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Reversible Binding of Nitric Oxide by a Salivary Heme Protein from a Bloodsucking Insect

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The bloodsucking bug *Rhodnius prolixus* has a salivary vasodilator, previously characterized as a nitrovasodilator, with salivary smooth muscle–relaxing and antiplatelet activity. *Rhodnius* salivary glands are bright red owing to the abundance of heme proteins. Electron paramagnetic resonance and optical spectroscopic experiments indicated that the salivary vasodilator is a nitrosylheme protein with an Fe(III) heme that binds nitric oxide (NO) reversibly. Dilution of the protein in neutral pH promoted NO release. This protein thus appears to be the NO carrier that helps *R. prolixus* to feed on blood.

The bloodfeeding bug *R. prolixus* has a salivary vasodilator with the general properties of NO (*1*). Because NO has a half-life on the order of seconds in the presence of aerated solutions (*2*), the question arises as to the identity of the parent molecule that ultimately releases the vasodilator NO. On account of an abundance of heme proteins that give *Rhodnius* salivary glands a deep cherry color (*3*), we tested the hypothesis that the *Rhodnius* vasodilator is a nitrosylheme protein that not only binds but also can readily release NO.

Optical spectroscopy of native *Rhodnius* salivary gland homogenates at pH 5.0 and 7.35 indicated typical heme spectra, with a Soret band maximum at 422 nm. After dilution, this maximum shifted to 406 nm only at the more alkaline pH, indicating a change in ligation of the heme in the alkaline pH range (Fig. 1). This dilution shift was rapid, and the resulting spectrum was stable for at least 30 min at room temperature.

We reasoned that if the heme protein was in equilibrium with free NO gas, then addition of a neutral gas to this solution should displace the NO and change the spectra. Indeed, when the native, concentrated homogenate at pH 7.4 was exposed to an argon atmosphere (*4*), the Soret maximum shifted from 422 to 406 nm, indicating volatile ligand displacement by argon. When this unliganded heme protein was subsequently exposed to NO for 1 min, the Soret maximum shifted back to 422 nm, identical to the original spectrum of the native protein (Fig. 2). When reexposed to argon, the Soret maximum of the reconstituted nitrosylheme protein again shifted to 406 nm, indicating loss of the NO group once more (Fig. 2). The same shift of the Soret maximum was also produced by dilution of the reconstituted nitrosylheme protein at alkaline pH. However, this shift did not occur at pH 5.0, a result identical to that obtained for the native homogenate shown in Fig. 1. These results support the hypothesis that the native homogenate contains a nitrosylheme protein with relatively low affinity for NO.

The Soret band shifts shown in Figs. 1 and 2 are similar to those observed for Fe(III) heme proteins, for example, oxidized myoglobin (metMb; 409.5 nm) to metMbNO (420.5 nm), and are opposite to the shifts observed for Fe(II) heme proteins, for example Mb (435.0 nm) to MbNO (421.5 nm) (*5*). The Soret band shifts are not consistent with either Fe(II) or Fe(III) spectral shifts of cytochrome P450 (*6*).

To investigate further the pH effect on the spectral changes after dilution of the homogenate, we used a simple model of two heme protein fractions that either absorb maximally at 406 nm (and with spectrum

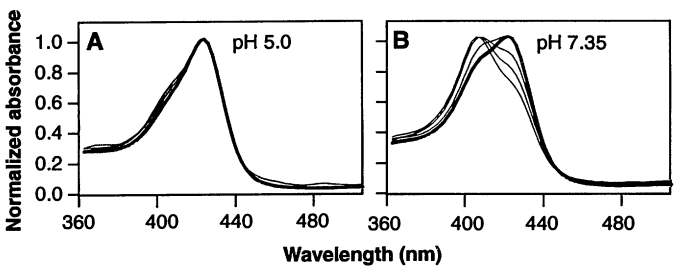

Fig. 1. Normalized spectra of *R. prolixus* salivary gland homogenates serially diluted at pH 5.0 (**A**) or 7.35 (**B**). The most concentrated sample is shown with a thicker line. Ten pairs of glands [in 20 μl of 10 mM sodium phosphate buffer and 150 mM NaCl (pH 7.2)–phosphate-buffered saline (PBS)] were added to 0.5 ml of buffer (0.1 M citrate buffer (pH 5.0) or 0.1 M Hepes buffer (pH 7.35), and spectra at 2-nm intervals were recorded in a diode array spectrophotometer (Hewlett-Packard 8452A) from 360 to 500 nm. The samples were then serially diluted twofold in the same buffer to obtain the various spectra shown. Absorbance of the concentrated samples was 1.2 absorbance units for a cuvette of path length 1 cm. The same results were obtained when acetate was used instead of citrate and phosphate instead of Hepes as buffer.

