDNA cloned in pQE60 (Qiagen) into *E. coli* BL21(pREP4-*gsp*). This strain co-expresses the unspecific phosphopantetheinyltransferase Gsp from *Bacillus brevis* (22) and is frequently used to produce activated NRPSs by expression in *E. coli*. Assuming that Ebony mimics NRPS activity, amino acid activation and binding as thioester of affinity-purified His$_6$-tagged protein was investigated separately. Activation experiments followed the established strategy in determining specificity of amino acid selection by ATP/PP$_i$ exchange (17). This assay, which monitors the reverse reaction of binding, the formation of [³²P]ATP from aminoacyladenylate and [³²P]PP$_i$, was used to examine the forward reaction involving specific amino acid selection and activation as aminoacyladenylate. In all of the assays, the formation of [³²P]ATP was determined after an incubation period of 15 min. As putative substrates, we tested the 20 proteinogenic L-amino acids in groups of three to six as well as β-alanine, γ-aminobutyric acid (GABA), propylamine, and propionic acid as single substrates. Because this assay affirms only activation of the cognate amino acid as aminoacyladenylate independent of subsequent binding as thioester, it could be performed with all of the three enzyme preparations. The results obtained were identical with S2 cell-expressed, *E. coli* expressed wild type, and *E. coli* expressed Ser[611]-Ala mutant Ebony (data not shown), which confirms again that amino acid
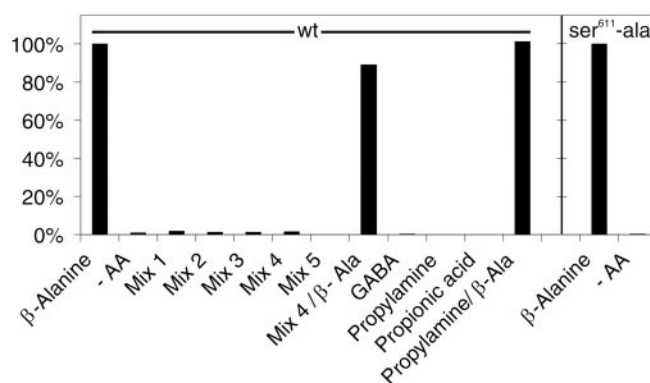

FIG. 5. **Amino acid selectivity of Ebony as determined by ATP/PP$_i$ exchange.** Radioactivity measured in an assay including *E. coli* expressed WT-Ebony (*wt*) and Ser[611]-Ala mutant Ebony (*ser[611]-ala*). Counts per minute obtained with β-alanine are set as 100%. Counts per minute measured using alternative substrates are calculated as the percentage of the β-alanine value and visualized as *bars*. Respective substrates are shown below each bar. −*AA* indicates the control assay without amino acid. Substrate activity of amino acids was tested in mixtures indicated as follows: *Mix 1*: Ala, Gly, Thr, and Ser; *Mix 2*: Pro, Leu, and Val; *Mix 3*: His, Phe, Asp, and Asn; *Mix 4*: Lys, Arg, Cys, Met, Tyr, and Ile; *Mix 5*: Gln, Trp, and Glu.

activation and thioesterification are independent processes. The presented experiments were performed using either *E. coli* expressed wild type or Ser[611]-Ala mutant Ebony protein. The highest amount of radioactivity incorporated into ATP was obtained when β-alanine was used as a substrate. The mean value from at least three separate measurements with several independent enzyme preparations was defined as 100% (Fig. 5). All of the proteinogenic L-amino acids that were used in combinations of 3–6 were below 2% of this value. Also, substances structurally related to β-alanine such as GABA, propylamine, and propionic acid did not serve as substrate for the adenylation process of Ebony. However, if β-alanine was added to one of the amino acid mixtures or to propylamine, [³²P]ATP formation increased to above 90% (Fig. 5). Adenylation activity of Ser[611]-Ala mutant Ebony was indistinguishable from wild type enzyme (Fig. 5). Taken together, among all of the substances tested, only β-alanine was selected as substrate and was bound as aminoacyladenylate by Ebony.

