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A Non-reactive Glutamine Residue of α_2-Antiplasmin Promotes Interactions with the Factor XIIIa Active Site Region

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Plasma Factor XIII (FXIII) exists as the heterotetramer A_2B_2 composed of two catalytic A-subunits and two carrier B-subunits, whereas cellular FXIII only consists of A_2. Zymogen FXIII is activated by thrombin in the presence of calcium generating a transglutaminase that catalyzes the formation of γ-glutamyl-ε-lysyl cross-links in the fibrin clot network [1]. Key substrates for FXIIIa include the fibrin α and γ chains, α_2-antiplasmin, and fibronectin [2].

α_2-antiplasmin is originally secreted as a 464 residue protein having an N-terminal Met [3]. The antiplasmin-cleaving enzyme (APCE) catalyzes cleavage of the P12-N13 amide bond resulting in a protein with an N-terminal Asn (N1-α_2AP). This proteolyzed form accounts for 70% of the circulating α_2-antiplasmin [4]. FXIIIa cross-links Q2 of N1-α_2AP to K303 of the fibrin(ogen) α-chain. This tethered α_2-antiplasmin serves as a potent inhibitor of the fibrinolytic agent plasmin [5].

Although a number of FXIIIa substrates have been identified, it is challenging to predict whether a particular glutamine will be reactive [2]. Fully understanding the substrate specificity of FXIIIa for glutamine containing substrates remains an important goal in the transglutaminase field. Phage display studies and FXIIIa substrate comparisons have suggested that the residues surrounding the reactive glutamine are not part of a strict consensus sequence [6, 7].

The N-terminal segment of N1-α_2-AP provides an effective model system for further probing the roles of individual amino acid positions. Our previous kinetic studies with the N1-α_2 AP peptide (^{1}NQEQVSPLTLLKLGN^{15}) revealed that the FXIIIa active site region is sensitive to changes around the ^{2}QEQ^4 region [8]. When both Q residues are present, FXIIIa will only target Q2. A subtle glutamine to asparagine substitution at Q4 leads to increases in K_m for N1-α_2 AP (1–15, Q4N). This Q4N role was recently confirmed by Pénzes et al [9]. Additional investigations are thus warranted for assessing the exact role that the Q4 position plays in regulating binding and catalysis at Q2. For the current project, N1-α_2-AP Q4X peptides containing E, M, S, A, L, P, or K substitutions were examined kinetically.

The studies were carried out using recombinant human cellular FXIII expressed in *Saccharomyces cerevisiea* [10]. A batch amount of FXIII was thrombin-activated to FXIIIa and the quantity of FXIIIa active sites assessed with 2-^{14}C iodoacetamide. The coupled

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The authors state that they have no conflict of interest

stopped kinetics UV assay modified by Cleary et al.[8] was then used to determine individual kinetic parameters. In such assays, FXIIIa catalyzes release of ammonia from glutamine containing peptides and then reacts with glycine-ethylester. Glutamate dehydrogenase then utilizes the released ammonia to convert α-ketoglutarate to glutamate in an NADH dependent reaction. The N1-α_2AP peptide concentrations used in the kinetic assay ranged from 38–1200 μM. All peptides were synthesized by New England Peptide (Table 1) and stock concentrations determined by quantitative amino acid analysis (AAA Service Laboratory, Damascus, OR).

K_m, k_{cat}, and k_{cat}/K_m were determined for the seven Q4X peptides and the results compared to wild type (Table 1). The mean K_m values for the Q4X residues are presented from weakest to strongest binding interaction where (P, M) are statistically different from (E, L, S) ($P<0.05$). For k_{cat}, an almost opposite trend occurred with S generating the slowest catalytic turn-over and M the fastest ($P< 0.01$). The specificity constant takes both parameters into consideration. These k_{cat}/K_m rankings revealed that L and S exhibited the greatest substrate specificity value whereas P and M exhibited the least ($P<0.05$). As expected for peptides, the K_m values are higher than what would be anticipated for intact N1-α_2AP *in vivo*. Peptides have, however, shown much value for kinetically screening substrate positions with enzymes such as thrombin and plasmin [11].

