Human Factor Xa Bound Amidine Inhibitor Conformation by Double Rotational-Echo Double Resonance Nuclear Magnetic Resonance and Molecular Dynamics Simulations

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Double rotational-echo double resonance (double REDOR) NMR was used to investigate the conformation of a 13C-, 15N-, and 19F-labeled inhibitor (Berlex Biosciences compound no. ZK-806299) bound to human factor Xa. Conformationally dependent carbon–fluorine dipolar couplings were measured by 13C{19F} REDOR. Natural abundance carbon signals in the full-echo spectra were removed by 13C{15N} REDOR. Major and minor binding modes were suggested by the NMR data, but only the former had adequate signal to noise for distance determinations. Molecular dynamics simulations restrained by double-REDOR-determined intramolecular 13C–19F distances revealed two models for the dominant binding mode that are consistent with the NMR data. We conclude that ZK-806299 binds similarly to both FXa. Moreover, it appears to bind to FXa in a fashion previously demonstrated for ZK-807834, a more selective FXa inhibitor.

Introduction

Human factor Xa (FXa) is a 45 kDa enzyme belonging to the serine protease class. When complexed with membrane-associated factor Va, FXa proteolytically converts prothrombin to thrombin in the rate-limiting step of blood coagulation.1 Recent in vivo studies demonstrate that potent FXa inhibitors are efficacious in models of both venous and arterial thrombosis without causing an excessive increase in bleeding.2 Inhibitors having specificity for FXa over trypsin (a digestive enzyme that is also a serine protease), while maintaining selectivity against thrombin, have been reported.3 Rotational-echo double resonance (REDOR) solid-state NMR experiments and molecular dynamics (MD) simulations determined the trypsin-bound conformations of two such inhibitors.5 For trypsin-bound inhibitor ZK-806299 (1), MD calculations without REDOR restraints yielded a model with an unsymmetrical inhibitor conformation, whereas calculations with REDOR restraints indicated a relatively symmetrical structure having similar 13C–19F distances to the amidino carbon and amino carbon.5 The experiments reported here characterize the same inhibitor bound to FXa. There is only one crystal structure of a bis-(phenoxy)pyridine inhibitor bound to FXa.6 REDOR data coupled with molecular modeling yield probable binding modes of inhibitors (like 1) for which there are no crystal structures.

The complex of 1 that we examined was not crystalline. We prepared this sample using cryo- and lyoprotectants PEG 8000 and trehalose, which have been shown to maintain enzymes in their native state during freezing stress and during drying.7 Our prior work with trypsin5 indicated that the Ca2+ in our sample plus the FXa inhibitor caused sample melt-back during lyophilization. Inclusion of dextran at 1.34% (w/v) and primary and secondary drying process at low temperatures produced the fluffy cake we consider ideal. The slowly cooled protein complex is relaxed at the moment of freezing and is maintained in that state during primary drying (ice crystal sublimation) and secondary drying (removal of part of the residual liquid water always present in complex frozen material).

Experimental Methods

Solid-State NMR Sample Preparation. Human factor Xa purified from plasma was purchased from Enzyme Research Labs (South Bend, IN). The synthesis of specifically labeled 1 was described previously.3 All other reagents were obtained from Sigma Chemical Company (St. Louis, MO). The FXa (from various lots) was thawed and combined. Except where indicated, all subsequent procedures were at ice temperature.
was packed, under inert conditions, into a Varian/Chemag. The bath temperature was raised gradually over a 6-day period until the vacuum sensor near the flask monitored sublimation. The bath vacuum was applied to the flask to begin lyophilization. A lower bath temperature was used. After the solution was frozen, the bath temperature was raised to 4 °C and the RF power levels (to within 0.2%). The sample was cooled to below 8 °C in a low-temperature bath. Once this temperature was achieved, vacuum was applied to the flask to begin lyophilization. A vacuum sensor near the flask monitored sublimation. The bath temperature was raised gradually over a 6-day period until the almost completely dry cake was at room temperature. Lyophilization was continued for 2 additional days. The sample was packed, under inert conditions, into a Varian/Chemagnetics 5 mm thin-wall pencil rotor.

**Double REDOR.** Details of the pulse lengths, radio frequency (RF) power levels, probe, and spectrometer for 125 MHz 13C(15N or 19F) double REDOR NMR have been published. Briefly, the 2 ms matched cross-polarization transfer and dephasing pulses for 13C and 15N were performed at 50 kHz. Proton decoupling and 19F dephasing pulses were 100 kHz. Custom feedback circuitry was used to stabilize both the 2 Hz) and the RF frequency (to within 0.2%). The sample was cooled to below −6 °C during NMR experiments. The rotational resonance condition was avoided by 6250 Hz magic-angle spinning.