Substrate amino acid binding as thioester was next determined by incubation of *E. coli* produced wild type Ebony with [³H]β-alanine. Again, mean counts from at least three independent experiments were taken as 100% (Fig. 6*A*). Incubation with *E. coli* produced Ser[611]-Ala-mutated Ebony (data not shown) as well as the removal of ATP from the reaction mixture containing functional wild type Ebony (Fig. 6*A*) led to a 98% reduction. This result provides direct biochemical evidence that Ebony selects specifically the amino acid β-alanine, activates it as aminoacyladenylate, and binds it as thioester of the 4′-phosphopantetheinyl group linked to Ser[611].

Product Formation from β-Alanine-loaded Ebony—The reaction step of peptide bond formation and product release is reflected by the loss of radioactive counts from Ebony preloaded with [³H]β-alanine. This is caused by complete consumption of enzyme-bound [³H]β-alanine during the bond formation reaction. To identify compounds that are able to form a β-alanine conjugate, we incubated [³H]β-alanine-preloaded Ebony with various putative substrates (Fig. 6*A*). Dopamine gave rise to ~94% reduction of trichloroacetic acid-precipitable counts, a result that indicates an almost complete conversion into product. We next examined whether additional substrates would be accepted. We first tested alternative bioactive compounds also containing a planar ring structure linked to an ethylamino or hydroxyethylamino group, the dopamine-related neurotrans-
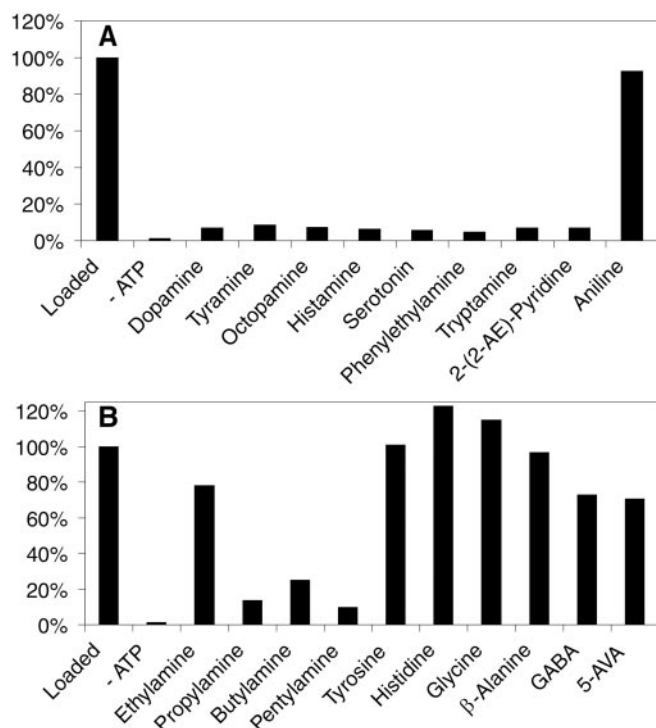
TABLE I
Product analysis using high resolution mass spectroscopy

product	structure	mass $[M+1]^+$ detected	mass $[M+1]^+$ calculated
β-alanyl-histamine		183.1249	183.1246
β-alanyl-tyramine		209.1299	209.1290
β-alanyl-dopamine		225.1239	225.1239
β-alanyl-octopamine		225.1227	225.1239
β-alanyl-serotonin		248.1388	248.1399

FIG. 6. *A and B, peptide bond formation activity of Ebony measured as release of* [³H]*β-alanine.* Radioactivity of [³H]β-alanine bound by Ebony in a loading reaction was set as 100% value. In a control reaction, ATP was omitted (−ATP). Conjugation efficiency of second substrates with [³H]β-alanine and concomitant release of product measured as reduction of counts per minute is shown as percentage of the starting value and indicated as *bar. 2-(2-AE)*-Pyridine, 2-(2-aminoethyl)-pyridine; *5-AVA*, 5-amino valerianic acid.

