Characterization of *Drosophila* Hemoglobin: Evidence for Hemoglobin-Mediated Respiration in Insects*

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*Running title:* *Drosophila* hemoglobin
*Footnotes:

Abbreviations used: BDGP, Berkeley Drosophila Genome Project; bp, base pairs; BSA, bovine serum albumin; EST, expressed sequence tag; Hb, hemoglobin; HRE, hypoxia-responsive sequence element; kb, kilo-base pairs; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gelelectrophoresis. UTR, untranslated region. Correspondence and requests for material should be addressed to T.H. (hankeln@molgen.biologie.uni-mainz.de) or T.B. (burmeste@mail.uni-mainz.de).
SUMMARY

In contrast to previous assumptions, the fruitfly *Drosophila melanogaster* possesses hemoglobin. This respiratory protein forms a monomer of about 17 kDa that is not exported into the hemolymph. Recombinant *Drosophila* hemoglobin displays a typical hexacoordinated deoxy-spectrum and binds oxygen with an affinity of 0.12 Torr. Four different hemoglobin transcripts have been identified, which are generated by two distinct promoters of the hemoglobin (*glob1*) gene, but are identical in their coding regions. Putative binding sites for hypoxia-regulated transcription factors have been identified in the gene. Hemoglobin synthesis in *Drosophila* is mainly associated with the tracheal system and the fat body. This suggests that oxygen supply in insects may be more complex than previously thought and may depend on hemoglobin-mediated oxygen transport and storage in addition to simple diffusion.
INTRODUCTION

The aerobic metabolism of most animals requires a sufficient supply of oxygen to internal tissues. Gas-exchange in insects and other terrestrial arthropods is mediated via trachea that enable the transport of oxygen from the atmosphere to the inner organs. For this reason, respiratory proteins have been regarded as unnecessary in most insects. Only in species that are adapted to an hypoxic environment were considered as sporadic exceptions: the aquatic larvae of the chironomid midges (1), some aquatic Hemiptera (2, 3) and the larvae of the botfly *Gasterophilus intestinalis* (4, 5), which live in the horse stomach, possess hemoglobins (Hbs). Insect Hbs may either occur freely dissolved in the hemolymph (Chironomidae) or accumulate in specialized tissues, mostly tracheal or fat body cells (6). Typically, the Hbs of these species reach concentrations in the millimolar range and thus may easily be identified due to the red color of the organs or the hemolymph. However, recently we showed that a hemoglobin gene is present and expressed in the fruitfly *Drosophila melanogaster* (7). The Hb gene of *Drosophila* (*glob1*) is located on chromosome 3 at position 89A13-B1. It shares significant sequence similarity with the extracellular Hbs from Chironomidae, but the highest identity score was found with the intracellular Hb of the botfly *G. intestinalis* (39% identity and 64% similarity of the amino acids).

The identification of an Hb in *Drosophila*, which was unexpected at the first glance, suggested the possibility that a respiratory protein is part of the standard genetic repertoire of insects. Such an assumption is supported by the molecular phylogenetic analysis, which shows a close association of the different insect Hbs, including the chironomid extracellular Hbs (7). The physiological role of intracellular Hbs, which are mostly expressed at low levels in many invertebrate taxa, is not well established. Intracellular Hbs may enhance the diffusional transport of oxygen from the extracellular medium to the mitochondria (8, 9). Others might be involved in oxygen sensing (10) or have enzymatic functions (11, 12). To understand the role of the
intracellular Hbs in insect physiology, we have investigated the gene organization, expression pattern, biochemical properties and ligand binding behavior of *D. melanogaster* Hb.

