

High Affinity Histamine-binding and Antihistaminic Activity of the Salivary Nitric Oxide-carrying Heme Protein (Nitrophorin) of *Rhodnius prolixus*

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Summary

The salivary glands of *Rhodnius prolixus* contain a nitrosyl-heme protein, named nitrophorin, that releases the vasodilatory and antiplatelet compound nitric oxide (NO). Because imidazole compounds such as histamine can interact with Fe(III) heme proteins, we investigated whether such substances could interact with *Rhodnius* nitrophorins. Both imidazole and histamine, but not histidine can produce full change of the difference spectra of the Soret band in the 1–3 μM concentration range (at a heme protein concentration of 0.4 μM). The apparent $K_{0.5}$ for the binding of histamine with the heme protein is below 1 μM . Furthermore, the complex histamine-heme protein does not dissociate after molecular sieving chromatography. To investigate whether histamine could displace NO from the native nitrosyl nitrophorins, histamine was added to the native heme proteins, leading to displacement of the bound NO as observed by changes in the absorption spectra as well as by the production of nitrite. Finally, the antihistamine effect of the heme protein was demonstrated by its inhibition of the histamine-provoked contractures of the guinea pig ileum. It is concluded that histamine, a common autacoid found at the site of injury and exposure to antigenic substances such as the site of feeding by hematophagous arthropods, can be scavenged by the nitrosyl nitrophorin of *R. prolixus*, which, in return, will release the vasodilatory and platelet inhibiting NO to counteract the host hemostatic response.

Blood sucking arthropods contain a diverse array of salivary antihemostatic substances that include anticoagulants, antiplatelet, and vasodilators (1). The blood sucking triatomine bug, *Rhodnius prolixus*, contains a salivary anti-factor VIII anticoagulant (2), large amounts of apyrase activity inhibits platelet aggregation, (3, 4), and a salivary nitrosyl compound further inhibits platelet function and induces vasodilation (5). This salivary antihemostatic cocktail, secreted into the host skin during the insect's probing and feeding, allows rapid location of blood vessels by the hungry bug (6).

Rhodnius salivary vasodilator consists of four closely related Fe(III)-heme proteins, or nitrophorins, which are found naturally loaded with nitric oxide (NO)¹ (5). Upon dilution in neutral or alkaline pH these heme proteins are able to release NO to the medium. It was thus postulated that vasodilation and inhibition of platelet aggregation by these nitrosyl compounds is brought about when the concentrated insect saliva is diluted (and NO is released) after injection into the vertebrate skin (5).

In addition to dilution alone, other factors could further increase dissociation of NO from the nitrosyl nitrophorins. Heme proteins can react with or bind a large number of

ligands, and Fe(III) heme proteins in particular can bind imidazolic compounds (7). For example, imidazole and imidazole derivatives inhibit the action of the heme protein thromboxane synthase (8, 9), imidazolic compounds inhibit NO synthase (10), whereas P450 cytochromes are inhibited by histamine and histamine analogs such as cimetidine (11, 12). We hypothesized that interaction of imidazolic compounds with *Rhodnius* NO-nitrophorins could favor displacement of the NO ligand from the heme moiety. Accordingly, we have investigated the interaction of histamine, an imidazolic compound likely to occur at sites fed upon by blood sucking insects, with the salivary NO-heme proteins of *R. prolixus*.

Materials and Methods

Organic reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Inorganic salts were all of American Chemical Standards grade. Water was obtained from a SepPack system from Millipore Corp. (Bedford, MA).

R. prolixus was reared and maintained at the Central Insectary Facility of the Center for Insect Sciences of the University of Arizona. All insects were fed on an anesthetized rabbit. Fifth instar insects starved from 4–10 wk were dissected to obtain the salivary glands. A plastic pestle was used to homogenize the glands inside a 1.5-ml conical plastic tube containing 100 μl of the indicated buffer. The supernatant of a 5 min centrifugation at 14,000 g was used for the experiments.

¹ Abbreviation used in this paper: NO, nitric oxide.