mitters tyramine and octopamine. Both compounds exhibited the same activity in the β-alanine release assay reducing trichloroacetic acid-precipitable radioactivity to ~6% of the starting value (Fig. 6A). We next tried to find out whether changing the ring structure from a benzyl ring to an imidazole ring would reduce activity. Replacing dopamine by histamine, the reduction of precipitable counts to ~5% was comparable with the previously employed substrates (Fig. 6A). Because there was no obvious difference in reactivity between a five- and a six-atom planar ring structure bound to an ethylamino group, we used the indol ring containing serotonin as substrate. Again, comparable activity leading to the release of 94% bound radioactivity was measured (Fig. 6A). The picture emerging by using physiologically active compounds was substantiated when biogenic amines were substituted by structurally related chemicals. Phenylethylamine and tryptamine could replace tyramine and serotonin, respectively, with the same efficiency of release of bound radioactivity. Also, the almost identical activity measured in the release assay when 2-(2-aminoethyl)-pyridine was used as substrate confirmed that the heterocyclic ring structure did not impair peptide bond-forming activity (Fig. 6A). However, when the amino group was placed close to the planar ring structure as in aniline for example, releasing activity was lost. We conclude from these results that a combination of an ethylamino or hydroxyethylamino group with a planar ring structure is sufficient to generate bond formation with β-alanine. We next investigated whether a primary amino group bound to an aliphatic chain would already allow β-alanine release. As shown in Fig. 6B, ethylamine had only little potential as substrate. With increasing length of the aliphatic chain as found in propylamine, butylamine, and pentylamine, a substantial portion of trichloroacetic acid-precipitable counts was released. Finally, we examined whether a negative charge con-

tained in a putative substrate would impair release activity. When we used tyrosine and histidine to replace tyramine and histamine, respectively, β-alanine release was not observed (Fig. 6B). Obviously, the carboxyl group hindered these compounds from serving as active substrates. If sterical interference at the primary amino group would be the major cause for inhibition, the aliphatic ω-amino carbonic acids glycine, β-alanine, GABA, and 5-amino valerianic acid (Fig. 6B) should exhibit a growing release activity with increasing distance of the carboxyl function from the amino group. A comparison of the results obtained with pentylamine and 5-amino valerianic acid, however, shows that a carboxyl group even in ω position substantially slows down product formation. Taken together, the preferred structure for β-alanine release is clearly an ethylamino or hydroxyethylamino group linked to an uncharged hydrophobic backbone. Among the active compounds tested in these assays, only the biogenic amines histamine, dopamine, tyramine, octopamine, and serotonin can be considered to play a major role in *Drosophila* physiology.

Release of trichloroacetic acid-precipitable counts from [³H]β-alanine-preloaded Ebony is an indirect measure of enzymatic activity but no direct proof of product formation. To ensure Ebony-catalyzed synthesis of the five predicted products β-alanyl-histamine, β-alanyl-dopamine, β-alanyl-tyramine, β-alanyl-octopamine, and β-alanyl-serotonin, we performed preparative product assays with β-alanine and the biogenic amines. These reactions were analyzed by high resolution mass spectroscopy. As a control, identical assays were performed and analyzed with the Ser[611]-Ala mutant replacing wild type Ebony. When using wild type Ebony, the mass signals of the expected products were detected, whereas the mass signals were absent when wild type Ebony was replaced by the Ser[611]-Ala mutant. These results are summarized in Table I.

DISCUSSION

Ebony Contains an NRPS-like Amino Acid Activation Module—Ebony similar to NRPSs belongs to the large family of aminoacyladenylate-forming enzymes (23). A relation to the thioesterification process, however, is in addition to NRPSs only present in the group of acyl carrier proteins including polyketide synthases and fatty acid synthases (24–26). The

homology between polyketide and fatty acid synthases and Ebony is limited to the core sequence element of the thiolation domain, which contains the invariant serine, the P-pant cofactor-mediated acyl carrier. Acyl carrier proteins, NRPSs, and Ebony need to be activated by P-pant cofactor transfer, which requires a corresponding transferase activity. Searching for two conserved amino acid sequence motifs detected in previously sequenced P-pant transferases (25), we indeed identified a reading frame in the *Drosophila* data base that showed a considerable homology to this conserved region. Expression of the corresponding putative P-pant transferase cDNA in *E. coli* gave rise to a protein that *in vitro* enhanced low level phosphantetheinylated S2 cell-derived Ebony activity depending on the presence of CoA comparable with the aforementioned Sfp.[2]

Our results demonstrate that the higher eucaryote *Drosophila* has preserved an amino acid activation mechanism that until now was considered to be specific for microbial NRPSs (27). Ebony combines this unique feature with a functional domain that allows peptide bond formation with a structurally constrained group of amines. However, a connection between two or more NRPS-like modules that enable the activation of amino acids and the formation of dipeptides has not yet been detected in higher eucaryotes even though genuine dipeptides such as β-alanyl-histidine (carnosine) have been shown to exist in vertebrates (28). Given the existence of a single NRPS-like activation domain in Ebony, nonribosomal synthesis of dipeptides in higher eucaryotes cannot generally be excluded. However, it would require two amino acid activation modules in addition to a functional domain for condensation of the two activated amino acids as well as a thioesterase activity for peptide release (29). Evidence that this complex structure of multimodular NRPS activity has been preserved through evolution to higher eucaryotes is still lacking.

