Purification and Properties of a Very High Density Lipoprotein from the Hemolymph of the Honeybee *Apis mellifera*†

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ABSTRACT: A larval-specific very high density lipoprotein (VHDL) has been isolated from the hemolymph of the honeybee *Apis mellifera*. VHDL was isolated by a combination of density gradient ultracentrifugation and gel filtration. The purified protein is a dimer of M_r 160 000 apoproteins as shown by chemical cross-linking with dimethyl suberimidate. N-Terminal sequence analysis indicates that the two polypeptide chains are identical. The holoprotein contains 10% lipid by weight and 2.6% covalently bound carbohydrate. A native M_r 330 000 species was obtained by gel permeation chromatography. Antiserum directed against VHDL was used to show that VHDL is distinct from other hemolymph proteins and appears to constitute a novel lipoprotein of unknown function. However, the lipoprotein is present in high amounts in hemolymph only at the end of larval life, suggesting a potential role in lipid transport and/or storage protein metabolism during metamorphosis.

The process of metamorphosis in insects is accompanied by dramatic alterations in morphology and physiology. These changes are oftentimes reflected in the protein components of hemolymph during specific life stages. Thus, during the late larval stages when food consumption is maximal, a class of proteins referred to as storage proteins (Levenbook, 1985) accumulates in hemolymph (up to 80 mg/mL; Kramer et al., 1980). Upon pupation, these proteins are taken up by fat body tissue, stored, and eventually degraded to provide amino acids for the production of adult protein structures (Levenbook & Bauer, 1984). All proteins of this type examined thus far exist as hexamers of 75 000–85 000-dalton subunits. A specific class of storage proteins referred to as arylphorins (Riddiford & Law, 1983; Telfer et al., 1983), which possess an exceptionally high content of aromatic amino acids has been described for a number of insect species, including the honeybee (Ryan et al., 1984a).

A second class of major hemolymph proteins, known as lipophorins, mediates lipid transport in all life stages (Chino, 1985; Ryan et al., 1984b). The lipophorin of larval (Robbs et al., 1985) and adult (Ryan et al., 1984b) honeybees has been studied and appears to be a multifunctional lipid transport vehicle containing a variety of lipid classes including diacylglycerol, phospholipid, cholesterol, triacylglycerol, free fatty acid, and hydrocarbon. In contrast to lipid transport in mammalian plasma, it seems that insects utilize a single multifunctional lipid transport vehicle to meet physiological lipid transport demands.

Upon closer examination of hemolymph proteins during larval–pupal metamorphosis in the honeybee, we have discovered a larval stage specific very high density lipoprotein (VHDL)[1] which possesses a developmental pattern similar to storage proteins and lipid components similar to those of lipophorin. As a first step toward understanding the role of VHDL in honeybee lipid transport and/or storage protein metabolism during metamorphosis, we report here its isolation and chemical and immunological properties.

Materials and Methods

Animals. Animals were reared in normally managed colonies of *Apis mellifera ligustica* Spin. Frames of honeycomb with the larvae were freed of adult bees and taken to the laboratory where individual larvae were carefully removed. Adults were harvested at the hive and transported in jars to the laboratory.

Hemolymph Collection and Purification of VHDL. Hemolymph from larval- and pupal-stage animals was collected as previously described (Ryan et al., 1984a), while adults were bled by the flushing-out method of Chino et al. (1981). In a typical VHDL preparation from fifth-instar larvae, the hemolymph sample (approximately 2.0 mL) was placed in 5 mL of phosphate-buffered saline (PBS: 0.10 M sodium phosphate, pH 7.0, and 0.15 M NaCl) containing 1 mM diisopropyl phosphorofluoridate and 5 mM glutathione. The sample was then centrifuged at 5000*g* for 10 min at 4 °C to remove hemocytes. The hemocyte-free hemolymph was then adjusted to a density of 1.31 g/mL by the addition of solid KBr and PBS. The 1.31 g/mL solution (20 mL final volume) was placed in a Beckman Quick-Seal tube, overlayered with a 1.16 g/mL KBr solution, and centrifuged at 50 000 rpm in a VTi50 rotor for 16 h at 10 °C (Haunerland & Bowers, 1986). The tube contents were then fractionated into 2-mL fractions and, after dialysis to remove KBr, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to determine the fractions containing VHDL. The peak fractions (10–14) were pooled, adjusted to 1.31 g/mL with KBr and PBS (20 mL final volume), placed in a Beckman Quick-Seal tube, overlayered with a solution of KBr (d = 1.23 g/mL), and centrifuged as described above. Following frac-

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[1] Abbreviations: VHDL, very high density lipoprotein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; Con A, concanavalin A; PTH, phenylthiohydantoin; HPLC, high-pressure liquid chromatography.

