Kinetic Analysis of Autotaxin Reveals Substrate-specific Catalytic Pathways and a Mechanism for Lysophosphatidic Acid Distribution

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Autotaxin (ATX), a secreted lysophospholipase D that hydrolyzes lysophosphatidylcholine (LPC) into lysophosphatidic acid (LPA), initiating signaling cascades leading to cancer metastasis, wound healing, and angiogenesis. Knowledge of the pathway and kinetics of LPA synthesis by ATX is critical for developing quantitative physiological models of LPA signaling. We measured the individual rate constants and pathway of the LPA synthesize cycle of ATX using the fluorescent lipid substrates FS-3 and 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl))-LPC. FS-3 binds rapidly ($k_1 \geq 500 \mu M^{-1} s^{-1}$) and is hydrolyzed slowly ($k_2 = 0.024 s^{-1}$). Release of the first hydrolysis product is random and rapid ($\geq 1 s^{-1}$), whereas release of the second is slow and rate-limiting (0.005–0.007 $s^{-1}$). Substrate binding and hydrolysis are slow and rate-limiting with LPC. Product release is sequential with choline preceding LPA. The catalytic pathway and kinetics depend strongly on the substrate, suggesting that ATX kinetics could vary for the various in vivo substrates. Slow catalysis with LPC reveals the potential for LPA signaling to spread to cells distal to the site of LPC substrate binding. An ATX mutant in which catalytic threonine at position 210 is replaced with alanine binds substrate weakly, favoring a role for Thr-210 in binding as well as catalysis. FTY720P, the bioactive form of a drug currently used to treat multiple sclerosis, displays both nucleotide phosphodiesterase activity (6) and a robust lysophospholipase D activity (lyso-PLD (7)). ATX phosphodiesterase activity is weak and is not considered relevant for in vivo function (7–9). Rather, the physiological activities of ATX have been attributed to synthesis of lysophosphatidic acid (LPA), a growth factor/chemokine that binds several endothelial differential gene family receptors (LPA1–5) (reviewed in Ref. 4), and initiates a variety of signaling cascades (4, 5) from lysophosphatidylcholine. ATX is the primary source of plasma LPA synthesis (10, 11).

The plasma LPC concentration (50–200 $\mu M$) is comparable with the $K_M$ value for steady-state LPC hydrolysis by ATX (7, 12–15). ATX binds LPC product more strongly than LPC substrate (12, 16), which has led to the hypothesis that product feedback inhibition regulates ATX activity and LPA production in vivo (16). However, rapid degeneration of serum LPA by lipid phosphate phosphohydrolase 1 (LPP1) (17, 18) would diminish LPA product inhibition of ATX.

Rapid LPA degradation upon release from ATX also limits the effective target area of newly synthesized LPA, such that LPA signaling is restricted to within the diffusional area of the ATX-lipid complex from substrate binding locations. If LPC binding, hydrolysis, and LPA product release are rapid, LPC release and downstream signaling would be local (i.e. limited to sites of LPC binding). If, however, LPC substrate binding were more rapid than LPC release and bound LPC/LPA could diffuse, thereby spreading LPA signaling to distal sites and cells. Recent in vivo studies show that competitive inhibition of ATX accelerates LPA degradation (19), consistent with the possibility of global ATX/LPA signaling via exclusion from LPP1.

In this study, we measured the individual rate constants and pathway of the LPA synthesize cycle of ATX using the fluorescent lipid substrates FS-3 (20) and LPC labeled at the fatty acid chain with NBD (NBD-LPC), using steady-state and transient kinetic methods. Our results and analysis indicate that the catalytic...
pathway and kinetics of ATX depend strongly on the substrate identity, suggesting that ATX could display different kinetic profiles for the various *in vivo* substrates. The overall catalytic cycle of ATX with LPC substrate is slow and favors long range LPA signaling by ATX distal to the site of LPC substrate binding.

**MATERIALS AND METHODS**

**Reagents**—All reagents were the highest purity commercially available. The fluorescent phospholipid analog FS-3 (20) was purchased from Echelon Biosciences (Salt Lake City, UT); fatty acid-labeled NBD lauroyl (12:0)-LPC (NBD-LPC) and fatty acid-labeled Top Fluor-LPA (TF-LPA) came from Avanti Polar Lipids (Alabaster, AL), and pNP-TMP was from Sigma. Substrates were freshly dissolved in assay buffer. Steady-state product release was assayed from changes in fluorescence intensity (FS-3, \( \text{NBD-LPC steady-state hydrolysis was measured by equilibrating FS-3 with ATX and equilibrating until completion of the reaction (judged by color change) and then lyophilizing the reaction mixture. The powder was dissolved in methanol and passed over a silica gel column equilibrated in methanol. The absorbance (494 nm) of eluted product was used to estimate the binding affinity (Eq. 1) and the substrate Michaelis-Menten constant, e.g., for free ATX.

**Kinetic Measurements**—All experimental measurements were performed in assay buffer. Steady-state product release catalyzed by ATX (100–200 nM) was assayed from changes in fluorescence intensity (FS-3, \( \lambda_{ex} = 485 \) and \( \lambda_{em} = 520 \)) using a SpectraMax Gemini XS plate reader at 25° and 37°C (21) or from changes in absorption at 405 nm (pNP-TMP) (20). FS-3 or NBD-LPC steady-state hydrolysis was measured by equilibrating with ATX (0.2–2 \( \mu M \)) and quenching the reaction at various time points with an equal volume of formic acid (3 M). NBD-LPC substrate and hydrolysis products were separated by HPLC over a C18 column under conditions used for NBD-LPA purification. FS-3 substrate and hydrolysis products were separated by HPLC over a C18 column with a water/acetonitrile/TFA gradient (60/40 mix of 94.9:5.0:1 and 9.9:90:0.1 and ending with a 40/60 mix), and quantitating by absorbance at 510 nm. A standard curve of known FS-3 and FP-3 was used to confirm all experimental measurements were made in the linear range of detection sensitivity.

