Human activated protein C (APC) is a key component of a natural anticoagulant system that regulates blood coagulation. *In vivo*, the catalytic activity of APC is regulated by two serpins, α1-antitrypsin and the protein C inhibitor (PCI), the inhibition by the latter being stimulated by heparin. We have identified a heparin-binding site in the serine protease domain of APC and characterized the energetic basis of the interaction with heparin. According to the counter-ion condensation theory, the binding of heparin to APC is 66% ionic in nature and comprises four to six net ionic interactions. To localize the heparin-binding site, five recombinant APC variants containing amino acid exchanges in loops 37, 60, and 70 (chymotrypsinogen numbering) were created. As demonstrated by surface plasmon resonance, reduction of the electropositive character of loops 37 and 60 resulted in complete loss of heparin binding. The functional consequence was loss in heparin-induced stimulation of APC inhibition by PCI, whereas the PCI-induced APC inhibition in the absence of heparin was enhanced. Presumably, the former observations were due to the inability of heparin to bridge some APC mutants to PCI, whereas the increased inhibition of certain APC variants by PCI in the absence of heparin was due to reduced repulsion between the enzymes and the serpin. The heparin-binding site of APC was also shown to interact with heparan sulfate, albeit with lower affinity. In conclusion, we have characterized and spatially localized the functionally important heparin/heparan sulfate-binding site of APC.

The protein C pathway is a functionally important anticoagulant system that regulates blood coagulation *in vivo*. The key component of this pathway is the vitamin K-dependent protein C (1–3). Protein C circulates in plasma as a zymogen to a serine protease that has anticoagulant properties. Protein C is a multidomain molecule that is composed of two disulfide-linked chains. The light chain comprises a γ-carboxyglutamatic acid domain and two epidermal growth factor (EGF)-like domains, whereas the heavy chain is composed of the activation peptide and a serine protease domain (4). Protein C is activated on endothelial cells by thrombin bound to thrombomodulin. Activated protein C (APC) regulates blood coagulation by cleaving and inhibiting two cofactors, activated factor V (FVa) and activated factor VIII (FVIIIa) (5), which serve as phospholipid-membrane-bound cofactors to factor Xa (FXa) and factor IXa (FIXa), respectively. FXa is the enzyme that activates prothrombin to thrombin, whereas FIXa converts FX to its active form (1, 6).

*In vivo*, the proteolytic activity of APC is regulated by two serpins, namely α1-antitrypsin and protein C inhibitor (PCI) (7, 8). Inhibition of APC by PCI is potentiaded by the glycosaminoglycan heparin, whereas the inhibition by α1-antitrypsin is insensitive to the presence of heparin. Structurally, heparin is heterogeneous in nature and is composed of long, highly negatively charged, unbranched polysaccharide chains. It is hypothesized that heparin binds to both PCI and APC during PCI-mediated inhibition of APC, thus guiding the encounter of these proteins via a template mechanism (9, 10). We therefore expect the formation of a ternary complex similar to the one suggested between antithrombin, heparin, and thrombin (11, 12). Recently, some residues in APC were implicated to interact directly with heparin during the PCI-induced inhibition of APC (13, 14), but a more complete definition of a heparin-binding site(s) in APC and energetic characteristics of the heparin interaction with APC were lacking.

Binding of heparin to proteins is usually ionic in nature, involving the side chains of clustered basic amino acids on the protein and negatively charged groups on the heparin molecule. Amino acid sequence patterns such as XBBXBX and XBBXBBXXB (B denotes basic, and X denotes nonbasic residues) are potential heparin recognition sites (15, 16). Alternatively, the basic residues may be located far apart in the linear sequence but are topological neighbors in the three-dimensional structure (17–20). In the three-dimensional structure of APC, a basic cluster of amino acids is located on loops 37, 60, and 70 (chymotrypsinogen nomenclature) (see Fig. 1) (21–23). Heparin-binding sites sharing similar distribution of charged amino acids are present on the surface of many proteins that bind heparin, e.g. hepatocyte growth factor (24). Multiple-sequence and structural alignments of APC with other serine proteases formed the basis for our mutagenesis strategy, aimed at identification of the APC heparin-binding site.

