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Biophysical Chemistry 117 (2005) 255 – 261

Biophysical Chemistry

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Mutagenesis studies toward understanding the mechanism of the cofactor function of thrombomodulin ☆

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Received 11 May 2005; received in revised form 7 June 2005; accepted 8 June 2005
Available online 20 June 2005

Abstract

Thrombomodulin (TM) is as essential cofactor in protein C activation by thrombin. To investigate the cofactor effect of TM on the P3-P3′ binding specificity of thrombin, we prepared a Gla-domainless protein C (GDPC) and an antithrombin (AT) mutant in which the P3-P3′ residues of both molecules were replaced with the corresponding residues of the factor Xa cleavage site in prethrombin-2. TM is known to interact with GDPC, but not AT in the complex. Thrombin did not react with either mutant in the absence of a cofactor. While the thrombin-TM complex also did not react with the AT mutant, it activated the GDPC mutant with a normal k_{cat}, but an ~4-fold impaired K_m value. Further studies revealed that the active-site directed inhibitor *p*-aminobenzamidine acts as a competitive inhibitor of both wild-type and GDPC mutant in reaction with the thrombin-TM complex. These results suggest that the interaction of the P3-P3′residues of GDPC with the active-site pocket of the thrombin-TM complex makes a dominant contribution to the binding specificity of the reaction. Moreover, the observation that the GDPC mutant, but not the AT mutant, functions as an effective substrate for the thrombin-TM complex suggests that GDPC interaction with the thrombin-TM complex may be associated with the alteration of the conformation of the P3-P3′residues of the substrate.

Keywords: Thrombin; Thrombomodulin; Protein C; Antithrombin; Factor Xa; Mutagenesis

1. Introduction

Protein C is a vitamin K-dependent plasma serine protease zymogen that upon conversion to activated protein C (APC)[1] by thrombin in complex with thrombomodulin (TM) down-regulates the coagulation cascade by degrading factors Va and VIIIa by limited proteolysis [1–4]. In addition to TM, the activation of protein C by thrombin requires the cofactor function of Ca^{2+} [1]. Interestingly, however, while Ca^{2+} stimulates protein C activation by thrombin in the presence of TM, it potently inhibits the zymogen activation in the absence of TM [5]. The Ca^{2+}-binding site responsible for altering the zymogenic properties of protein C has been localized to the 70–80 loop (chymotrypsinogen numbering [6]) in the protease domain of the zymogen, the same loop which is also a Ca^{2+}-binding site in trypsin [7,8]. Kinetics and direct binding studies have indicated that Ca^{2+} binding to this loop induces a conformational change in the activation peptide of protein C [9–11]. There are two acidic Asp residues at the P3 and P3′sites of protein C. It is thought that these residues in the Ca^{2+}-stabilized conformation make inhibitory interactions with the active-site pocket of thrombin, and that a cofactor function for TM is to overcome these inhibitory interactions [1,9]. The mechanism by which TM overcomes the inhibitory effect of Ca^{2+} in protein C activation by thrombin is not well understood. An attractive hypothesis has been that TM binding to thrombin alters the conformation of the

☆ The research discussed herein was supported by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (HL 62565 and HL 68571 to ARR).
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doi:10.1016/j.bpc.2005.06.002

active-site pocket of thrombin, thereby facilitating the docking of the Ca^{2+}-altered conformation of protein C into the catalytic pocket of the protease [1].

Recently, the crystal structure of thrombin in complex with the epidermal growth factor-like domains 4, 5, and 6 of TM (TM456) was resolved [12]. Unexpectedly, the structural data indicated that TM4 domain of the cofactor does not contact thrombin in the complex. Subsequent molecular modeling and mutagenesis data suggested that TM4 may provide a secondary binding site for protein C in the activation complex [12–14]. Although the question of the allosteric effect of TM on the active-site pocket of thrombin could not be resolved unequivocally because of thrombin having a tripeptidyl inhibitor in the active-site, nevertheless, the results indicated that protein C interaction with TM in the activation complex is critical for recognition and activation of the zymogen by thrombin [12–15].

