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Regulation of α-Chymotrypsin Catalysis by Ferric Porphyrins and Cyclodextrins

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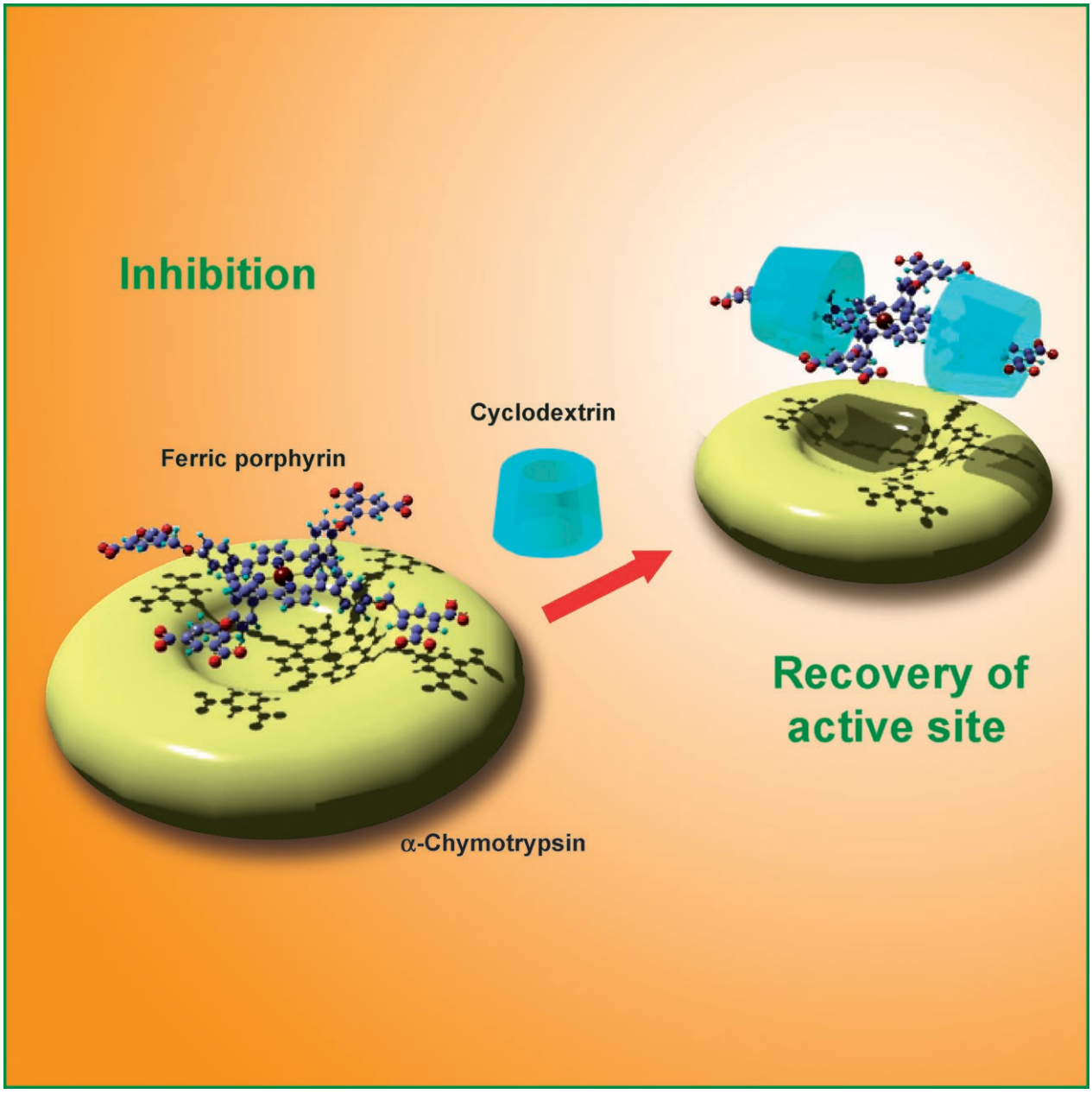

Abstract: Positively charged α-chymotrypsin (ChT) formed a 1:1 complex with negatively charged 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) (FeTPPS) in phosphate buffer at pH 7.4 through electrostatic interaction. In spite of the large binding constant ($K=4.8\times10^5\,\text{M}^{-1}$), FeTPPS could not completely inhibit the catalysis of ChT in the hydrolysis of the model substrate, *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA). The degree of inhibition (60%) was saturated at 1.6 equivalents of FeTPPS, which indicates that covering of the active site of ChT by FeTPPS was insufficient. The enzymatic activity lowered by FeTPPS was entirely recovered for the freshly prepared sample when the porphyrin on the protein surface was detached by per-*O*-methylated β-cyclodextrin (TMe-β-CD), which formed a stable 1:2 inclusion complex with FeTPPS ($K_1=1.26\times10^6\,\text{M}^{-1}$, $K_2=6.3\times10^4\,\text{M}^{-1}$). FeTPPS gradually induced irreversible denaturation of ChT, and the denatured ChT further lost its catalytic ability. No repairing effect of TMe-β-CD was observed with irreversibly denatured ChT. A new reversible inhibitor, 5,10,15,20-tetrakis[4-(3,5-dicarboxyphenylmethoxy)phenyl]porphyrinato iron(III) (FeP8M), was then designed, and its inhibitory behavior was examined. FeP8M formed very stable 1:1 and 1:2 FeP8M/ChT complexes with ChT, the K_1 and K_2 values being 2.0×10^8 and $1.0\times10^6\,\text{M}^{-1}$, respectively. FeP8M effectively inhibited the ChT-catalyzed hydrolysis of SPNA (maximum degree of inhibition = 85%), and the activity of ChT was recovered by per-*O*-methylated γ-cyclodextrin. No irreversible denaturation of ChT occurred upon binding with FeP8M. The kinetic data support the observation that, for nonincubated samples, both inhibitors did not cause significant conformational change in ChT and inhibited the ChT activity by covering the active site of the enzyme.

