Characterization of Saxitoxin Binding to Saxiphilin, a Relative of the Transferrin Family That Displays pH-Dependent Ligand Binding†

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ABSTRACT: Saxiphilin is a 91 kDa saxitoxin-binding protein that is homologous to members of the transferrin family of Fe^{3+}-binding proteins noted for pH-dependent release of Fe^{3+}. The mechanism of toxin binding to purified native saxiphilin from the bullfrog (*Rana catesbeiana*) was studied using [^{3}H]saxitoxin. At pH 7.4 and 0 °C, [^{3}H]saxitoxin binds to a single site on saxiphilin with a K_D of ~0.2 nM. The pH dependence of [^{3}H]saxitoxin binding follows a one-site titration curve in the range of pH 9–4 with maximal binding from pH 9 to 7 and half-inhibition at pH 5.7. Inhibition of toxin binding at low pH is the combined result of a decrease in the rate of toxin association and an increase in the rate of toxin dissociation. The dependence of the apparent rate constants for [^{3}H]saxitoxin association and dissociation on [H^+] can be accounted for by a four-state model of allosteric interaction between the toxin-binding site and a single titratable residue of saxiphilin with a pK_a of 7.2 in the toxin-free form and 4.3 in the toxin-bound form. From 0 to 25 °C, the temperature dependence of [^{3}H]saxitoxin binding to saxiphilin is characterized by $\Delta H° = -8.3$ kcal mol^{-1}, $\Delta S° = 13.8$ cal mol^{-1} K^{-1}, and activation energies of 22.5 kcal mol^{-1} for dissociation and 11.1 kcal mol^{-1} for association. Binding of [^{3}H]saxitoxin to saxiphilin is competitively inhibited with low affinity by a variety of divalent metal and lanthanide cations. Inhibition of toxin binding by the carboxyl-methylating reagent trimethyloxonium is prevented by pre-equilibration with [^{3}H]saxitoxin, implicating the presence of one or more carboxyl groups in the binding site. Functional similarities suggest that the saxitoxin-binding site of saxiphilin is located in an interdomain cleft analogous to the location of one of the two homologous Fe^{3+}-binding sites of transferrins. On the basis of residue substitutions between saxiphilin and transferrins, it is proposed that the saxitoxin-binding site is located in the carboxy terminal lobe of saxiphilin and that binding is modulated by protonation of a conserved histidine residue.

Saxiphilin is a soluble protein that binds saxitoxin (STX)[1] with high affinity and specificity. Saxiphilin from the North American bullfrog (*Rana catesbeiana*) has been studied most extensively (Mahar et al., 1991; Li & Moczydlowski, 1991; Li et al., 1993); however, similar activity is present in a variety of ectothermic vertebrates (L. Llewellyn, J. Lynch, P. Bell, and E. Moczydlowski, unpublished results). Using [^{3}H]STX binding as an assay, saxiphilin was purified from bullfrog plasma and identified as a 91 kDa protein related to transferrin (Li & Moczydlowski, 1991). The ligand, STX, is a potent neurotoxin with a structure distinguished by two cyclized guanidinium groups (Figure 2). STX or "paralytic shellfish poison" is produced by certain cyanobacteria and dinoflagellates and accumulated by numerous invertebrate and vertebrate species in marine environments (Hall et al., 1990). Neurotoxicity of STX is a result of the blockage of voltage-dependent sodium channels that mediate the depolarizing phase of action potentials. The large (~210 kDa) α-subunit of sodium channels contains one extracellular binding site for STX that is located in an external vestibule close to the entrance of the ion-selective pore (Catterall, 1992).

In contrast to sodium channels, the physiological function of saxiphilin is presently unknown. Partial sequencing of the native protein (Li & Moczydlowski, 1991) provided information that led to the isolation from bullfrog liver of a full-length cDNA clone encoding saxiphilin (Morabito & Moczydlowski, 1994). The primary sequence confirmed that saxiphilin is homologous to members of the transferrin family of Fe^{3+}-binding proteins, which are also known as siderophilins. For example, pairwise alignment of saxiphilin with human lactoferrin shows 44% sequence identity at the amino acid level, similar 2-fold internal homology, and apparent conservation of 14 disulfide bonds. However, the presence of a unique insertion of 144 residues, the substitution of most of the conserved Fe^{3+}-coordination residues, and a lack of $^{55}Fe^{3+}$ binding discriminate saxiphilin from other members of the transferrin family (Li et al., 1993; Morabito & Moczydlowski, 1994).

The transferrin family includes serum transferrin, lactoferrin, ovotransferrin, and melanotransferrin. These latter proteins exhibit bicarbonate-dependent binding of Fe^{3+}, with a high affinity, K_D, for Fe^{3+} estimated to be ~10^{-20} M for human transferrin (Aisen et al., 1978). Serum transferrin sequesters, transports, and ultimately delivers Fe^{3+} to eukaryotic cells by the process of receptor-mediated endocytosis

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[1] Abbreviations: BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STX, saxitoxin; TMO, trimethyloxonium tetrafluoroborate; Tris, tris(hydroxymethyl)aminomethane.

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(Dautry-Varsat, 1986). The related protein lactoferrin serves as a bacteriostatic agent in milk and other secretions by limiting free Fe^{3+} required for growth of microorganisms (Griffiths & Bullen, 1987). Ovotransferrin (conalbumin) performs a similar function in egg white of birds. Melanotransferrin is an Fe^{3+}-binding protein associated with the plasma membrane of melanoma cells; however, its exact function is unknown (Rose et al., 1986; Baker et al., 1992). The crystal structures of transferrin and lactoferrin are characterized by two homologous lobes that each contain a ferric ion coordinated by two tyrosines, one histidine, one aspartic acid, and one bicarbonate anion cofactor that is also hydrogen-bonded to a conserved arginine residue (Bailey et al., 1988; Anderson et al., 1989). An important aspect of the function of serum transferrin is the pH-dependent release of Fe^{3+} in the range of pH 6–4 (Lestas, 1976; Van Renswoude et al., 1982). Physiologically, after endocytosis of serum transferrin bound to the transferrin receptor, acidification of the internal endosome compartment by an H^+-ATPase triggers the release of Fe^{3+} from transferrin for biosynthesis of other iron-containing proteins.

The identification of saxiphilin as a structural relative of the transferrin family suggests that such proteins may have other biochemical functions besides their known role in iron metabolism and homeostasis. To pursue the function of saxiphilin, this study is an initial investigation of the mechanism of STX binding to saxiphilin as compared to the known mechanism of Fe^{3+} binding to transferrin. We find that native saxiphilin has a binding stoichiometry of one [^{3}H]STX-binding site per molecule, in contrast to the two Fe^{3+}-binding sites of most transferrins. The pH dependence of [^{3}H]STX binding to saxiphilin is similar to the pH dependence of Fe^{3+} binding to serum transferrin and is consistent with an equilibrium between high- and low-affinity conformations of the binding site controlled by protonation of a histidine residue. We also find that certain divalent and lanthanide metal cations competititvely inhibit [^{3}H]STX binding to saxiphilin with low affinity, which implies that the STX-binding site contains residues that form a weak metal ion-binding site. These similarities suggest that STX binds to one lobe of saxiphilin in a manner analogous to that of transferrin and lactoferrin, in which Fe^{3+} is bound in a clawlike fashion between two flexible domains connected by a hinge region (Bailey et al., 1988; Anderson et al., 1989). Particular amino acid residues that may be involved in STX binding and the pH dependence of saxiphilin are suggested on the basis of structural homology to transferrin.

MATERIALS AND METHODS

Materials. [^{3}H]STX labeled by ^3H_2O exchange (Ritchie et al., 1976) was purchased from Amersham and standardized as described (Moczydlowski et al., 1988). The working specific activity was 25 400 cpm/pmol using Ecoscint scintillation fluid (National Diagnostics). Stock solutions of STX (Calbiochem) were diluted in 1 mM citrate buffer (pH 5.0). Mops, Mes, and Hepes were obtained from Sigma, and Tris base was from American Bioanalytical. Heparin-Sepharose CL-6B, PBE 118, Polybuffer 96, and Pharmalyte 8–10.5 ampholytes were from Pharmacia LKB. AG50W-X2 cation exchange resin (100–200 mesh, H^+ form) was purchased from Bio-Rad. Trimethyloxonium tetrafluoroborate (TMO) was from Aldrich. Chloride salts of metal cations and lanthanides were from Alfa. Other chemicals were reagent grade from J. T. Baker. Water was deionized with a Milli-Q system from Millipore.

Purification and Stability of Saxiphilin. Saxiphilin was purified from bullfrog (*R. catesbeiana*) plasma by modification of the procedure of Li and Moczydlowski (1991). The chromatofocusing resin was PBE 118 rather than PBE 94. After chromatofocusing, ampholytes were removed by gel permeation chromatography on a column of Sephacryl S-300 (1.4 × 30 cm) equilibrated with 1 mM Hepes-NaOH, 100 mM NaCl (pH 7.4). To avoid inactivation by freeze–thawing, aliquots of purified saxiphilin were diluted 1:1 with 40% glycerol in 1 mM Hepes-NaOH, 100 mM NaCl (pH 7.4) and quick frozen in liquid N_2. Thawed saxiphilin was also stabilized by addition of 0.1 mg/mL BSA in all assays and dilutions of the protein. In all experiments, protein which had been frozen only once was used to ensure maximal activity. Protein concentration of pure saxiphilin was based on quantitative amino acid analysis performed by the Yale Protein Chemistry Facility and corrected for proline, cysteine, and tryptophan which were assumed to be 10.7% by weight according to the known amino acid composition (Morabito & Moczydlowski, 1994). Saxiphilin concentrations were calculated from the protein molecular weight of 90 818 deduced from saxiphilin cDNA (Morabito & Moczydlowski, 1994).