To further investigate the cofactor function of TM in protein C activation by thrombin, we decided to prepare a Gla-domainless protein C (GDPC) and an antithrombin (AT) mutant in which the P3-P3′ residues of both molecules were replaced with the identical residues of the factor Xa recognition site in prethrombin-2, which is known to be a very poor recognition site for thrombin [16]. The reactivity of thrombin with these mutants was evaluated in both the absence and presence of the cofactors TM456 and heparin and the active-site directed inhibitor *p*-aminobenzamidine (PAB). The results suggest that protein C may be docked into the active-site pocket of thrombin with an altered conformation in the presence of TM.

2. Experimental procedures

2.1. Materials

The chromogenic substrate, Spectrozyme PCa (SpPCa) was purchased from American Diagnostica (Greenwich, CT) and S2266 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH). A full-length high affinity heparin with an average molecular mass of ~21,000 (~70-saccharides) was a generous gift from Dr. Steven Olson (University of Illinois-Chicago). Human plasma protein factors Va and Xa were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Polybrene and *p*-aminobenzamidine (PAB) were purchased from Sigma (St. Louis, MO).

2.2. Recombinant proteins

The methodology for the construction, expression and purification of wild-type Gla-domainless protein C (GDPC) and antithrombin (AT) as well as mutants of both proteins in which the P3-P3′ residues of the activation peptide of the zymogen or the reactive site loop of the serpin were replaced with the corresponding residues of the second factor Xa cleavage site in prothrombin (^{320}Asp-Gly-Arg-Ile-Val-Glu^{325}, named *GDPC/Proth-2* and *AT/Proth-2*) has been described [9,16]. Following confirmation of the accuracy of the mutagenesis, both zymogen and serpin derivatives were expressed in human 293 cells using RSV-PL4 expression/purification vector system as described [9]. The TM fragment containing the epidermal growth factor-like domains 456 (TM456) was expressed in the same vector system as described [9]. Prethrombin-2 (prothrombin lacking both Gla and kringles 1 and 2 domains) was expressed and purified as described [16]. All proteins were purified to homogeneity by immunoaffinity chromatography using the Ca^{2+}-dependent monoclonal antibody HPC4 as described [9,16]. All recombinant proteins were tested for homogeneity by SDS–PAGE.

2.3. Zymogen activation

The initial rate of wild-type or mutant GDPC activation by thrombin was measured in both the absence and presence of Ca^{2+}, EDTA and TM456 as described [9,17]. In the absence of TM456, the concentration dependence and time course of wild-type or mutant GDPC (1–20 μM) activation by thrombin (5–50 nM) was studied in 0.1 M NaCl, 0.02 M Tris–HCl, pH 7.4 (TBS) containing 1 mg/mL BSA, 0.1% polyethylene glycol (PEG) 8000 in the presence of either 5 mM Ca^{2+} or 1 mM EDTA in 96-well assay plates. At different time intervals, thrombin activity was quenched by 1 μM AT and the rate of protein C activation was measured from the cleavage rate of SpPCa (200 μM) at 405 nm by a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described [17]. The experimental conditions in the presence of TM456 were the same with the exception that the activation by thrombin (1.0 nM) was carried out in the presence of a saturating concentration of TM456 (200 nM) and increasing concentrations of either GDPC or GDPC/Proth-2 (0.2–86 μM).