**MATERIALS AND METHODS**

**Cloning and purification of recombinant *Drosophila* Hb.** The *Drosophila* Hb coding region (EMBL/GenBank™ accession number NM_079645) was cloned into the pET3a expression plasmid using the *Nde*I and *Bam*HI restriction sites. The recombinant plasmid was transformed into *E. coli* strain BL21(DE3)pLysS. Cells were grown at 25°C in TB medium containing 200 µg/ml ampicillin, 30 µg/ml chloramphenicol and 1 mM δ-amino-levulinic acid. Hb expression was induced at A₆₀₀ = 0.8 by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. After 14-18 hrs, the reddish bacteria were harvested and dissolved in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM dithiotreitol. The bacteria were broken with three freeze-thaw cycles, followed by sonication. The debris was removed by two centrifugation steps (10 min 10,000 × g and 60 min 105,000 × g). Further purification was achieved by 40 and 90 % (NH₄)₂SO₄ precipitation. The 90 % pellet, which contains the crude Hb pellet, was dissolved in 50 mM Tris-HCl, pH 8.5, dialyzed over night and loaded onto a Fast Flow DEAE-Sepharose (AMERSHAM PHARMACIA) column equilibrated in the same buffer. After washing of the unbound material, the Hb was eluted with 200 mM NaCl. The Hb fractions were concentrated by AMICON filtration (PM10) and passed through a Sephacryl S200 column. The final Hb fractions were pooled, concentrated and stored at –20°C until use.

**Spectra and ligand binding kinetics.** Absorption spectra of recombinant *D. melanogaster* Hb were measured for the various forms and conditions using an SLM-Aminco DW2000 spectrophotometer. Ligand-binding experiments were performed on recombinant Hb in 100 mM potassium phosphate pH 7.0 at 25°C. Kinetics were measured by flash photolysis (13), using laser pulses of 10 ns at 532 nm (QUANTEL, France), with detection at 436 nm. The kinetics were studied for
samples equilibrated with air or CO. The oxygen dissociation rate was determined from the kinetics after photolysis of samples equilibrated under a mixed oxygen-CO atmosphere. The transition from the hexacoordinated deoxy form or the oxy form to the CO bound form was measured by stopped flow (BIOLOGIC, Claix, France) by mixing a deoxygenated Hb sample with a solution equilibrated under CO. These kinetics involve the competition of ligands, and simulations were made taking into account the on/off rates for each ligand, the solvent concentration of the external ligand, and the absorption coefficients at the wavelength of detection. The equations for the binding of both ligands were numerically integrated to obtain the time course for the overall reaction. The oxygen affinity in equilibrium was determined independently, by measuring full spectra at different partial pressures of oxygen for samples in a tonometer.

**Western blotting.** Antibodies against recombinant *Drosophila* Hb were raised in rabbits. For Western blotting, total protein extracts were separated by gel electrophoresis on a 14% acrylamide gel in the presence of SDS. The proteins were transferred to nitrocellulose by the semi-dry method. The filters were blocked in 2% non-fat dry milk in TBST (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.3% Tween-20) for several hours and subsequently incubated over night with the antibodies (1:10,000) diluted in TBST/2% milk. Goat anti-rabbit IgGs conjugated with alkaline phosphatase were used as secondary antibody. Detection was performed using nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Immuno-staining.** *Drosophila* larvae and adults were anesthetized and fixed overnight at 4°C in BT-fix (4% sucrose, 0.12 M CaCl₂, 0.1 M NaH₂PO₄, pH 7.4) supplemented with 4% paraformaldehyde, and subsequently rinsed in 2× BT-fix. Mounted 15 µm cryosections were rinsed in PBS (7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 130 mM NaCl), blocked with 30% BSA/PBS, and incubated with the anti-*Drosophila* Hb antibody (1:500). The specimens were washed 3× 5 min in PBS and incubated in the dark for 1 h with anti-rabbit antibodies coupled with horse
radish peroxidase. After washing 3 × 5 min with PBS, specific staining was detected by comparison with control slices (without first antibody).