To remove native NO from the salivary heme protein, salivary gland homogenates were dialyzed for 8 h against 5 mM Tris-HCl, pH 8.3, and further dialyzed against several washes of water for the next 24 h. A shift of the Soret band from 422–404 nm maximum confirmed the removal of NO (5).

Purification of the main heme protein isoform was done as described by Champagne, D., R. H. Nussenzeig, and J. M. C. Ribeiro (manuscript submitted for publication). Briefly, 300 pairs of glands were submitted to chromatofocussing on a Mono P column (Pharmacia LKB, Piscataway, NJ) equilibrated with 25 mM triethanolamine buffer, pH 8.3, flowing at 0.5 ml/min. 2 ml of the homogenate diluted in the same buffer was injected, followed by elution with a 10:1 dilution of polybuffer 96 (Pharmacia LKB) adjusted to Ph 7.0. Absorbance at 280 and 413 nm was monitored. Four different heme proteins were resolved by this protocol. The main heme protein, responsible for 40–50% of the total amount of salivary heme protein, was further purified by a MacroSphere strong cation exchange column (4.3 × 250 mm; Alltech Associates Inc., Deerfield, IL) equilibrated with 20 mM sodium acetate pH 5.0. Pooled fractions (1.5–2 ml) from the chromatofocusing columns were diluted with water to a volume of 5 ml and the pH was adjusted to 5.0. After injection into the column at a flow rate of 0.5 ml/min, a gradient from 20 mM sodium acetate pH 5.0 to the same buffer containing 1 M NaCl was initiated after 10 min, the gradient being completed for 1 h of injection. The isolated peak was dialyzed against water and freeze dried. The resulting material had no remaining reactive nitrogen groups, as measured by the Griess reagent (13).

Heme content from heme proteins were measured by the pyridine hemochromogen assay. 75 μ l of the protein solution in water were mixed with 20 μ l of pyridine and 2 μ l of 5 N KOH, mixed, and added crystals of dithionite. After rapid mixing, the spectra was taken and the difference in absorbance from 556 to 700 nm was used to estimate heme content with a millimolar extinction coefficient of 32 absorbance units/cm (7).

Molecular sieving chromatography was done using a TSK G2000SW column (7.5 mm × 60 cm; TosoHaas, Montgomeryville, PA) perfused at 1ml/min with NaCl 0.15 M and sodium phosphate, pH 6.8. The eluent absorbance at 413 nm was determined. Fractions were collected at 0.5 min intervals. Nitrite was measured by the Griess method (13).

The guinea pig ileum bioassay was performed isotonicly using Tyrode's solution at 37°C and bubbled with O₂ (96%) and CO₂ (4%) (14).

Optical absorption spectra were measured with a spectrophotometer (lambda-19; Perkin-Elmer Cetus, Norwalk, CT). Spectra were stored to a computer disk, and difference spectra were determined with a spreadsheet program.

Electron paramagnetic resonance (EPR) spectra was recorded on an X-band spectrometer equipped with a helium cryostat (Bruker ESP-300E; Oxford Instruments Inc., Columbia, MD). Conditions were as follows: power attenuation, 20 dB; modulation frequency, 100 KHz; Modulation amplitude 3.2 G, receiver gain, 1.25 × 10⁵ or larger as necessary, resolution, 1,024 points; time constant 82 ms; and sweep width, 5,000 G.

All animals used in these experiments were treated according to approved protocols reviewed by the University of Arizona Institutional Animal Care and Use Committee.

Results

Binding of Histidine, Histamine, and Imidazol to Rhodnius Salivary Heme proteins. To investigate whether binding of im-

idazolic compounds could occur with the salivary heme proteins of *R. prolixus*, we measured the absorption spectrum of the Soret band region of dialyzed salivary homogenates (0.4 μ M heme equivalent) in the presence or absence of several concentrations of imidazole, histamine, and histidine. The difference spectra indicate that at 1 μ M of either imidazole or histamine a change of the spectra was noticeable and almost saturated, but histidine led to a spectral change still increasing at the higher concentration used (1 mM) (Fig. 1).