2.4. Thrombin reaction with the antithrombin mutant

In the absence of heparin, thrombin (2 nM) free or in complex with TM456 (200 nM) was incubated with the AT mutant (2–5 μM) in TBS containing 0.1 mg/mL BSA, 0.1% PEG 8000 and 2.5 mM Ca^{2+} (TBS/Ca^{2+}) at room temperature for 5–15 h. In the presence of a full-length high affinity heparin (~70-saccharides), the reaction conditions were the same except that thrombin was incubated with 0.8–4 μM AT/Proth-2 and catalytic levels of heparin (0.01–1.6 μM). All reactions were carried out in 50 mL volumes in 96-well polystyrene plates at room temperature. After a period of time (30–300 min, depending on the rate of reactions), 50 mL of the chromogenic substrate (400 μM SpPCa) in TBS containing 1 mg/mL Polybrene was added to each well and the remaining enzyme activity was measured with a V_{max} Kinetics Microplate Reader as described above. The observed pseudo-first-order rate

constants (k_{obs}) were determined by computer fitting of the time-dependent change of the protease activity to a first-order rate equation and the second-order association rate constants were obtained from the slopes of linear plots of k_{obs} vs. the concentration of the AT-heparin complex as described [16]. In inhibition reactions where the k_{obs} values exhibited a saturable dependence on the concentration of the AT-heparin complex, data were analyzed according to a hyperbolic equation as described [18].

2.5. Competitive kinetic assays

The competitive effect of *p*-aminobenzamidine (PAB) on both the amidolytic and proteolytic activities of the thrombin-TM456 complex was evaluated. For the amidolytic activity assays, the hydrolysis of increasing fixed concentrations of S2266 (15–2000 μM) by thrombin (6–8 nM), alone or in complex with TM456 (200 nM), was monitored in the presence of increasing fixed concentrations of PAB (0–320 μM) in TBS/Ca^{2+}. In the proteolytic activity assay, the activation of increasing fixed concentrations of GDPC (0.5–20 μM) or GDPC/Proth-2 (1.0–66.7 μM) by thrombin (5–10 nM) in complex with TM456 (200 nM) was monitored in the presence of increasing fixed concentrations of PAB (0–320 μM) in TBS/Ca^{2+} at room temperature. The K_i, K_m, and V_{max} values were determined by global fitting of the kinetic data to a competitive binding equation as described [19].

3. Results

3.1. Characterization of mutants

The initial rate of wild-type and mutant GDPC activation by thrombin was studied in both the absence and presence of TM456 and Ca^{2+}. In agreement with previous results, thrombin was a slow activator of GDPC in the presence of Ca^{2+} (Fig. 1A, open circles). Under the experimental conditions described under the legend of Fig. 1, thrombin did not activate GDPC/Proth-2 with a detectable rate (closed circles). In contrast to thrombin, factor Xa did not activate wild-type GDPC with a detectable rate; however, it activated GDPC/Proth-2 with a rate that was only 2-fold slower than the rate of wild-type GDPC activation by thrombin (Fig. 1B). It is known that the activation of GDPC by thrombin in the absence of TM is inhibited by Ca^{2+} [1]. In agreement with previous data [9], GDPC activation by thrombin in the presence of EDTA was markedly improved, however, the activation of the GDPC mutant by thrombin in the presence of EDTA was not affected (data not shown). These results suggest that grafting the P3-P3′ residues of the factor Xa cleavage site in prethrombin-2 have switched the specificity of protein C so that the mutant functions as a substrate for factor Xa, but not thrombin. Factor Xa activation of the