Transient kinetic measurements were performed with an Applied Photophysics SX.20MV-R stopped-flow apparatus equipped with polarizers and thermostated at 25 °C (±0.1). Time courses of fluorescence (\( \lambda_{ex} = 485 \), emission monitored through 515 long pass colored glass filters for FS-3 and NBD-LPC) intensity and anisotropy change were monitored by rapidly mixing lipid substrates or products (Fig. 1) with varying concentrations of ATX. Inner filter effects are negligible at the fluorophore concentrations used.

Time courses of total fluorescence intensity (\( FI \)) change under pseudo-first order conditions (e.g., ATX >> [lipid]) were generally fitted to a sum of exponentials (e.g., single or double). Time courses of fluorescence intensity change in which pseudo-first order conditions were not fulfilled were fitted to the following quadratic expression (Equation 1) that accounts for total ATX ([P] \(_{t} \)) and FP-3 ([L] \(_{t} \)) concentrations (supplemental material) and in which \( F_{0} \) is the base-line fluorescence; \( FI_{eq} \) is the maximal fluorescence at equilibrium; \( k_{off} \) is the observed rate constant,

\[
FI(t) = FI_{eq} - (FI_{eq} - FI_{0}) \left( \frac{r - 1}{r - e^{k_{off}t}} \right) \tag{Eq. 1}
\]

and Equation 2,

\[
r = \frac{[P]_{t} + [L]_{t} + \frac{k_{off}}{K_{on}} - \sqrt{([P]_{t} + [L]_{t} + \frac{k_{off}}{K_{on}})^{2} - 4[P]_{t}[L]_{t}}}{[P]_{t} + [L]_{t} + \frac{k_{off}}{K_{on}} + \sqrt{([P]_{t} + [L]_{t} + \frac{k_{off}}{K_{on}})^{2} - 4[P]_{t}[L]_{t}}}
\tag{Eq. 2}
\]

Time courses of fluorescence anisotropy change were globally fitted with time courses of total fluorescence intensity change as described by Otto et al. (22) and Henn et al. (23).

Equilibrium FP-3 (choline equivalent product) binding was measured from changes in fluorescence intensity (\( \lambda_{ex} = 485 \) nm and \( \lambda_{em} = 520 \) nm) of equilibrated samples containing 300 nm FP-3 and a range of ATX concentrations. Choline binding was assayed from inhibition of ATX steady-state phosphodiesterase and lyso-PLD activities quantitated from time courses of fluorescence intensity (FS-3, \( \lambda_{ex} = 485 \) and \( \lambda_{em} = 520 \)) or absorbance (pNP-TMP, \( \lambda_{ex} = 485 \) and \( \lambda_{em} = 520 \)) (20) change after mixing ATX (100 nm) and FS-3 (4 \( \mu M \)) or pNP-TMP (1500 \( \mu M \)) substrates, respectively, with a range of choline. Choline product inhibition was fitted to the competitive inhibition Equation 3,

\[
v = \frac{k_{cat}[E][S]}{K_{I} + I} + [S] \tag{Eq. 3}
\]

in which \( v \) is the initial velocity of steady-state product release; \( k_{cat} \) is the maximum catalytic turnover rate; \( S \) is lipid substrate; \( I \) is inhibitor; and \( K_{I} \) and \( K_{S} \) are the inhibitor equilibrium binding affinities and the substrate Michaelis-Menten constant, respectively, for free ATX.

The binding affinity of LPA for ATX was measured by equilibrium fluorescence anisotropy titration and inhibition of ATX steady-state phosphodiesterase activity. Equilibrium titrations were done by measuring the [ATX] dependence of...
the TF-LPA fluorescence anisotropy and intensity (λ<sub>ex</sub> = 485, λ<sub>em</sub> = 504 nm) with a Photon Technologies Alphascan fluorimeter equipped with polarizers. NBD-LPA was titrated into a steady-state reaction of ATX (100 nM) and pNP-TMP (1500 μM), and the ATX catalytic activity assayed from the rate of change in absorbance at 405 nm was calculated using ε = 18.5 mM<sup>-1</sup> cm<sup>-1</sup> (25). Data were fitted to the competitive inhibition Equation 3.

Inhibition of the steady-state activity of ATX with pNP-TMP substrate was also measured with varying concentrations of unlabeled oleoyl-LPA and analyzed using the mixed inhibition Equation 4,

$$v = \frac{k_{\text{cat}}[E][S]}{K_s + [S] \left(1 + \frac{I}{K_I}\right)} \quad \text{(Eq. 4)}$$

where $K_{I,\text{comp}}$ and $K_{I,\text{uncomp}}$ are the binding affinities of the inhibitor for ATX and the ATX-substrate complex, respectively.

The FTY720P binding constant and inhibition type were determined from inhibition of ATX steady-state lyso-PLD activity as used to measure choline binding, with the addition of 1 mg/ml BSA. FTY720P binding was fitted to the uncompetitive inhibition equation, where $K_I$ is the affinity of inhibitor for substrate bound ATX as shown in Equation 5,

$$v = \frac{k_{\text{cat}}[E][S]}{K_s + [S] \left(1 + \frac{I}{K_I}\right)} \quad \text{(Eq. 5)}$$

Kinetic Modeling—The reaction Scheme 1 of the ATX catalytic cycle (notation is for LPC hydrolysis for simplicity) was used to interpret and model experimental data acquired from transient kinetic experiments.

The rate constants are numbered ($i = 1, 2, \ldots$) such that they describe progression through the cycle with $k_i$ representing the forward reaction and $k_{-i}$ representing the corresponding reverse reaction. We present experimental evidence below of multistep FP-3 (choline equivalent) binding, but present it as a
single biochemical transition in Scheme 1 for simplicity. Kinetic simulations and fitting of experimental data were done using Kintek Global Kinetic Explorer (Kintek Co.).