High Affinity Binding of Histamine to Rhodnius Nitrophenol. Difference spectra with increasing additions of histamine from 10 nM to 10 μ M to dialyzed salivary gland homogenates (0.4 μ M heme equivalent), or with the purified major salivary heme protein (at the same concentration) indicated a high affinity binding with $K_{0.5}$ of 0.102 ± 0.024 and 0.050 ± 0.020 μ M (Mean \pm SE, $n = 3$) for the total homogenate or the pure major heme protein, respectively (Fig. 2). Further dialysis of salivary homogenates treated with 200 μ M histamine against 500 volumes of water for 4 d with two changes of water a day failed to return the spectrum from the ligated (411 maximum) to the unligated (404 nm maximum) species (not shown), indicating a tight binding of histamine to the heme. We also conclude that spectral changes induced by histamine in the Soret absorption band are similar in both total salivary homogenate and in the pure dominant nitrophenol.

Spectral Properties of the Histamine-Nitrophenol Complex. Binding of imidazolic compounds to high spin ferric heme changes the optical spectrum to that of a low spin, hexacoordinated iron heme species (15). Optical spectra of the dialyzed homogenate with or without histamine indicate a spectrum consistent with changes from a high spin to that of a low spin ferric heme (Fig. 3). Additional evidence for the formation of a low spin ferric heme is obtained from EPR spectra of the dialyzed homogenates with or without 1 mM histamine added to the sample. A loss of the signal at $g = 6$ is observed, but no new signals appeared in the $g = 2.8$ – 3.0 , 2.3 , and 1.5 – 1.7 regions, as expected for a hexacoordinated, low spin ferric heme protein (16) (Fig. 4). Similar results were obtained upon addition of cyanide, which is well known to form the low spin ferric state (not shown) (17, and references therein). It thus appears that the low spin ferric species formed by addition of histamine (or cyanide) produces a species that exhibits fast relaxation of its EPR signal, even at 4.2 K. Weak, broad, or nearly unobservable signals are sometimes observed for low spin ferric heme proteins, even at very low temperatures (18, and Walker, F. A., unpublished observations). The loss of the $g = 6$ signal upon addition of histamine, coupled with the optical spectral changes that are indicative of formation of a low spin ferric state, are strongly supportive of the formation of this type of species.

Displacement of NO by Histamine Binding to NO-loaded Nitrophenols. To investigate whether binding of histamine could displace the NO from the native nitrosyl heme protein, we measured the absorption spectra of the native protein before and immediately after (1 min) addition of 50 μ M histamine. The spectra, which displayed a Soret maximum of 422 nm (previously reported as the NO heme protein ad-