In the present study, site-directed mutagenesis and recombinant human protein C expression were used to localize the heparin-binding site in APC. A cluster of lysines located on loops 37 and 60 was found to be crucial for heparin binding. Characterization of the binding of heparin to APC under...
Interaction of Heparin and PCI with APC

**Experimental Procedures**

**Materials**—Restriction endonucleases were obtained from New England Biolabs or Fermentas. Dulbecco's modified Eagle's medium, Waymouth's medium, and fetal calf serum were obtained from Life Technologies, Inc. Cell cultureware was purchased from Falcon, vitamin K$_1$ was from Hoffmann La Roche, hyogromycin B was from Calbiochem, Q-Sepharose Fast Flow, heparin-Sepharose, and PD-10 columns were from Amersham Pharmacia Biotech. The chromogen substrate S-2366 (t-pyroglutamyl-t-prolyl-t-arginine-p-nitroanilin), specific for protein C, was obtained from Chromogenix (Sweden), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) were from ICN. Human α-thrombin was prepared from plasma-purified prothrombin, as described (25). Basic fibroblast growth factor (bFGF) was from Sigma. Heparan sulfate (bovine) was obtained from Sigma. Preparations of unfractionated heparin (Heparin-M) and dextran sulfate (0.5 mg of each) were dissolved in 200 μl of 0.1 M MES, pH 5.6. EZ-link biotin hydrazide and N-ethyl-N-(dimethylamino)propylcarbo- diimide were added to a final concentration of 2.5 and 0.5 mM, respectively. After a 6-h incubation at room temperature with constant shaking, the biotinylated glycosaminoglycans were desalted on a PD-10 column (Amersham Pharmacia Biotech) equilibrated in water, then freeze-dried, and dissolved in 0.3 M NaCl.

**Biotinylation of Heparin and Heparan Sulfate**—Heparin or heparan sulfate (0.5 mg of each) was dissolved in 200 μl of 0.1 M MES, pH 5.6. EZ-link biotin hydrazide and N-ethyl-N-(dimethylamino)propylcarbo- diimide were added to a final concentration of 2.5 and 0.5 mM, respectively. After a 6-h incubation at room temperature with constant shaking, the biotinylated glycosaminoglycans were desalted on a PD-10 column (Amersham Pharmacia Biotech) equilibrated in water, then freeze-dried, and dissolved in 0.3 M NaCl.

**Binding of Activated Protein C to Immobilized Heparin and Heparan Sulfate**—Heparin and heparan sulfate were immobilized under pseudo-first-order conditions. In brief, WT and variant APCs by PCI in the absence of heparin or heparan sulfate was measured by surface plasmon resonance using BIAcore 2000. Biotinylated, unfraccionated heparin and heparan sulfate were immobilized in flow cells 2 and 3, respectively, of a streptavidin sensor chip. Functional integrity of the chip-bound heparan sulfate was shown by the fact that it bound bFGF with high affinity. APC (5–150 μg/ml) was injected at a flow rate of 30 μl/min at various NaCl concentrations (80–200 mM) into flow cells containing heparin. In addition, APC (150 μg/ml) was injected at 30 μl/min in a flow cell containing immobilized heparan sulfate. Flow cell 1 without any immobilized glycosaminoglycan was used as control. To investigate whether fluid phase heparan sulfate could compete with immobilized heparin for the binding to APC, 0.5 mg/ml heparan sulfate was included in the flow buffer. The derived BIAcore sensograms were evaluated with BIAevaluation 3.0 software to calculate association (k$_{on}$) and dissociation (k$_{off}$) rate constants.

**Affinity Chromatography on Heparin-Sepharose**—WT and variant APCs in 20 mM Tris-HCl, 50 mM NaCl, pH 7.4 buffer were applied on heparin-Sepharose, and bound APC was eluted with a linear NaCl gradient from 0.05 to 0.4 M. APC and NaCl concentrations were measured by hydrolisis of chromogenic substrate S-2366 hydrolysis and flame photometry, respectively (13).

**Electrostatic Potential**—The side chains of variant APC molecules were replaced interactively using InsightII and Biopolymer, and all structures were energy-minimized using the simulation program Discover (Bioysm-MSI). The electrostatic potential isosurfaces were computed with DelPhi as part of the Bioysm-MSI-modeling suite (inner/outer dielectrics 4/80; physiological ionic strength, formal charges) for APC molecules and for the highly negatively charged heparin molecule.