RESULTS

Steady-state FS-3 Hydrolysis—Time courses of fluorescence increase corresponding to steady-state FS-3 (Fig. 2A) hydrolysis by ATX at 25 °C are linear over the time scale examined (20) with rates that depend hyperbolically on the [FS-3] (Fig. 2C) yielding a $K_M$ value of 1.1 μM and a $k_{cat}$ value of 0.002 ± 0.001 s$^{-1}$ (Table 1). The $k_{cat}$ was confirmed by measuring the relative amounts of FS-3 and FP-3 by HPLC (Fig. 2C). The linear time courses are consistent with the lack of product inhibition during steady-state hydrolysis of FS-3 over the concentration range examined and indicate that the equilibrium binding affinity of ATX for both products must be weak. At 37 °C, time courses of FS-3 hydrolysis by ATX display a distinguishable lag phase that increases with FS-3 concentration (Fig. 2B). Fits to the linear regime of the time courses representing steady-state hydrolysis yield a $k_{cat}$ value that is ~2-fold more rapid and a $K_M$ value that is ~4-fold weaker at 37 °C than at 25 °C (Table 1).

Single Turnover of FS-3 Hydrolysis—Single turnover kinetic measurements (i.e. [enzyme] > [substrate]) were performed by simultaneously recording time courses of fluorescence intensity and anisotropy change in a stopped-flow to determine the rate constant of the biochemical transitions limiting FP-3 product release during ATX cycling. FS-3 and FP-3 have higher anisotropy and lower fluorescence intensity when bound to ATX than when free in solution because of the presence of the quencher moiety (Fig. 1, top panel).

The initial anisotropy of the experimental data observed after mixing varying amounts of ATX with 50–100 nM FS-3 is higher than that of free FS-3 in solution (Fig. 3A), indicating that a phase associated with FS-3 binding to ATX is completed in the instrument dead time (<2 ms). A lower limit of 500 μM$^{-1}$ s$^{-1}$ can be placed on the second order association rate constant for ATX-FS-3 complex formation ($k_{11}$; Table 1) because it is essentially completed within 2 ms at all [ATX] examined. This value is rapid but 10–20 times slower than the theoretical maximal value for encounter of molecules free in solution calculated using the Smoluchowski Equation 6,

$$k_{collision} = 4 \pi (D_A + D_B)(r_A + r_B) \frac{N_A}{1000}$$

and radii of 20 Å for FS-3 and 65 Å for ATX.

The observed time courses of fluorescence intensity and anisotropy change are biphasic and globally (22, 23) fitted to two

TABLE 1

| Parameter | Value | Method of determination |
|-----------|-------|-------------------------|
| $k_{cat}$ 25 °C | 0.002 s$^{-1}$, 0.005 s$^{-1}$ | Steady state (Fig. 2), simulation/calculation (Fig. 7) |
| $k_{cat}$ 25 °C | 0.004 s$^{-1}$ | Steady state (Fig. 2) |
| $k_{cat}$ 37 °C | 4.5 μM | Steady state (Fig. 2) |
| $K_M$ 37 °C | 0.91 μM | Steady state (Fig. 2) |
| $k_{cat}$ | 500 μM$^{-1}$ s$^{-1}$ to 10,000 μM$^{-1}$ s$^{-1}$ | Single turnover (Fig. 3) |
| $k_{cat}$ | 4.55 s$^{-1}$ to 9.100 s$^{-1}$ | Simulation (Fig. 7) |
| $k_2$ | 0.024 s$^{-1}$ | Single turnover/chemical cleavage (Figs. 3 and 4) |
| $k_3$ | >1 s$^{-1}$ | Single turnover/chemical cleavage (Figs. 3 and 4) |
| $k_4$ | >0.005 s$^{-1}$ | Single turnover/cleavage (Figs. 3 and 4) |
| $k_{cat}$ | >0.007 s$^{-1}$ | Single turnover/chemical cleavage (Figs. 3 and 4) |
| $k_{cat}$ | >0.04 s$^{-1}$ | Single turnover/cleavage (Fig. 7) |
| $k_{cat}$ | >100 μM | Product binding (Fig. 5) |

FIGURE 2. Steady-state hydrolysis of FS-3 by ATX. A, time courses of steady-state hydrolysis of 1, 2, and 4 μM FS-3 by 250 nM ATX at 25 °C. Solid lines are the best fits of the data to linear functions. B, time courses of steady-state hydrolysis at 37 °C with 50 nM ATX and 0.5, 2, 4, and 15 μM FS-3. Solid lines are the best fit by an exponential decay followed by a linked linear regime (24). C, [FS-3] dependence of the steady-state FS-3 hydrolysis rate at 25 °C (filled squares) and 37 °C (filled circles). The lines represent the best fits to rectangular hyperbola yielding an FS-3 $K_M$ value of 4.5 (± 0.3) μM and a $k_{cat}$ of 3.50 (± 0.08) × 10$^{-3}$ s$^{-1}$ at 37 °C and a $K_M$ value of 1.1 (± 0.1) μM and a $k_{cat}$ of 1.83 (± 0.03) × 10$^{-3}$ s$^{-1}$ at 25 °C. The filled triangle corresponds to measurements made by HPLC at 25 °C. Uncertainty bars represent one S.D. from the mean. AU, arbitrary units.
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![Graphs](image)

FIGURE 3. Single turnover of ATX with FS-3 substrate. A time courses of fluorescence intensity and anisotropy changes after mixing 2 μM ATX with 100 nM FS-3. The smooth lines through the data represent the best fit to a double exponential (Intensity) and corrected double exponential (Anisotropy, see “Materials and Methods”). The arrow indicates the anisotropy of free FS-3 and FP-3 in solution, as well as the fluorescence intensity of free FS-3. B, time courses of fluorescence intensity changes after mixing (α-ε) 50 nm FS-3 with 2.1, or 0.5 μM ATX. Solid lines are the best fits to double exponentials; dotted lines are best fits to simulation based on Scheme 1 and the constants provided in Table 1. C, [ATX] dependence of the observed single turnover fast rate constant. The solid line represents the best fit of the data to a rectangular hyperbola. D, [ATX] dependence of the observed single turnover slow rate constant. The solid line represents the best fit of the data to a horizontal line.