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tionation and pooling of the VHDL peak fractions, the sample was concentrated by ultrafiltration (Amicon) and applied to a 1.5 × 170 cm column of AcA 22 (LKB) equilibrated in PBS with collection of 2.6-mL fractions at a flow rate of 10 mL/h. The VHDL-containing fractions were pooled and stored at 4 °C under a N_2 atmosphere or lyophilized after exhaustive dialysis against deionized H_2O.

Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gradient gel electrophoresis was performed according to Laemmli (1970). Except where indicated, a 4–12% acrylamide gradient separating gel and 2.5% stacking gel were employed. Electrophoresis was performed at 30-mA constant current until the bromophenol blue dye reached the base of the gel.

Carbohydrate Analysis. The carbohydrate content of VHDL was estimated by the phenol–sulfuric acid method of Dubois et al. (1956). Further characterization of the carbohydrate moiety was accomplished by fluorescein isothiocyanate conjugated concanavalin A (FITC–Con A) staining of VHDL following SDS–PAGE and transfer to nitrocellulose paper as previously described (Ryan et al., 1985a). Sugar composition was determined by gas–liquid chromatography. A sample of purified VHDL (2.6 mg) was hydrolyzed in 2 N HCl at 100 °C for 2 h followed by preparation of alditol acetates (Grimes & Greegor, 1976). Ten micrograms of 2-deoxyglucose was employed as internal standard, and samples were separated on a Hewlett-Packard 5700A gas chromatograph with a $^1/_8$th in. × 6 foot column of 3% (w/w) OV 225 on Supelcoport.

Lipid Analysis. Total lipid was extracted from a known amount of VHDL protein by the method of Bligh and Dyer (1959). The extract was dried under a stream of nitrogen and the lipid content determined gravimetrically. Twenty-five micrograms of dipentadecanoylphosphatidylcholine and 10 μg each of pentadecanoic acid, dipentadecanoin, and tripentadecanoin were added to the VHDL lipid extract, and then the sample was subjected to thin-layer chromatography (silica gel G plates developed in hexane–diethyl ether–formic acid, 70:30:2). The bands corresponding to phospholipid, diacylglycerol, free fatty acid, and triacylglycerol were scraped from the plate, eluted with chloroform–methanol (2:1), saponified in 5% KOH in methanol (w/v), and methylated with 14% boron trifluoride in methanol (w/v). Following extraction, the fatty acid methyl esters were chromatographed on a Varian Model 4600 gas chromatograph equipped with a flame ionization detector. Samples were separated on a 60 m OB-1 0.25-mm fused silica column and integrated with a Varian CDS-401 integrator. Sterol content was determined according to the procedure of Bowman and Wolf (1962) after lipid extraction of VHDL.

Chemical Cross-Linking. Cross-linking of VHDL was performed in 0.2 M triethanolamine buffer, pH 8.3 (Davies & Stark, 1970). One hundred microliters of a 1 mg/mL solution of VHDL was incubated with increasing amounts of dimethyl suberimidate as described by Ryan et al. (1985b). The final cross-linker concentration ranged from 0 to 0.5 mg/mL. After incubation for 1.5 h with shaking, the reaction was stopped by the addition of SDS–PAGE sample treatment buffer (Laemmli, 1970) and boiling. The samples were then separated on a 3–8% acrylamide gradient slab gel.

Amino Acid Analysis. The amino acid composition of purified VHDL was performed as previously described (Ryan et al., 1985b).

Determination of the N-Terminal Amino Acid Sequence. Honeybee VHDL was subjected to automated Edman degradation (Edman & Begg, 1967) using the Beckman 890M sequencer. The individual PTH derivatives were subsequently analyzed with the Beckman 110 HPLC system fitted with a C-18 reversed-phase column. The column was eluted with a linear gradient consisting of (A) 10% acetonitrile with 0.02 M sodium acetate and (B) 100% acetonitrile. The eluates were monitored by a 3390A Hewlett Packard integrator. The sample load was 2 nmol of VHDL (in 50% acetic acid), and the recovery of the first residue was 56%. Repetitive yields were consistently >95%.