Keywords: cyclodextrins · electrostatic interactions · enzyme catalysis · inhibitors · supramolecular chemistry

Introduction

α-Chymotrypsin (ChT) is a serine protease ($M_r=25\,000$, pI = 8.7)[1] whose crystal structure has been examined in detail.[2] ChT has a hydrophobic pocket as the active site, which is surrounded by cationic residues such as arginine (Arg) and lysine (Lys) (Figure 1). The positive amino acid residues are located on the circumference of a circle with a diameter of 3.7 nm, whose center is the active site of the enzyme. As the cationic and, hence, hydrophilic residues in the protein are exposed to the aqueous bulk phase, ChT can bind various artificial, anionic effectors to lead to an alteration of enzymatic activity. Hamilton and co-workers found a distinct inhibition effect of an octa-anionic calixarene bound to cationic ChT through complementary Coulomb interactions.[3] A similar effect was realized by gold nanoparticles fabricated by anionic carboxylates,[4] anionic micelles,[5] an anionic polymer,[6] and anionic dendrimers.[7] These inhibitors showed high abilities in binding to ChT ($K=10^6$–$10^7\,\text{M}^{-1}$) and strong inhibition effects (the degrees of inhibition were over 90%). The idea of the utilization of Coulomb

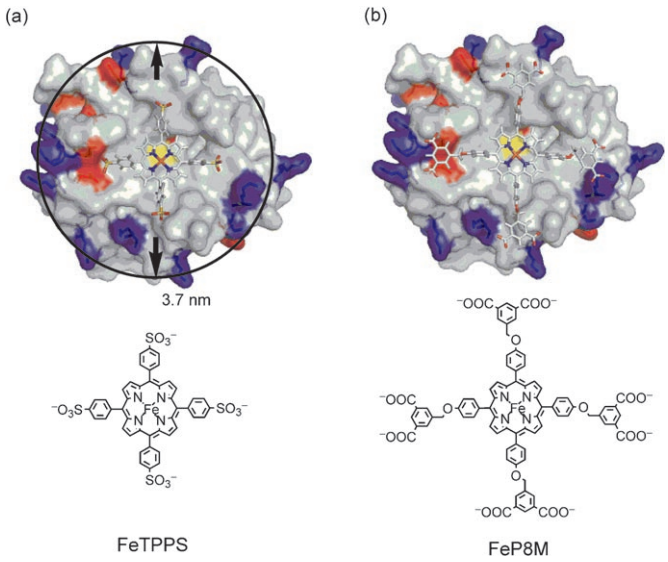

Figure 1. X-ray structure of ChT (PDB 4CHA) and relative sizes of a) FeTPPS and b) FeP8M. Blue and red represent the basic (Arg and Lys) and acidic amino acid residues (Asp and Glu), respectively.

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interactions for protein recognition by small, multivalent anionic molecules was first presented by Clark-Ferris and Fisher[8] and was developed by Hamilton and co-workers.[9,10] From the viewpoint of the "regulation" of enzymatic activity, it is desirable to recover the catalysis of an enzyme inhibited by an effector by an appropriate repairing agent. The addition of a large amount of inorganic salt that weakens the Coulomb interaction between the enzyme and the inhibitor is one of the applicable methods for this purpose.[4d,6,7a] However, a high concentration of inorganic salt alters the structure of the enzyme, which leads to a change in the enzymatic activity[11] and to difficulty in understanding the results. Another method is the detachment of the anionic effector by using a cationic amphiphile.[4b] However, certain cationic additives tend to enhance the enzymatic activity of ChT,[5,12] which leads to the same problem as the inorganic salts. Electronically neutral repairing agents are preferable for fundamental studies on the regulation of ChT catalysis and for use in vivo. Although such a requirement is simple, no example has been reported with a nonionic and less toxic repairing agent. We applied the ChT-catalyzed hydrolysis of *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) as a model reaction and invented a new method for regulating the protein functionality by using anionic water-soluble tetraphenylporphyrins as the inhibitors and per-*O*-methylated cyclodextrins as the nonionic and less toxic repairing agents. At first, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) (FeTPPS) was used as the inhibitor, in which the longest distance between the carboxylate groups (1.9 nm) is much shorter than the diameter of the circle that encompasses the cationic amino acid residues of ChT on its circumference (Figure 1). However, FeTPPS is a promising effector because this porphyrin is known to interact strongly with neutral heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMe-β-CD).[13] TMe-β-CD was expected to be a repairing agent that detaches FeTPPS from the protein surface. 5,10,15,20-Tetrakis[4-(3,5-dicarboxyphenylmethoxy)phenyl]porphyrinato iron(III) (FeP8M) is another inhibitor; the distance between the opposite carboxylate groups (2.8 nm) is longer than that of FeTPPS (Figure 1). FeP8M was expected to bind to ChT more strongly than FeTPPS. The results indicate that the present methodology is principally useful for the regulation of ChT catalysis, although a more precise molecular design is necessary for use in vivo.