Measurement of [^{3}H]STX Binding. [^{3}H]STX bound to saxiphilin was measured in 100 μL aliquots applied to 1 mL minicolumns of AG50W-X2 resin (100–200 mesh, converted to $Tris^+$ form and pre-equilibrated with 20 mM Tris-HCl, pH 7.4, 10 mg/mL BSA). Rapid elution (10 s) of bound [^{3}H]-STX into scintillation vials was achieved after addition of sample and wash (0.5 mL of 20 mM Tris-HCl, pH 7.4) by applying pressure to the column with a syringe. At neutral pH, this cation exchange resin selectively binds free [^{3}H]-STX and not the [^{3}H]STX/saxiphilin complex which is larger than the exclusion limit (<2700 MW). Binding isotherms for Scatchard analysis were obtained by varying total [^{3}H]-STX concentration from 0.05 to 10 nM. Samples for equilibrium binding assays were allowed to equilibrate for at least 1 h. Nonspecific binding was determined with 10 μM unlabeled STX. All binding experiments were performed at 0 °C except for studies of the temperature dependence of [^{3}H]STX binding (Figure 5) where sample temperature was maintained with a circulating water bath. Binding assays are based on means of duplicate determinations that did not differ by more than 10%.

A stock buffer mixture of Tris (0.5 M)/Mes (0.25 M)/acetic acid (0.25 M), adjusted with tetramethylammonium hydroxide or HCl, was used in studies of the pH dependence of [^{3}H]STX binding. This buffer mixture is designed to maintain a relatively constant ionic strength throughout the pH range of 4–9 (Ellis & Morrison, 1982). The final buffer concentration in the assay was either 20 or 100 mM in Tris, as noted in figure legends. For binding experiments where pH was varied to pH < 6.5, the minicolumns were pre-equilibrated with 100 mM Tris-HCl (pH 7.4) to maintain constant recovery of saxiphilin. Reported pH values for the buffers were measured at assay concentrations and temperatures with a Corning Model 150 ion analyzer.

Dissociation and Association Time Course. For dissociation, saxiphilin was first equilibrated with 4.8 nM [^{3}H]STX at the desired assay conditions. After removing two aliquots (100 μL) to determine the initial value of bound [^{3}H]STX, 10 μM STX was added to begin exchange with the radioligand. Aliquots (100 μL) were removed at various time intervals and applied to cation exchange columns to determine the time course of dissociation. The association time course was similarly determined by initiating the reaction with 19.2 nM

[^{3}H]STX after pre-equilibration of ~0.5 nM saxiphilin at assay conditions for 30 min. The equilibrium level of binding was established by following the reaction for periods up to 1 h.

Effect of Divalent Metals and Lanthanide Cations on [^{3}H]-STX Binding. Binding inhibition titrations were performed with chloride salts of various divalent metal and lanthanide cations in the presence and absence of 100 mM $NaHCO_3$, using 20 ng/mL saxiphilin (~0.2 nM), 100 mM NaCl, 4.8 nM [^{3}H]STX, and 100 mM Mops-NaOH at a final pH of 7.45. Samples were incubated for 1 h before assay of bound [^{3}H]STX. For experiments with Pr^{3+}, sample incubation was also extended to 6 h. Association and dissociation kinetics of [^{3}H]STX binding in the absence or presence of 10 mM $PrCl_3$ and 100 mM $NaHCO_3$ were determined as described above with 34 ng/mL saxiphilin (~0.4 mM) in 100 mM NaCl, 100 mM Mops-NaOH (pH 7.4). In all experiments with varying concentrations of metal chlorides in the absence or presence of $NaHCO_3$, the assay mixture was maintained at pH 7–7.4 throughout the titration. No precipitation was observed in the assay samples.

Effect of TMO Modification on [^{3}H]STX Binding. Since TMO is hydrolyzed in water (MacKinnon & Miller, 1989), saxiphilin was exposed to this reagent as rapidly as possible after it was dissolved. Various amounts of TMO (2–14 mg) were weighed into tubes that were flushed with N_2 and sealed. Aliquots (200 μL) of a buffered solution containing saxiphilin, BSA, and NaCl were added to each tube and vortexed. After 10 min, [^{3}H]STX plus water was added to a final volume of 250 μL. Final conditions for the assay were 63 ng/mL (~0.7 nM) saxiphilin, 19.2 nM [^{3}H]STX, 100 mM NaCl, 100 mM Tris-HCl, pH 8.6. After equilibration for 30 min, bound [^{3}H]-STX was measured in duplicate 115 μL aliquots. Control binding was defined with an identical sample without TMO. Nonspecific binding was measured in a sample with 10 μM STX. For ligand protection experiments, saxiphilin was first pre-equilibrated with 19.2 nM [^{3}H]STX for 30 min in the assay buffer before exposure to TMO for 10 min. The TMO experiment of Figure 9 was performed four times with similar results.

Miscellaneous. SDS–PAGE (7% T, 2.7% C) was performed according to Laemmli (1970) with a water-cooled slab gel (0.75 mm × 12 cm × 16 cm) apparatus. Samples for electrophoresis were heated at 90 °C in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol. Gels were stained with Coomassie brilliant blue R-250 according to Diezel et al. (1972). B_{max} and K_D values for a one-site binding model were obtained by Scatchard analysis using the LIGAND computer program (Munson & Rodbard, 1980) obtained from Biosoft. Parameter fits to binding competition curves, exponential kinetics, and a four-state model of the pH dependence of [^{3}H]STX binding were obtained with the nonlinear fitting utility of Sigmaplot 4.1 (Jandel Scientific).

RESULTS

Stability and Stoichiometry of [^{3}H]STX Binding to Saxiphilin. Preparations of saxiphilin used for this study were virtually homogeneous as judged by a single major band on SDS–PAGE (Figure 1A). As noted previously (Li et al., 1993), the apparent molecular weight of saxiphilin estimated by SDS–PAGE is in good agreement with the protein molecular weight of 90 818 deduced from the cDNA sequence (Morabito & Moczydlowski, 1994). To ensure maximal activity for determination of toxin-binding stoichiometry, the

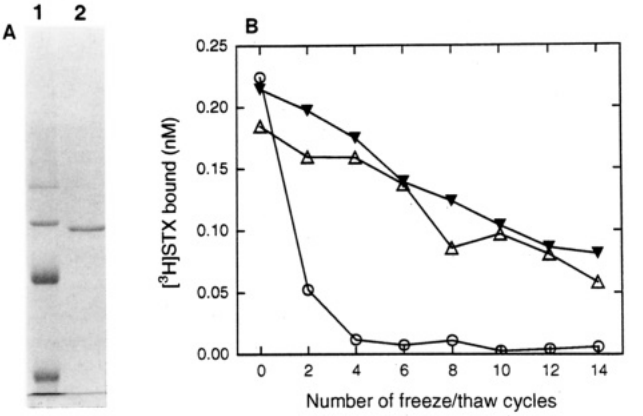


FIGURE 1: Sensitivity of purified saxiphilin to freeze–thawing. (A) SDS–PAGE (7% total acrylamide) of saxiphilin used for determination of [^{3}H]STX-binding stoichiometry. Lane 1, 1.7 μg each of M_r standards (top to bottom): β-galactosidase (116 000), phosphorylase b (97 400), bovine serum albumin (67 000), bovine carbonic anhydrase (29 000); lane 2, 0.8 μg of saxiphilin. (B) Effect of freeze–thawing on [^{3}H]STX-binding activity of saxiphilin and partial protection by glycerol and ethylene glycol. A sample of saxiphilin (27 ng/mL) (~0.3 nM) without cryoprotectant (○) or with 20% glycerol (▼) or 20% ethylene glycol (△) was subjected to repeated cycles of freeze–thawing and assayed for [^{3}H]STX-binding activity.

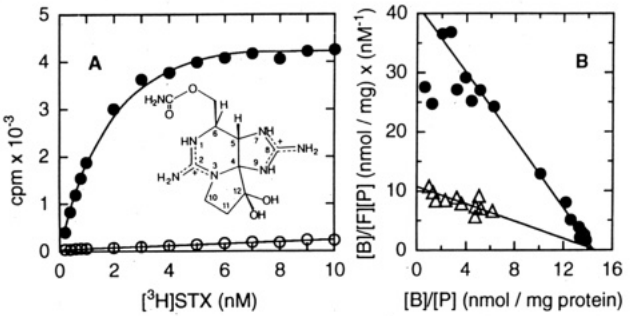


FIGURE 2: Stoichiometry and K_D of [^{3}H]STX binding to pure saxiphilin. (A) Binding isotherm obtained with increasing concentrations of total [^{3}H]STX in the presence of 200 mM NaCl, 10 mM Mops-NaOH (pH 7.45), and 120 ng/mL saxiphilin. Samples were assayed in the absence (●) or presence (○) of 10 μM STX to assess nonspecific binding. The insert shows the structure of STX. (B) Scatchard transformation of the data in panel A (●) is fit with a K_D of 0.35 ± 0.02 nM and a B_{max} of 14.5 ± 0.2 nmol/mg of protein. Results are also shown for a similar [^{3}H]STX titration at pH 5.55 (△) in the presence of 100 mM Tris/50 mM Mes/50 mM acetic acid, 100 mM NaCl and fit with a K_D of 13.6 ± 3.6 nM and a B_{max} of 14.7 ± 2.9 nmol/mg of protein.

stability of purified saxiphilin was investigated. Figure 1B shows that saxiphilin is subject to denaturation by freeze–thawing as noted by complete loss of [^{3}H]STX-binding activity after four repetitive cycles of freezing in liquid N_2. This loss of activity was attenuated by addition of 20% glycerol or ethylene glycol to the freezing buffer, but these cryoprotectants did not completely eliminate this form of denaturation (Figure 1B). To minimize such loss of activity, saxiphilin was aliquoted in small volumes in buffer containing 20% glycerol and thawed only once before use.