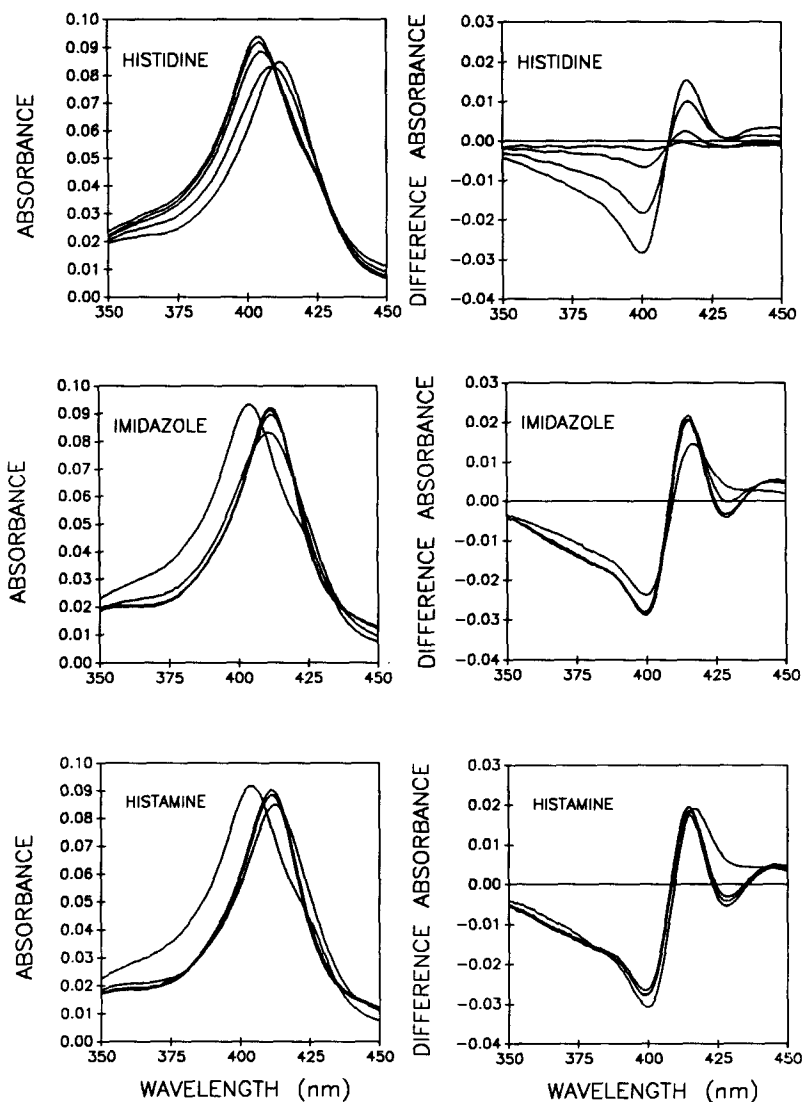


Figure 1. Spectral changes (*left*) and difference spectra (*right*) after successive additions of imidazole compounds to give 1, 10, 100, and 1,000 μM to dialyzed salivary gland homogenates of *R. prolixus*. Spectra were performed with homogenate to give 0.4 μM heme, 0.15 M NaCl, and 10 mM sodium phosphate, pH 7.3. Imidazole compound concentrated solutions were adjusted to pH 7.3 and added to the cuvette with a 10- μl syringe. The native protein representing no histamine added contains a maximum at 404 nm and is represented by the spectra most shifted to the UV region.

duct) (5), shifted to 411 nm (Fig. 5), typical of the spectrum shown by the low spin histamine heme protein complex (Fig. 3). The visible part of the spectra also indicates change in the α and β bands, as well as disappearance of a band at 620 nm upon addition of histamine. Control spectra on homogenates not receiving histamine continued to show a 422 nm maximum for up to 20 min (not shown). Furthermore, when the homogenates were submitted to molecular sieving chromatography and the main heme protein optical spectral peaks were scanned, the control homogenate indicated a mixture of the heme proteins containing or not containing NO (with maxima at 422 and 404 nm, respectively), but the histamine-treated homogenate had an absorption maximum at 411 nm indicating a tight binding of histamine even after separation of the free ligands by chromatography (Fig. 6). These results indicate that histamine displaces NO from the heme binding site and further support the conclusion that histamine binding to the heme protein(s) of *Rhodnius* is of high affinity.

Antihistamine Property of Rhodnius Nitrophorin. Because the

high affinity binding of histamine could result in an inhibition of histamine activity on smooth muscle bioassays at physiologically relevant concentrations, we tested the effect of both dialyzed homogenate and pure major salivary heme protein on the contractures of the guinea pig ileum induced by histamine. In the presence of dialyzed homogenate containing the equivalent of one pair of glands/ml or less, ($\leq 100 \mu\text{g}$ of protein/ml), or pure major heme protein equivalent to that of 0.2 pairs of glands/ml, significant inhibition of histamine contractures were observed (Fig. 7).