Abstract in Japanese:

カチオン性の α-キモトリプシン (ChT) は pH 7.4 のリン酸緩衝溶液中、アニオン性の 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron(III) (FeTPPS) と静電相互作用により、安定な 1:1 錯体を形成した。大きな結合定数 (K = 4.8 × 10^5 M^{-1}) にも関わらず、モデル基質である *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) の加水分解に対する ChT の触媒効果を完全には阻害しなかった。阻害率 (60 %) は 1.6 当量の FeTPPS の添加により飽和し、このことより FeTPPS は ChT の活性点を完全に覆わないものと思われた。作製直後の試料に対しては、FeTPPS と 1:2 包接錯体 (K_1 = 1.26 × 10^6 M^{-1}, K_2 = 6.3 × 10^4 M^{-1}) を形成するパー *O*-メチル化 β-シクロデキストリン (TMe-β-CD) を添加することによって、FeTPPS が ChT 表面から剥がされるために、ChT の触媒活性は完全に回復した。FeTPPS は徐々に ChT の不可逆的な変性を誘起し、ChT のさらなる活性阻害をもたらした。変性した ChT に対しては、TMe-β-CD の回復効果が認められなかった。そこで、新たな阻害剤である 5,10,15,20-tetrakis[4-(3,5-dicarboxyphenylmethoxy)phenyl]porphyrinato iron(III) (FeP8M) を分子設計し、その阻害効果を検討した。FeP8M は ChT と非常に安定な 1:1 および 1:2 FeP8M/ChT 錯体を形成し、K_1 および K_2 はそれぞれ 2.0 × 10^8 および 1.0 × 10^6 M^{-1} であった。FeP8M は SPNA の ChT 触媒加水分解反応を効率よく阻害し（最大阻害率＝85 %）、ChT の触媒活性はパー *O*-メチル化 γ-シクロデキストリンの添加によって回復した。FeP8M は ChT の不可逆的な変性を誘起しなかった。速度論の結果も、未熟成な試料については、両阻害剤とも ChT の大きな構造変化を引き起こさず、触媒の活性点を一部覆うことにより、ChT の活性を阻害するという結論を支持した。

Results and Discussion

Interactions between ChT and Metalloporphyrins

The binding constant (K) for the complexation of ChT with FeTPPS was determined in 5×10^{-3} M phosphate buffer at pH 7.4 and 25 °C by a UV/Vis-spectroscopic-titration method. The regular spectral changes of FeTPPS with isosbestic points were recorded upon the addition of ChT (see Supporting Information). FeTPPS exists in diaqua form ($(H_2O)_2$FeTPPS) at low pH. The diaqua complex dissociates into a monohydroxo complex ((OH^-)FeTPPS) that immediately changes to the μ-oxo dimer as the pH increases.[13] The pK_a value of FeTPPS for the equilibrium between $(H_2O)_2$FeTPPS and the μ-oxo dimer was determined to be 7.6 in 5×10^{-3} M aqueous $NaClO_4$. The analysis of the titration curve with a theoretical equation for the 1:1 complexation provided a K value of $(4.8\pm0.3)\times10^5$ M^{-1}. The continuous-variation method also supports the formation of the 1:1 FeTPPS/ChT complex. The μ-oxo dimer, which coexists with $(H_2O)_2$FeTPPS, dissociates into the monomer as $(H_2O)_2$FeTPPS binds to ChT. Table 1 shows the effect of buffer concentration on K. The marked decrease in K with increasing ionic strength of the system demonstrates that Coulomb interaction is the main binding force in the present system.

Table 1. Effect of buffer concentration on binding constant for the complexation of Fe^{III}TPPS with ChT and binding constants for the complexation of Zn^{II}TPPS and Mn^{III}TPPS with ChT at 25 °C.

Porphyrin	[Phosphate buffer] [M]	K [M^{-1}]
Fe^{III}TPPS	5×10^{-3}	4.8×10^5
Fe^{III}TPPS	1×10^{-2}	2.2×10^5
Fe^{III}TPPS	2×10^{-2}	6.8×10^4
Fe^{III}TPPS	5×10^{-2}	3.0×10^4
Fe^{III}TPPS	1×10^{-1}	1.7×10^4
Fe^{III}TPPS	5×10^{-1}	3.7×10^3
Zn^{II}TPPS	5×10^{-3}	1.6×10^4
Mn^{III}TPPS	5×10^{-3}	5.5×10^3

There is metal selectivity in the complexation of ChT with metalloporphyrins (Table 1). The K value decreases in the order Fe^{III}TPPS (4.8×10^5 M^{-1}) > Zn^{II}TPPS (1.6×10^4 M^{-1}) >