Reviewing the kinetic parameters for the N1-α_2AP (1–15) Q4X peptides suggests that the k_{cat}/K_m trend follows that of K_m. Furthermore, there is not a large spread in k_{cat}/K_m often due to compensatory K_m and/or k_{cat} effects. This property can be seen with the Q4S, Q4L, and Q4E peptides. Their sequences provide the strongest binding contributions (lowest K_m), but at the same time, the weakest turnover (lowest k_{cat}). The native Q4 lies in the latter half of the peptide series. This amino acid exhibits weaker binding interactions (higher $K_{m)}$ and more effective turnover (higher k_{cat}) than Q4S, L, or E. The kinetic results suggest that the FXIIIa subsite for the Q4X residue of α_2-antiplasmin must be relatively broad. One region must effectively accommodate polar residues whereas another region must accept a long nonpolar side chain.

The TRANSDAB website [7] records FXIIIa substrates and reveals an array of residues for the substrate position in question. There are, however, some interesting trends. Leucine (L) is the most prevalent residue and also one of the better candidates for N1-α_2-AP. Other dominant players include Q and the residues G, K, and R that were probed by Gorman and Folk in a β-casein derived peptide [12]. The additional P, M, A, E, and K that were probed in the current project are also physiological. The S which produced the strongest kinetics for the N1-α_2-AP peptides and the N which hindered the K_m [8, 9] have not yet been documented.

A recent phage display study provided valuable new results related to FXIIIa substrate specificity [6]. The Hitomi group reported a preferred sequence of QxxΦxWP (x = non-conserved, Φ = hydrophobic). The most reactive peptide segments place M, P, and K at the second x position. Interestingly, these phage display studies have selected agents which favor k_{cat}. This effect may occur because they monitored incorporation of lysyl mimics into Q-containing candidates as opposed to following initial loss of ammonia from the Q.

A preliminary review of FXIIIa substrate sequences suggests that the residue located two positions beyond the reactive glutamine is not conserved. A more in-depth kinetic evaluation indicates that this position makes important contributions toward promoting effective FXIIIa-substrate interactions (K_m). Major differences in k_{cat}/K_m across the peptide series were, however, not observed. Further knowledge on the residues that surround the reactive glutamines will be highly valuable for characterizing FXIIIa substrate specificity and for designing specific inhibitors against this transglutaminase.

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Table 1

Kinetic Parameters for FXIIIa Catalyzed Reaction with N1-α_2-Antiplasmin Based Peptides

Substrate	N1-α_2AP Peptide Sequence	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m (sec^{-1} μM^{-1})
α_2AP (Q4P)	^{1}NQE**P**VSPLTLLKLGN^{15}	181 ± 35	574 ± 121	0.315 ± 0.09
α_2AP (Q4M)	^{1}NQE**M**VSPLTLLKLGN^{15}	233 ± 16	508 ± 43	0.459 ± 0.05
α_2AP (Q4)	^{1}NQE**Q**VSPLTLLKLGN^{15}	175 ± 15	459 ± 50	0.382 ± 0.05
α_2AP (Q4K)	^{1}NQE**K**VSPLTLLKLGN^{15}	157 ± 30	429 ± 93	0.365 ± 0.10
α_2AP (Q4A)	^{1}NQE**A**VSPLTLLKLGN^{15}	156 ± 7.0	336 ± 23	0.463 ± 0.04
α_2AP (Q4E)	^{1}NQE**E**VSPLTLLKLGN^{15}	108 ± 6.7	222 ± 14	0.486 ± 0.04
α_2AP (Q4L)	^{1}NQE**L**VSPLTLLKLGN^{15}	143 ± 14	200 ± 38	0.717 ± 0.16
α_2AP (Q4S)	^{1}NQE**S**VSPLTLLKLGN^{15}	104 ± 5.8	142 ± 28	0.733 ± 0.15

The underlined Q is FXIII reactive and the residue in bold is being substituted across the series. Studies were carried out using the coupled UV-vis assay of Cleary et al. [8] and the kinetic parameters were calculated from Lineweaver Burk plots. Sigma Plot derived means and standard errors of the means are reported. The N1-α_2AP Q4X peptides were examined in triplicate or in duplicate. The presence of statistical difference was tested by applying mixed linear models to account for unequal sample sizes (SAS Proc Mixed, SAS Institute). Individual pair wise comparisons revealed differences that were statistically significant ($P<0.05$ and <0.01). Values that exhibited such properties tended to occur between sets of strongest versus weakest members of a particular kinetic parameter series.