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equal to a dilute protein exposed to argon) or 422 nm (and with spectrum equal to the concentrated NO exposed protein) and assumed an experimental spectrum to be the result of both contributions. The fraction of each liganded or unliganded heme protein could then be calculated from the ratios of absorbance at 422 and 406 nm. Accordingly, we derived Hill plots for dilutions made at various pH's (Fig. 3A) and calculated the apparent $K_{0.5}$ for each curve (the intercept in the x-axis), which resulted in a secondary plot (Fig. 3B). This second plot suggests the presence of an ionizable group with a pK_a of 6.5.

To further confirm that the change in the spectra of the native homogenate was due to the removal of endogenous NO, we exposed the homogenate at pH 7.4 to argon for times ranging from 5 min to 3 hours and measured both the spectral characteristics and vasodilatory activity. Vasodilatory activity was progressively lost as the homogenate changed from a maximum absorbance of 422 to 406 nm (Fig. 3C). However, some residual vasodilatory activity on the order of 15 to 20% still remained after the heme protein appeared to be depleted of NO. Because all vasodilatory activity in *Rhodnius* saliva behaves as NO (*1*), we investigated whether nitrosothiol groups could account for this residual activity. We determined the presence of nitrosothiol groups in *Rhodnius* salivary glands by measuring the NO in the presence and absence of added Hg^{2+} ions (*7*), which gave NO_2^- equivalents of 310 ± 0.9 and 282 ± 2.6 pmol per pair of glands, respectively (mean ± SE; n = 3), indicating about a 10% contribution of nitrosothiol groups to the salivary vasodilator. These groups could act as a temporary buffer for NO released from the heme protein (*8*). We conclude that the majority (>80%) of the NO vasodilatory activity in *Rhodnius* is associated with the heme protein.

The affinity of most Fe(II) heme proteins

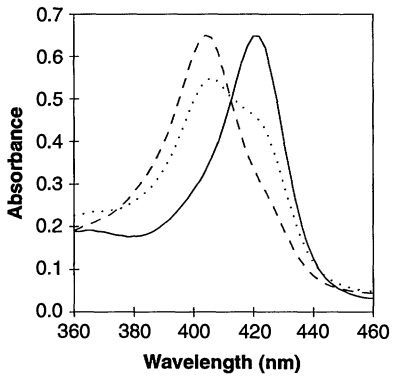

Fig. 2. Spectra of *Rhodnius* salivary gland homogenates after exposure to argon (dashed line), followed by exposure to NO (solid line) and then by further exposure to argon (dotted line). *R. prolixus* salivary gland homogenates [1000 pairs per milliliter in 50 μl of 150 mM NaCl and 10 mM sodium phosphate (pH 7.2)] were equilibrated with argon for 2 hours and to light for 2.5 min (*4*) and then diluted to 10 pairs per milliliter in 100 mM Hepes buffer (pH 7.4). Spectra were taken as in Fig. 1A. The same sample was then exposed briefly to NO and then to argon for 5 min.