The fast and slow phases of FP-3 release correspond to dissociation through the upper and lower product release pathways in Scheme 1. Although there was some variability in the relative amplitudes of slow and fast phases, they are approximately equal, indicating comparable partitioning through the upper and lower pathways (i.e. product release pathway is random). The observed fast phase rate constant depends hyperbolically on the [ATX] (Fig. 3C) with a maximum (0.024 s⁻¹) that is significantly faster than the steady-state turnover rate (k_cat, 0.002 s⁻¹); the slow phase rate constant depends weakly on the [ATX] and has a value (~0.007 s⁻¹) approximately equal to the k_cat. Therefore, the fast phase corresponds to flux through the upper pathway of Scheme 1, with LPA analog product release contributing to the slow steady-state turnover rate, and the slow phase is flux through the bottom pathway (see supplemental material for mathematical analysis). The [ATX] at half-maximal saturation of the observed fast phase rate constant (K_{α,3}) is a composite of the equilibria preceding fluorescent product release (i.e. substrate binding K_s and hydrolysis K_p) that can be treated as a rapid equilibrium. Below, we show that the K_{α,5} corresponds to substrate binding (K_s) and the maximal observed fast phase rate constant at saturating [ATX] corresponds to hydrolysis of bound FS-3. It is likely that this transition represents a conformational change preceding and limiting rapid hydrolysis of bound FS-3 rather than chemical cleavage itself. A slow hydrolysis and a slow transition followed by a rapid hydrolysis are kinetically equivalent. We therefore treat them as a single biochemical transition.

Chemical Cleavage of FS-3—The rate constant of bound FS-3 hydrolysis (chemical cleavage) was measured by mixing ATX with FS-3 under single turnover conditions, quenching the reaction at various times with formic acid to release bound FS-3 and hydrolysis products and quantifying the FS-3 and hydrolysis product, FP-3, concentrations. This assay measures all hydrolysis products (bound and free) and therefore measures the chemical cleavage, although the spectroscopic assays presented thus far report only free FP-3 released from ATX after hydrolysis of FS-3. The observed rate constant of chemical cleavage in the presence of...
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FIGURE 5. FP-3 binding to ATX. A, time courses of fluorescence change after mixing 300 mM FP-3 with 4 μM ATX or buffer. The smooth lines through the data represent the best fit to a single exponential (+ATX) or a line (Buffer). B, [ATX] dependence of the observed FP-3 binding rate constant. The second order rate constant determined from the slope of the best fit (solid line) is 0.004 μM⁻¹ s⁻¹. Uncertainty bars represent one S.D. C, [ATX] dependence of FP-3 fluorescence intensity at equilibrium. The solid line is a linear fit to the data. The linearity of the data over the range examined indicates that the FP-3 affinity is >>10 μM. AU, arbitrary units.

Saturation of ATX (12–18 μM) is −0.008 s⁻¹ (Fig. 4), comparable with the observed fast rate constant of −0.02 s⁻¹ at the same ATX concentration measured spectroscopically (Fig. 3C). The similarity of the chemical cleavage rate constant with the fast rate constant in FP-3 release obtained with a different assay indicates that the fluorescent product release in the fast phase of the single turnover measurement (Fig. 3) is rapid and limited by hydrolysis of bound FS-3 at 0.024 s⁻¹. Essentially 100% of the FS-3 is hydrolyzed (Fig. 4), indicating that either the hydrolysis equilibrium constant largely favors product formation (K₆ > 10) and/or a transition(s) after hydrolysis (product release) is rapid and irreversible.

Fluorescent FP-3 Product Binding and Release—FP-3 binding increases the fluorescence intensity of the choline analog hydrolysis product (FP-3; Fig. 5A). Time courses of fluorescence intensity change after mixing ATX with FP-3 follow single exponentials with observed rate constants that depend linearly on the ATX concentration over the range examined (Fig. 5B), yielding a second order association rate constant (kₒ in Scheme 1) of 0.004 μM⁻¹ s⁻¹ from the slope of the linear fit to the ATX concentration-dependent observed rate constants. We note that measurements made at low [ATX] do not fulfill pseudo-first order conditions, so time courses were fitted to Equation 1 derived in supplemental material and yielded essentially identical results. The intercept, often used to estimate the apparent dissociation rate constant (kₐ in Scheme 1) despite it being subject to uncertainty, is −0.006 s⁻¹ and comparable with the slow observed rate constant of a single turnover (0.007 s⁻¹).

The ratio of rate constants yields an apparent FP-3 binding affinity with ATX (K₆) of ~1 μM. However, the binding affinity measured by equilibrium titration (Fig. 5C) suggests a weaker affinity of >10 μM. Consistent with weak FP-3 binding, single turnover measurements indicate that FP-3 binds with >5 μM affinity (described earlier). In addition, the lack of product inhibition in the steady-state time courses indicates that FP-3 binds more weakly than FS-3.

The discrepancy between the FP-3 affinity measured by equilibrium titrations and calculated from the ratio of apparent association and dissociation rate constants can be reconciled by invoking multistep binding and an additional product-bound intermediate as defined by the two-step FP-3 binding mechanism shown in Scheme 2,

\[
\text{ATX-FP-3} \xrightarrow{k_{fo}} \text{ATX} + \text{FP-3}
\]

in which (ATX-FP-3) is a low fluorescence, nonspecific collision or other intermediate complex that isomerizes to the high fluorescence ATX-FP-3 state. According to Scheme 2, the kₒ of association (right to left in Scheme 2) depends hyperbolically on [ATX]. Our observed rate constants of ATX binding to FP-3 appear to depend linearly on the [ATX] over the limited range examined (Fig. 5B), indicating that the maximal observed rate constant is >0.04 s⁻¹. The intercept of the observed rate constant corresponds to the reverse isomerization rate constant (k₋₆ ~ 0.006 s⁻¹) in Scheme 2. The affinity of ATX for FP-3 thus greatly increases from the initial binding affinity (estimated Kₒ > 100 μM) to the isomerized form (estimated K₋₆ < 0.15 μM), with an overall binding affinity (Kₒ/K₋₆) of ~15 μM.