**Kinetics of APC Inactivation by PCI in the Absence and Presence of Heparin or Heparan Sulfate**—The rate of inhibition of WT and mutant APCs by PCI in the absence of heparin or heparan sulfate was measured under pseudo-first-order conditions. In brief, WT and variant APCs were incubated (between 0 to 120 min) with a 10-fold molar excess of human plasma PCI at room temperature in 20 mM Tris-HCl, 0.10 M NaCl, 5 mM CaCl$_2$, pH 7.5, containing 0.1% bovine serum albumin. Chromogenic substrate S-2366 was added to a final concentration of 0.2 mM, and the rate of substrate hydrolysis was measured with a V$_{max}$ kinetic microplate reader (Bio-TEK instruments). First-order rate constants (k) were calculated as ln[A]t/ln[A]0, where [A]t is the fractional protease activity remaining relative to the uninhibited control, t is the time of measurement, and [A]0 is the PCI concentration.

In the presence of unfraccionated heparin (M$_t$ from 5,000 to 30,000; LEO) the rate of APC inactivation was too fast to be measured under the conditions that are described above. Therefore, PCI (7.5–200 nM at final concentration) was mixed with APC (30 nM) and 1 IU/ml (equal to 6.6 μg/ml) unfractionated heparin in a final volume of 100 μl. After defined time periods, the reaction was stopped by the addition of 2.5 mg/ml Poly- 
A. The residual APC activities were determined by adding 50 μl of S-2366 (final concentration was 0.2 mM), and the ΔΔA at 405 nm was measured. The following equation was used to calculate the k$_{2}$ value.

\[ k_2 = \frac{1}{(|A_0| - |B_0|)} \ln \left( \frac{|B|}{|A|} \right) \]  

(Eq. 1)

The proteins were detected using a polyclonal antibody against human protein C (No. 370 DAKO) followed by an alkaline phosphatase-conjugated swine anti-rabbit antibody (No. 306 DAKO), and BCIP (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (NBT) substrate was used for development.

**Activation of Protein C and Catalytic Activity against Small Substrates**—Purified WT protein C or protein C variants were incubated with human α-thrombin (10:1 mol/mol) in 20 mM Tris-HCl, 150 mM NaCl, pH 7.4, at 37 °C for 2 h in the presence of 5 mM EDTA. A substrate-heparin-Sepharose column was used to remove unreacted protein. The reaction mixture. APC concentrations were estimated by measurement of absorbance at 280 nm. Amidolytic activities were determined using chromogenic substrate S-2366 (20 mM Tris-HCl, 150 mM NaCl, pH 7.4 at 37 °C) in a microplate reader (ELX800U from Bio-TEK instruments) and expressed in absorbance change at 405 nm (ΔA/min). The concentration of chromogenic substrate S-2366 ranged between 0.1 and 3 μM, and the concentrations of APC ranged between 1 and 50 nM. K$_{m}$ and V$_{max}$ values were obtained from Lineweaver-Burk plots, and k$_{cat}$ was calculated from the Michaelis-Menten equation (27, 28).