Figure 2A shows raw data from a binding titration of a sample of pure saxiphilin with increasing concentrations of [^{3}H]STX. The low level of "nonspecific" binding measured in the presence of 10 μM unlabeled STX is characteristically observed with highly purified saxiphilin. The corresponding Scatchard plot of Figure 2B shows that [^{3}H]STX-binding data obtained at pH 7.45 and 0 °C are consistent with a homogeneous class of sites with K_D = 0.35 ± 0.02 nM and B_{max} = 14.5 ± 0.2 nmol/mg of protein. A similar experiment with a different preparation of saxiphilin gave values of K_D

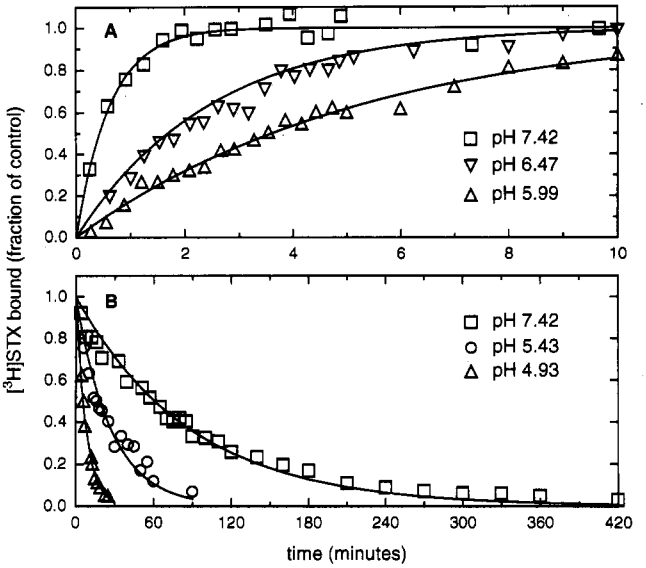


FIGURE 3: Effect of pH on association and dissociation kinetics of [^{3}H]STX binding to saxiphilin. (A) Time course of [^{3}H]STX association at three different pH values was determined with 19.2 nM [^{3}H]STX and 46 ng/mL (~0.5 nM) saxiphilin in the presence of 100 mM NaCl, 100 mM Tris/50 mM Mes/50 mM acetic acid buffer adjusted to pH 5.99 (△), 6.47 (▽), and 7.42 (□). (B) Time course of [^{3}H]STX dissociation at three different pH values was determined after equilibrating 4.8 nM [^{3}H]STX and 27 ng/mL saxiphilin (~0.3 nM) for 30 min in the same buffer adjusted to pH 4.93 (△), 5.43 (○), and 7.42 (□). Binding data are normalized to the equilibrium value at long times (A) or the initial value (B). Data points at each pH value are fit to a single exponential with rate constants: (A) pH 5.99, 3.2×10^{-3} s^{-1}; pH 6.47, 6.85×10^{-3} s^{-1}; pH 7.42, 15.5×10^{-3} s^{-1}; (B) pH 4.93, 21.3×10^{-4} s^{-1}; pH 5.43, 4.9×10^{-4} s^{-1}; pH 7.42, 1.44×10^{-4} s^{-1}. Some overlapping data points have been omitted to relieve crowding.

$= 0.16 \pm 0.02$ nM and $B_{max} = 13.9 \pm 0.4$ nmol/mg of protein. The mean value of B_{max} based on 10 determinations is 14.1 ± 1.4 nmol of [^{3}H]STX bound/mg of protein. The theoretical value of B_{max} for a protein with a molecular mass of 90.8 kDa is 11.0 nmol/mg for one STX-binding site and 22.0 nmol/mg for two STX-binding sites. Since the linear Scatchard plot indicates one class of binding sites in the protein preparation, we conclude that the B_{max} data are consistent with one [^{3}H]-STX-binding site per saxiphilin molecule. The discrepancy between the measured B_{max} value of 14.1 nmol/mg and the theoretical value of 11.0 nmol/mg is probably due to calibration errors in the specific activity of [^{3}H]STX and/or the measurement of protein concentration. On the basis of the structural homology of saxiphilin to members of the transferrin family (Morabito & Moczydlowski, 1994) which bind two Fe^{3+} ions, this stoichiometry suggests that only one of the two homologous lobes of saxiphilin contains a functional STX-binding site.

Effect of pH on the Kinetics of [^{3}H]STX Binding. Binding of [^{3}H]STX to saxiphilin is inhibited by a decrease in pH. Scatchard analysis (Figure 2B) indicates that the K_D of [^{3}H]-STX is 13.6 ± 3.6 nM at pH 5.55 (0 °C) which is ~40-fold lower affinity than the K_D of 0.35 nM at pH 7.45. However, the extrapolated B_{max} value of [^{3}H]STX binding does not appear to be altered at low pH (Figure 2B). This indicates that the number of available binding sites does not depend on [H^+] and that the decrease in affinity must be due to altered kinetics of [^{3}H]STX binding.

The reduction in affinity for [^{3}H]STX at low pH is the combined result of a decrease of the association rate (Figure 3A) and an increase of the dissociation rate (Figure 3B). The time course of association of [^{3}H]STX to saxiphilin was measured under pseudo-first-order conditions with the concentration of ligand (19.2 nM [^{3}H]STX) approximately 20-fold greater than the concentration of binding sites. Under these conditions, the association time course was closely approximated by a single exponential, giving an apparent rate constant of $k_p = 15.5 \times 10^{-3}$ s^{-1} at pH 7.42 (Figure 3A). Reduction of the pH to 6.47 and 5.99 resulted in a 2.3- and 4.8-fold decrease in the apparent association rate, respectively. When the dissociation time course of [^{3}H]STX was measured by the rate of exchange with excess unlabeled STX, the kinetics were well-described by a single-exponential process under a wide range of conditions (e.g., Figure 3B). At pH 7.42 and 0 °C, the dissociation of [^{3}H]STX from saxiphilin occurs with a halftime of ~80 min corresponding to a rate constant of $k_d = 1.44 \pm 0.03 \times 10^{-4}$ s^{-1}. This rate is enhanced by a factor of 3.4- and 14.8-fold at pH 5.43 and 4.93, respectively. The observed pseudo-first-order association rate constant (k_p) for [^{3}H]STX at pH 7.42 may be converted to a bimolecular association rate constant (k_a), using the familiar expression derived from the rate expression for a reversible bimolecular reaction: $k_p = [STX]k_a + k_d$ (Fersht, 1985). This gives a value of $k_a = 8.0 \times 10^5$ s^{-1} M^{-1}. Using the ratio of k_d/k_a to calculate the equilibrium dissociation constant at pH 7.42 gives $K_D = 0.18$ nM, which is close to the value (0.16–0.35 nM) derived from Scatchard analysis at this pH. This agreement between kinetic and equilibrium measurements further supports the assumptions of a single binding site for [^{3}H]STX and first-order kinetics.

Mechanism of Inhibition of [^{3}H]STX Binding to Saxiphilin by H^+. Figure 4A is a plot of the equilibrium concentration of bound [^{3}H]STX measured at various pH values from 9 to 4.7. This experiment shows that [^{3}H]STX binding is essentially constant in the range of pH 9–7. Further acidification to pH 4 results in a progressive inhibition of [^{3}H]STX binding to a level near the limit of detectibility. Fitting of the pH titration in the presence of 200 mM NaCl to the Hill equation, $y = B_{max}K_{0.5}^n/([H^+]^n + K_{0.5}^n)$, gives a value of $n = 1.0$ for the Hill coefficient and $pK_{0.5} = 5.7$. Similar behavior ($n = 1.0$; $pK_{0.5} = 5.4$) with a small shift of the titration curve to lower pH is observed at 600 mM NaCl (Figure 4A). This comparison shows that the pH dependence is rather insensitive to ionic strength in this range of NaCl concentration. The data of Figure 4A also indicate that the effect of pH is not due to the ionization state of the ligand. STX has two cyclized guanidinium groups (Figure 2). The C-2 guanidinium group of the six-membered ring has a pK_a of 11.3, and the C-8 guanidinium group of the five-membered ring has a pK_a of 8.2 (Rogers & Rapoport, 1980; Shimizu et al., 1981). Since both of these guanidinium groups are completely protonated over the range of pH 7–4, inhibition of [^{3}H]STX binding by decreasing pH is most likely due to protonation of saxiphilin. The lack of a significant decrease in the level of [^{3}H]STX binding in the range of pH 8–9 also implies that protonation of the C-8 guanidinium of STX is not required for high-affinity binding to saxiphilin.