Calculations of Reaction Rate Constants and Steady-state Rates—In the supplemental material we provide derivations of expressions used to analyze the experimental data according to the minimal ATX catalytic cycle reaction mechanism depicted in Scheme 1. Scheme 1 treats choline analog release from the bottom pathway as a single transition. This approximation is justified because formation of the encounter complex (Scheme 2) is much faster than isomerization (k₋₆ ~ kₒ), and thus overall binding can be treated as a single kinetic transition.

Time courses of ATX catalysis under single turnover conditions are composed of three chemical relaxations in the form of exponentials (λ₁ − λ₃), one of which depends on ATX concentration, according to Equation 7 (see supplemental material for derivation),

\[
\begin{align*}
\lambda₁ &= k₁ + k₅ \\
\lambda₂ &= \frac{k₆k₇[ATX]}{k₆[ATX] + k₋₆ + k₂} \\
\lambda₃ &= k₅ \\
\end{align*}
\]
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Only two exponentials are observed in the experimental time courses (Fig. 3) as follows: a fast phase with an observed rate constant ($\lambda_{XY}$) that depends hyperbolically on ATX concentration, and a slow phase ($\lambda_{YX}$) that is independent of ATX concentration and comparable with the apparent FP-3 dissociation rate constant. The first phase (i.e. $k_{cat}$) is limited by the slow chemical cleavage step preceding (i.e. $k_3 + k_5 \gg k_2$). The maximum fast phase observed rate constant ($\lambda_{XZ}$) corresponds to the forward hydrolysis rate constant ($k_3$). The slow phase rate constant ($\lambda_{YZ}$) reflects the choline analog release rate constant from ATX-C ($k_6$).

The minimum reaction scheme depicted in Scheme 1 accounts for experimental time courses under multiple turnover conditions, including the observation of a lag phase at 37 °C (Fig. 2B). Time courses of product formation (e.g. choline analog FP-3) from ATX catalysis under multiple turnover conditions are predicted by Equation 8 (see Scheme 1 and supplemental material for definitions of constants),

$$[FP-3] 
= \frac{(k_6(\lambda_{XZ} - k_4) - (k_5k_6 - k_5k_2))\eta((k_6 - \eta)(\lambda_{XZ} - k_4) - k_5k_6 + 2k_5^2\eta\sigma)}{\eta\lambda_{XZ}(\lambda_{XZ} - \lambda_{XZ})} (1 - e^{-\lambda_{XZ}t}) 
+ \frac{(k_6(\lambda_{XZ} - k_4) - (k_5k_6 - k_5k_2))\eta((k_6 - \eta)(\lambda_{XZ} - k_4) - k_5k_6 + 2k_5^2\eta\sigma)}{\eta\lambda_{XZ}(\lambda_{XZ} - \lambda_{XZ})} (1 - e^{-\lambda_{XZ}t}) 
+ (k_3 + k_5)k_5k_6\sigma$$

(Eq. 8)

The first two terms are exponentials with their amplitudes in opposite signs that describe the approach to steady state. The third term is linear with respect to time and corresponds to steady-state catalysis. At 25 °C, the first two exponentials are not observed in the experimental data, presumably because they are too rapid to detect, and time courses of product formation are linear (Fig. 2A). In contrast, experimental time courses at 37 °C display a prominent lag phase (Fig. 2B), indicating that the exponential phases are slow enough to be detected experimentally. The existence of the lag phase arises because the exponential terms have comparable constants (24).

The predicted time course of FP-3 product formation (Equation 8) allows us to define the maximum turnover rate ($k_{cat}$) and Michaelis constant ($K_D$) in terms of fundamental rate constants (Scheme 1). Because the fast and slow phase amplitudes under single turnover conditions are comparable, flux through the top and bottom product release pathways is also comparable, requiring $k_3 \approx k_5 \approx 0.007$ s$^{-1}$ (Fig. 3). We emphasize that this approximation is supported by the experimental data as follows: if $k_3 \gg k_5$, the maximum steady-state rate ($k_{cat}$) would be (partially, if $k_5$ was not $\gg k_2$) limited by hydrolysis ($k_2 + k_{-2}$; because $k_5$ is rapid, see above) and approach a value of $0.02$ s$^{-1}$, which is about 1 order of magnitude faster than the experimental measured $k_{cat}$. As a result, the $k_{cat}$ value is approximated by (supplemental material) Equation 9,

$$k_{cat} = \frac{k_2k_4}{k_4 + k_2} \approx \frac{k_5k_6}{k_6 + k_2}$$

(Eq. 9)

The $k_{cat}$ value predicted by the calculated rate constants and Equation 9 is $0.005$ s$^{-1}$, in reasonable agreement with the experimentally determined value of $0.002$ s$^{-1}$ (Fig. 2 and Table 1). The $K_M$ value approximated to Equation 10 (supplemental material) is 0.2 µM.

$$K_M = \frac{k_4}{k_4 + k_2}$$

(Eq. 10)

This value differs ~5-fold from the experimentally determined value of 1.1 µM. Although this difference could reflect experimental uncertainty, the deviation is significant and may indicate that additional intermediates not included in Scheme 1 exist and/or that the approximation of irreversible product release made in our modeling does not apply.