**Interaction of Heparin and PCI with APC**—Heparin and PCI were incubated under pseudo-first-order conditions. In brief, WT and variant APCs by PCI in the absence of heparin or heparan sulfate was measured by surface plasmon resonance using BIAcore 2000. Biotinylated, unfraccionated heparin and heparan sulfate were immobilized in flow cells 2 and 3, respectively, of a streptavidin sensor chip. Functional integrity of the chip-bound heparan sulfate was shown by the fact that it bound bFGF with high affinity. APC (5–150 μg/ml) was injected at a flow rate of 30 μl/min at various NaCl concentrations (80–200 mM) into flow cells containing heparin. In addition, APC (150 μg/ml) was injected at 30 μl/min in a flow cell containing immobilized heparan sulfate. Flow cell 1 without any immobilized glycosaminoglycan was used as control. To investigate whether fluid phase heparan sulfate could compete with immobilized heparin for the binding to APC, 0.5 mg/ml heparan sulfate was included in the flow buffer. The derived BIAcore sensograms were evaluated with BIAevaluation 3.0 software to calculate association (k$_{on}$) and dissociation (k$_{off}$) rate constants.
[A]₀ and [B]₀ were the initial concentrations of APC and PCI, respectively, and \( x \) was the molar concentration of APC-PCI complexes formed after time \( t \); \( k_2 \) was calculated from data points demonstrating 15–85% proteinase inhibition. All experiments were performed in triplicates. To determine the effect of heparan sulfate on the rate of inhibition of APC by PCI, PCI (60 nM final concentration) was mixed with APC (30 nM final concentration) and increasing concentrations of heparan sulfate (0–100 mg/ml) in a final volume of 1 ml. Under these conditions, maximum stimulation of inhibition was observed at concentrations \( \geq 50 \text{ mg/ml} \). At intervals, the reactions were terminated by the addition of 2 mg/ml Polybrene. Residual activity of APC was determined by the addition of 50 ml of S-2466, and the \( DA \) at 405 nm was measured. The \( k_2 \) values were calculated as described for heparin from data points demonstrating 15–85% proteinase inhibition. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Quantitative and Qualitative Analysis of APC Variants—To evaluate the involvement of positively charged amino acid residues in loops 37, 60, and 70 in the binding of heparin to APC, five protein C variants were constructed by site-directed mutagenesis (Fig. 1). According to our structural analysis, four of the variants were expected to have reduced affinity toward heparin (K37S/K38Q/K39Q, K62N/K63D, K37S/K38Q/K39Q/K62N/K63D, R74Q), whereas one mutant was created with the intention of increasing the heparin affinity (E60aS/S61R). Since the target amino acid residues are solvent-exposed on loop structures and naturally occurring in related serine proteinases, it should be expected that the above amino acid substitutions will not alter the folding of APC or damage its catalytic machinery. Furthermore, because the newly introduced residues are similar in size to the original ones, these mutations can be considered as conservative, and they should be better tolerated in the structure than, for instance, small hydrophobic alanine substitutions. Clustering of three to five alanine residues could create a destabilizing solvent-exposed hydrophobic patch no longer able to form hydrogen bonds with water molecules or could induce misfolding due to the high helical propensity of this amino acid.

The various protein C cDNA variants were used to transfect the eukaryotic cell line HU293, and stable cell lines were established. The different protein C variants demonstrated similar expression levels (2.7 to 6.7 mg/liter). The proteins were purified with overall recoveries of 35–40%, and the isolated proteins were more than 90% pure, as estimated by SDS-PAGE. On SDS-PAGE, the protein C variants migrated at similar positions as WT protein C (Fig. 2). Under nonreducing conditions, the different protein C variants migrated as single bands. After reduction of disulfide bridges, the 41-kDa heavy chains and the 21-kDa light chains were observed in addition to the 62-kDa single chain forms of protein C. Under reducing conditions, the protein C variants migrated as single bands. After reduction of disulfide bridges, the 41-kDa heavy chains and the 21-kDa light chains were observed in addition to the 62-kDa single chain forms of protein C.
conditions, for all recombinant proteins a small amount of single chain protein C remained. After activation, the different recombinant protein variants demonstrated similar amidolytic activities (k_m and k_cat) (Table I). The equally high expression levels observed for all the different protein C variants and their full amidolytic activities after activation together with the above structural considerations suggest that the recombinant proteins were correctly folded and could be used for further functional characterizations.

**Binding of APC Variants to Immobilized Heparin**—Binding of WT APC and APC variants to immobilized heparin was analyzed with surface plasmon resonance using a BIAcore 2000 (Table II). At physiological NaCl concentration, the dissociation constant (K_d) of WT APC for heparin was 0.32 μM. The R74Q variant showed slightly lower affinity (K_d = 0.54 μM), whereas E60aS/S61R bound more tightly (K_d = 0.19 μM) to heparin than WT APC (Table II, Fig. 3). There was no detectable binding of K37S/K38Q/K39Q, K62N/K63D, and K37S/K38Q/K39Q/K62N/K63D to immobilized heparin molecules. To qualitatively confirm the results obtained by BIAcore, WT APC and the five APC variants were applied on heparin-Sepharose, and bound protein was eluted with a linear NaCl gradient. The results agreed well with those obtained by the BIAcore analysis. Thus, removal of the positively charged residues at positions 37/38/39 or 62/63 or 37/38/39/62/63 yielded diminished or undetectable binding to the heparin-Sepharose (results not shown). In contrast, the E60aS/S61R variant bound tighter to the heparin column than WT APC and was eluted at higher NaCl concentrations. These results strongly suggest that the heparin-binding site of APC is entirely dependent on the presence of a few positively charged residues on loops 37 and 60 (Fig. 4).