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$Mn^{III}TPPS$ ($5.5\times10^{3}\,M^{-1}$). As the net charges of $Fe^{III}TPPS$ and $Zn^{II}TPPS$ in neutral aqueous solution are −3 and −4, respectively, the strength of the Coulomb interaction does not account for the difference in the *K* values between these two metalloporphyrins. Axial coordination might be another reason for the metal selectivity. In aqueous solution, monomeric FeTPPS exists only in the diaqua form ($(H_2O)_2FeTPPS$) because (OH^-)FeTPPS immediately changes to the μ-oxo dimer.[13,19] If the axial ligand (L) interferes with the interaction between (L)FeTPPS and ChT, the complex formed by the free base, H_2TPPS, and ChT should be more stable than that for FeTPPS. The *K* value determined for H_2TPPS ($6.5\times10^{4}\,M^{-1}$), however, was smaller than that for FeTPPS by one order of magnitude. Therefore, H_2O as the axial ligand does not explain the metal selectivity. Although there is no direct evidence, coordination of a histidine (His) residue of ChT to FeTPPS might be a reason for the characteristically large *K* value for the ChT/FeTPPS system. There are two His residues at the 40- and 57-positions in ChT. Both His residues can bind with FeTPPS. The much smaller *K* value for the ChT/$Mn^{III}TPPS$ pair is understandable because of the lower ability of the manganese(III) porphyrin to bind imidazole in a protic polar solvent.[20]

To examine the structural change in ChT upon binding with FeTPPS, the CD spectral changes of ChT were recorded as a function of FeTPPS concentration (Figure 2a). No distinct CD spectral change was observed when each CD spectrum was recorded immediately after sample preparation; however, the CD spectrum of the ChT/FeTPPS complex changed progressively (see below). CD spectroscopy clearly points out that FeTPPS does not induce a significant change in the secondary structure of ChT unless the mixture is incubated.

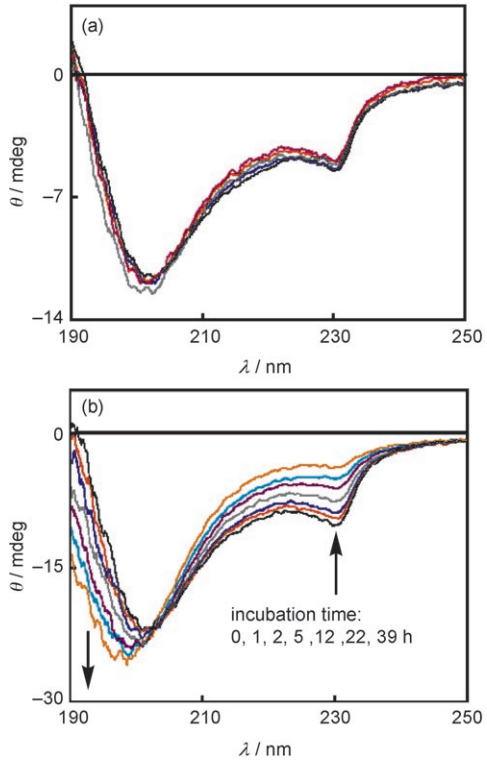

Figure 2. a) CD spectral changes of ChT (5.0×10^{-6} M) upon addition of FeTPPS and b) progressive CD spectral changes of ChT (1.0×10^{-5} M) in the presence of FeTPPS (1.0×10^{-5} M) in 5×10^{-3} M phosphate buffer at pH 7.4 and 25 °C.

Inhibition of ChT Activity by FeTPPS and Recovery by TMe-β-CD

FeTPPS inhibited the ChT-catalyzed hydrolysis of a chromogenic model substrate, SPNA. The hydrolysis was followed by monitoring of the absorbance (*A*) at 410 nm, and then the initial velocity ($\Delta A/\Delta t$) was determined. The effect of FeTPPS on the ChT-catalyzed hydrolysis of SPNA is shown in Figure 3, which also shows the relative activities for the

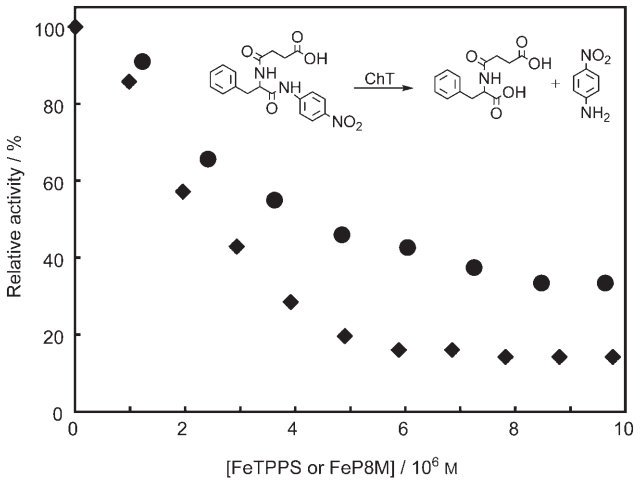

Figure 3. Inhibition profiles for ChT-catalyzed hydrolysis of SPNA in 5×10^{-3} M phosphate buffer containing 3% (*v*/*v*) ethanol at pH 7.4 and 25 °C. $[ChT]_0=5\times10^{-6}$ M, $[SPNA]_0=4\times10^{-4}$ M. FeTPPS (●) and FeP8M (◆) were used as the inhibitors.