Kinetic Simulations of FS-3 Substrate Reactions—Kinetic simulations confirm that the experimentally determined rate and equilibrium constant values (Scheme 1) and analysis reliably account for the experimental single turnover time courses with FS-3 substrate (Fig. 3B) and allow us to refine the overall steady-state parameters. Kinetic simulations of steady-state time courses with $k_4 = 0.002$ s$^{-1}$ predict a small amplitude burst phase that is not observed in the experimental data. Global fitting (25) of steady-state time courses yields a steady-state $k_{cat}$ of 0.005 s$^{-1}$ and $k_4$ of 0.007 s$^{-1}$ (Table 1). We emphasize that the minimal ATX cycle mechanism (Scheme 1) does not represent a unique solution, and more complex models could also account for the experimental data.

The experimentally determined constants defined in Scheme 1 indicate that FS-3 product release from ATX is randomly through the two pathways, i.e. flux through the top and bottom pathways of product release occur with equal probability, and as a result, LPA analog is released before choline analog approximately half of the time. Such a random product release mechanism predicts two phases of LPA analog release from ATX, a rapid phase completed in ~40 s and a slower phase of equal amplitude occurring over ~500 s.

Steady-state NBD-LPC Hydrolysis—Time courses of steady-state NBD-LPC hydrolysis measured by HPLC (Fig. 6A) are linear over the time range measured (Fig. 6B) with rates that depend hyperbolically on NBD-LPC concentration (Fig. 6C), yielding a $K_D$ value of 308 ± 195 µM and a $k_{cat}$ values of 0.056 ± 0.011 s$^{-1}$. The $K_M$ value is comparable with values of 100–250 µM measured with various LPA substrates (7, 12) and considerably weaker than that of FS-3 substrate (1.1 µM, Table 1). The $k_{cat}$ is ~20-fold faster with NBD-LPC than with FS-3.

Single Turnover of NBD-LPC Hydrolysis—Time courses of NBD-LPC hydrolysis under single turnover conditions display a single phase associated with a fluorescence intensity reduction and anisotropy increase (Fig. 7A), interpreted as NBD-LPC binding (i.e. ATX-LPC complex formation). The total intensity and anisotropy changes are globally well fitted to a single relaxation (Fig. 7A) (22, 23), consistent with both signals monitoring the same biochemical transition(s). The observed rate constant depends linearly on the ATX concentration, yielding an apparent second order association rate constant ($k_1$) of 0.003 ± 0.0007 µM$^{-1}$ s$^{-1}$ for LPC binding to ATX from the slope of the
An ATX affinity of $6.9 \pm 3.4 \mu M$ for TF-LPA, an LPA analog with a fluorophore different from NBD, was determined by globally fitting the [ATX] dependence of the fluorescence anisotropy (Fig. 9B) and intensity (Fig. 9A). This affinity is consistent with the NBD-LPA affinity measured from direct binding measurements and inhibition of the ATX pNP-TMP hydrolysis activity. This observation suggests that contributions to the LPA binding affinity originating from the fluorophore are likely to be small.

The binding affinity of unlabeled oleoyl-LPA (18:1) was measured from inhibition of the ATX steady-state pNP-TMP hydrolysis activity. Oleoyl-LPA is a mixed inhibitor of ATX, with affinities of $2.0 \pm 0.50 \mu M$ for ATX alone and $5.0 \pm 1.3 \mu M$ for the ATX-nucleotide complex (Fig. 10). The observed mixed inhibition indicates that unlabeled oleoyl-LPA can bind at a site distal to the active site, possibly the potential secondary LPA-binding site identified in the crystal structure (28, 29). FP-3 and NBD-LPA appear to bind weakly to this secondary site, as indicated from FS-3 multiple and single turnover kinetic analysis (Fig. 3 and supplemental material), and undetectable substrate binding to the active site ATX mutant, T210A (see below).

Choline weakly inhibits the phosphodiesterase and lyso-PLD activities of ATX with substrate analogs pNP-TMP and FS-3, and the inhibition was fit to the competitive inhibition equation, yielding an apparent competitive binding constant ($K_I$) of $\sim 0.3 \mu M$ (Fig. 11). Ammonium chloride also inhibits ATX activity and is equally efficient as choline ($K_I \sim 0.3 \mu M$, see Fig. 11). Increasing the solution ionic strength with NaCl also inhibits ATX activity, but it is slightly less effective ($K_I \sim 1 \mu M$; data not shown). The weak choline affinity for ATX renders both choline release steps ($k_3$ and $k_6$) in Scheme 1 essentially irreversible.
Kinetic Simulations of NBD-LPC Substrate Reactions—The solutions derived for FS-3 in the supplementalmaterial do not apply to NBD-LPC because substrate binding is slow and, in some cases, rate-limiting. We therefore employed kinetic simulations and fitting of the experimental data (25) to assess the reaction mechanism of NBD-LPC hydrolysis. Kinetic simulations using the rate and equilibrium constants (Table 2) confirm that the experimentally determined parameters and Scheme 1 account for the NBD-LPC single turnover and steady-state time courses (Figs. 6 and 7). The simulations overlay the experimental multiple turnover and single time courses extremely well (Figs. 6 and 7) and predict a steady-state $K_M$ value of 263 μM and $k_{cat}$ value of 0.04 s$^{-1}$ consistent with those determined experimentally. Fig. 12 shows simulated bound and free NBD-LPA species changing with time.

The NBD-LPA hydrolysis and choline release rate constants were not measured in this study. However, one of these transitions is partially rate-limiting and contributes to the slow $k_{cat}$. The simulation indicates the NDB-LPC hydrolysis rate constant $k_2 \sim 0.024$ s$^{-1}$ is necessary to account for the experimental $k_{cat}$ value. This value is the same as the observed FS-3 hydrolysis rate constant, a reaction likely limited by conformational rearrangement.

![Figure 8. Kinetics and affinity of NBD-LPA binding to ATX. A, time courses of NBD-LPA binding to 10 μM (left) and 7 μM (right) ATX. Solid lines are the best fits to single exponentials. B, [ATX] dependence of the observed NBD-LPA association rate constant. The solid line is the best fit to a line yielding $k_4 = 3.4 \pm 0.4$ s$^{-1}$ from the slope. C, [NBD-LPA] dependence of the rate of steady-state pNP-TMP (1500 μM) hydrolysis by ATX (100 nM) in the presence of NBD-LPA. The solid line is the best fit to the competitive inhibition equation (Equation 3) yielding a $K_I$ of 4.9 ± 2.5 μM.