Minor changes from the published mirror symmetric, single-refocusing pulse, double REDOR sequence are shown in Figure 1. Instead of using two sequential experiments having two free induction decays (FIDs) each, the four FIDs were acquired in a single experiment (Figure 1). The 13C(19F) REDOR FIDs (dephased and full echo) were immediately followed by 13C(15N) REDOR FIDs (dephased and full echo). No REDOR evolution occurs during the single rotor cycle on either side of the center refocusing pulse. The two pairs of dephasing-pulse loops on each side of the central refocusing pulse are shown as square brackets (Figure 1). The inner pair of loops (Figure 1, solid brackets) contained 8 rotor cycles for all experiments, corresponding to 16 rotor cycles of evolution. The outer pair of loops (Figure 1, dashed brackets) contained 16, 12, or 16 rotor cycles (which added an additional 16, 24, or 32 rotor cycles of evolution, respectively). Fluorine pulses in both loops were on for 19F dephasing (FID 1). Only the inner-loop pulses were turned on for 15N dephasing (FID 3) to selectively dephase signals from 12C within 2 Å of a 13C label. Neither 19F nor 15N pulses were on during accumulation of the full-echo signals (FID 2 and FID 4). Total evolution time was the same for all four FIDs.

To increase sensitivity, the two full-echo spectra were summed. Each dephased spectrum was multiplied by 2 and subtracted from the summed full-echo spectrum to create the REDOR difference spectra. The REDOR difference spectra contain signals only from 13C that are dipolar-coupled to either 15N or 19F. The 13C(15N) difference spectrum (ΔS) shows the 13C signals of FXa without interference from natural abundance signals in the protein. It is the reference for distance determinations using 13C(19F) ΔS. To simplify data analysis, the first spinning sidebands (ssb) of the REDOR difference spectra were folded into the centerbands.

The folded difference spectra were fit by Gaussians (Kaleidagraph, Synergy Software, Reading, PA; error bars are included in fit output) and integrated (MacNMR, Tecmag, Inc. Houston, TX; no error analysis available). When parameters consistent with the experiments were used, Simpson simulations with and without 19F homonuclear coupling (1006 Hz) had a maximum deviation of 3% from each other. Because 3% is small relative to the experimental error, we used three spins (observed 13C and two 19F dephasing spins) with no homonuclear coupling in our REDOR dephasing calculations.

**Computational Methods.** REDOR-constrained models were built using the FXa catalytic chain coordinates and conformations of FXa trypsin models 2a and 2b. Computer methods were similar to those used for trypsin models except for these minor changes: (i) no chloride counterions were added; (ii) the temperature coupling constant for the protein was 0.1 ps; (iii) the half-harmonic restraints on the water cap were 0.5 kcal mol−1 Å−2; (iv) restraints of 10 kcal mol−1 Å−2 were placed on the two hydrogen bonds from the S1 Amino acid to the carboxy group of Asp 189 (chymotrypsin numbering scheme) to keep the inhibitor in the pocket. The two starting models converged after 1 ns of MD. To generate the three allowed inhibitor conformations, the phenyl ring in the S4 pocket was flipped to point the m-methylamino group toward Phel74 instead of toward Tyr99 and MD was repeated. Theoretical REDOR dephasing curves for the preliminary models were calculated and compared with the experimental data. The 13C−19F distance restraints were adjusted to improve the match of the calculated dephasing with the experimental data. A second cycle of MD (500 ps) yielded models whose calculated dephasing curves agreed with the data within experimental error.

**Results and Discussion**

**Line Assignments and REDOR Dephasing.** The 125 MHz 13C(15N or 19F) REDOR spectra of the FXa complex of FXa(1) with 40 rotor cycles of dipolar evolution are shown in Figure 2. The 15N labels allow separation of labeled 13C signals from the natural-abundance 13C background (Figure 2, center spectrum and upper left inset). The 164.4 and 45 ppm peaks have line widths of 1.8 and 2.7 ppm, respectively (Figure 2, center). They are assigned to amide and amine carbons of 1 in the S1 and S4 binding sites of FXa, respectively. The broad shoulder of the amidine signal (Figure 2, upper left) is difficult to quantitate because several combinations of position and width give satisfactory fits. When a 2.1 ppm line centered at 168 ppm was used and when two Gaussians were fitted, the amidine shoulder accounted for about 30% of the amidine signal. By analogy to the trypsin/symmetric inhibitor results, this implies that about 30% of the asymmetric inhibitors complexed to FXa adopt a second conformation that perturbs the chemical shift of the amidine. One possibility is that the methylamino group binds in the S1 pocket, where it could form a hydrogen bond with Ser195. Although there is no precedent for
all carbons within about 12 Å of a 19F. This includes if both orientations of the amine in the S4 site indicated amidine 13C second ssb at 65 ppm (Figure 2, middle).