**RNA preparation and RT-PCR.** Total RNA was isolated using the guanidinium-isothiocyanate method (14). For the RT-PCR experiments cDNA was synthesized with the Superscript™ II reverse transcriptase (GIBCO-BRL Life Technologies, Karlsruhe, Germany) using a specific primer, and amplified by PCR using nested primers. Appropriate water and extraction controls have been included in all analyses. 5' RACE assays were carried out with a series of nested oligonucleotide primers using the kit from GIBCO-BRL Life Technologies. The PCR products were purified from agarose gels and directly sequenced. EST (expressed sequence tag; 15) sequences were obtained from the Berkeley Drosophila Genome Project (BDGP) database using the BLAST algorithm (16).

**Whole-mount in situ-hybridization.** Digoxigenin-labeled anti-sense RNA probes were produced using the ROCHE in vitro-transcription kit according to the manufacturer's instructions. Non-radioactive in situ-hybridization with embryos, larvae and adult *Drosophila* was essentially performed according to the method of Tautz and Pfeifle (17). Briefly, the embryos were fixed for 20 min in 50% heptan, 5.5% formaldehyde in PBT (7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 130 mM NaCl, 0.1% Tween-20), washed with methanol and stored in ethanol at -20°C. The tissues of larval and adult specimens were fixed 10 min in 4% paraformaldehyde in PBS. After 3 × 5 min washing with PBT, the samples were post-fixed for 20 min in 5% formaldehyde in PBT. Prehybridization was carried out in 50% formamide, 5 × SSC, 0.1% Tween-20 for about 2 h at 55°C, and hybridization with labeled anti-sense RNA probes were performed at 65°C over night in the same solution. Sense-probes were used as negative controls. The samples were washed for 15 min at 65°C in hybridization solution, 15 min in 50% hybridization solution/50% PBT, and 5 × 10 min in PBT. The specimens were incubated at room temperature with an anti-digoxigenin antibody coupled with alkaline phosphatase (90 min 1:1,000 in PBT) and washed 3 × 10 min in
PBT. Detection was carried out using nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, and specimens were post-fixed in 3.7% formaldehyde in PBT, washed with PBT and stored in 70% glycerol.

**RESULTS**

**Ligand binding properties.** The *Drosophila* Hb was recombinantly expressed in the bacterial pET3a system and purified by ammonium-sulfate precipitation and chromatography. The protein elutes as a monomer in size exclusion chromatography of about 17 kDa (not shown). The absorbance spectra were recorded between 350 and 710 nm (Fig. 1A). The ferrous deoxy form of *Drosophila* Hb exhibits a spectrum that is typical of a hexacoordinated Hb, as illustrated by large amplitudes of the $\alpha$ band (561 nm) and the Soret band (426.5 nm), plus a smaller peak at 531 nm. The oxy-form displays the typical Hb-like pattern with absorption maxima at 419, 541 and 576 nm; the maxima of the CO-form are at 422, 542 and 572 nm.

The binding of CO and O$_2$ to the photodissociated *Drosophila* Hb is a biphasic process (Fig. 1B), with a rapid phase reflecting the competitive binding of the external ligand and an intrinsic protein ligand, most likely the distal E7-histidine. The slower phase corresponds to the globin fraction in which the distal histidine as an internal ligand is bound to the iron atom. The replacement of the histidine by CO occurs on a much slower timescale, essentially limited by histidine dissociation, which has a time coefficient of 25 ms (from stopped flow measurements) or 30 ms by the flash photolysis technique. The CO association rate is best determined at high levels of CO (1 atm), where the kinetics has little contribution from the histidine; at sufficiently low levels of CO, histidine binding is favored and one observes essentially the His on rate.