![Figure 9. Equilibrium binding of TF-LPA and ATX. A, [ATX] dependence of TF-LPA (300 nM) fluorescence intensity. The solid line is the best global fit of the fluorescence intensity and anisotropy data to a rectangular hyperbola yielding a TF-LPA binding affinity of $6.9 \pm 1.3$ μM (Table 2). B, [ATX] dependence of TF-LPA (300 nM) fluorescence anisotropy. The solid line is the best global fit of the fluorescence intensity and anisotropy data to a rectangular hyperbola.

![Figure 10. Inhibition of ATX pNP-TMP hydrolysis activity by oleoyl (18:1)-LPA. Steady-state pNP-TMP hydrolysis was measured in the absence (upper curve) or presence (lower curve) of 4 μM LPA. The data favor a mixed inhibition mechanism with an LPA affinity of 2.0 ± 0.50 μM for free ATX and an affinity of 5.0 ± 1.3 μM for the ATX substrate complex.

| Parameter       | Value         | Method of Determination          |
|-----------------|---------------|----------------------------------|
| $K_M$           | 308 ± 195 μM  | Steady state (Fig. 2)            |
| $k_{cat}$       | 0.056 ± 0.011 s$^{-1}$ | Steady state (Fig. 2)         |
| $k_{cat}$       | 263 μM        | Simulations                      |
| $k_1$           | 0.04 s$^{-1}$  | Simulations                      |
| $k_1$           | 0.03 ± 0.0007 μM$^{-1}$ s$^{-1}$ | Single turnover (Fig. 3)       |
| $k_2$           | 0.055 ± 0.004 s$^{-1}$ | Single turnover (Fig. 3)       |
| $k_3$           | > 1 μM        | Simulations                      |
| $k_4$           | > 1 μM        | Simulations                      |
| $K_5$           | 9.2 \pm 1 μM  | NBD-LPA binding (Fig. 8)         |
| $K_6$           | 4.9 ± 2.5 μM  | pNP-TMP competition (Fig. 8)     |
| $K_7$           | 6.9 ± 3.4 μM  | pNP-TMP competition (Fig. 11)    |
| $K_8$           | < 1 μM        | Equilibrium titration (Fig. 9)   |
| $K_9$           | > 1 μM        | Single turnover (Fig. 3)         |
| $K_{10}$        | < 1 μM        | Single turnover (Fig. 3)         |
| $K_{11}$        | > 1 μM        | pNP-TMP competition (Fig. 10)    |
| $K_{12}$        | > 1 μM        | pNP-TMP competition (Fig. 11)    |
| $K_{13}$        | 2.0 ± 0.50 μM | pNP-TMP competition (Fig. 10)    |
| $K_{14}$        | 5.0 ± 1.3 μM  | pNP-TMP competition (Fig. 10)    |
| $K_{15}$        | 4.9 ± 2.5 μM  | pNP-TMP competition (Fig. 11)    |
| $K_{16}$        | 6.9 ± 3.4 μM  | pNP-TMP competition (Fig. 11)    |
Arrangement of the ATX-lipid complex. Slow LPC hydrolysis at ~0.024 s\(^{-1}\) could partially limit turnover.

Analysis of T210A ATX Mutant—The ATX T210A mutant has been proposed to bind but not hydrolyze lipid substrates (26, 27). ATX T210A displayed undetectable steady-state hydrolysis activity with the nucleotide, pNP-TMP, or FS-3 (data not shown), consistent with previous studies (8, 27). No changes in the anisotropy of FS-3 were detected upon mixing with 2 \(\mu\)M ATX T120A (data not shown) suggesting that inhibition arising from substitution of threonine 210 with alanine results from compromised substrate binding as well as hydrolysis. The lack of NBD-LPC binding in the presence of 6 \(\mu\)M Thr-120-ATX (data not shown) corroborates the conclusion that Thr-120 plays a role in lipid binding and that this effect is not specific to the FS-3 substrate system.

Inhibition by FTY Inhibitor—The ATX inhibitor, FTY720P, partially inhibits the steady-state hydrolysis of FS-3 in an uncompetitive manner (i.e. lowers \(k_{cat}\) and \(K_m\); Fig. 13A) with an affinity (\(K_I\)) for FS-3-bound ATX of 2.3 ± 0.3 \(\mu\)M (Fig. 13, A and B). Some basal ATX activity exists in the presence of saturating FTY720P (Fig. 13B). To identify the biochemical transition inhibited by FTY720P, a single turnover experiment with FS-3 was performed in the absence and presence of 5 \(\mu\)M FTY720P (Fig. 13C). Substoichiometric FTY720P (5 \(\mu\)M) inhibits ATX (15 \(\mu\)M) activity by 90% under steady-state conditions (Fig. 13B) and inhibits ATX activity substantially under single turnover conditions (Fig. 13C).

Kinetic simulations were performed to determine a binding model that accounts for this level of inhibition. The observation that FTY720P inhibits ATX activity under substoichiometric conditions (with free ATX in excess) indicates that FTY720P binds more strongly to ATX with bound FS-3/products rather than apo-ATX. Purely competitive (i.e. FTY720P binds only apo-ATX) or noncompetitive/mixed inhibition (i.e. FTY720P binds apo-ATX and an ATX with bound FS-3/products complex) would have minimal effect on catalysis under these conditions because free excess ATX in solution would sequester most of the available FTY720P, thereby diminishing the overall inhibition of the small ATX fraction that is bound with substrate undergoing normal catalysis. Kinetic simulations and global fitting of data with and without FTY720P (Fig. 13C) favor a model in which FTY720P inhibits FS-3 hydrolysis, i.e. uncompetitive inhibition.
**Kinetic Analysis of Autotaxin**

**DISCUSSION**

*Substrate-specific Kinetics of ATX—*Transient kinetic analysis presented in this work indicates that ATX catalysis follows a random and rate-limiting product release pathway with FS-3 substrate. FS-3 binds rapidly and is cleaved slowly (~0.02 s⁻¹), although hydrolysis is not rate-limiting. Such a slow rate constant for chemical cleavage suggests major conformational rearrangement of ATX-FS-3 is required for hydrolysis. It is likely that this transition represents isomerization of the ATX-FS-3 complex to a hydrolysis-competent conformation of ATX-FS-3. The overall cycling of ATX \( k_{cat} \) reflects contributions from FS-3 hydrolysis and release of the second product along both dissociation pathways.