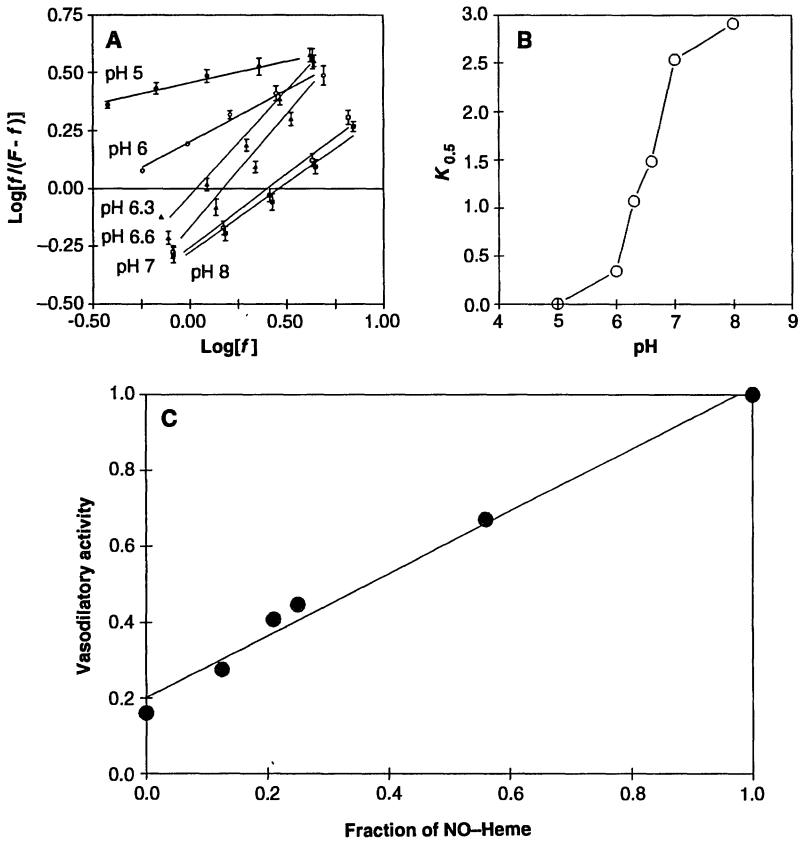

Fig. 3. (**A**) Hill plots obtained from serial dilutions of *R. prolixus* salivary homogenates at the indicated pH. F is the total concentration of heme protein, and f is the concentration of the unligated heme protein. Results are given as the mean ± SE (n = 5). (**B**) Secondary plot of the apparent $K_{0.5}$ as a function of the pH. Concentrations are in pairs of glands per milliliter. The most concentrated sample was at 20 pairs of glands per milliliter. (**C**) Vasodilatory activity of *Rhodnius* salivary homogenate [ten pairs of glands in 100 μl of PBS (pH 7.5)] as a function of the fraction of nitrosylheme protein remaining after exposure to argon for various times. The fraction of nitrosylheme protein in each portion was calculated as described in the text. Vasodilatory activity was measured on rabbit aortic rings constricted with noradrenaline (*1*) and was compared with doses of the untreated homogenate.

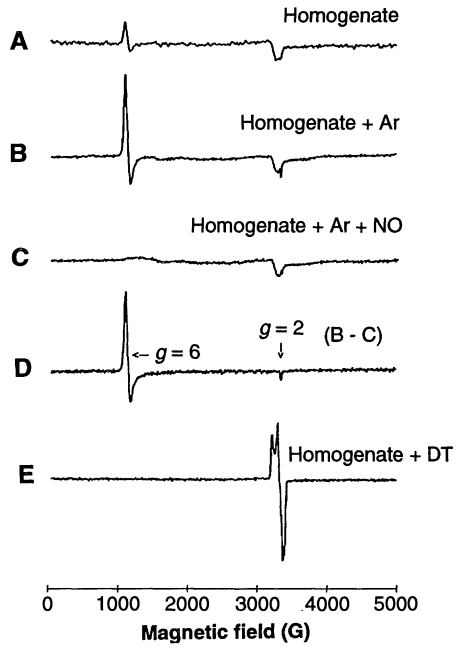

Fig. 4. EPR spectra of 100 pairs of *Rhodnius* salivary glands obtained in 125 μl of PBS (**A**) before argon equilibration; (**B**) after equilibration in an argon atmosphere for 4 hours; and (**C**) after equilibration of (B) with NO for 2 min. (**D**) is the difference spectrum, that is, (B − C). (**E**) is the homogenate as in (A) treated with dithionite (DT) to reduce Fe(III) to Fe(II). All spectra are in the same scale, except (E), which is shown reduced 3× in amplitude. The spectra were recorded on a Bruker ESP-300E X-band spectrometer equipped with a helium cryostat (Oxford Instruments). Conditions were as follows: Power attenuation, 30 dB; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; receiver gain, 1.25 × 10^5; resolution, 1024 points; time constant, 82 ms; and sweep width, 5000 G.

studied to date for NO is extremely high, with binding constants on the order of 10^{11} M^{-1} (9). Furthermore, the kinetics of dissociation are very slow: The rate constant for the release of bound NO from human hemoglobin is on the order of 10^{-5} s^{-1} at 20°C and neutral pH (9). However, Fe(III) heme proteins bind NO with much less affinity, with binding constants in the range of 10^3 to 10^5 M^{-1}, and the kinetics of dissociation are faster (k_{off} = 1 to 40 s^{-1}) (*10*). Thus, an Fe(III) heme protein would be a much better carrier of NO if release of this effector after dilution is required. On the basis of the spectral changes observed (Figs. 1 and 2), it appears likely that an Fe(III) heme protein may be involved in the *Rhodnius* salivary vasodilator.