The reported 13C peak in FXa/1, and was also close to a 19F of 1, its 13C(19F) ΔS could not be distinguished from that due to 1. The size of this error cannot exceed 1% of the full-echo signal (Figure 2, bottom) at the frequencies specified by the 13C(15N) ΔS spectrum (Figure 2, center). An error of this size is negligible compared to the error arising from the experimental signal to noise. The 13C(19F) ΔS/13C(15N) ΔS values for FXa/1 at multiple evolution times are shown in Figure 3. The amine carbon has slightly higher values than the amidine carbon in FXa/1 (the reverse is true in trypsin/1), but otherwise, the FXa/1 and trypsin/1 results are similar. These differences translate into small distance differences (≤0.4 Å) between the complexes of 1 with FXa and trypsin. Although the amide carbon and amine carbon signals in the 13C(15N) ΔS have two components (Figure 2, center and upper left inset), the corresponding signals in the 13C(19F) ΔS were too weak for reliable fitting to two Gaussians. Instead, the peak position and width of the larger Gaussian (Figure 2, upper left, thick line) were used for single Gaussian fits. The reported 13C(19F) ΔS/13C(15N) ΔS ratios (Figure 3) are an average of (i) integration over the entire amidine or amine 13C signal and (ii) single Gaussian fits that exclude the smaller, broad components of both lines (Figure 2). The two methods yield ratios that agree within the signal to noise of the data.

The error bars (Figure 3) show the uncertainty range from the Gaussian fits only. At 40 rotor cycles (Figure 3), the data averages appear centered within the error bars because integration and Gaussian fitting gave nearly the same values. For the 32 and 48 rotor-cycle experiments, however, the agreement between integration and Gaussian fitting was not as good, so the displayed (Gaussian only) error ranges appear slightly skewed from the data averages.

The amine-13C peak in FXa/1 has a shorter T2 relaxation time (5.3 ± 0.1 ms) than was observed in...
trypsin/1 (12.5 ± 1.9 ms). The T2 relaxation times of the amidine-¹³C peak in FXa/1 (8.1 ± 1.1 ms) and trypsin/1 (9.1 ± 0.8 ms) are similar. Both smaller sample size and faster T2 relaxation for the amidine carbon peak reduced the sensitivity of REDOR experiments for FXa/1. Thus, even with the higher magnetic field used in the experiments on FXa/1, more scans were required than for the experiments on trypsin/1 to achieve comparable signal to noise.5

Modeling of the Inhibitor in FXa and Comparison with Trypsin Binding Models. Two models (designated A and B) with the amidine-carbon of 1 in the S1 binding site of FXa that are consistent with the double-REDOR NMR data are shown in Figure 4. The biggest difference between the models is the orientation of the (aminomethyl)phenyl moiety. The ¹³C–¹⁹F distances of 1 in FXa models A and B are given in Table 1.

The two models of trypsin/1 arise from the amine group interacting with polar areas at either the top or the bottom of S4.5 The trypsin-bound inhibitor structures, when applied to FXa, converged after dynamics and minimization. This is understandable: In FXa, the top of S4 is hydrophobic (Phe 174), so the amine prefers the bottom. The bottom of S4 in FXa (Glu 97 and Tyr 99) is even more electronegative than in trypsin (carboxyls of Thr 98 and Asn 97). The strong electronegative region of the F.Xa S4 pocket attracts basic groups.

The amine in both FXa models is perpendicular to the phenyl ring, facing up toward the solvent. This allows some solvent access to the amine groups while still partially burying the hydrophobic benzyl portions in the hydrophobic S4 pocket. In FXa/1 model A (Figure 4, top), the amine of 1 is hydrogen bonded to the carbonyl of Glu 97. This hydrogen bond appears in 1EZQ,16 but is not present in other published structures of FXa inhibitors, including our trypsin/1 models.5 Although the amino group in FXa/1 model B (Figure 4, bottom) lacks a direct hydrogen bond, this group is in nearly the same position as in model A. The NH3+ to O distance is 2.8 Å in model A and 4.2 Å in model B. The overall architecture is similar to the crystal structures of the most potent (<1 nM) factor Xa inhibitors (1FJS,6 1EZQ,16 1KSN,17 1MQ5, 1MQ6).5

Other binding modes could exist which are also consistent with the experimental restraints. Nevertheless, crystal structures of trypsin complexed with other dibasic inhibitors have displayed binding modes similar to those shown in Figure 4.19 Coordinates for the two FXa/1 models have been deposited in the Brookhaven Protein Data Bank20 with identification code 1MSX.

Properties of the S1 and S4 Binding Sites. Structure–activity data are available from a number of FXa inhibitor templates containing arylamidines. In general, (i) there is flexibility with respect to the nature of the second basic group, and (ii) substitution of the amidine group presumed to bind in the S1 pocket results in several orders of magnitude loss of potency.3ab,21 The difficulty in predicting the S4 preference from the analysis of substrate cleavage sites (in which glutamic acid is found several times) has been discussed.22 Basic group binding to the hydrophobic “cation hole” at S4 has been seen in the crystal structures of other small-molecule inhibitors of factor Xa.6ab,22 The importance of the alignment of the salt bridge between the benzamidine and Asp1899 strongly suggests that the binding mode(s) of compound 1 to FXa will preserve this critical interaction. We conclude that 1 binds similarly to both FXa and trypsin, and binds to FXa in the same general manner as revealed in the X-ray structure of the more selective inhibitor ZK-807834.6

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