As for other Hbs, there is a higher affinity for CO, although oxygen association is about 5 times faster than CO-binding ($64 \times 10^6$ M$^{-1}$ s$^{-1}$ and $13 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively). These
conditions allow use of a mixed O$_2$/CO atmosphere to study competition between these two external ligands. After photodissociation, a certain fraction will bind oxygen, followed by a slow replacement reaction for the return to the CO form. As for the case of histidine, this experiment allows a determination of both the oxygen on and off rate; the off rate was 1/s as compared to 1.1/s from the stopped flow method. The rate coefficients are summarized in Table 1. The overall oxygen affinity calculated for *Drosophila* Hb from the kinetic measurements is 0.18 Torr (flash photolysis technique) or 0.14 Torr with the stopped flow rates, as compared to 0.12 Torr for direct measurements using the tonometer.

**Structure of the *Drosophila* Hb gene and mRNA.** The amino acid coding region of the *Drosophila* Hb gene (*glob1*) is distributed on three exons, with introns at codon positions D7.0 and G7.0 (7). Additional database analyses revealed the presence of a total of about hundred ESTs from various *Drosophila* cDNA libraries, which are available at the Berkeley Drosophila Genome Project (BDGP, http://www.fruitfly.org). Ignoring a few putative sequencing errors, these EST clones are identical within the *glob1* coding region, but display striking differences at the 5' ends. Comparison with the published *Drosophila* genomic sequence (18) demonstrates the presence of three additional exons in the 5' UTR, which are differentially spliced into four distinct transcripts (Fig. 2). Transcript A is composed by the small exon 1 (56 bp) plus the three coding exons 4 to 6, transcript B contains an additional exon of 182 bp (#2), which is separated from exon 1 by a 59 bp intron. Transcripts C and D start with the third exon and use an alternative promoter, which is located in the second intron about 2 kb downstream of the first promoter. These transcripts only differ in the length of their first exon (#3), which has two different splice donor sites at its 3' end.

The expression pattern of the different transcripts was investigated by Northern-blotting (data not shown) and RT-PCR experiments (Fig. 3). For unknown reasons, transcript B, which is represented by only 2 cDNAs from the adult head out of about hundred EST sequences, was not
identified in these analyses. In both, the larvae and adults, transcripts A, C and D were detected. In embryos, we could only find transcripts A and C, while the EST dataset contains few embryonic entries for transcript B. No differences between male and female adults have been observed. The transcription start site of transcript A, which is the major form in all investigated stages, was determined by 5’-RACE (Fig. 2). Comparison with the genomic sequences revealed the presence of a TATA box 28 bp upstream of the start site. The start site of transcripts C and D was identified from the EST databases, and turned out to be identical with that predicted by the computer. A TATA box-like sequence (CATAAA) was found 30 bp upstream of this transcriptional start site.

The 7 kb genomic region of the *glob1* gene was scanned for the presence of putative hypoxia-responsive sequence elements (HREs). Such HREs in hypoxia-regulated mammalian genes are not positionally fixed relative to the transcription unit and are defined by the combined presence (in direct or inverted orientation) of binding sites for hypoxia-inducible transcription factor HIF-1 (‘core’ sequence: RCGTG) and the EPO sequence 5’-CACAG-3’ (19). Several closely spaced motif combinations (either two potential HIF-binding sites or one HIF- plus one EPO-site) have been detected in the *Drosophila* globin gene region and await further characterization by functional assays (Fig. 2).

**Detection of the Drosophila Hb mRNA and protein.** In situ-hybridization experiments were performed on different developmental stages using Digoxigenin-labeled anti-sense RNA probes encompassing the complete *glob1* coding region (Fig. 4; Table 2). Labeled sense RNA was used in control experiments and resulted in either no or only diffuse background-staining (not shown). Embryos of stages 1 through 5 showed the presence of *Drosophila* Hb mRNA in the whole embryo (Fig. 4A), whereas no staining was observed in stages 6 through 10. Expression of Hb mRNA in the yolk sac and in vitellophages was observed in later developmental periods (stages 11 through 15) (Fig. 4B). Starting with embryonic stage 14, the Hb is expressed in the
developing fat body (Fig. 4C). Tracheal expression was found in stage 15 and older embryos. In this stage, additional staining of the pharynx was observed. In the larvae, Hb mRNA is expressed in fat body cells (Fig. 4D), and the terminal cells of the tracheal system (Fig. 4E). In the adult fly, Hb expression was observed in the tracheoles and terminal cells (Fig. 4F), as well as in fat body cells that are associated with organs such as the gonads (Fig. 4G). Hb is also expressed in late oogenesis (Fig. 4H).