SPNA hydrolysis as a function of FeTPPS concentration. The degree of inhibition was saturated at 8 equivalents of FeTPPS, and no complete inhibition was achieved (the maximum degree of inhibition was 63%), which indicates that ChT with FeTPPS embedded still has enzymatic activity. The free-base porphyrin, H_2TPPS, also acted as an inhibitor, as did FeTPPS (see Supporting Information), which indicates that the coordination of the His57 residue at the active site of ChT to FeTPPS does not account for the inhibition effect of this metalloporphyrin. It is considered, therefore, that covering of the active site of ChT by the porphyrin leads to the inhibition.

The enzymatic activity of ChT weakened by FeTPPS was completely recovered by the addition of electronically neutral TMe-β-CD. Figure 4 shows the recovery behavior of TMe-β-CD in the ChT-catalyzed hydrolysis of SPNA inhibited by FeTPPS. For the freshly prepared sample, 2 equivalents of FeTPPS to ChT inhibited the hydrolysis to 40% rel-

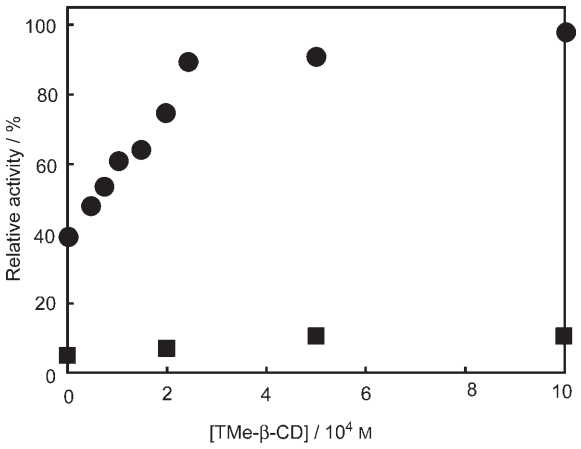

Figure 4. Recovery of ChT activity by TMe-β-CD for freshly prepared (●) and incubated (■) samples. $[ChT]_0 = 5 \times 10^{-6}$ M, [FeTPPS] = 1×10^{-5} M, $[SPNA]_0 = 4 \times 10^{-4}$ M in 5×10^{-3} M phosphate buffer containing 3% (*v*/*v*) ethanol at pH 7.4 and 25 °C. The incubation time was 36 h.

ative activity. The addition of 5 equivalents of TMe-β-CD to FeTPPS resulted in complete recovery of ChT activity. It is known that TMe-β-CD forms extremely stable 1:1 and 2:1 TMe-β-CD/FeTPPS complexes ($K_1 = 1.26 \times 10^6$ M^{-1}, $K_2 = 6.3 \times 10^4$ M^{-1}).[21] The inclusion of FeTPPS by TMe-β-CD triggered the detachment of FeTPPS on the surface of ChT. An increase in the ionic strength of the system is a common way of detaching anionic effectors from the surface of a cationic protein.[3c,d,6] However, such a method cannot be applied in vivo. Our method might be useful in vivo because TMe-β-CD is a less toxic, chemically modified cyclic oligosaccharide.

Incubation Effect

The relative activity of ChT for SPNA hydrolysis in the presence of 2 equivalents of FeTPPS was around 40%. The relative activity decreased to 7% when the mixed solution of ChT/FeTPPS was incubated for 38 h (Figure 5). TMe-β-CD could not restore the activity of ChT in the incubated solution. Such an incubation effect can be interpreted in terms of irreversible denaturation of ChT caused by FeTPPS. Figure 2b shows the CD spectral changes of ChT in phosphate buffer at pH 7.4 containing 1 equivalent of FeTPPS as a function of incubation time. ChT shows a characteristic CD band at 230 nm that is a measure of ChT activity.[22] Upon incubation of the solution of ChT/FeTPPS, the negative CD band at 230 nm diminished, and the negative CD band at 198 nm due to the random-coil structure of ChT strengthened. Figure 6 shows the progressive changes in the induced CD spectrum of FeTPPS bound to ChT during incubation. The minus-to-plus bisignate CD Cotton effect was observed at the Soret band of FeTPPS, and the CD signal intensities gradually increased with incubation time. Such a bisignate CD Cotton effect can be interpreted in terms of the asymmetrical overlapping of two porphyrin rings.[23] These CD spectral changes suggest that the irreversible denaturation of ChT is induced by aggregation of ChT initiated by binding with FeTPPS. FeTPPS probably binds to ChT gradually and slightly withdraws a hydrophobic part of ChT, thus leading to slow self-aggregation of ChT through hydrophobic interaction. Self-aggregation of ChT is known to occur during denaturation.[24,25]

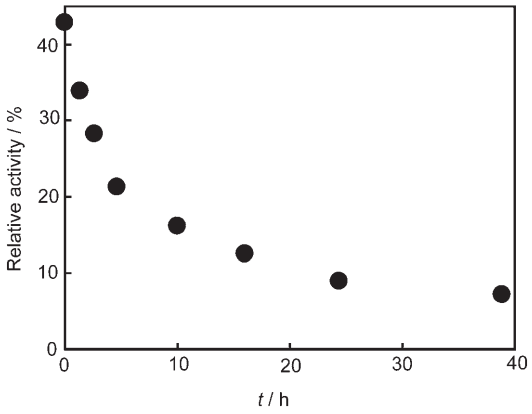

Figure 5. Effect of incubation time on ChT (5×10^{-6} M) activity inhibited by FeTPPS (1×10^{-5} M) in 5×10^{-3} M phosphate buffer containing 3% (*v*/*v*) ethanol at pH 7.4 and 25 °C. The activity of ChT inhibited by FeTPPS was further lowered upon incubation.