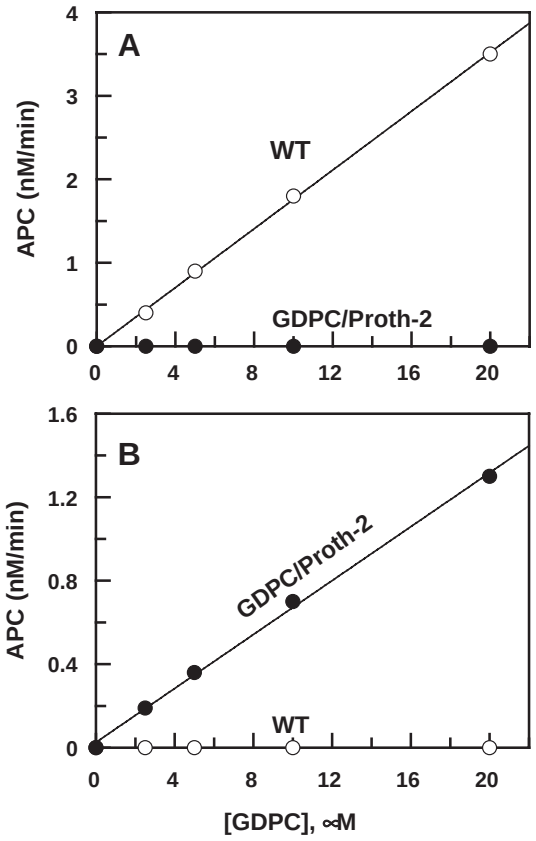

Fig. 1. Concentration dependence of wild-type and mutant *GDPC/Proth-2* activation by thrombin and factor Xa. (A) The initial rate of wild-type GDPC (○) or GDPC/Proth-2 (●) activation by thrombin (50 nM) was measured in TBS/Ca^{2+}. Following incubation for 35 min at room temperature, the thrombin activity was neutralized by AT, and the rate of activated GDPC generation was determined by an amidolytic activity assay as described under "Experimental procedures". (B) The same as A, except that factor Xa (50 nM) was used to activated the zymogens. The plots are representative experiments out of 2 independent and reproducible measurements.

mutant was stimulated 2–3-fold by Ca^{2+} (data not shown). No significant differences in GDPC/Proth-2 activation by factor Xa was noticed in either the absence or presence of factor Va, suggesting that factor Va may have no major allosteric effect on the active-site pocket of factor Xa in the P3-P3′ binding sites of the protease (data not shown). These results are consistent with the literature [20].

Next, the initial rate of GDPC activation by thrombin was evaluated in the presence of TM456. Unlike thrombin alone, the thrombin-TM456 complex activated the GDPC mutant with a k_{cat} value (~0.14 s^{-1}) that is essentially identical to the corresponding value for the wild-type GDPC measured under the same conditions (Fig. 2). However, relative to $K_{m(app)}$ of wild-type GDPC (~5 μM), the corresponding value for the mutant (~21 μM) remained ~4-fold impaired. It should also be noted that the thrombin-TM456 activation kinetic data indicated that the affinity of Ca^{2+} for interaction with the protein C mutant was impaired ~10-fold (data not shown). This result is consistent with our previous observation showing that the activation peptide of

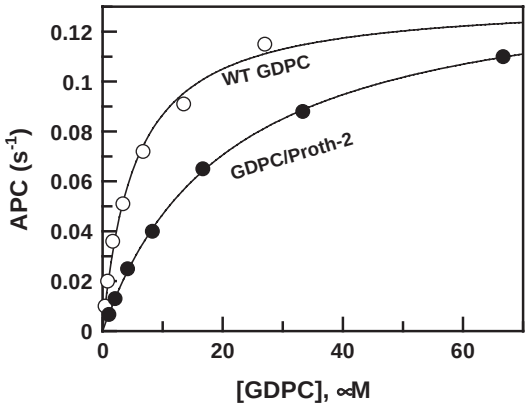

Fig. 2. Concentration dependence of wild-type and mutant GDPC activation by thrombin in the presence of TM. The initial rate of different concentrations of wild-type GDPC (○) or GDPC/Proth-2 (●) activation by thrombin (1 nM) in complex with TM456 (200 nM) was measured in TBS/Ca^{2+}. The thrombin activity was neutralized by AT, and the rate of activated GDPC generation was determined as described under "Experimental procedures". The plots are representative experiments out of 2–3 independent and reproducible measurements.

protein C is energetically coupled to the Ca^{2+} binding site of the protein [10,11].