Discussion

Fe(II) heme proteins bind NO very strongly and, indeed, hemoglobin is used as a pharmacological tool to inhibit the effects of NO/EDRF in vitro (19). However, Fe(III) heme proteins bind NO with much less affinity (20) and could function as carriers for this unique gas. We indeed showed that NO could dissociate from the native heme protein upon di-

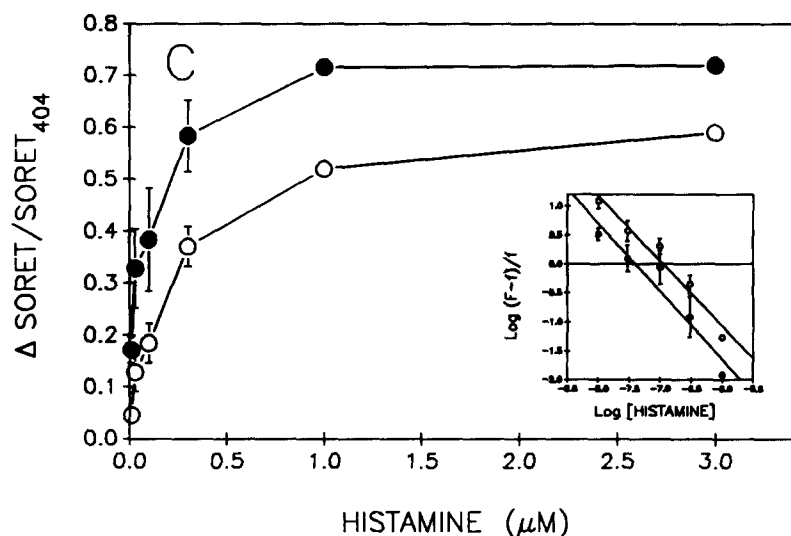
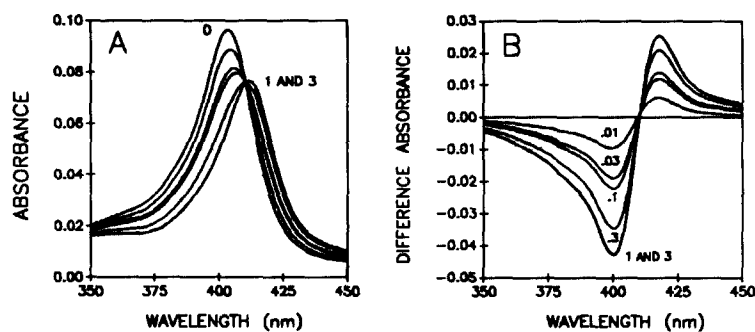


Figure 2. Spectral changes induced by the titration of the salivary heme proteins of *R. prolixus* with histamine. (A) Spectra derived from successive additions of histamine to give final concentrations of 10, 30, 100, 300, 1,000, and 3,000 nM to the pure main nitrophenol at a heme concentration of 0.4 μM . The native protein representing no histamine added contains a maximum of 404 nm and is represented by the spectra most shifted to the UV region. (B) Difference spectra of the results presented in A. (C) Plot of the maximum difference spectra (as shown in B) as a function of the added histamine concentration. (Solid symbols) Performed with pure salivary heme protein I. (Open symbols) Performed with total dialyzed homogenate in conditions similar to A. Inset, Hill plots derived from the plots in C, where F is the total heme protein and f is the fraction of the heme protein bound with histamine, as derived from plot C¹⁰, assuming total conversion of the heme protein to bound form at 3 μM histamine. When f is equal 0.5F, then $\log(F-f)/f$ equals 0. Intercepts at 0 values of $\log(F-f)/f$ thus indicate $K_{0.5}$ values. Symbols and bars are the mean \pm SE of three experiments. Other conditions are as in Fig. 1.

lution at pH 7.35, and that this heme protein was a Fe(III) heme protein (5). We report here that histamine can bind with a high affinity to the heme protein, that this binding displaces native NO from the heme pocket, and that the *Rhodnius* heme protein can display an antihistaminic activity.