The dependence of the rate of [^{3}H]STX association and dissociation on [H^+] (Figure 3) requires that both the STX-bound and STX-free states of saxiphilin are sensitive to protonation. A Hill coefficient of $n = 1$ for the effect of [H^+] on the fractional occupancy of saxiphilin by [^{3}H]STX (Figure 4A) further suggests that this pH dependence is mediated by a single titratable group of saxiphilin. The simplest model that can explain these observations is a negative allosteric interaction between the protonation of a single amino acid residue of saxiphilin and the STX-binding site. According to

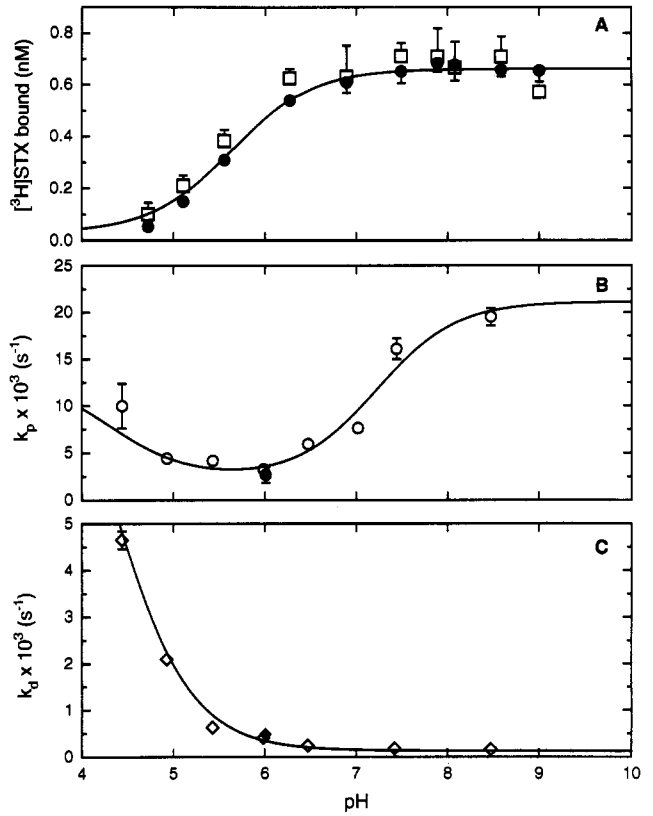
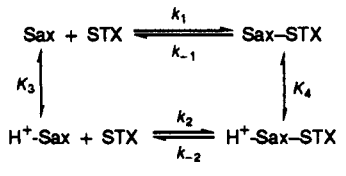


FIGURE 4: Analysis of the pH dependence of [³H]STX binding to saxiphilin. (A) Equilibrium measurement of bound [³H]STX after incubation of 5 nM [³H]STX at pH 9–4 in the presence of 200 mM NaCl (●) and 38 ng/mL (~0.42 nM) saxiphilin. The pH buffer was 20 mM Tris/10 mM Mes/10 mM acetic acid. Results for a similar experiment at higher ionic strength (600 mM NaCl) are also shown (□). (B) Apparent pseudo-first-order rate constant for association (k_p) of [³H]STX as a function of pH. k_p (○) was measured at various pH values as described in Figure 3A. (C) Apparent rate constant for dissociation (k_d) of [³H]STX as a function of pH. k_d (◇) was measured at various pH values as described in Figure 3B. Errors were evaluated from the fitting statistics of a single time course (B and C) or from the standard error of the mean of three experiments (A). Solid lines correspond to a theoretical fit obtained by simultaneous fitting of the data sets from A (200 mM NaCl), B, and C to eqs 1–3 derived from the scheme as discussed in the text. The best-fit parameters are listed in Table 1. Solid symbols in B and C correspond to measurements made on a different saxiphilin preparation.

this model, a certain residue of saxiphilin can be protonated in either the STX-free or STX-bound form. Protonation of the STX-free form results in an H⁺-form of the protein that has lower affinity for STX and a slower apparent association rate. Similarly, protonation of the STX-bound form of saxiphilin also destabilizes STX binding and results in an H⁺-saxiphilin–STX ternary complex that has a faster dissociation rate of STX, giving rise to pH-dependent dissociation of [³H]STX. Thus, the pH dependence of [³H]STX binding can be explained by the following four-state scheme, where Sax refers to the unprotonated form of saxiphilin and H⁺-Sax refers to the protonated form:



In the above scheme, k_1 and k_2 are the bimolecular association rate constants for STX binding to the unprotonated and protonated forms of saxiphilin, respectively. The dissociation constants k_{-1} and k_{-2} are the respective rate constants for STX dissociation. K_3 and K_4 are the equilibrium dissociation constants for protonation of the STX-free and STX-bound forms of saxiphilin, respectively. To investigate whether this scheme is sufficient to describe the kinetics and pH dependence of STX binding, pseudo-first-order association (k_p) and dissociation (k_d) rate constants for [³H]STX were measured at various pH values from 8.5 to 4.5 from the time course of binding as illustrated in Figure 3. Results shown in Figure 4B,C indicate that k_p follows a biphasic dependence on pH with an apparent minimum near pH 5.5 and that k_d increases in a monotonic fashion with decreasing pH.

Assuming that the K_3 and K_4 protonation reactions are in rapid equilibrium compared to the slower STX-binding steps, the dependence of k_p on [H⁺] can be derived from the rate expression for association of [³H]STX to the combined unliganded states, Sax and H⁺-Sax, to obtain

$$k_p = [^3\text{H-STX}]\left(\frac{k_1 + k_2[H^+]/K_3}{1 + [H^+]/K_3}\right) + \left(\frac{k_{-1} + k_{-2}[H^+]/K_4}{1 + [H^+]/K_4}\right) \quad (1)$$

Similarly, the dependence of k_d on [H⁺] as derived from the rate expression for the dissociation of [³H]STX from the combined states, Sax–STX and H⁺-Sax–STX, is given by

$$k_d = \frac{k_{-1} + k_{-2}[H^+]/K_4}{1 + [H^+]/K_4} \quad (2)$$

Since the equilibrium concentration of [³H]STX bound has also been measured as a function of pH (Figure 4A), the scheme can be used to predict this relationship. The following equation derived from the scheme expresses the concentration of bound [³H]STX ([bound]) as a function of the total concentration of binding sites ($[\text{bound}]_{max}$), the free [³H]-STX concentration, and the equilibrium constants (K_3, K_4, and $K_1 = k_{-1}/k_1$):

$$[\text{bound}] = \frac{[\text{bound}]_{max}[^3\text{H-STX}]}{[^3\text{H-STX}] + K_1(1 + [H^+]/K_3)/(1 + [H^+]/K_4)} \quad (3)$$

For the purpose of fitting the data in Figure 4 to eqs 1–3, the equilibrium constant K_3 can be eliminated from eqs 1 and 3 by making use of the microscopic reversibility relation, $K_3 = K_1K_4/K_2$. Equations 1–3 thus describe the theoretical behavior of the data in Figure 4 on the basis of the above scheme and five independent kinetic parameters (k_1, k_{-1}, k_2, k_{-2}, and K_4). To obtain estimates for these parameters, the data in Figure 4A–C were simultaneously fit to eqs 1–3 using a nonlinear least-squares procedure. The resulting best-fit parameters are summarized in Table 1, and theoretical curves using these values are shown as solid lines through the data in Figure 4A–C. According to the scheme and the parameters of Table 1, the unprotonated form of saxiphilin has a high affinity for STX with an equilibrium constant of $K_1 = 0.12$ nM, whereas the protonated form has a low affinity of $K_2 = 102$ nM. The amino acid residue that determines the observed pH dependence is expected to have a pK_a (pK_3) of 7.22 in the STX-free form and 4.29 (pK_4) in the STX-bound form.

Effect of Temperature upon [³H]STX Binding. The temperature dependence of [³H]STX binding was investigated

Table 1: Binding Parameters for a Four-State Model of pH-Dependent Binding of STX to Saxiphilin[a]

parameter	value	units
k_{-1}	$1.3 \pm 1.0 \times 10^{-4}$	s^{-1}
k_1	$1.1 \pm 0.1 \times 10^{6}$	$s^{-1}\ M^{-1}$
k_{-2}	$1.1 \pm 0.9 \times 10^{-2}$	s^{-1}
k_2	$1.1 \pm 0.3 \times 10^{5}$	$s^{-1}\ M^{-1}$
K_1	1.2×10^{-10}	M
K_2	1.0×10^{-7}	M
K_3	6.0×10^{-8}	M
K_4	$5.1 \pm 0.6 \times 10^{-5}$	M

[a] Data in Figure 4A–C were simultaneously fit to eqs 1–3 derived from the scheme using k_{-1}, k_1, k_{-2}, k_2 and K_4 as independent variables. Error estimates correspond to standard errors obtained from the fit.

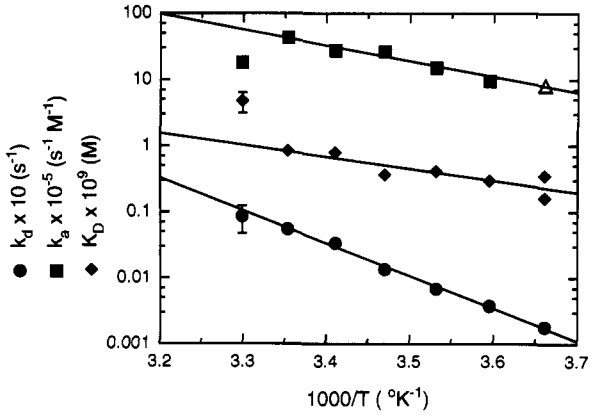


FIGURE 5: Arrhenius and van't Hoff plots of [^{3}H]STX binding to saxiphilin at pH 7.4. K_D values (◆) were determined from Scatchard analyses performed at 5 °C intervals between 0 and 30 °C. k_d (●) values were similarly determined from the dissociation time course at various temperatures. k_a values (■) were calculated from the measured k_d and K_D values according to $k_a = k_d/K_D$. One k_a value (△) at 0 °C was also measured experimentally from the association time course.

by measuring the dissociation rate constant, k_d, and the equilibrium K_D at 5° intervals over the temperature range of 0–30 °C at pH 7.4. The apparent bimolecular association rate constant at these temperatures was obtained from the relationship $k_a = k_d/K_D$. The results are presented as combined van't Hoff ($\ln K_D$ vs T^{-1}) and Arrhenius plots ($\ln k$ vs T^{-1}) in Figure 5. These plots are well-described by linear functions in the temperature range of 0–25 °C. At 30 °C, the measured K_D deviated from linear behavior, suggesting either a temperature-dependent transition or possibly thermal inactivation. Of the three parameters, k_d exhibited the highest temperature dependence with a 4.3-fold increase from 0 to 10 °C. For this same temperature increase, k_a and K_D increased by 1.7- and 2.2-fold, respectively. The solid line through the K_D values in Figure 5 is a fit to the equation $\ln K_D = \Delta H°/RT - \Delta S°/R$, using $\Delta H° = -8.3 \pm 2.0$ kcal mol^{-1} and $\Delta S° = 13.8 \pm 7.2$ cal mol^{-1} K^{-1}. The solid lines through the k_d and k_a values are fit to the equation $\ln k = \ln A - E_a/RT$ using $E_a = 22.5 \pm 1.0$ kcal mol^{-1} for k_d and 11.1 ± 2.1 kcal mol^{-1} for k_a. At 0 °C, $T\Delta S°$ is equal to 3.8 kcal mol^{-1}, which indicates that the enthalpic term predominants over the entropic term in the free energy change of binding ($\Delta G° = \Delta H° - T\Delta S°$). However, as noted by Dickinson et al. (1993), it is difficult to interpret such parameters mechanistically since absolute values of binding free energy and entropy depend on the choice of a reference state, normally 1 M concentration.