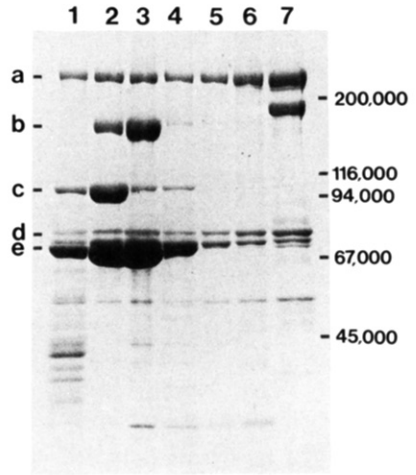

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of worker honeybee hemolymph at different stages of development. Samples were electrophoresed in a 4–12% acrylamide gradient slab at 30-mA constant current until the tracking dye reached the base of the gel. Lane 1, third-instar larval hemolymph; 2, fifth-instar larval hemolymph; 3, prepupa hemolymph; 4, day 3 pupa hemolymph (days of development); 5, day 7 pupa hemolymph; 6, newly eclosed adult; and 7, older adult hemolymph (>2 weeks). All samples were taken from workers (female). Bands identified by a letter are identified as (a) apolipophorin I, (b) VHDL, (c) M_r 105 000 protein, (d) apolipophorin II, and (e) storage proteins.

Immunology. Antibodies directed against VHDL were prepared in a New Zealand white rabbit. One milligram of VHDL was emulsified in Freund's complete adjuvant (1.0 mL) and injected intramuscularly into the hind limbs. After 4 weeks, a booster injection (1 mg of VHDL in Freund's incomplete adjuvant) was administered. Two weeks later, the rabbit was bled through a vein in the ear. The obtained serum was aliquoted (1 mL) and stored at −80 °C in the presence of 0.02% NaN_3. Double radial immunodiffusion was performed according to Ouchterlony (1968), and precipitin lines were visualized by staining with Coomassie blue.

Molecular Weight Determination. The native molecular weight of VHDL was determined by gel permeation chromatography performed at 25 °C. A 1.5 × 170 cm column of AcA 22 was calibrated with molecular weight standards (Bio-Rad) and a semilog plot of molecular weight vs. elution volume utilized. The molecular weight of the VHDL apoprotein was determined by SDS–PAGE. A semilog plot of the relative migration of VHDL apoprotein vs. that of known standards (Bio-Rad) was employed.

RESULTS

Hemolymph Protein Profiles. When hemolymph is drawn from worker honeybees during different life stages and subjected to SDS–PAGE, major changes in the protein pattern are observed (Figure 1). Included in these changes is the appearance of an M_r 160 000 polypeptide (Figure 1, band b) as the animal progresses through larval development and into pupation. Adults lack this protein, but older "foraging" workers contain a M_r 185 000 protein specific to that life stage (Figure 1, lane 7). We believe this to be the egg yolk protein

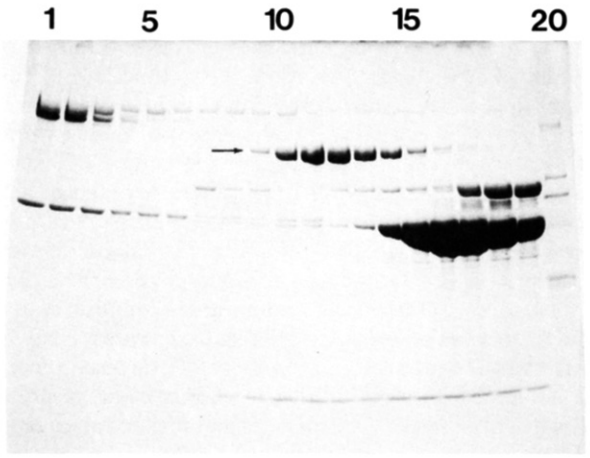

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of honeybee hemolymph following the initial density gradient ultracentrifugation step. Following ultracentrifugation, the contents of the tube were fractionated from the top into 2-mL fractions. After dialysis, aliquots of each fraction were applied to the gel (lanes 1–19), and electrophoresis was performed as described in Figure 1. Lane 20 is the molecular weight standards. Arrow indicates position of VHDL.