Heparin is derived from mast cells, and it is unlikely that APC interacts with heparin under normal physiological conditions if heparin is not administered as a therapeutic. Heparan sulfate present on the surface of endothelial cells is the glycosaminoglycan that is more likely to interact with circulating APC. For this reason, we tested the binding of APC to immobilized heparin with surface plasmon resonance, and BIAevaluation 3.0 software was used to calculate association (k_a) and dissociation (k_d) rate constants from the sensograms. APC variants K37S/K38Q/K39Q, K62N/K63D, and K37S/K38Q/K39Q/K62N/K63D demonstrated no binding to the heparin-coated SA-chip. Therefore, no k_a and k_d rate constants could be calculated for these APC variants.

**Table I**

| Enzyme | K_m (μM) | k_cat (s^-1) | k_cat/K_m (s^-1) |
|--------|----------|--------------|------------------|
| Wild type | 0.91 ± 0.02 | 70 ± 1 | 76.9 |
| K62N/K63D | 0.95 ± 0.01 | 78 ± 5 | 82.1 |
| K37S/K38Q/K39Q | 0.98 ± 0.04 | 75 ± 7 | 76.5 |
| K37S/K38Q/K39Q/K62N/K63D | 0.93 ± 0.01 | 71 ± 1 | 76.3 |
| E60aS/S61R | 0.93 ± 0.08 | 70 ± 6 | 75.3 |
| R74Q | 0.90 ± 0.02 | 76 ± 8 | 84.4 |

**Table II**

| APC | K_d (μM) | k_a (s^-1) | k_off (s^-1) |
|-----|----------|------------|--------------|
| [NaCl] = 150 mM | ΔG = -8.8 (kcal/mol) | 6.1 × 10^3 | 3.3 × 10^-3 |
| R74Q | 0.54 | 103 | 8.4 × 10^3 |
| [NaCl] = 150 mM | ΔG = -8.5 (kcal/mol) | 7.8 × 10^3 | 1.5 × 10^-3 |
| E60aS/S61R | 0.19 | 10^3 | 3 × 10^-3 |
| [NaCl] = 150 mM | ΔG = -9.1 (kcal/mol) | 7.8 × 10^3 | 1.5 × 10^-3 |
Table III

| APC        | \(K_d^{\text{obs}}\) | \(k_{\text{on}}\) | \(k_{\text{off}}\) |
|------------|----------------------|------------------|------------------|
| Wild type  | 0.075 μM             | 5.7 x 10^{-4}    | 4.3 x 10^{-3}    |
| [NaCl] 80 mM | \(\Delta G = -9.6\) kcal/mol |                   |                  |
| Wild type  | 0.92                | 2.6 x 10^{4}     | 8.4 x 10^{-3}    |
| [NaCl] 150 mM | \(\Delta G = -8.8\) kcal/mol |                   |                  |
| Wild type  | 1.9                 | 0.6 x 10^{4}     | 11.6 x 10^{-3}   |
| [NaCl] 200 mM | \(\Delta G = -7.7\) kcal/mol |                   |                  |
| Wild type  | 47                  | 0.1 x 10^{4}     | 47.7 x 10^{-3}   |
| [NaCl] 300 mM | \(\Delta G = -6\) kcal/mol |                   |                  |