NBD-LPC substrate, in contrast, has very slow substrate binding, which is rate-limiting at low substrate concentrations. Hydrolysis (estimated as ~0.02 s⁻¹ from kinetic simulations) is partially rate-limiting at high substrate concentrations. Choline and LPA release are rapid. The experimental data cannot distinguish between random or sequential LPA and choline product release. The LPA product affinity is high enough that significant rebinding can occur, potentially resulting in competitive feedback inhibition.

There are significant differences in ATX catalysis with FS-3 and LPC substrates. The differences presumably originate from modification of the choline moiety in FS-3 (Fig. 1); the choline analog in FS-3 has a much higher affinity for ATX than choline itself (Figs. 5 and 11). Tighter binding could contribute to the slower choline analog dissociation observed with FS-3 substrate and influence flux through the bottom product release pathway (i.e. LPA analog released before choline analog).

ATX catalysis depends strongly on the substrate identity and could thus have substantially diverse activities and functions with different in vivo substrates. ATX has very low substrate specificity, as evidenced by its ability to hydrolyze both nucleotides and lysophospholipids in its active site. The lack of substrate discrimination could be balanced by substrate-specific kinetics tailored for distinct physiological activities. LPC and sphingosylphosphorylcholine are two identified in vivo ATX substrates (7, 13, 30), although several more lysophospholipids are hydrolyzed by ATX in vitro (12, 31). The kinetics and pathway of ATX catalysis with sphingosylphosphorylcholine could differ from those of the two substrates analyzed here, thereby yielding different in vivo signaling activities.

*Mechanism for Dispersion of Synthesized LPA—*Free LPA is rapidly degraded by lipid phosphate phosphohydrolase 1 (LPP1). For synthesized LPA to spread from the site of ATX binding, mobility of ATX before LPA release is essential. LPA release from ATX is slow and occurs on the tens of seconds time scale (Fig. 12).

Although the mobility of ATX-lipid complex in vivo is uncertain and depends on numerous factors and extracellular location (i.e. blood, interstitial space, and cerebrospinal fluid), an approximation of the ATX signaling range can be evaluated by considering particular extremes. We consider free diffusion as a first approximation. The average distance a molecule diffuses over a given time \( t \) in three dimensions is determined by its diffusion coefficient \( (D) \) according to Equation 11,

\[
(d) = \sqrt{6Dt}
\]  

An ATX diffusion coefficient \( (D) \) of \( 3.6 \times 10^{-7} \) cm² s⁻¹ in water (viscosity, \( \eta = 1 \times 10^{-3} \) pascals) and \( (T = 25°C) \) was calculated from the Stokes-Einstein Equation 12,

\[
D = \frac{k_BT}{6\pi\eta r}
\]  

using an approximate ATX radius \( (r) \) of ~65 Å (50–100 Å depending on orientation) from the crystal structure (28). A comparable diffusion coefficient of \( 5.3 \times 10^{-7} \) cm² s⁻¹ was estimated from the molecular mass, using the empirical Equation 13 (33),

\[
D = \frac{8.34 \times 10^{-6}T}{\eta M^{1/3}}
\]

in which \( M \) is the molecular mass of ATX.

These parameters yield a mean displacement of ~65 μm for the ATX-lipid complex before LPA dissociates, indicating the potential for LPA signaling to spread to cells distal to the site of LPC substrate binding by ATX. ATX in blood will naturally disperse to a much greater extent. Conversely, interactions with the extracellular matrix (34) or integrin receptors on cell surfaces (29) would diminish this range.

*Analysis of T210A Mutant—*The ATX mutant T210A does not hydrolyze lipid substrates (26, 27). High resolution ATX crystal structures (28, 29) reveal that Thr-210 coordinates one of the two catalytic zinc ions and hydrogen bonds with the phosphate or sulfate in the active site (26–29), consistent with a role in chemical cleavage of lipid substrates. However, the T210A substitution also compromises substrate binding, as indicated by the lack of a change in FS-3 anisotropy with 2 T210A substitution also compromises substrate binding, as indicated by the lack of a change in FS-3 anisotropy with 2 Å resolution ATX crystal structures (28, 29) is also weak, as this interaction is likely to be independent of the active site mutation.

*Inhibition by FTY720P—*FTY720P is the bioactive form of FTY720, a drug currently used to treat multiple sclerosis (35). ATX plays a critical role in demyelination and neuropathic pain, as well as the migration of lymphocytes out of lymph nodes and into the peripheral circulation (2, 36–39). The ATX/LPA pathway may therefore be a second molecular target of FTY720 (in addition to the S1PR) in multiple sclerosis treatment.

FTY720P has been reported to act as a competitive inhibitor of ATX (32). The reduction in \( k_{cat} \) in our experiments favors an uncompetitive inhibitory mechanism, with FTY720P binding more strongly to the ATX-lipid complex than free ATX. Potent inhibition of ATX catalysis under single turnover conditions by substoichiometric FTY720P (Fig. 13C) supports this interpretation. This uncompetitive mechanism allows FTY720P to inhibit ATX in the presence of lipid substrates, in contrast to competitive inhibitors, whose effectiveness lessens when substrate is available.
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