Inhibition of [^{3}H]STX Binding by Divalent Metal and Lanthanide Cations. By all indications, binding of [^{3}H]STX to saxiphilin is a chemically specific interaction. The only class of organic molecules found to competitively inhibit this binding reaction is STX and naturally occurring or synthetic derivatives of STX that differ by small chemical substituents (Mahar et al., 1991). In the course of the present experiments, we found that a variety of other molecules that structurally resemble STX, such as adenosine, 8-aminoguanine, 8-aminoguanosine, creatinine, folic acid, uric acid, and xanthine, do not affect [^{3}H]STX binding to saxiphilin when tested at concentrations in the range of 1–10 mM (L. Llewellyn, unpublished results). Such observations emphasize that the structural requirements for ligand binding to saxiphilin are stringent.

Since saxiphilin is homologous to transferrin, an important question is whether it can bind metal ions. Thus far, attempts to directly demonstrate transferrin-like binding of $^{55}Fe^{3+}$ have been unsuccessful (Li et al., 1993). To further examine this question, various divalent metal and lanthanide cations were tested for their ability to inhibit binding of [^{3}H]STX both in the presence and absence of $NaHCO_3$. As summarized in Figures 6 and 7, a variety of such metal ions display inhibition in the millimolar range. For example, Figure 6A shows that Zn^{2+} inhibits [^{3}H]STX binding with a $K_{0.5}$ of 67 mM. Such inhibition is not merely an effect of ionic strength since $MgCl_2$ tested up to 200 mM (Figure 6A) and NaCl tested up to 1 M (not shown) have no effect on the control level of [^{3}H]STX binding. Furthermore, the data of Figures 6 and 7 show that certain lanthanide cations such as Eu^{3+}, Tb^{3+}, and Nd^{3+} are more effective inhibitors of [^{3}H]STX binding than the tested transition metals (Co^{2+}, Ni^{2+}, and Zn^{2+}) with a $K_{0.5}$ of ~6 mM. This inhibition is not strictly dependent on added HCO_3^-, as noted by the similar $K_{0.5}$ values measured with or without 100 mM $NaHCO_3$ (Figure 7). However, several of the lanthanides such as Tb^{3+}, Pr^{3+}, and La^{3+} do show a 2–10-fold enhancement of affinity in the presence of HCO_3^-. Some of the metal ions exhibited Hill coefficients (n) greater than 1.0 in displacement titrations, suggesting the participation of more than one ion (e.g., $n = 1.5$ for $ZnCl_2$; Figure 6A). However, it is possible that such behavior may also be related to slow equilibration as described below for Pr^{3+}.

In the case of Pr^{3+}, an unusual phenomenon was observed. If saxiphilin is incubated for 1 h with [^{3}H]STX and increasing concentrations of Pr^{3+}, practically no binding inhibition is observed with up to 100 mM $PrCl_3$ in the absence of added HCO_3^- (Figure 6B). However, effective displacement by Pr^{3+} ($K_{0.5} = 6$ mM) is observed in the presence of 100 mM HCO_3^- (Figure 6A). When assayed after equilibration for 1 h, $NaHCO_3$ promotes the inhibition of [^{3}H]STX binding in the presence of 100 mM Pr^{3+} in a concentration-dependent fashion (Figure 6C). The unusually steep nature of the HCO_3^- titration curve (Figure 6C) suggested that the effect of HCO_3^- might reflect a synergistic effect on the rate at which Pr^{3+} is able to displace [^{3}H]STX rather than highly cooperative binding of HCO_3^-. Indeed, if Pr^{3+} is incubated for 6 h with saxiphilin and [^{3}H]STX in the absence of HCO_3^-, the titration curve for Pr^{3+} approaches that observed for a 1 h incubation with 100 mM HCO_3^- (Figure 6B). These results indicate that equilibration of Pr^{3+} with saxiphilin is slow in the absence of HCO_3^- and that HCO_3^- acts synergistically to enhance the rate of equilibration of the metal ion.

In the presence of 10 mM $PrCl_3$ and 100 mM $NaHCO_3$, Scatchard analysis revealed a low-affinity K_D of 4.6 nM for [^{3}H]STX binding without a change in B_{max}, indicative of a competitive interaction between the toxin and the lanthanide cation (data not shown). To further examine the mechanism of inhibition of [^{3}H]STX binding by lanthanide cations, the effect of 10 mM Pr^{3+} plus 100 mM $NaHCO_3$ on the time

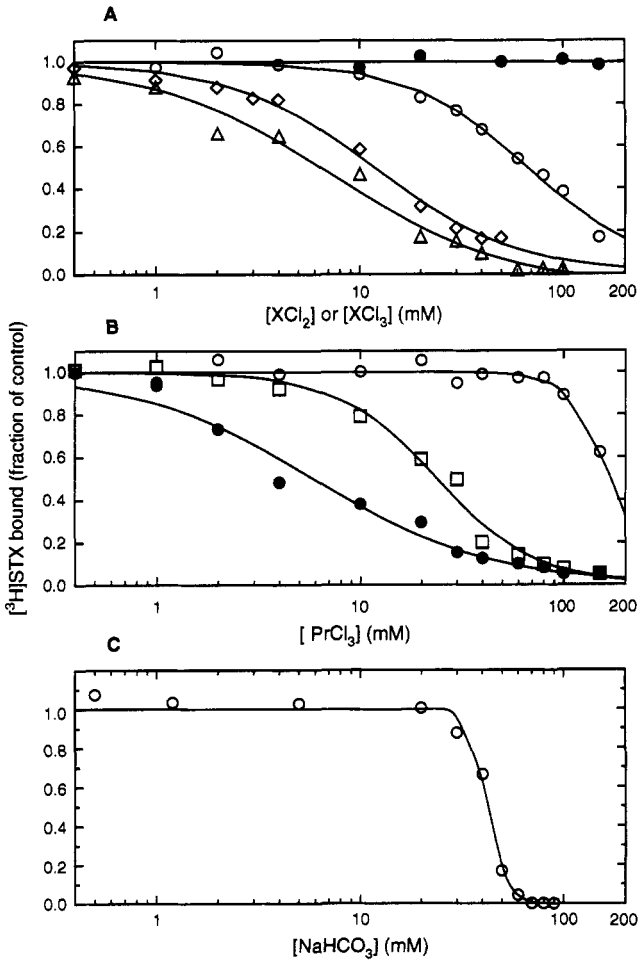


FIGURE 6: Inhibition of [^{3}H]STX binding to saxiphilin by various divalent and trivalent cations. (A) Effect of (△) $EuCl_3$, (◇) $TbCl_3$, (○) $ZnCl_2$, and (●) $MgCl_2$ on equilibrium binding of [^{3}H]STX. Saxiphilin (20 ng/mL, ~0.22 nM) was preincubated for 1 h in the presence of 4.8 nM [^{3}H]STX, 100 mM NaCl, 100 mM $NaHCO_3$, 100 mM Mops-NaOH (pH 7.45), and the indicated concentrations of di(tri)valent cations. [^{3}H]STX binding is normalized to the control value in the absence of metal ions. Except for $MgCl_2$, the solid lines are fit to the Hill equation, $y = K_{0.5}^n/([X]^n + K_{0.5}^n)$, using $n = 1.10$ ($EuCl_3$), 1.24 ($TbCl_3$), and 1.50 ($ZnCl_2$) and $K_{0.5} = 6.0 \pm 0.7$, 11.7 ± 0.6, and 66.6 ± 2.6 mM, respectively. (B) Effect of $PrCl_3$ measured after a 1 h incubation (○), effect of $PrCl_3$ measured after a 6 h incubation (□), and effect of $PrCl_3$ plus 100 mM $NaHCO_3$ measured after a 1 h incubation (●). For $PrCl_3$ plus $NaHCO_3$, the solid line fit to the Hill equation is drawn according to $n = 1.0$ and $K_{0.5} = 5.8$ ± 0.7 mM. (C) Titration of $NaHCO_3$ in the presence of 100 mM $PrCl_3$ assayed after 1 h incubation.