Our experimental data now show that Ebony uses a novel two-step reaction mechanism including amino acid activation and binding followed by peptide bond formation. The procedure of amino acid activation and binding resembles that of NRPSs. Peptide bond formation and product release require a nucleophilic attack of an incoming primary amine that must meet the observed structural prerequisites. This is different in multimodular NRPSs in two ways. 1) The nature of peptide bond forming amino acid is predetermined by the specificity of a second adenylation domain within the multimodular enzyme, and 2) in NRPSs, a condensation domain located between the modules is essential for peptide bond catalysis. Such a condensation domain is missing in Ebony. Instead, a C-terminal domain (Fig. 2) with a yet unknown function seems to be responsible for catalyzing the nucleophilic attack of the primary amines (with relaxed substrate specificity) on the activated carboxyl thioester group of β-alanine. The mechanism of this reaction and that of dipeptide product release are still unknown.

Putative Ebony Function in Neurotransmitter Metabolism—
Ebony is expressed in diverse tissues at different times during development (11). Nervous system activity has been predicted from the behavioral and visual phenotype of the mutant but was only recently confirmed by activity staining of an *ebony-lacZ* fusion gene transformant and by immunocytochemistry (11, 13). The puzzling fact that evidence for dopaminergic neurons in the lamina was lacking (30) led to experiments that revealed that Ebony is involved in β-alanyl-histamine formation in the eye (12). The capacity of capturing the biogenic amines histamine, dopamine, tyramine, octopamine, and serotonin that clearly fulfill different functions in *Drosophila* to β-alanine might reflect a key function of Ebony at specific sites of the body (6, 11).

Here we provide evidence that Ebony is indeed capable of binding biogenic amines including histamine to β-alanine. Therefore, it is plausible to assign to Ebony a function in histamine neurotransmitter metabolism at the photoreceptor synapse of the eye. Because histamine synthesis (31) as well as metabolic degradation (10) in the eye is relatively slow, the almost infinite transmitter supply must be maintained by a fast re-uptake system (32, 33). Therefore, at the synapse where transmitter removal excites the postsynaptic cell by disinhibition, a mechanism of fast retraction of histamine from the synaptic cleft is essential. Interestingly, in illuminated barnacle photoreceptor preparations, [³H]histamine was concentrated over the photoreceptor terminals, whereas after incubation in the dark, the label was found at the glia (33). This observation lends support to the concept that at darkening a fast clearance of transmitter out of the synaptic cleft would be achieved by transport of histamine into the surrounding glia where it could be trapped by Ebony via β-alanine binding. The model requires that β-alanine is sufficiently loaded in the glia to prime Ebony for histamine capture and a biochemical pathway that allows the subsequent reuse of the withdrawn histamine in the photoreceptor. Although both histamine transport into photoreceptor as well as into glia has been reported previously (32, 33), it remains to be investigated whether a mechanism exists that darkening and concomitant reduction of histamine release shifts uptake toward glia followed by immediate inactivation by β-alanine binding.

Fast histamine removal from the synaptic cleft is essential for the function of arthropod photoreceptor synapses that operate with tonic release of histamine. *In vitro* product formation from [³H]β-alanine-loaded Ebony with histamine or any of the other biogenic amine substrates was already completed within 10 s, the shortest time point that could be determined under standard assay conditions.[2] This time point is still far away from the reaction velocity expected for a function in neurotransmitter inactivation. Beyond this point, reaction velocity may differ among the biogenic amines serving as substrate. Determination of V_{max} and K_m values of individual biogenic amines requires specific analytical methods operating in the millisecond range. They will disclose whether Ebony can fulfill the kinetic prerequisites for neurotransmitter inactivation.

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