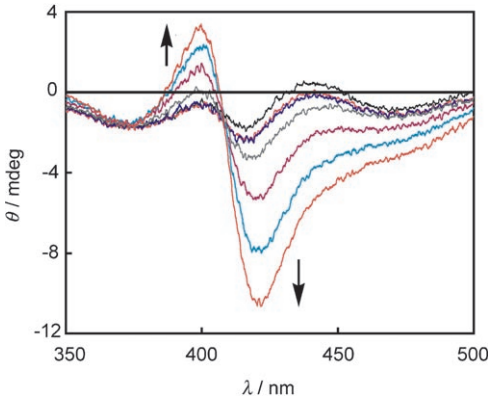

Figure 6. Effect of incubation time on the induced CD spectrum of FeTPPS (1×10^{-5} M) bound to ChT (1×10^{-5} M) in 5×10^{-3} M phosphate buffer at pH 7.4 and 25 °C. Incubation time = 0, 1, 2, 5, 12, 22, and 39 h.

As TMe-β-CD did not restore the activity of denatured ChT, the FeTPPS-induced denaturation should be irreversible. On the basis of the UV/Vis spectrum, it was found that FeTPPS (λ_{max} of FeTPPS bound to ChT = 410 nm) was detached from the surface of ChT by forming the inclusion complex with TMe-β-CD (λ_{max} of the 2:1 TMe-β-CD/FeTPPS complex = 415 nm).[13] However, the activity as well as the CD spectrum of ChT did not return to original levels by TMe-β-CD. A very small amount of FeTPPS (e.g., 1 equiv of FeTPPS) led to the irreversible denaturation of ChT. To denature ChT by urea, a well-known denaturing agent, several molar amounts of urea must be added.[24,26] Relative to urea, FeTPPS is a strong denaturant whose de-

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naturation ability is similar to that of carboxylate-embedded nanoparticles.[4c]

An Improved Inhibitor

Although FeTPPS and TMe-β-CD acted as the inhibitor and the repairing agent, respectively, during the ChT-catalyzed hydrolysis of SPNA, FeTPPS did not show sufficiently strong inhibitory power and induced the irreversible denaturation of ChT. Next, we designed a new inhibitor, octaanionic FeP8M, in which the distance between the opposite carboxylate groups is 2.8 nm (Figure 1). The expectation for FeP8M is as follows. FeP8M might bind to ChT through Coulomb interactions that are stronger than those for the FeTPPS/ChT complexation because of the well-matched distance between the CO_2^- groups of FeP8M for interacting with the cationic parts of ChT. Furthermore, electrostatic repulsion between CO_2^- groups of the FeP8M molecules that are not used for association with ChT might interfere with the self-aggregation of ChT, which would result in prevention of the irreversible denaturation of ChT. pH titration monitored by UV/Vis spectroscopy showed the pK_a value for the diaqua–monohydroxo (or diaqua–μ-oxo-dimer) equilibrium of FeP8M to be 8.4 in 5×10^{-3} M aqueous $NaClO_4$. To evaluate the K value for the complexation of FeP8M with ChT, the UV/Vis spectral changes of FeP8M were recorded as a function of ChT concentration (see Supporting Information). The titration curve could not be analyzed by the equation for the 1:1 complexation, but could be analyzed with the equation for the 1:1 and 2:1 ChT/FeP8M complex formation to give K_1 and K_2 values of $(2.0\pm0.4)\times10^8$ and $(1.0\pm0.5)\times10^6\,M^{-1}$, respectively. As expected, the binding affinity of FeP8M for ChT is much higher than those of FeTPPS ($K=4.8\times10^5\,M^{-1}$), polyanionic calixarenes ($1.23\times10^6\,M^{-1}$),[3] a dendrimer ($5\times10^5\,M^{-1}$),[7b] and gold nanoparticles (10^6–$10^7\,M^{-1}$).[4e]

The relative activities of ChT for SPNA hydrolysis as a function of FeP8M concentration are shown in Figure 3. The inhibition behavior of FeP8M is more distinct than that of FeTPPS. Even so, the inhibition by FeP8M was saturated at 1 equivalent of FeP8M, at which the degree of inhibition was 85%. Klaikherd et al. studied the effect of anionic dendrimers on ChT catalysis and reported that anionic dendrimers inhibit the catalysis for an anionic substrate but accelerate the ChT-catalyzed hydrolysis of a cationic substrate.[7a] A similar substrate–charge dependency was observed for amide hydrolysis catalyzed by ChT sitting on the gold nanoparticles functionalized by an anionic amino acid amphiphile.[4g] These results suggest that an effector with a flexible skeleton does not strictly block the active site of ChT. The cationic substrate seems to be gathered by the anionic effector and slipped into the hydrophobic pocket (active site) of ChT, thus leading to enhanced substrate capture. In contrast, electrostatic repulsion causes poor capture of the anionic substrate by ChT.[4g] In the case of FeP8M, however, the anionic parts of this inhibitor are located far from the active site of ChT (Figure 1). Therefore, blocking of the active site of ChT by the rigid porphyrin skeleton should be the reason for the inhibition by FeP8M as well as FeTPPS.