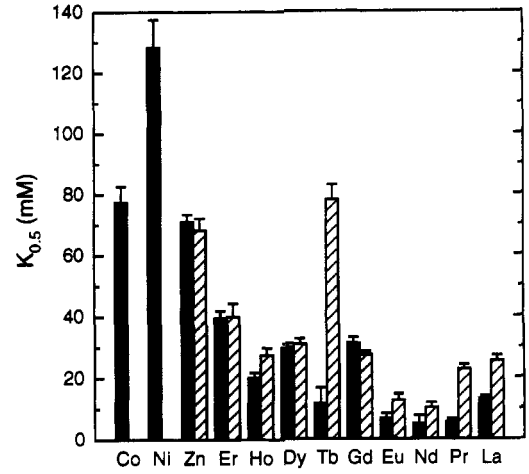


FIGURE 7: The concentration ($K_{0.5}$) of various divalent metal and lanthanide cations that produced 50% inhibition of [^{3}H]STX binding as determined in Figure 6. Solid bars correspond to results obtained in the presence of 100 mM $NaHCO_3$, and cross-hatched bars were obtained in the absence of $NaHCO_3$. Binding was measured after equilibration for 1 h except in the case of $PrCl_3$ which was equilibrated for 6 h. The effect of bicarbonate was not tested for Co^{2+} and Ni^{2+}.

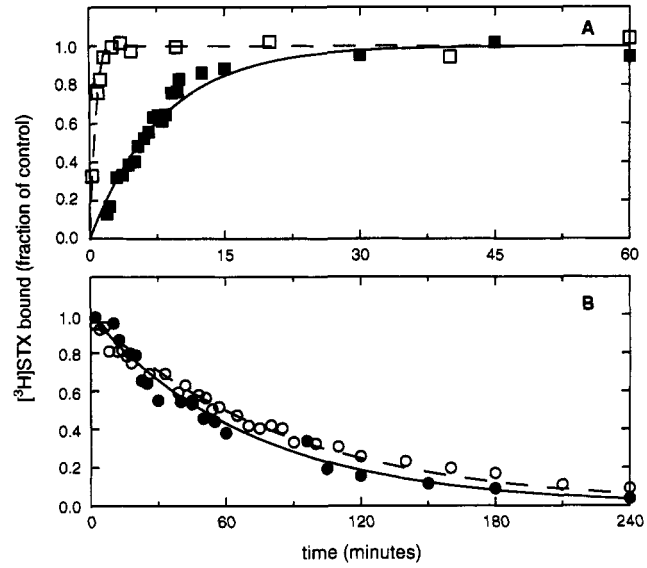


FIGURE 8: Effect of $PrCl_3$ plus $NaHCO_3$ on the kinetics of [^{3}H]STX binding to saxiphilin. The association time course (A) and the dissociation time course (B) of [^{3}H]STX was determined in the absence (open symbols) or presence (filled symbols) of 10 mM $PrCl_3$ plus 100 mM $NaHCO_3$. Other conditions: 19.2 nM [^{3}H]STX, 34 ng/mL saxiphilin (~0.38 nM), 100 mM NaCl, 100 mM Mops-NaOH (pH 7.4). Binding data are normalized to values at long times (A) or initial values (B). Dashed lines (control) and solid lines ($PrCl_3$ plus $NaHCO_3$) are fits to a single exponential using rate constants of (A) 13.4×10^5 (□) and 1×10^5 s^{-1} M^{-1} (■) and (B) 2.07×10^{-4} (○) and 2.7×10^{-4} s^{-1} (●). Some overlapping data points have been omitted to relieve crowding.

course of [^{3}H]STX association and dissociation was studied. The results of Figure 8 show that the reduced affinity for [^{3}H]STX in the presence of Pr^{3+}/HCO_3^- is due to a large decrease in the toxin association rate with virtually no effect on the toxin dissociation rate. Thus, inhibition of [^{3}H]STX binding by lanthanides is very different from that of H^+, which greatly accelerates the dissociation rate as well as slows the association of [^{3}H]STX. The results of Figure 8 are consistent with a model in which a low-affinity metal cation-binding site is formed by amino acid residues that directly participate in STX binding. If binding of a lanthanide ion and STX are mutually exclusive, Pr^{3+} would only affect the association rate of [^{3}H]STX and not the toxin dissociation rate, since the two ligands would never be simultaneously bound.

Inhibition of [^{3}H]STX Binding by TMO Modification. Trimethyloxonium is a protein modification reagent that methylates carboxyl groups of aspartate and glutamate residues in a rather specific manner (Parsons et al., 1969; Raber et al., 1979). As shown in Figure 9, treatment of saxiphilin for 10 min with increasing concentrations of TMO resulted in the complete inhibition of [^{3}H]STX binding. However, when the prebound complex of saxiphilin and [^{3}H]-STX was treated with TMO in the same fashion, virtually complete protection was observed (Figure 9). These results suggest that carboxyl groups of saxiphilin are involved in STX binding. It is likely that the observed inhibition of [^{3}H]STX binding by TMO results from the methylation of aspartate and/or glutamate residues of saxiphilin that participate in hydrogen bonds with the bound toxin molecule.

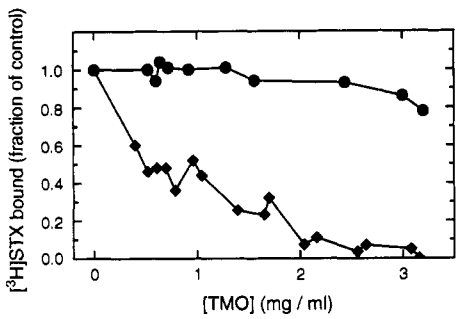


FIGURE 9: Effect of chemical modification by TMO on [^{3}H]STX binding to saxiphilin. Saxiphilin (91 ng/mL, ~1 nM) was pre-exposed to the indicated concentrations of TMO for 10 min in the absence (◆) or presence (●) of 19.2 nM [^{3}H]STX before assay of [^{3}H]STX binding. Other conditions: 100 mM NaCl, 100 mM Tris-HCl, pH 8.6. Binding data are normalized to the value in the absence of TMO (0.46 pmol of [^{3}H]STX bound).

DISCUSSION

This paper describes the functional characteristics of ligand binding to a newly recognized member of the transferrin family. Saxiphilin was the first protein to be identified that is homologous to transferrin (Morabito & Moczydlowski, 1994) but does not contain at least one fully intact Fe^{3+}/HCO_3^--binding site formed by the five highly conserved signature ligands of Asp63(392), Tyr95(426), Arg124(456), Tyr188-(517), and His249(585) as numbered according to the sequence of human transferrin (Yang et al., 1984) in the N(C)-lobes. As noted in Table 2, saxiphilin contains only one of these known Fe^{3+} ligands at position Asp60, which is equivalent to Asp63 of human transferrin. Two other transferrins, human melanotransferrin (Rose et al., 1986; Baker et al., 1992) and a transferrin from the tobacco hornworm (Bartfield & Law, 1990), have both lost one functional Fe^{3+} site in the C-lobe. However, it is not known whether the loss of Fe^{3+} binding in one lobe of these latter two proteins corresponds to a new function such as the binding of a different ligand. Recently, a 79 kDa monomeric protein purified from pig plasma has also been characterized as a novel member of the transferrin family that does not bind iron (Wuebbens et al., 1994). This latter protein was originally described as a specific inhibitor of the CAII isozyme of carbonic anhydrase (Roush & Fierke, 1992) and later found to be related to transferrin after purification and sequencing. The example of saxiphilin and the porcine inhibitor of carbonic anhydrase support the possibility that transferrins may comprise a diverse protein family. This leads to an important biochemical question that has only begun to be addressed—how many members of the transferrin family are there and what are their functions?

In the case of saxiphilin, STX may be potentially considered as a substitute ligand for Fe^{3+}; however, much less is known about the physiological function of saxiphilin in comparison to transferrin and the transferrin receptor cycle of Fe^{3+} metabolism (Crichton, 1991; Dautry-Varsat, 1986). Besides the lack of Fe^{3+}-binding residues, a unique function for saxiphilin is also suggested by the presence of a 144-residue insertion that has not been found in other members of the transferrin family (Morabito & Moczydlowski, 1994). Although saxiphilin and transferrin are clearly divergent members of a gene family, several of the functional characteristics of saxiphilin described in this paper are reminiscent of transferrin behavior. Most notable among these similarities is the pH dependence of [^{3}H]STX binding (Figure 4A) which occurs over a pH range that is about 0.5 pH unit more basic than that observed for the displacement of Fe^{3+} from human serum transferrin (Princiotto & Zapolski, 1975; Lestas, 1976; Mazurier & Spik, 1980; Baker et al., 1992). This finding supports the hypothesis that saxiphilin may also function in the internalization of a ligand by endocytosis, since a decrease in pH is a common physiological mechanism by which receptor-bound ligands are released in endosomes (Dautry-Varsat, 1986).

The 1:1 stoichiometry and the monoexponential kinetics of the [^{3}H]STX-binding reaction indicate that saxiphilin has only one STX-binding site in comparison to the two Fe^{3+}-binding sites of most known transferrins. [^{3}H]STX binding to saxiphilin also readily occurs without the addition of a cofactor such as HCO_3^- which is required for physiological binding of Fe^{3+} to transferrins (Schlabach & Bates, 1975; Harris & Aisen, 1989). The study of the kinetics of Fe^{3+} exchange to or from transferrin proteins generally requires a synergistic anion (e.g., HCO_3^- and nitrilotriacetic acid) and a chelator or Fe^{3+} acceptor (e.g., EDTA and desferrioxamine) to observe Fe^{3+} release. The one-site system and the absence of a cofactor greatly simplify quantitative analysis of the pH dependence of [^{3}H]STX binding to saxiphilin in comparison to transferrins.