Western blot analysis shows a single band in the range of 17 kDa in larvae, adult heads and adult abdomen (Fig 5A). No other protein was stained in these analyses. Immuno-staining experiments on larval and adult tissues confirm the in situ data, and show hemoglobin expression in the trachea and fat body tissues. The strongest staining was found in the fat body of the adult head (Fig. 5B-E).

**DISCUSSION**

The presence of an Hb in *D. melanogaster* (7) is surprising because, among insects, only those species living under pronouncedly hypoxic conditions were thought to require a respiratory protein. Ironically, even after determination of the complete genomic sequences of *D. melanogaster* (18), the occurrence of a hemoglobin-like gene in this species was denied (20). Here we have shown that the *Drosophila* Hb exhibits ligand binding properties and expression patterns that resemble those of other known insect globins (6). These similarities suggest a common, conserved function of the intracellular Hbs in insect physiology and lead us to conclude that insect respiration may be more complex than previously thought.

*Drosophila* Hb is a hexacoordinated globin with high oxygen affinity. Recombinantly expressed *Drosophila* Hb is a monomer with a *M,* of about 17 kDa (Fig. 5A). Only a single Hb
gene was detected in the genome of this species, as evident from Southern blot experiments (data not shown) and comparison with the complete *D. melanogaster* genome sequence (18). Other insect Hbs vary in their quaternary structures, ranging from monomers to hexamers (6). The dimeric *Gasterophilus* Hb consists of at least two distinct polypeptides that are encoded by different genes (5). More than 30 different Hb genes are present in *Chironomus thummi* and related species (21, 22) and encode both monomeric and homodimeric extracellular Hb variants.

Like some animal (23-28) and the plant non-symbiotic Hbs (29), the Fe$^{2+}$ in the heme of the Hb of *Drosophila melanogaster* is hexacoordinated even in the absence of an external ligand. Based on a sequence alignment (7), the proximal (F8) and distal (E7) residues of *Drosophila* Hb are histidines, indicating a His-Fe-His binding scheme. This is in contrast to the intracellular Hb of *G. intestinalis* and the extracellular Hbs of the chironomids, which are penta-coordinated (5, 30, 31). The hexacoordinated state of the *Drosophila* Hb results in an apparently slow binding of external ligands, since the displacement of the distal His (E7) is required. The flash photolysis data show that ligand association to the pentacoordinated form is quite fast (Table 1). However, the observed oxygen affinity of the Hb is much lower than in the pentacoordinated form due to competition with the internal protein ligand. The kinetics are thus similar to those of vertebrate neuroglobin (Ngb) (26). The resulting high oxygen affinity of *Drosophila* Hb (0.12 Torr) is fully comparable to the values measured for the intracellular *Gasterophilus* Hb (5), as well for several variants of chironomid Hbs (30, 31) (Table 1).

**Differential splicing of the *Drosophila* Hb gene.** The structure of the *Drosophila* Hb gene (*glob1*) is complex with a total of six exons that are used to create at least four different transcripts with an identical coding region (exons 4-6), but different 5' UTRs (Fig. 2). The functional role of this organization is largely unknown. The unequal distribution of ESTs in different cDNA libraries that have been prepared by the BDGP suggests a differential expression of transcripts. For example, the adult head cDNA libraries contain ESTs of transcripts A and B,
but no transcripts C or D, whereas the libraries from embryos mainly contain transcripts C and D. Our RT-PCR experiments (Fig. 3) indicate the presence of multiple transcripts throughout fly development, possibly in different relative amounts. The results may also be explained by tissue-specific usage of alternate splice forms, which has not yet been studied in detail. The differences between transcripts A and B vs. C and D probably reflect the presence of two alternate promoters that might be differentially controlled.