The extent of the reactivity of AT/Proth-2 and prethrombin-2 with thrombin was also evaluated in the presence of TM. AT/Proth-2 has been fully characterized in the past and is shown to be folded properly, having near normal reactivity with factor Xa in both the absence and presence of heparin [16]. Unlike the activation of GDPC/Proth-2, TM456 did not improve the reactivity of thrombin with AT/Proth-2 in either the absence or presence of Ca^{2+}. Similarly, thrombin, in either the absence or presence of TM456 did not activate prethrombin-2 with a detectable rate (data not shown). Since no data could be obtained for the thrombin reaction with either AT/Proth-2 and prethrombin-2 in either the absence or presence of TM456, these studies did not clarify whether or not TM has an allosteric effect on the conformation of the P3-P3′binding residues in the active-site cleft of thrombin. Nevertheless, the results indicated that the interaction of the substrate with TM in the ternary complex is required for the cofactor function of TM since among the three molecules GDPC/Proth-2, prethrombin-2 and AT/Proth-2, only GDPC/Proth-2 acted as a substrate for the thrombin-TM456 complex. Thus, substrate interaction with TM somehow facilitates the docking of the non-optimal P3-P3′residues into the catalytic pocket of thrombin. This could occur if the active-site pocket of thrombin and/or P3-P3′ residues of the GDPC mutant have altered conformations in the ternary complex. It should be noted that the same P3-P3′ residues may be folded in different structural frameworks in each mutant protein. Unfortunately, however, the lack of reactivity of thrombin with any one of the mutants in the absence of a cofactor did not allow speculation about this important question.

AT/Proth-2 reacted slowly with thrombin in the presence of a high affinity heparin. Unlike the inhibition of thrombin by AT in the presence of heparin, which is very rapid and may only be analyzed by rapid kinetic methods [21], the rate of inhibition of thrombin by AT/Proth-2 in the presence of heparin was very slow, thus it could be analyzed by simple conventional kinetic methods (Fig. 3). Thus, the concentration dependence of the heparin–AT/Proth-2 inactivation of thrombin revealed a K_D value of ~500 nM for the first step of the reaction and a rate constant of $k=7.2 \pm 0.5\times10^{-4}$ s^{-1} for the stable covalent complex formation (Fig. 3). Relative to wild-type AT ($k=\sim8$ s^{-1}) [21], the rate of stable complex formation with AT/Proth-2 has been impaired by 4-orders of magnitude. No reactivity for thrombin was detected with AT/Proth-2 in complex with the high affinity pentasaccharide, suggesting that the mutant serpin has a very poor reactive site loop sequence for recognition by thrombin in either the native or activated conformations. Comparisons of the rate constants of the thrombin reaction with GDPC/Proth-2 (0.14 s^{-1}) in the presence of TM456 and AT/Proth-2 in the presence of heparin suggest that TM improves the rate (k_{cat}) of the mutant GDPC activation by thrombin ~200-fold. Such a dramatic difference between the reactivities of thrombin with two mutant target molecules having identical P3-P3′ residues cannot be readily understood unless the active-site pocket of thrombin and/or the P3-P3′ residues of GDPC/Proth-2 have altered conformations in the ternary protease-cofactor-substrate complex.

3.2. Competitive kinetic studies

It has been demonstrated that active-site dependent interactions primarily determine the catalytic specificity, and exosite dependent interactions determine the binding specificity of coagulation proteases in the presence of cofactors [22,23]. To investigate the contribution of each one of these factors to the determination of the specificity of

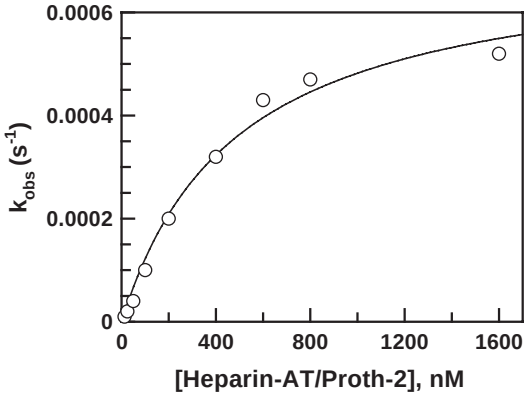

Fig. 3. Dependence of the pseudo-first order (k_{obs}) rate constants of the thrombin inhibition on the heparin–AT/Proth-2 complex concentrations. The k_{obs} values were determined from the time-dependent inhibition of thrombin at different concentrations of the mutant AT-heparin complex (*x*-axis) in TBS/Ca^{2+} at room temperature as described under "Experimental procedures". The plots are representative experiments out of 2 independent and reproducible measurements.