To further confirm the importance of the Fe(III) heme protein, we carried out experiments using electron paramagnetic resonance (EPR) spectroscopy. Fe(III) heme proteins have an odd number of electrons that exist in a high-spin form when pentacoordinated and typically give rise to EPR spectral features at spectroscopic splitting factors (*g*) of 6 and 2 (*11*, *12*). Addition of NO to an Fe(III) heme protein produces an even-electron species that is EPR silent (*12*). The EPR spectra of *Rhodnius* salivary gland homogenates before and after exposure to an argon atmosphere and after subsequent exposure to NO are shown in Fig. 4 (*13*). Only a weak signal was observed for the homogenate before argon equilibration, but strong signals at $g = 6$ and $g = 2$ were observed after argon equilibration (Fig. 4, B and D). These signals disappeared after subsequent exposure of the homogenate to NO. The small signal at 3200 G ($g = 2.08$) that does not change with argon or NO equilibration is probably produced by a nonheme-based radical that accounts for ~5% of the intensity of the high-spin Fe(III) signal. The observation that the shape of this $g = 2.08$ signal does not change after NO equilibration eliminates a nitrosyliron(II) center from consideration because that odd-electron species gives rise to very characteristic EPR spectral features in precisely the same region of the EPR spectrum (*14*). This characteristic is shown in Fig. 4E, where dithionite has been added to the untreated homogenate solution to produce the Fe(II)NO species.

Chromatofocusing of whole-gland homogenates on a Mono P column yielded three major heme proteins, with isoelectric points (pI's) of 7.97, 7.53, and 6.94. The reduced proteins had apparent masses of 26.0, 23.1, and 23.4 kD and were present in amounts of 192, 57.6, and 44.0 pmol per gland pair, respectively (*15*). Taken together, these three proteins account for at least 90% of the total heme protein content of the glands. Preliminary results indicate that all three proteins display identical NO-binding properties, which closely match the properties of the whole-gland homogenates. The summed content of these proteins (293 pmol per gland pair) also matches the total content of NO, 282 pmol per pair, measured in the absence of Hg^{2+}.

Nitric oxide has recently been identified as an important signal molecule involved in the regulation of vascular tone in vertebrates (*2*); its presence in *Rhodnius* saliva suggests a remarkable case of convergent evolution. The *Rhodnius* heme proteins are well suited to enhance feeding success by unloading the vasodilatory NO as they are diluted in the host bloodstream; further, NO exhibits antiplatelet activity and may help inhibit the formation of the platelet plug (*2*). Although NO synthesis is related to a variety of functions in vertebrates (*2*), it has previously been reported only once in another invertebrate (*16*). It remains to be seen whether the ability to produce NO is a phylogenetically old trait or whether it has arisen independently in different lineages.

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Regulation of TCR Signaling by CD45 Lacking Transmembrane and Extracellular Domains

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The CD45 protein is a transmembrane tyrosine phosphatase that is required for normal T cell receptor (TCR)–mediated signaling. A chimeric complementary DNA encoding the intracellular enzymatically active portion of murine CD45 preceded by a short amino-terminal sequence from $p60^{c\text{-}src}$ was transfected into $CD45^-$ T cells. Expression of this chimeric protein corrected most of the TCR signaling abnormalities observed in the absence of CD45, including TCR-mediated enhancement of tyrosine kinase activity and Ca^{2+} flux. Thus, the enzymatically active intracellular portion of CD45 is sufficient to allow TCR transmembrane signaling.

Stimulation of T cells through the antigen-specific receptor (TCR) initiates increases in tyrosine phosphorylation (*1*), phosphatidylinositol hydrolysis, and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (*2*). Studies of T cell lines that are deficient in expression of CD45 have established that this transmembrane molecule, whose intracytoplasmic domains have intrinsic tyrosine phosphatase activity (*3*, *4*), participates in coupling the TCR to these activation events (*5*, *6*). Studies of CD45-deficient variants of a murine T cell line, YAC-1, have shown that CD45 expression is inversely related to spontaneous tyrosine phosphorylation of a number of substrates, including the ζ chain of the TCR. In contrast to the typical rapid $[Ca^{2+}]_i$ elevation exhibited by the YAC-1 wild-type

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