**Hb-expression in Drosophila is similar to other insects** – The principal sites of Hb synthesis in larval and adult *Drosophila* are the cells of the fat body and the tracheal system. Although the total Hb concentration is comparatively low in *Drosophila*, this distribution mirrors the pattern in other insects with more pronounced Hb expression: synthesis of intracellular Hbs in fat body and tracheal cells was observed in the botfly *G. intestinalis* (4), as well as in the backswimmers (Hemiptera) *Buenoa confusa* (2), *Anisops pellucens* and *A. assimilis* (3). The extracellular Hbs of the Chironomidae are also synthesized in fat body cells (6, 32). Moreover, the presence of hemoglobin in chironomid oocytes has been described (33) and may be equivalent to the *in situ* signal, which we obtained in *Drosophila* oogenesis. The absence of Hb mRNA in embryos of stages 6 through 10 suggests that maternally derived message is responsible for the staining in early embryos (stages 1 through 5). Embryonic Hb synthesis starts in stage 11 in the vitellophages and later in the fat body.

**Function of intracellular hemoglobins in Drosophila and other insects.** There is little difficulty to associate the expression of Hbs at high levels with their function in oxygen storage and transport (6). The total expression level of the *Drosophila* Hb, however, appears to be lower compared to the classical insect globin models. Nevertheless, similar oxygen affinity (Table 1), tissue distribution (Figs. 4 and 5) and the phylogenetic history (7) suggest similar functions. Thus, the transport and storage of oxygen should be considered as the most likely role of insect Hbs, even under vastly normoxic conditions. The Hb in the tracheoles and terminal cells of the
trachea may facilitate a permanent oxygen flow from the air in the tracheal space into the tissues. The oxygen demand of the mitochondria may be the responsible for further \( O_2 \) delivery into the metabolically active cells. The strong Hb expression in the fat body cells that surround the adult organs of \textit{Drosophila}, in particular the adult brain, may be associated with the local storage of oxygen for these metabolically highly active tissues. Insect species that live under permanent hypoxic conditions such as the aquatic backswimmers (2, 3) and the \textit{G. intestinalis} larvae (4, 5) have reacted with a genetically fixed enhancement of Hb expression in the fat body and the tracheal organ. It is tempting to relate the presence of an intracellular Hb to the enhanced tolerance of and recovery from anoxia (as compared to vertebrates) observed in several insect taxa (34). In particular, \textit{Drosophila} embryos and larvae are surprisingly resistant towards prolonged periods of experimentally induced hypoxia (35, 36), which may \textit{e.g.} be of adaptive value when the fly eggs and larvae compete with microorganisms for a limiting supply of oxygen within fermenting fruits.

Although the present data strongly support the primary role of the \textit{Drosophila} Hb as a respiratory protein involved in oxygen storage or transport, other possible functions must still be taken into account. For example, it is conceivable that intracellular insect hemoglobins may serve as an oxygen sensor, which for example may be involved in the regulation of tracheal growth (37). Since tracheal growth under hypoxia is stimulated by a signaling pathway involving nitric oxide (NO) (36), the Hb might also be instrumental in supplying the NO synthase with an oxygen reserve needed for NO synthesis. \textit{Drosophila} as a prime model organism will provide the tools for further investigations of lowly expressed intracellular hemoglobins.