precursor vitellogenin (Harnish & White, 1982). Also, present in all life stages is the high-density lipoprotein lipophorin (Robbs et al., 1985), indicated by the presence of the M_r 250 000 apoprotein apolipophorin I (apoLp-I) and the M_r 80 000 apoprotein apolipophorin II (apoLp-II) (Figure 1, bands a and d). The profile during the larval and pupal stages is dominated by protein bands (Figure 1, band e) in the M_r 75 000–80 000 range. These bands represent subunits of the M_r 500 000 hexameric storage proteins (Ryan et al., 1984a). Another major band (Figure 1, band c) of M_r 105 000, which has yet to be investigated, appears to be specific to the immature stages. It is evident from Figure 1 that the M_r 160 000 protein has a developmental profile distinct from most other major hemolymph proteins in that it is abundant only during the late larval stage. A similar developmental profile for the M_r 160 000 protein was obtained for males (drones) (data not shown).

Purification of VHDL. Hemolymph collected from worker larvae (late fifth instar) was subjected to density gradient ultracentrifugation (see Materials and Methods) to float lipophorin, as shown by SDS–PAGE of fractions separated on the basis of density (Figure 2). In addition to the expected lipophorin apoproteins apoLp-I and -II (M_r 250 000 and 80 000, respectively), the M_r 160 000 protein floated to a position (arrow, Figure 2) in the gradient intermediate between that of lipophorin and the remainder of the hemolymph proteins, suggesting it is a very high density lipoprotein (VHDL). Fractions enriched in VHDL were subsequently pooled, adjusted to 1.31 g/mL, and subjected to a second density gradient ultracentrifugation step (see Materials and Methods). Fractionation of the material from the second density gradient ultracentrifugation and analysis of the major protein peak by SDS–PAGE indicated VHDL was nearly pure; the only contaminant was a low molecular weight component. The latter was quantitatively removed by gel permeation chromatography. Following this step, SDS–PAGE (Figure 3) showed that a homogenous protein had been obtained. The molecular weight of the holoprotein was estimated by gel permeation chromatography to be ~330 000, while the apoprotein size was determined to be 160 000 daltons by gradient gel electrophoresis in the presence of SDS. The apoprotein stoichiometry in native VHDL was assessed by chemical

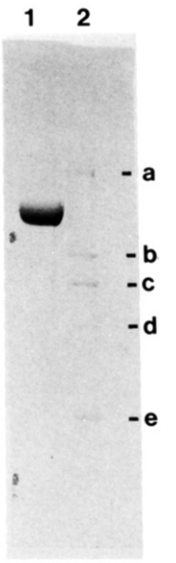

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified VHDL. Acrylamide gradient slab gel electrophoresis was performed as described in the legend to Figure 1. Lane 1, 20 μg of VHDL; lane 2, molecular weight standards including (a) myosin (M_r 200 000), (b) β-galactosidase (M_r 116 000), (c) phosphorylase *b* (M_r 94 000), (d) bovine serum albumin (M_r 67 000), and (e) ovalbumin (M_r 45 000).

cross-linking. The results of this experiment revealed that under a broad range of protein:cross-linker ratios, native VHDL exists as a dimer of M_r 160 000 apoproteins.

Lipid Composition. The behavior of VHDL, when subjected to density gradient ultracentrifugation, suggested the presence of lipid in the holoprotein. Following density gradient ultracentrifugation, an equilibrium density of 1.26 g/mL was determined by measuring the density of individual fractions by refractometry. Extraction of VHDL with organic solvents and gravimetric measurement indicated that native VHDL is composed of 10% lipid. Further characterization of the lipid components indicated a large percentage of polar lipid (72.5% phospholipid) with lesser amounts of free fatty acids (11.9%), sterol (7.3%), diacylglycerol (4.6%), and triacylglycerol (3.7%). As with nearly all other insect lipoproteins reported to date (Beenakkers et al., 1986; Chino & Kitazawa, 1981), the major neutral glyceride was diacylglycerol. The predominant acyl chains of all glycerolipids and free fatty acids were palmitate, stearate, and oleate.

Carbohydrate Analysis. The presence of covalent mannose-containing oligosaccharide residues on VHDL was confirmed by positive FITC–Con A staining of the VHDL apoprotein following separation by SDS–PAGE and electrophoretic transfer to nitrocellulose paper. Colorimetric determination of the carbohydrate of VHDL indicated a carbohydrate content of 2.6%. Compositional analysis of the sugar residues by gas–liquid chromatography indicated the presence of mannose–*N*-acetylglucosamine–glucose in a ratio of 9:2:1.