Considering the difference between a one-site and two-site system, the equilibrium pH dependence of [^{3}H]STX binding to saxiphilin (Figure 4A) most closely resembles the experimentally resolved pH dependence of Fe^{3+} binding to the N-terminal lobe of human serum transferrin (Lestas, 1976). The pH dependence of Fe^{3+} binding to native diferric–transferrin titrates in the range of pH 6–4, but displays a characteristically biphasic titration curve that is known to reflect slightly higher affinity of Fe^{3+} binding to the "acid-stable" C-lobe as compared to the "acid-labile" N-lobe (Aisen et al., 1978). In contrast to serum transferrin and saxiphilin, the pH dependence of Fe^{3+} binding to human lactoferrin occurs over a pH range that is approximately 2 pH units more acidic (Mazurier & Spik, 1980; Baker et al., 1992; Day et al., 1992). The greater acid stability of Fe^{3+} binding to lactoferrin has been proposed to reflect a primary function of maintaining a low concentration of free Fe^{3+} in secretions such as milk to limit the growth of microorganisms rather than a role in Fe^{3+} delivery to cells (Dewan et al., 1993). Studies of the pH dependence of Fe^{3+} dissociation from transferrin have previously suggested that protonation of a functional group with a pK_a of ~7 serves as a triggering mechanism in the release of Fe^{3+} (Chasteen & Williams, 1981; Thompson et al., 1986). In the case of lactoferrin, protein–protein interactions between the N-lobe and the C-lobe are also important in stabilizing the release of Fe^{3+} to pH (Legrand et al., 1990; Day et al., 1992).

Chemical modification studies of serum transferrin have shown that ethoxyformylation of histidine residues with diethylpyrocarbonate markedly stabilizes serum transferrin to the pH-dependent dissociation of Fe^{3+} (Thompson et al., 1986). Comparison of various transferrin sequences led Thompson et al. (1986) to implicate the homologous residues His207 in the N-lobe and His535 in the C-lobe of human transferrin as the histidine residues that trigger the release of Fe^{3+} from each respective lobe upon protonation. These residues are generally conserved in both lobes of several serum transferrins but are replaced by Glu in the N-lobe and Asp in the C-lobe of the more acid-stable human lactoferrin (see Table 2). Noting that these residues are located near the Fe^{3+}-binding site and the hinge region involved in domain closure, Anderson et al. (1989) also suggested that they may play a role in pH dependence. Melanotransferrin, which

Table 2: Sequence Comparison Adjacent to Residues That Bind Fe^{3+} and HCO_3^- in Transferrins for Human Serum Transferrin (Stf),[a] Human Lactoferrin (Ltf),[b] and Saxiphilin (Sax)[c]

N-lobe[d]	Asp63	Tyr95	Arg124	Tyr188	His207	His249
	*	*	! ! !!	*	+	*
Stf	DAVTLDAG	TFYYAVA	HTGLGRSAG	YFGYSGAF	DVAFVKHSTI	YPSHTVVA
Ltf	DAVTLDGG	THYTAVA	HTGLRRTAG	YFSYSGAF	DVAFIRESTV	VPSHAVVA
Sax	DAMFLDSG	TCHYTVA	HSGVSKTDG	YYGNYGAF	DVAFLRSTAL	VPAGTVVT
C-lobe[d]	**Asp392**	**Tyr426**	**Arg456**	**Tyr517**	**His535**	**His585**
	*	*	! ! !!	*	+	*
Stf	DAMSLDGG	AGYFAVA	HTAVGRTAG	YYGYTGAF	DVAFVKHQTV	APNHAVVT
Ltf	DAMSLDGG	EGYLAVA	HTAVDRTAG	YYGYTGAF	DVAFVKDVTV	APNHAVVS
Sax	DAVKLEVQ	GTLRAVA	HTGVGDIAG	YYGNQGAF	DVAFVPHTVV	IPPPAIVT

[a] Human transferrin sequence (Yang et al., 1984). [b] Human lactoferrin sequence (Rey et al., 1990). [c] Saxiphilin sequence (Morabito & Moczydlowski, 1994). [d] Functionally significant residues are numbered from the amino terminus of human transferrin. * = Residues that directly coordinate Fe^{3+} in human lactoferrin (Anderson et al., 1989) and rabbit transferrin (Bailey et al., 1988). ! = Residues that hydrogen bond with HCO_3^- in lactoferrin (Anderson et al., 1989). + = Histidine residues proposed to mediate the pH dependence of human transferrin (Thompson et al., 1986).

contains only one functional Fe^{3+}-binding site in its N-lobe, exhibits a transferrin-like pH dependence in the range of pH 6–5 (Baker et al., 1992) and also has a conserved His residue homologous to His207 of transferrin. Table 2 shows that saxiphilin has a Ser residue (N-lobe) and a His residue (C-lobe) in this position. The pH-dependent kinetics of [^{3}H]-STX binding to saxiphilin (Figure 4) are consistent with a pK_a of 7.22 for the residue that mediates inhibition in the toxin-free state. This pK_a value is typical of the histidine imidazole group. If the hypothesis of Thompson et al. (1986) is correct, then the corresponding His679 residue in the C-lobe of saxiphilin may be the functional group responsible for the distinctive transferrin-like pH dependence of saxiphilin. Dewan et al. (1993) have also identified a unique interaction between two highly conserved lysine residues in the crystal structure of the N-lobe of chicken transferrin that is proposed to act as a pH-sensitive triggering mechanism for opening of the binding cleft and Fe^{3+} release. Saxiphlin is one of the few transferrins that lack these two lysine residues and also an analogous Lys–Asp–Arg interaction in the C-lobe. These particular sequence differences may also be important in determining the pH dependence of the different members of the transferrin family.

According to our analysis of the data in Figure 4, the pH dependence of STX binding to saxiphilin may be regarded as an example of an antagonistic ligand interaction (Weber, 1975) between the binding of STX and H^+ at distinct sites on saxiphilin. Using the parameters of Table 1, the coupling free energy between STX and H^+ for this interaction ($\Delta\Delta G^\circ = RT \ln(K_2/K_1) = RT \ln(K_4/K_3)$) is calculated to be 3.9 kcal/mol. This is one of the largest values of coupling free energy reported for experimentally accessible ligand-binding interactions of soluble proteins, which generally fall in the range of ±2 kcal/mol (Cantor & Shimmel, 1980). Since Fe^{3+}-binding transferrins are known to undergo large conformational changes identified as open and closed forms of the two domains that form the Fe^{3+}-binding site (Anderson et al., 1990; Grossmann et al., 1992), it is conceivable that this large coupling energy reflects a similar conformational change of saxiphilin. In this case, the reversible protonation of free saxiphilin (K_3 reaction in the above-mentioned scheme) might actually represent a more complex equilibrium of closed and open conformations of the STX-binding site, which exist in both protonated and unprotonated forms.

The enthalpy change ($\Delta H^\circ = -8.3$ kcal mol^{-1}) measured from the temperature dependence of the K_D of [^{3}H]STX binding (Figure 5) indicates that the forward binding reaction of the toxin to saxiphilin is exothermic. Direct calorimetric studies of the binding of the Fe–NTA complex to chicken transferrin in the presence of bicarbonate have shown that ligand binding to transferrin is a complex process that occurs in two distinct stages (Lin et al., 1991). The first rapid stage is an exothermic contact binding of the Fe–NTA complex analogous to that inferred here for STX binding to saxiphilin, and the second stage reflects more complex thermodynamic behavior of the slower exchange of NTA and bicarbonate. With respect to activation energy, the temperature dependence of the first-order dissociation rate constants for Fe^{3+} removal from each lobe of human transferrin is very similar to that measured here for dissociation of STX from saxiphilin. Kretchmar and Raymond (1986) found that dissociation of Fe^{3+} from the C-lobe has an activation energy of 20 kcal mol^{-1}, nearly identical to the 22.5 kcal mol^{-1} value found for saxiphilin. The temperature dependence of Fe release from the N-lobe of transferrin is more complex and exhibits a discontinuity with a value of 21 kcal mol^{-1} below 12 °C and 15 kcal mol^{-1} above 20 °C (Kretchmar & Raymond, 1986). Comparison of the present data with the temperature dependence of [^{3}H]STX binding to the guanidinium toxin receptor site of sodium channels also shows very similar behavior and absolute values of binding enthalpy and activation energies for dissociation and association. For example, binding of [^{3}H]STX to sodium channels from rat brain synaptosomes is characterized by $\Delta H^\circ = -6.0$ kcal mol^{-1}, $E_a = 18.8$ kcal mol^{-1} for k_d, and $E_a = 10.9$ kcal mol^{-1} for k_a, as calculated from the data of Weigele and Barchi (1978). In this regard, it is interesting that two structurally distinct proteins exhibit similar absolute binding affinity and binding thermodynamics

for the same neurotoxin. It remains to be seen whether this coincidence reflects similar molecular interactions in the mechanism of binding.

The low affinity for various metal ions detected by inhibition of [^{3}H]STX binding (Figure 7) is quite distinct from the behavior of transferrins, which have been found to tightly bind a variety of transition metals and lanthanides (Harris & Aisen, 1989). If we hypothesize that the evolutionary process that led to saxiphilin involved the transformation of an Fe^{3+}-binding site of an ancestral transferrin into a binding site for STX, then inhibition by metal ions may reflect the remaining vestige of a formerly functional Fe^{3+} site. The competitive kinetics of [^{3}H]STX binding to saxiphilin observed in the presence of Pr^{3+} (Figure 8) is consistent with an overlapping location of a low-affinity site for Pr^{3+} and a high-affinity site for STX. The curious effect of HCO_3^- on the equilibration of Pr^{3+} with saxiphilin (Figure 6B) is also vaguely reminiscent of the synergistic effect of HCO_3^- and other anions in promoting the binding of metal ions to transferrin. The phenomenon of anion-assisted binding of metal ions is one of the unique characteristics of transferrins. It is possible that this phenomenon also reflects the evolutionary relationship of saxiphilin and transferrin. The observations on metal ion inhibition and the evidence from TMO modification that carboxylate groups form part of the STX-binding site (Figure 9) together suggest that one or several glutamate or aspartate residues form hydrogen bonds with STX and alternatively serve as ligands to form a weak metal ion-binding site in the absence of STX. An analogy to the sodium channel binding site for STX can also be drawn for this finding, since TMO modification is known to abolish guanidinium toxin binding to sodium channels (Reed & Raftery, 1976; Doyle et al., 1993). STX binding in sodium channels is also known to behave competitively with respect to monovalent alkali cations and various divalent metal ions (Schild & Moczydlowski, 1991; Doyle et al., 1993).