Notably, the incubation did not have any effect on the ChT-catalyzed hydrolysis of SPNA in the presence of FeP8M, whereas a significant incubation effect was found in the FeTPPS/ChT system. Preincubated solutions of a mixture of ChT and FeP8M showed the same enzymatic activities as nonincubated ones, which indicates that FeP8M does not induce the denaturation of ChT. No CD spectral change was recorded when the ChT/FeP8M mixed solution was incubated for a long time (see Supporting Information). On the basis of the K_1 and K_2 values for the complexation of FeP8M with ChT, only the 1:1 complex is meaningful under these conditions. Therefore, the denaturation process of the 1:1 complex must be considered. In this complex, some carboxylate groups of FeP8M might not be used for electrostatic binding with ChT. These remaining carboxylate groups seem to play an important role in the stabilization of ChT by preventing the self-aggregation of ChT. This behavior of FeP8M resembles the effect of the oligo(ethylene glycol) chain in functionalized gold nanoparticles[27] and that of an amphiphilic polystyrene with carboxymethyloxy and benzyloxy groups on the phenyl rings.[6] We assume that the hydrophobic interaction between the porphyrin ring and ChT induces an initial but slight unfolding of the protein followed by extended self-aggregation of the protein that results in irreversible denaturation. The free carboxylate groups of FeP8M bound to ChT might inhibit the extended self-aggregation of the slightly denatured ChT.

The catalysis of ChT inhibited by FeP8M was recovered upon the addition of TMe-β-CD and TMe-γ-CD (Figure 7). The relative activity of ChT, diminished by FeP8M to 15%, was recovered to 67 and 57% upon the addition of TMe-β-CD (1000 equiv to FeP8M) and TMe-γ-CD (43 equiv), respectively. UV/Vis spectroscopic titrations produced K_1 and K_2 values of (63 ± 8) and $(7.5\pm1.1)\times10^5\,M^{-1}$, respectively, for the complexation of FeP8M with TMe-β-CD (see Supporting Information). Meanwhile, the K_1 and K_2 values for TMe-γ-CD were $(9.9\pm0.7)\times10^5$ and $(4.1\pm0.4)\times10^4\,M^{-1}$, respectively. Although TMe-γ-CD, which has a larger cavity than TMe-β-CD, was the much better repairing agent, no complete recovery of ChT activity was achieved. The energy-minimized structure of the FeP8M/TMe-β-CD complex is shown in Figure 8. As the lengths of the peripheral substituents of FeP8M (1.2 nm) are much longer than the depth of the cyclodextrin cavity (≈0.84 nm), the terminal carboxylate groups that protrude from the cyclodextrin cavity as well as the carboxylate groups of the phenyl groups that are not included by the cyclodextrin can interact with the cationic parts of ChT, thus resulting in weak inhibition. The FeP8M/ChT complex is so stable ($K_1=2\times10^8$, $K_2=1\times10^6\,M^{-1}$) that a large amount of the cyclodextrin is needed to withdraw FeP8M from the ChT surface. Furthermore, the depth of the cyclodextrin cavity is unchangeable. Reluctantly, a polycationic cyclodextrin, heptakis(6-amino-6-deoxy)-β-cyclodextrin in protonated form (per-NH_3^+-β-CD), was then used in place of the neutral per-*O*-methylated cy-

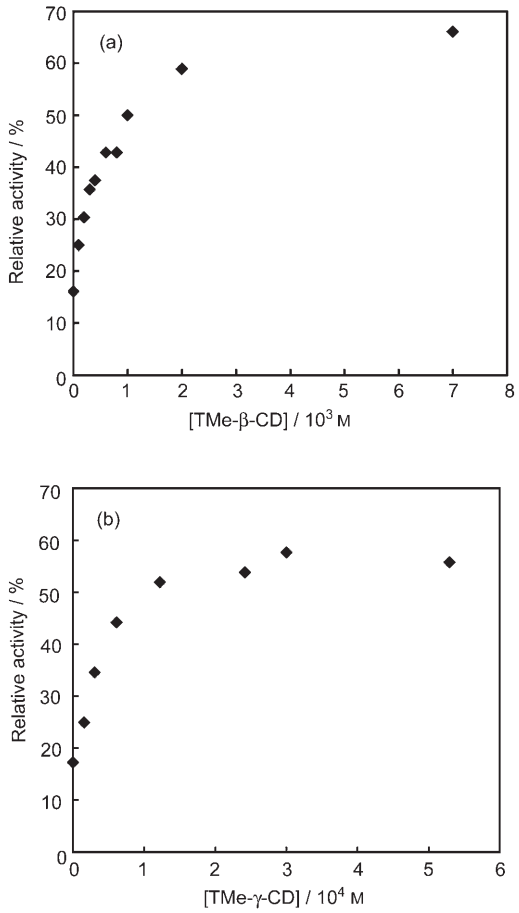

Figure 7. Recovery behavior of a) TMe-β-CD and b) TMe-γ-CD toward ChT (5×10^{-6} M) activity inhibited by FeP8M (7×10^{-6} M) in 5×10^{-3} M phosphate buffer containing 3% (v/v) ethanol at pH 7.4 and 25°C.