Amino Acid Analysis and N-Terminal Sequence. The amino acid composition of VHDL is presented in Table I. The VHDL holoprotein contains two apoproteins of M_r 160 000. N-Terminal sequence analysis of the apoproteins (Figure 4) yielded only a single sequence, indicating that the two VHDL apoproteins are identical.

Immunology. Antibodies directed against purified VHDL were used in experiments to assess the purity of VHDL and its presence or absence during different life stages or in different sexes. Double radial immunodiffusion of purified VHDL and anti-VHDL serum produced a single sharp precipitin line (Figure 5). A single precipitin line was also

Table I: Amino Acid Composition of Honeybee VHDL

amino acid	mol %[a]	amino acid	mol %[a]
aspartate[b]	13.5	isoleucine	7.6
threonine	6.6	leucine	8.6
serine	6.9	tyrosine	3.4
glutamate[b]	7.0	phenylalanine	2.8
proline	3.4	histidine	5.8
glycine	4.2	lysine	7.9
alanine	4.7	arginine	3.6
valine	8.7	cysteine[c]	2.3
methionine	2.9	tryptophan	nd[d]

[a] Data derived from analysis of duplicate samples hydrolyzed for 24, 48, and 72 h in 6 N HCl in vacuo, 110 °C. [b] Includes acid plus amide. [c] Duplicate samples were performic acid oxidized and then hydrolyzed for 24 h in 6 N HCl in vacuo. [d] Not determined.

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                    5                   10

NH2-Val-Ala-Pro-Phe-Pro-His-Gly-Lys-Leu-Val-

                   15                   20

Thr-Tyr-Lys-Tyr-Ile-Ala-Asp-Val-Lys-Ala-

                   25

Gly-Val-Asp-Pro-Ser-Leu-Val
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FIGURE 4: Covalent structure of VHDL N-terminal segment. Details are given under Materials and Methods. The sequence was determined in a single automated Edman degradation run using 2 nmol of intact VHDL.

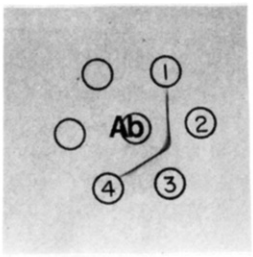

FIGURE 5: Double radial immunodiffusion analysis of honeybee hemolymph samples and purified VHDL vs. anti-VHDL serum. One percent agar plates were utilized, and immunodiffusion was allowed to proceed for 24 h at room temperature. The plate was stained with Coomassie blue. Center well, anti-VHDL serum; peripheral wells: (1) second-instar larval hemolymph; (2) late fifth-instar larval hemolymph; (3) purified VHDL; (4) adult hemolymph.

observed when whole fifth-instar larval worker hemolymph was utilized, indicating the presence of a single reactive antigen at this life stage. Fusion of the two precipitin lines (whole fifth-instar hemolymph and purified VHDL) indicates identity. The lack of observable precipitin lines in second-instar larval hemolymph or adult hemolymph is in agreement with the gel electrophoretic results (Figure 1) which suggested that VHDL appears and disappears from hemolymph on a timed developmental schedule. Identical results were obtained with hemolymph samples from workers (female) and drones (male), indicating VHDL is not a sex-specific protein.

Discussion

Hemolymph protein profiles from larvae of holometabolous insects are usually dominated by a high molecular weight class of proteins referred to as "storage proteins" (Levenbook, 1985). Although several immunologically distinct classes of storage proteins have emerged recently (Riddiford & Law, 1983; Ryan et al., 1985b), a common structural theme of these proteins is a hexameric quaternary arrangement of M_r 75 000–85 000 subunits. While slight variations in the timetable of hemolymph appearance of these proteins have been noted, invariably storage proteins are present late in larval life and are absent or greatly reduced in hemolymph from mature adults. Levenbook and Bauer (1984) have shown that labeled amino acids derived from [^{14}C]phenylalanine-labeled *Calliphora erythrocephala* arylphorin are utilized in the production of adult protein structures during metamorphosis. Thus, storage proteins synthesized during feeding stages can provide amino acid reserves which are utilized during the quiescent nonfeeding pupal stage to produce adult proteins. Honeybees appear to be unique because in addition to classical storage proteins (Ryan et al., 1984a), the hemolymph of fifth-instar larvae and prepupae contains a component of M_r 160 000 (as determined by SDS–PAGE) that follows a pattern of development which resembles that of the storage proteins. Given the apparent developmental relationship between this protein and storage proteins and its potential physiological importance in metamorphosis and development, the protein was purified, and its physical and chemical properties were investigated. A purification scheme based on density gradient ultracentrifugation provided a rapid and efficient method for purification of significant amounts of this protein.