The interaction of histamine with the salivary heme protein as shown by combined optical and electron paramagnetic resonance spectroscopy indicates the change from a high spin Fe(III) to that of a low spin Fe(III) species. This behavior reflects a change in the coordination number of iron

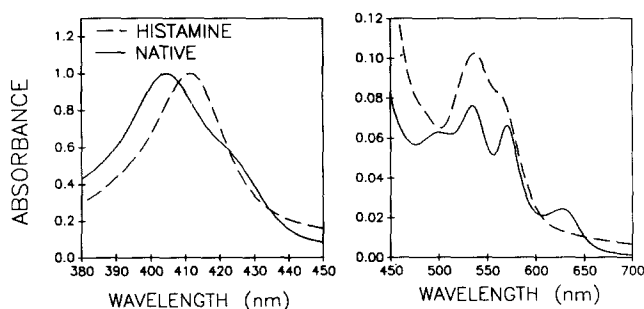


Figure 3. Near UV (left) and visible (right) spectra of dialyzed native homogenate in the absence or in the presence of 50 μM histamine. Maximum of 404 nm for the native heme protein changes to 411 in the presence of histamine.

from five to six, and strongly indicates that the added heme ligand is a strong field ligand, such as a nitrogen donor, as would be expected for binding of the imidazole nitrogen to the heme. Although the optical and EPR spectral changes

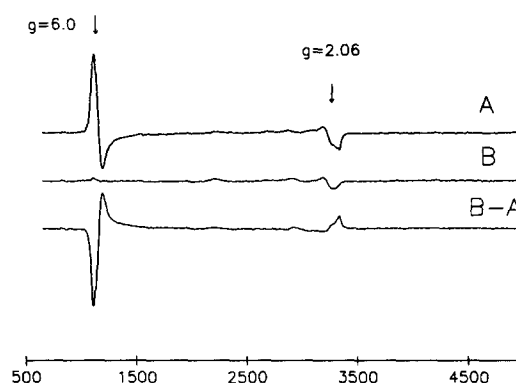


Figure 4. EPR spectra of 100 pairs of salivary glands (~ 10 mg protein) in 125 μl of 0.15 M NaCl and 10 mM sodium phosphate, pH 7.3 (~ 0.1 mM heme concentration) in the absence and in the presence of 0.2 mM histamine or 0.2 mM potassium cyanide. The small signals at $g = 2.9, 2.3,$ and 1.7 in the absence of added ligand are indicative of a small amount of low spin ferric heme in the NO-depleted protein sample. However, these signals do not change significantly when histamine is added, and no new signal appears, suggesting that a "fast-relaxing" low spin ferric species has been produced.

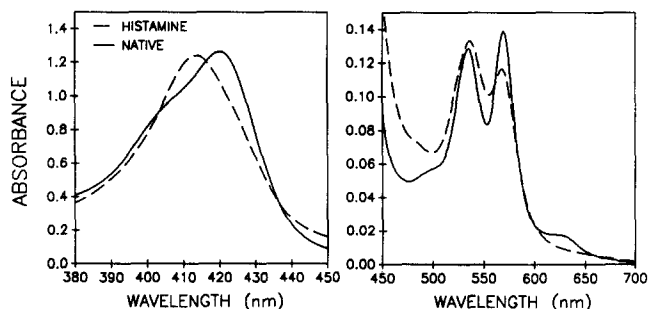


Figure 5. Near UV (*left*) and visible (*right*) spectra of fresh native homogenate (containing endogenously bound NO) in the absence or in the presence of 50 μM histamine. Maximum of 422 nm for the native heme protein changes to 411 in the presence of histamine.

in themselves cannot provide information concerning the stability of the complex formed, they do indicate that an imidazole nitrogen is coordinated to the sixth position on the heme. It is tempting to suggest that the fact that the EPR spectrum of this complex is not observable at 4.2 K indicates that the geometric relationship of the two imidazole planes of the proximal histidine and the added histamine are perpendicular to each other. Such perpendicular alignment of