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REFERENCES


FIGURE LEGENDS

**Fig. 1.** Ligand binding of recombinant *Drosophila* Hb (A) Absorption spectra equilibrated under oxygen (dotted line), CO (dashed line) or nitrogen ("deoxy", solid line). All measurements were performed at pH 7 and 25 °C. (B) Ligand recombination kinetics after photodissociation. The kinetics are biphasic, with a rapid competition of CO and His for (pentacoordinated Fe$^{2+}$) binding sites, followed by a slow replacement of the fraction binding His (Fe$^{2+}$-His ⇔ Fe$^{2+}$-CO).

**Fig. 2.** Structure of the *Drosophila* Hb gene (*glob1*) and its transcripts. The sequences were compiled from the data of Burmester and Hankeln (7) and the published *Drosophila* genome (Adams *et al.*, 2000). The mRNAs were deduced from 5'-RACE experiments and from the BDGP database (http://www.fruitfly.org). The open reading frame is hatched. The distribution of potentially relevant hypoxia-responsive sequence elements in the gene region is shown. The closed circles show the HIF-1 consensus binding sites 5'-RCGTG-3', the open circles the strictly conserved HIF-1 core-sequences 5'-CGTG-3'; the closed squares represent sites matching 4 of 5 bases of the EPO-binding sequence (5'-CACAG-3') adjacent to HIF-1. The start and stop codons, and the TATA-boxes are indicated.

**Fig. 3.** Detection of different Hb transcripts by RT-PCR. Specific primers were used to amplify transcripts A, C, and D (see text) using RNA from different developmental stages. Abbreviations: e, embryos; l, larvae; a, adults; A, C, D, transcripts A, C, D.

**Fig. 4.** Detection of Hb mRNA by *in situ*-hybridization. A, stage 5 embryo; B, stage 12 embryo; C, stage 16 embryo; D, larval fat body; E, larval brain; F, adult gut; G, spermatheca; H, ovary. The presence of Hb-mRNA is indicated by blue to purple staining. The abbreviations are: Y, yolk sac; F, fat body, T, tracheoles and terminal cells; S, spermatheca.
**Fig. 5.** Detection of Hb protein. (A) Western blotting: C, control (recombinantly expressed *Drosophila* Hb); L, larvae; A, adult abdomen; H, adult head. (B-E) Cryo-sections of the adult head. B and D, controls (no primary antibody); C and E, immuno-staining with anti-hemoglobin antibodies. The fat body, which shows strong labelling, is shown at higher magnification (D, E). The scale bars correspond to 100 μm (B, C) and 50 μm (D, E).
Table 1. Ligand binding kinetics of Drosophila Hb and other selected Hbs

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<th>$k_{on}$ $(O_2)$</th>
<th>$k_{off}$ $(O_2)$</th>
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The on and off rates were measured at 25°C, using the flash photolysis technique; the histidine on rate of Drosophila Hb was 550/s and off rate 30/s. The stopped flow method gave a faster histidine dissociation rate of 40/s and thus a lower ratio of rates $k_{on}/k_{off} = 15$, a dimensionless quantity for the internal (histidine) ligand. Note that the ratio of oxygen binding rates provides the intrinsic ligand affinity of the pentacoordinated form: $K_5 = k_{on}/k_{off}$ in units /M.$^1$ In the case of hexacoordinated Hbs, the observed oxygen affinity depends on the competition with the protein ligand (histidine in this case) $K_{obs} = K_5/(1+K_{His})$. The $P_{50}$ values for the oxygen affinity was determined from equilibrium measurements; the kinetically derived values for Drosophila Hb would be 0.18 torr (by flash photolysis) or 0.14 torr with the stopped flow dissociation rate; the value for mouse neuroglobin is 1.2 torr.$^2$ Extracellular hemoglobin.
**Table 2. Summary of Hb expression in *Drosophila***

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Characterization of drosophila hemoglobin: evidence for hemoglobin-mediated respiration in insects
Thomas Hankeln, Viviane Jaenicke, Laurent Kiger, Sylvia Dewilde, Guy Ungerechts, Marc Schmidt, Joachim Urban, Michael Marden, Luc Moens and Thorsten Burmester

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