protein C activation by thrombin in the presence of TM, the amidolytic and proteolytic activity of the thrombin-TM456 complex was evaluated in the presence of *p*-aminobenzamidine (PAB). It is previously shown that PAB is a competitive inhibitor of the tripeptidyl substrate cleavage and noncompetitive inhibitor of the macromolecular substrate cleavage by the coagulation activation complexes [22,23]. A noncompetitive mode of inhibition of the coagulation protease-cofactor complexes by PAB has been interpreted to indicate that exosite-dependent interactions primarily determine the binding specificity of the coagulation reactions [22,23]. In agreement with the literature, PAB was a classical competitive inhibitor of S2266 cleavage by thrombin and the thrombin-TM456 complex with K_i values of 45±4 μM and 28±2 μM, respectively (Fig. 4A, shown only in the presence of TM456). Similar to S2266, PAB also acted as a classical competitive inhibitor of both wild-type and mutant GDPC activation by the thrombin-TM456 complex (Figs. 4B and C). These results suggest that unlike other coagulation proteases, active-site dependent interactions with protein C make a significant contribution to the productive thrombin-protein C complex formation in the presence of TM. A similar competitive effect for PAB in protein C activation by the thrombin-TM complex was reported in a recent study [24].

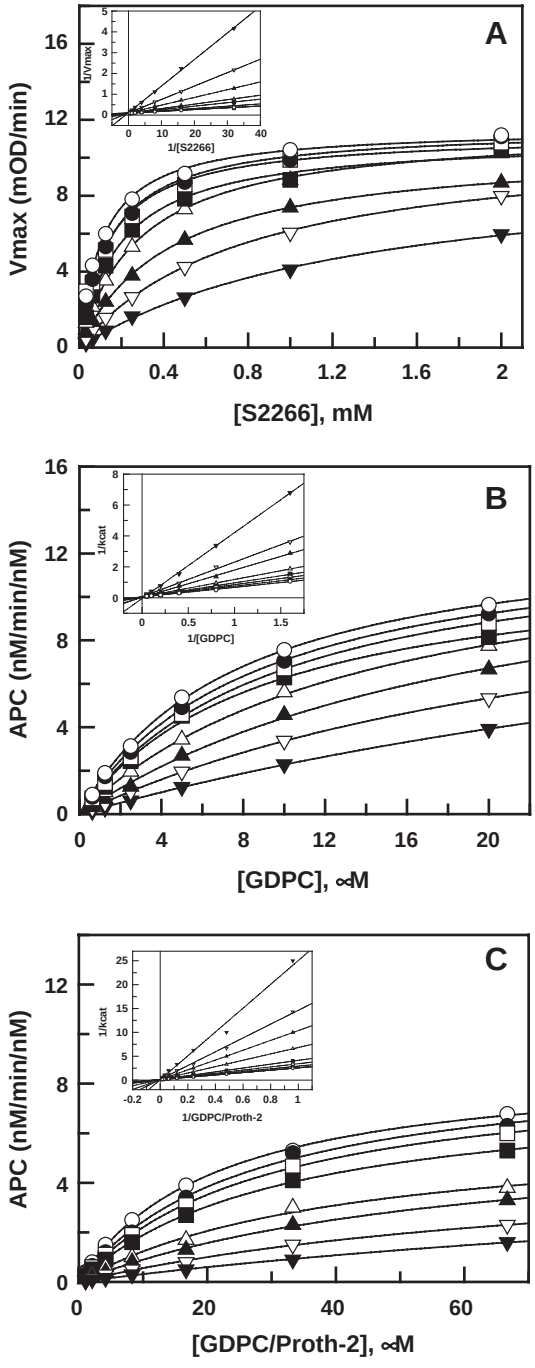

Fig. 4. The competitive effect of PAB on the amidolytic and proteolytic activity of the thrombin-TM456 complex. (A) The amidolytic activity of thrombin (7.5 nM) in complex with TM456 (200 nM) toward fixed and different concentrations of S2266 (*x*-axis) was monitored in the presence of different concentrations of PAB [0 (○), 5 μM (●), 10 μM (□), 20 μM (■), 40 μM (△), 80 μM (▲), 160 μM (▽), and 320 μM (▼)] in TBS/Ca^{2+}. Global computer fit of kinetic data to a competitive equation yielded the constants: $V_{max}/[E]$=9.5±0.1 s^{-1}, K_m=130±6 μM, and K_i=27.9±1.7 μM. (B) The proteolytic activity of thrombin (5 nM) in complex