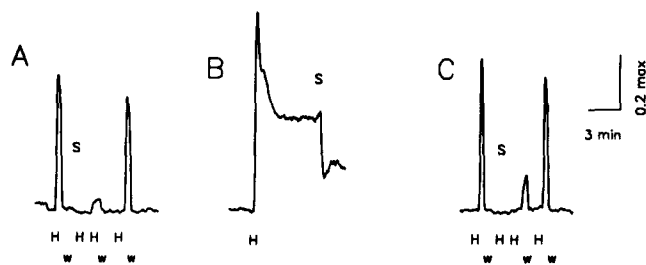


Figure 7. Antihistamine effect of dialyzed salivary gland homogenate (*A* and *B*) or pure salivary heme protein I (*C*). *H* indicates addition of 0.25 μM histamine (*A*) and (*C*), or 0.5 μM histamine (*B*). *S* indicates addition of (*A*) dialyzed homogenate to give the equivalent of one pair of glands/ml (*A*), (*B*) dialyzed homogenate to give 0.2 pairs of glands/ml or (*C*) 20 μg of pure salivary heme protein I. *W* indicates when the preparation was washed with fresh saline solution. One pair of glands contain $\sim 100 \mu\text{g}$ of protein, half of which is composed by four similar heme proteins, of which salivary protein I constitutes half of the total heme protein pool.

the imidazole planes has been shown to produce a broad, difficult to observe, single-featured EPR spectrum with the single *g*-value significantly greater than 3.0 (21), and similar spectra have been observed for the b cytochromes of mitochondrial Complex III, the b,cI complex (18).

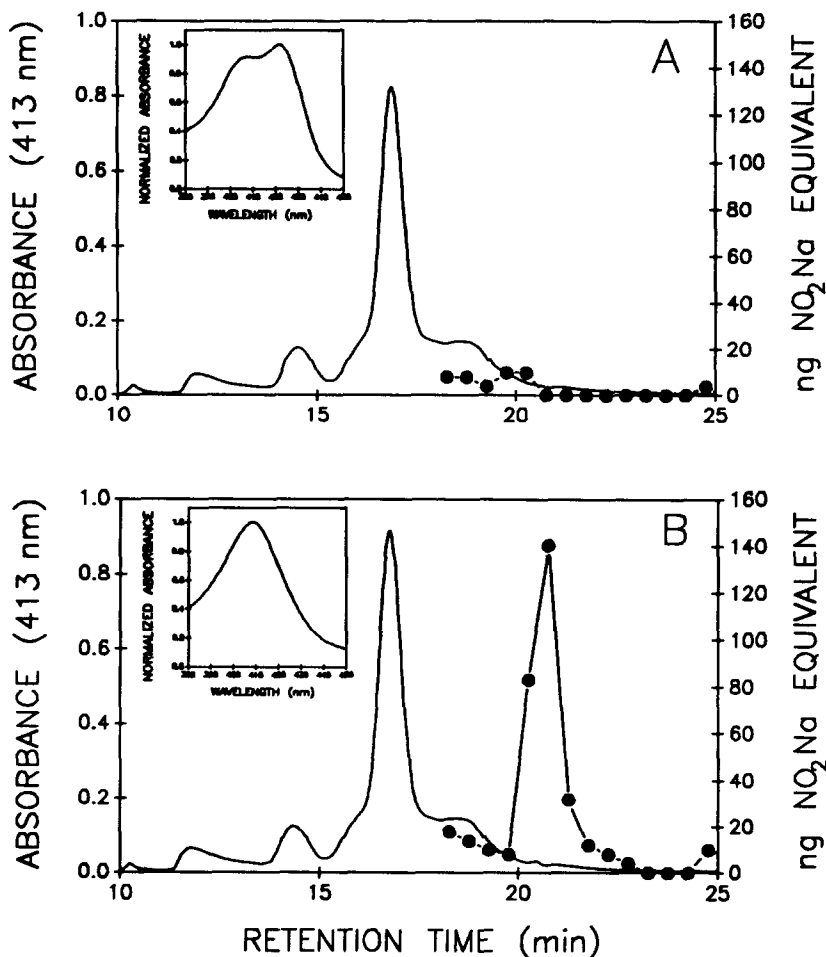


Figure 6. Molecular sieving chromatography of 20 pairs of freshly homogenized salivary glands of *R. prolixus* (containing endogenously bound NO) in 100 μl of buffer in the absence (*A*) and in the presence (*B*) of 1 mM histamine. Histamine was present in the 100 μl sample alone, but not in the column perfusing buffer. The *inset* represents the near UV absorbance of the fraction containing the main Soret absorbing fraction (16.5–17-min retention time), with maxima at 422 and 411 nm for the native and histamine-treated homogenates, respectively. The *solid symbols* represent the nitrite measured by the Griess method. The nitrite peak shown in the presence of histamine has the same retention time as authentic nitrite, injected in the absence of histamine or homogenate. Controls with histamine alone injected into the column did not give any detectable product by the Griess reagent.