The observed \(K_d\) (\(K_d^{\text{obs}}\)) is related to the nonionic equilibrium dissociation constant (\(K_d^{\text{nomic}}\)) through the relationship log \(K_d^{\text{obs}}\) = log \(K_d^{\text{nomic}}\) + \(\Psi Z Na^+\). The plot of log \(K_d^{\text{obs}}\) versus log [salt] is linear, and the value of \(Z\) can be calculated from the slope. The formation of Z electrostatic interactions between WT APC and heparin is accompanied by the displacement of \(\Psi Z\)-condensed counter-ions from the heparin. \(\Psi\) is the effective fraction of counter-ion bound per heparin negative charge, which has been estimated to be 0.8 (11). The slope (0.8 x \(Z = 4.86\)) provides the effective number of purely ionic interactions formed between WT APC and heparin. Thus, about 4–6 ionic interactions seem to be involved in this process. At 1.0 mM NaCl, ionic interactions were neutralized, and log \(K_d^{\text{obs}}\) = log \(K_d^{\text{nomic}}\), which we estimate to be 5600 μM. Because the \(K_d\) for the APC-heparin interaction at physiological salt concentration (0.92 μM) was estimated to be 0.85, 66% of the binding free energy (\(\Delta G\)) was estimated to be ionic, with the remaining 34% nonionic. The \(\Delta G\) of WT APC binding to heparin at physiological salt concentration (150 mM) was estimated to be \(-8.5\) kcal/mol, with the nonionic and ionic contributions to this interaction \(-3\) kcal/mol and \(-5.7\) kcal/mol, respectively. Thus, the WT APC binding to heparin is mainly electrostatic in nature just like the binding of heparin to thrombin (\(K_d^{\text{obs}} = 6–10\) μM, about 80% ionic) (11) or to mucus proteinase inhibitor (\(K_d^{\text{obs}} = 0.05\) μM; >80% ionic) (34).

There is a considerable effect of salt on \(K_d^{\text{obs}}\) for the APC-heparin interaction (Table III), and the corresponding \(\Delta G\) val-


In conclusion, we have characterized the structural and energetic basis of a functionally important heparin-binding site in the heparin/heparan sulfate-stimulated inhibition of APC. This binding site is functionally important in the heparin/heparan sulfate-stimulated inhibition of APC by its serpin inhibitor PCI.

It is noteworthy that the now demonstrated heparin/heparan sulfate-binding site in APC is located on another part of the protease domain as compared with the heparin binding exosite II of thrombin (Fig. 1). Similarly, PCI binds heparin on the other side (around helix H) as compared with the heparin binding D-helix of antithrombin (10, 36, 37). Therefore, the helix H area of PCI should be the topological neighbor of APC loops 37 and 60 in the Michaelis-like complex.

In conclusion, we have characterized the structural and energetic basis of a functionally important heparin-binding site in the serine protease domain of APC. This binding site is functionally important in the heparin/heparan sulfate-stimulated inhibition of APC by its serpin inhibitor PCI.

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| APC | Presence of heparin, $k_2$ ($\times 10^6$ M$^{-1}$ s$^{-1}$) | Absence of heparin, $k_2$ ($\times 10^6$ M$^{-1}$ s$^{-1}$) | Acceleration of APC inhibition |
|-----|-------------------------------------------------|---------------------------------|-----------------------------|
| Wild type | 8.7 ± 0.1 | 0.7 ± 0.1 | 805 |
| R74Q | 7.4 ± 0.9 | 0.4 ± 0.01 | 541 |
| E60aS/S61R | 7.9 ± 1.5 | 8.7 ± 0.5 | 11,012 |
| K62N/K63D | 46.5 ± 0.2 | 0.4 ± 0.1 | 186 |
| K37S/K38Q/K39Q | 16.1 ± 0.1 | 0.2 ± 0.2 | 124 |
| K62N/K63D/K37S/K38Q/K39Q | 175 ± 0.1 | 0.2 ± 0.1 | 11 |

**FIG. 5.** PCI-catalyzed inhibition of APC. Time courses of inhibition of WT and variant APCs by PCI determined in the absence (A) and presence (B) of heparin. In the absence of heparin, PCI (20 nM) was incubated for 30 min at room temperature with a 10-fold molar excess of human plasma PCI in 20 mM Tris-HCl, pH 7.5, containing 0.1% bovine serum albumin. Chromogenic substrate S-2386 was added, and the rate of substrate hydrolysis was measured. In the presence of heparin, 7.5 nM PCI was mixed with APC (30 nM) and 1 IU/ml unfractionated heparin. E60aS/S61R (●), K62N/K63D (○), and R74Q (■) were inhibited equally fast as WT APC (●) by PCI, whereas higher rates of inhibition were detected for K37S/K38Q/K39Q (△) and K37S/K38Q/K39Q/K62N/K63D (□). Each data point shows the mean value of three experiments, independently performed.
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