with TM456 (200 nM) toward increasing concentrations of GDPC (*x*-axis) was monitored in the presence of different concentrations of PAB [0 (○), 5 μM (●), 10 μM (□), 20 μM (■), 40 μM (△), 80 μM (▲), 160 μM (▽), and 320 μM (▼)] in TBS/Ca^{2+}. Global computer fit of kinetic data to a competitive equation yielded the constants: $V_{max}/[E]$=0.17±0.01 s^{-1}, K_m=5.7±0.4 μM, and K_i=30.5±1.7 μM. (C) The same as (A) with the exception that the proteolytic activity of the thrombin-TM456 toward increasing concentrations of GDPC/Proth-2 (*x*-axis) was monitored in the presence of different concentrations of PAB. Global computer fit of kinetic data to a competitive equation yielded the constants: $V_{max}/[E]$=0.14±0.01 s^{-1}, K_m=19.4±1.1 μM, and K_i=19.4±1.1 μM. Inset, the double reciprocal plots of $1/V_{max}$ versus $1/[S]$ are presented to show the mode of inhibition in all three panels. The values for both panels (A) and (B) are averages of 3 independent experiments±S.E. and those for panel (C) are from a single experiment due to the limited material.

4. Discussion

TM accelerates protein C activation by thrombin in the presence of Ca^{2+} more than 1000-fold by improving both the K_m (~10-fold) and k_{cat} (~100-fold) of the reaction [1]. The mechanism of the cofactor function of TM has been under investigation for many years, but it is still not well understood. What has been fairly well established is that the residues of the activation peptide of protein C, in particular the acidic residues at the P3 and P3′ sites, are inhibitory for docking into the catalytic cleft of thrombin in the presence of Ca^{2+} [9]. Direct binding studies have supported a conformational change in the activation peptide of protein C upon interaction with Ca^{2+} [10,11]. Thus, a role for TM in the activation complex has been speculated to be the alleviation of the inhibitory interaction of the activation peptide with the active-site cleft of thrombin by an allosteric mechanism [9,25]. Results of several mutagenesis, spectral and kinetic studies have