Rhodnius salivary heme protein has a unique high affinity for histamine. Our apparent $K_{0.5}$ values calculated from Hill plots (Fig. 2) can only be taken qualitatively, as they indicate a value that is lower than the amount of protein present in the cuvette at the time of measurement ($0.4 \mu\text{M}$, giving a Soret maximum of 0.1 absorbance units/cm). This indicates that the actual value is smaller than the protein concentration used in the assay. Additionally, the stability of the complex, as indicated by the permanence of the Soret absorbance maximum of 411 nm (typical of the heme protein-histamine complex) after gel filtration, further indicates a high affinity of binding, as is also the case of our inability to restore the spectrum of the hemoprotein to the unligated species after extensive dialysis of the heme-histamine complex. Whatever the real value of $K_{0.5}$, it is more than one order of magnitude lower than previously reported for other heme proteins. Indeed cytochrome P450 has an affinity for histamine of $16 \mu\text{M}$ (12). Similarly, the affinity reported for thromboxane synthase for imidazole and histamine were of $46 \mu\text{M}$ and $>500 \mu\text{M}$, respectively (based on inhibition of thromboxane B2 synthesis) (8). It is possible that this high affinity for histamine represents some basic property that is required for an efficient NO carrying Fe(III) heme protein, or that it represents an independent evolution designating yet another function that might help the insect feeding success.

Histamine can be found at sites of injury after its release from platelets (22), or from mast cells belonging to hosts previously sensitized by the arthropod salivary antigens (23). Histamine content in different mammal platelets vary; human and rabbit platelets store about equal amounts of serotonin and histamine (22). Released histamine produces burning pain and itching on the host, activating its defensive responses which may ruin the insect's feeding or its life (24, 25). Neutralization of histamine action at the site of feeding is thus of advantage to the survival of the bug.

We have previously reported that the saliva of *R. prolixus* contained antihistaminic and antiserotonin activities (26). At that time, however, we could not characterize the nature of the inhibitor and the effects described could be jointly caused by both the salivary NO (which has spasmolytic activity in smooth muscle, reference 19) and unligated heme protein. Results presented in Fig. 7, which used salivary homogenates extensively dialyzed against alkaline buffer, or pure heme protein devoid of NO (the NO is lost during the extensive purification procedures), indicate that antihistaminic activity can be verified by the heme protein alone without NO. These data are consistent with the high affinity binding of histamine to the heme, and are observed with the equivalent of less than one pair of salivary gland homogenates/ml of Tyrode's solution (Fig. 7). This compares with the loss of up to 90% of the salivary protein and ingestion of $300 \mu\text{l}$ of blood by a Vth instar nymph (3), or a salivary concentration of 0.9 pairs \times 0.3/ml or 2.7 pairs of glands/ml of ingested blood. Although NO may physiologically antagonize some of the effects of histamine in the hemostatic response, the salivary heme protein may add to the removal of histamine at the feeding site. The vertebrate hemostatic response is a complex and redundant physiological phenomenon (1) and it is not surprising that insects that survive by counteracting this system have also developed a sophisticated and redundant array of antihemostatic activities.

Although of a speculative nature, the results presented here can also provide some insights into other systems. To the extent that high affinity histamine binding may be a general property of ferric heme proteins that interact with NO, the effects of histamine on guanylate cyclase and NO synthase (two heme proteins that interact with NO through a heme group, reference 19) should be investigated, mainly taking into consideration the fact that histamine has recently been proposed as an intracellular mediator (27-29).

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