Crystallographic analysis of lactoferrin has identified a large internal cavity adjacent to the Fe^{3+}-binding site with an approximate diameter of 10 Å (Anderson et al., 1989). Anderson et al. (1989) proposed that this cavity allows for the binding of larger organic anions that are known to substitute for HCO_3^- in transferrin. In principle, a cavity of this size would be large enough to accomodate STX (approximate size, 8.5 Å × 4.4 Å × 6.9 Å) and may be the analogous location of the toxin-binding site in saxiphilin. On the basis of the available data and homology to transferrin, we propose that the C-lobe cavity of saxiphilin is the most probable location of the STX-binding site. The N-lobe is an unlikely candidate because the 144-residue insertion unique to saxiphilin in this lobe occurs directly in the hinge region shown to mediate the opening and closing reaction of the Fe^{3+}-binding site in lactoferrin (Anderson et al., 1989, 1990). A large insertion at this location would be expected to perturb the conformational dynamics of the N1 and N2 subdomains necessary for ligand binding within the interdomain cleft. As noted above, the presence of the conserved His679 residue in the C-lobe of saxiphilin (Table 2) provides a plausible candidate residue for the pH dependence of [^{3}H]STX binding. In considering the various substitutions of Fe^{3+}-site residues (Table 2), the C-lobe of saxiphilin has an Asp residue in place of the Arg456 residue of transferrin that hydrogen bonds with HCO_3^- (Anderson et al., 1989). Saxiphilin also has a conservative substitution of Glu for Asp392 in the C-lobe of transferrin that coordinates directly with Fe^{3+}. The presence of these latter carboxyl ligands in the binding site for STX could explain the sensitivity of [^{3}H]STX binding to modification by TMO (Figure 9) and may also account for the ability of lanthanide cations to bind weakly and competitively displace [^{3}H]STX (Figure 7). With respect to the lanthanides, it is interesting that only the C-lobe of transferrin is capable of binding Nd^{3+} and Pr^{3+} (Luk 1971; Harris et al, 1981). Such clues lead us to suspect that the C-lobe of saxiphilin contains the STX-binding site. This hypothesis can now be tested by site-directed mutagenesis of recombinant saxiphilin which is currently in progress.

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REFERENCES

Aisen, P., Leibman, A., & Zweier, J. (1978) *J. Biol. Chem. 253*, 1930–1937.

Anderson, B. F., Baker, H. M., Norris, G. E., Rice, D. W., & Baker, E. N. (1989) *J. Mol. Biol. 209*, 711–734.

Anderson, B. F., Baker, H. N., Norris, G. E., Rumball, S. V., & Baker, E. N. (1990) *Nature 344*, 784–787.

Baker, E. N., Baker, H. M., Smith, C. A., Stebbins, M. R., Kahn, M., Hellström, K. E., & Hellström, I. (1992) *FEBS Lett. 298*, 215–218.

Bailey, S., Evans, R. W., Garratt, R. C., Gorinshy, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., & Watson, J. L. (1988) *Biochemistry 27*, 5804–5812.

Bartfield, N. S., & Law, J. H. (1990) *J. Biol. Chem. 265*, 21684–21691.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Part III, pp 874–878, W. H. Freeman, San Francisco.

Catterall, W. A. (1992) *Physiol. Rev. 72*, S15–S44.

Chasteen, N. D., & Williams, J. (1981) *Biochem. J. 193*, 717–727.

Crichton, R. R. (1991) *Inorganic Biochemistry of Iron Metabolism*, pp 1–263, Horwood, New York.

Dautry-Varsat, A. (1986) *Biochimie 68*, 375–391.

Day, C. L., Stowell, K. M., Baker, E. N., & Tweedie, J. W. (1992) *J. Biol. Chem. 267*, 13857–13862.

Dewan, J. C., Mikami, B., Hirose, H., & Sacchettini, J. C. (1993) *Biochemistry 32*, 11963–11968.

Dickinson, R., Franks, N. P., & Lieb, W. R. (1993) *Biophys. J. 64*, 1264–1271.

Diezel, W. G., Kopperschläger, G., & Hoffmann, E. (1972) *Anal. Biochem. 48*, 617–662.

Doyle, D. D., Guo, Y., Lustig, S. L., Satin, J., Rogart, R. B., & Fozzard, H. A. (1993) *J. Gen. Physiol. 101*, 153–182.

Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405–426.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., p 132, W. H. Freeman, New York.

Griffiths, E., & Bullen, J. J. (1987) in *Iron and Infection: Molecular, Physiological and Clinical Aspects* (Bullen, J. J., & Griffiths, E., Eds.) pp 171–209, John Wiley & Sons, New York.

Grossmann, J. G., Neu, M., Pantos, E., Schwab, F. J., Evans, R. W., Townes-Andrews, E., Lindley, P. F., Apell, H., Thies, W.-G., & Hasnain, S. S. (1992) *J. Mol. Biol. 225*, 811–819.

Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P. B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology* (Hall, S., & Strichartz, G., Eds.) pp 29–65, American Chemical Society, Washington, DC.

Harris, W. R., Carrano, C. J., Pecoraro, V. L., & Raymond, K. N. (1981) *J. Am. Chem. Soc. 103*, 2231–2237.

Harris, D. C., & Aisen, P. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) pp 239–371, VCH, New York.

Kretchmar, S. A., & Raymond, S. A. (1986) *J. Am. Chem. Soc. 108*, 6212–6218.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Legrand, D., Mazurier, J., Colavizza, D., Montreuil, J., & Spik, G. (1990) *Biochem. J. 266*, 575–581.
Lestas, A. N. (1976) *Br. J. Haematol. 32*, 341–350.
Li, Y., & Moczydlowski, E. (1991) *J. Biol. Chem. 266*, 15481–15487.
Li, Y., Llewellyn, L., & Moczydlowski, E. (1993) *Mol. Pharmacol. 44*, 742–748.
Lin, L.-N., Mason, A. B., Woodworth, R. C., & Brandts, J. F. (1991) *Biochemistry 30*, 11660–11669.
Luk, C. K. (1971) *Biochemistry 10*, 2838–2843.
MacKinnon, R., & Miller, C. (1989) *Biochemistry 28*, 8087–8092.
Mahar, J., Lukacs, G. L., Li, Y., Hall, S., & Moczydlowski, E. (1991) *Toxicon 29*, 53–71.
Mazurier, J., & Spik, G. (1980) *Biochim. Biophys. Acta 629*, 399–408.
Moczydlowski, E., Mahar, J., & Ravindran, A. (1988) *Mol. Pharmacol. 33*, 202–211.
Morabito, M. A., & Moczydlowski, E. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 2478–2428.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Groff, T., Racs, J., & Raftery, M. A. (1969) *Biochemistry 8*, 700–712.
Princiotto, J. V., & Zapolski, E. J. (1975) *Nature 255*, 87–88.
Raber, D. J., Gariano, P., Brod, A. O., Gariano, A., Guida, W. C., Guida, A. R., & Herbst, M. D. (1979) *J. Org. Chem. 44*, 1149–1154.
Reed, J., & Raftery, M. (1976) *Biochemistry 15*, 944–953.
Rey, M. W., Woloshuk, S. L., deBoer, H. A., & Pieper, F. R. (1990) *Nucleic Acids Res. 18*, 5288.
Ritchie, J. M., Rogart, R. B., & Strichartz, G. R. (1976) *J. Physiol. (London) 261*, 477–494.
Rogers, R. S., & Rapoport, H. (1980) *J. Am. Chem. Soc. 102*, 7335–7339.
Rose, T. M., Plowman, G. D., Teplow, D. B., Dreyer, W. J., Hellström, K. E., & Brown, J. P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1261–1265.
Roush, E. D., & Fierke, C. A. (1992) *Biochemistry 31*, 12536–12542.
Schild, L., & Moczydlowski, E. (1991) *Biophys. J. 59*, 523–537.
Schlabach, M. R., & Bates, G. W. (1975) *J. Biol. Chem. 250*, 2182–2188.
Shimizu, Y., Hsu, C., & Genanah, A. (1981) *J. Am. Chem. Soc. 103*, 605–609.
Thompson, C. P., Grady, J. K., & Chasteen, N. D. (1986) *J. Biol. Chem. 261*, 13128–13134.
Van Renswoude, J., Bridges, K. R., Harford, J. B., & Klausner, R. D. (1982) *Proc. Natl. Sci. U.S.A. 79*, 6186–6190.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1–83.
Weigele, J. B., & Barchi, R. L. (1978) *FEBS Lett. 91*, 310–314.
Wuebbens, M. M., Roush, E. D., DeCastro, C. M., & Fierke, C. A. (1994) *FASEB J. 8*, A1288.
Yang, F., Lum, J. B., McGill, J. R., Moore, C. M., Naylor, S. L., vanBragt, P. H., Baldwin, W. D., & Bowman, B. H. (1984) *Proc. Natl. Sci. U.S.A. 81*, 2752–2756.