supported such a mechanism of cofactor function for TM [17,25–27]. However, the recent crystal structure determination of the active-site inhibited thrombin-TM456 complex did not support the allosteric modulation of the active-site pocket of thrombin as the primary mechanism by which TM might promote the activation of protein C by thrombin [12]. This is because no major structural difference between the active-site pocket of a free and a TM-complexed thrombin was observed [12]. Another interesting finding of the structural data was the observation that TM4 of the cofactor did not come in contact with thrombin, but protruded out to possibly form a binding site for protein C, raising the possibility that a key function for TM is "substrate presentation" [12]. To further investigate this question, we measured the reactivity of thrombin with three mutants *GDPC/Proth-2*, *AT/Proth-2* and *prethrombin-2*, all of which have identical P3-P3′ residues, but only *GDPC/Proth-2* can interact with TM in the activation complex. While TM456 did not influence the reactivity of thrombin with either *prethrombin-2* or the *AT/Proth-2* mutant, it catalyzed the thrombin activation of the GDPC mutant with a rate that was comparable to that observed with the wild-type protein. These results suggested that TM interaction with both thrombin and protein C is essential for the activation reaction. Moreover, the observation that the non-optimal P3-P3′ residues of prethrombin-2 are effectively recognized by thrombin in complex with TM suggests that ternary complex formation possibly alters the conformation of the active-site pocket of the protease and/or the P3-P3′ residues of the substrate. It should, nevertheless, be noted that in a similar recent mutagenesis study, the substitution of the thrombin recognition site of TAFI, another substrate for the thrombin-TM complex, with the P3-P3′ residues of protein C resulted in a mutant which was poorly activated by the thrombin-TM complex [28]. The authors concluded that the cofactor function of TM has an insignificant allosteric effect on the active-site pocket of thrombin and that substrate presentation by TM primarily accounts for its cofactor function.

The concentration dependence of the heparin–AT/Proth-2 inactivation of thrombin revealed that the rate constant of stable complex formation has been impaired by 4-orders of magnitude. Noting that the cofactor effect of high molecular weight heparins is solely mediated through lowering of the dissociation constant for the formation of the initial enzyme-inhibitor encounter complex with a minimum effect on the rate constant (k) for formation of a stable and covalent complex (21), these results suggest that the mutant serpin has a very poor reactive site loop sequence for recognition by thrombin. On the other hand, the concentration dependence of GDPC/Proth-2 activation by thrombin-TM456 indicated that the protein C mutant was activated with a comparable k_{cat}, suggesting that the cofactor role of TM does not resemble heparin to merely provide a binding site for protein C on the activation complex. Thus, it is hard to understand how thrombin can react efficiently with a GDPC mutant that has the same poor recognition sequence around the scissile bond, unless the sequence is presented to thrombin with a significantly rearranged and altered conformation and/or the active-site pocket of thrombin has an altered conformation in the ternary complex. Consistent with a possible allosteric effect for TM on the substrate, a recent mutagenesis study utilized a panel of 77 Ala mutants of thrombin and mapped the epitope of thrombin recognizing protein C in the absence and presence of TM [29]. The subsequent temperature dependent protein C activation studies revealed that TM increases the rate of diffusion of protein C into the active site pocket of thrombin [29]. This previous study concluded that the cofactor function of TM allosterically alters the conformation of the activation peptide of protein C to facilitate its docking into the catalytic pocket of thrombin.

Recently, the active-site directed inhibitor PAB has been used in competitive kinetic studies to demonstrate that cofactor dependent exosite interactions primarily determine the binding specificity of coagulation reactions [22,23]. This has been evidenced by the observation that PAB acts as a competitive inhibitor of the cleavage of the tripeptidyl substrates by coagulation proteases, but as a noncompetitive inhibitor in reaction with their target macromolecular substrates [22,23]. However, PAB acted as a competitive inhibitor of both the amidolytic and proteolytic activity of the thrombin-TM456 complex with both wild-type and GDPC mutant. A similar competitive effect for PAB in protein C activation by the thrombin-TM complex was reported in a recent study [24]. These results suggest that the interaction of the P3-P3′ residues of protein C with the active-site pocket of thrombin makes a significant contribution to the productive assembly of the activation complex. Furthermore, the observation that the GDPC mutant with an extremely bad recognition sequence can act as an effective competitive inhibitor of PAB supports the hypothesis that the active-site pocket of thrombin and/or the P3-P3′residues of the substrate have undergone a conformational change in the ternary protease-cofactor-substrate complex. Finally, the observation that the *GDPC/Proth-2* mutant was recognized and activated by factor Xa, but not thrombin suggests that P3-P3′ residues may primarily determine the substrate specificity of other coagulation proteases in the absence of cofactors.

Acknowledgements

We would like to thank Audrey Rezaie for proofreading the manuscript.

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