The results of amino acid analysis and immunological studies demonstrate that VHDL is a distinct hemolymph protein unrelated to the other major hemolymph proteins. The presence of 10% lipid in the native protein places the protein in the very high density lipoprotein (VHDL) range. The lipid classes found in VHDL are the same as those of honeybee lipophorin but differ in relative amounts (Robbs et al., 1985). The rapid rise in the hemolymph concentration of VHDL just prior to metamorphosis may serve to supplement lipid transport processes normally accomplished by lipophorin. In addition to lipid, honeybee VHDL is composed of two identical apoproteins of M_r 160 000 with a native holoprotein of M_r 330 000. Furthermore, the presence of covalently bound high-mannose oligosaccharide was confirmed by lectin binding studies and compositional analysis. The presence of glucose residues on honeybee VHDL oligosaccharide, while unusual, is consistent with the presence of this residue on high-mannose oligosaccharides from hemolymph proteins of *Manduca sexta*.[2] Structure determination of VHDL oligosaccharide will be required before accurate comparisons with other known glucose-containing high-mannose oligosaccharides (Tsai et al., 1984) can be made. Also, it is interesting to note that nearly every major hemolymph protein in *A. mellifera* is glycosylated as judged by fluorescent lectin binding to hemolymph proteins separated by SDS–PAGE.

Anti-VHDL serum was monospecific for the lipoprotein. No cross-reaction of anti-VHDL was observed upon immunodiffusion with hemolymph from *Manduca sexta*. Indeed, honeybee VHDL has no counterpart in any insect species so far reported, with the possible exception of a blue chromoprotein from *Heliothis zea* (Haunerland & Bowers, 1986). The *H. zea* protein is a tetrameric very high density lipoprotein with 150 000-dalton apoproteins. While it is predominant in the late larval stage, there is no immunological relationship between the VHDL of *H. zea* and the honeybee.[3] The availability of monospecific antiserum will allow for precise monitoring of the appearance and fate of VHDL during ho-

[2] R. O. Ryan, D. R. Anderson, W. J. Grimes, M. A. Wells, and J. H. Law, unpublished experiments.

[3] R. O. Ryan, unpublished observation.

neybee development. Important questions regarding VHDL synthesis, potential uptake, and storage by tissue and its role in honeybee lipid metabolism can now be addressed.

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Intramolecular Cross-Linking of Myosin Subfragment 1 with Bimane†

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ABSTRACT: We previously showed that the fluorescent inter-thiol cross-linker dibromobimane (DBB) [Kosower, N. S., Kosower, E. M., Newton, G. L., & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3382–3386] cross-links two [50 and 20 kilodaltons (kDa)] of the three major fragments of myosin subfragment 1 (S-1); on intact S-1, DBB quenches tryptophans and inhibits all ATPases [Mornet, D., Ue, K., & Morales, M. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1658–1662]. Here we characterize the modification chemically: DBB cross-links Cys-522 (50 kDa) with Cys-707 (20 kDa), thereby sealing a large preexisting heavy-chain loop containing important functionalities. Cross-linking rate is insensitive to nucleotides, but apparently sterically, either monobromobimane or DBB reduces Ca^{2+}-ATPase to low, nonzero levels.

In a previous paper (Mornet et al., 1985), we reported that the inter-thiol cross-linker dibromobimane (DBB)[1] (Kosower et al., 1979) cross-links Cys-707 ("SH1") of the myosin head with a second thiol whose sequence position[2] was not identified except for ascertaining that it resided on the proteolytic fragment of S-1 known as "50 kDa" (Cys-707 resides on the proteolytic fragment, "20 kDa"). We showed that this intramolecular cross-linking seals off a myosin heavy-chain "loop" whose length was shown to exceed 44 residues (5 kDa).

This new modification of S-1 is interesting for several reasons: when in place, DBB (a) strongly accepts resonance

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[1] Abbreviations: S-1, chymotryptic subfragment 1 of myosin; DBB, dibromobimane; MBB, monobromobimane; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; M_r, molecular weight; LC_1, alkali light chain 1; LC_3, alkali light chain 2; SH, thiol residue; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; dansyl, 5-(dimethylamino)-1-naphthalenesulfonate.

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