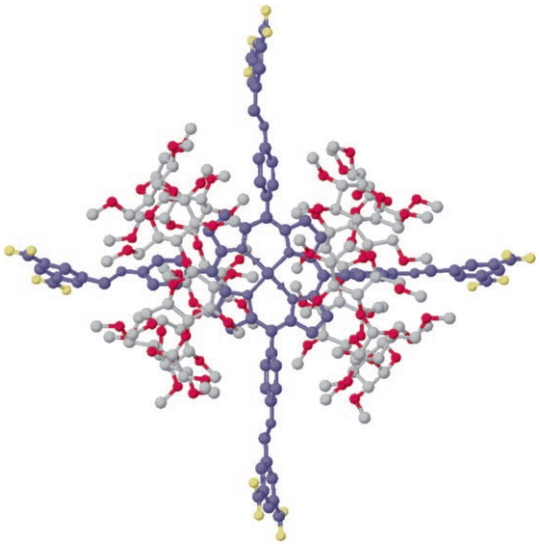

Figure 8. Energy-minimized structure of the 1:2 FeP8M/TMe-β-CD complex obtained from MM2 calculations with BioMedCAChe 6.0. Yellow represents the oxygen atoms of the carboxylate groups of FeP8M (blue).

clodextrins. The relative activity of ChT, lowered by FeP8M to 15%, was recovered to 75% upon the addition of 3.5 equivalents of per-NH_3^+-β-CD to FeP8M. The addition of 17 equivalents of per-NH_3^+-β-CD enhanced the catalysis of ChT to 160% relative activity. The pK_a values of the NH_3^+ groups of per-NH_3^+-β-CD are 6.9–8.5.[28] Therefore, both the NH_2 and NH_3^+ groups coexist under the experimental conditions. Per-NH_3^+-β-CD seems to detach FeP8M from the surface of ChT owing to the formation of the electrostatic association complex. The hyperactivation effect[5,12] of an excess amount of per-NH_3^+-β-CD might explain the enzymatic activity of ChT enhanced by per-NH_3^+-β-CD.

Kinetics

The enzymatic reaction [Eq. (1)] is kinetically represented by the Michaelis–Menten equation [Eq. (2)]:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} P + E \quad (1)$$

$$v = \frac{k_{cat}[E]_0[S]}{K_M + [S]} \quad (2)$$

in which S, E, ES, and P are the substrate, the enzyme, the enzyme–substrate complex, and the product, respectively, v is the rate of the reaction, k is the rate constant, and K_M is the Michaelis constant. We used Lineweaver–Burk plots [Eq. (3)] to determine k_{cat} and K_M for the ChT-catalyzed hydrolysis of SPNA in the absence and presence of the inhibitors.

$$\frac{1}{v_0} = \frac{K_M}{k_{cat}[E]_0[S]_0} + \frac{1}{k_{cat}[E]_0} \quad (3)$$

From the linear relationship between v_0^{-1} and $[S]_0^{-1}$, the k_{cat} and K_M values for the freshly prepared samples were evaluated from the vertical intercept and the slope, respectively. The reaction conditions in the presence of FeTPPS and FeP8M were arranged in such a way that the inhibition degree of each system was 50%. The results are summarized in Table 2. The k_{cat} values are almost the same in all cases, which means that ChT bound with either FeTPPS or FeP8M did not change its conformation. As $K_M = (k_{-1} + k_{cat})/k_1$, K_M corresponds to the dissociation constant of the enzyme–substrate complex if $k_{cat} \gg k_{-1}$. The K_M values for the inhibitory systems are 2.3–2.5 times higher than that for the system without the inhibitor. The kinetic data show that both

Table 2. Kinetic parameters for hydrolysis of SPNA by ChT (5×10^{-6} M) in the absence and presence of FeTPPS (5×10^{-6} M) and FeP8M (2.5×10^{-6} M) in 5×10^{-3} M phosphate buffer at pH 7.4 and 25°C.

System	10^6K_i [M][a]	10^3K_M [M]	k_{cat} [min^{-1}]
ChT	–	0.88±0.16	0.28±0.03
ChT/FeTPPS	2.1	2.04±0.14	0.27±0.01
ChT/FeP8M	5.0×10^{-3}, 1.0	2.39±0.49	0.26±0.01

[a] The inhibition constant represented by $K_i = [E][I]/[EI]$ corresponds to the inverse of the binding constant for the complexation of ChT with the inhibitor.

FeTPPS and FeP8M inhibit the binding of the substrate to ChT by covering the active site, and the conformational change of ChT caused by the iron porphyrin does not account for the inhibition.

Conclusions

We have proposed a new methodology for regulating the function of ChT. Rigid, planar, macrocyclic, amphiphilic porphyrinato iron(III) complexes, such as FeTPPS and FeP8M, inhibit the enzymatic activity of ChT. The inhibited activity of ChT, however, was restored by detaching the iron porphyrin by electronically neutral per-*O*-methylated cyclodextrin. In the ChT/FeTPPS system, the irreversible denaturation gradually proceeded, and TMe-β-CD, which is a strong remover of FeTPPS, did not restore the function of ChT. Meanwhile, FeP8M did not induce the irreversible denaturation of ChT; therefore, this iron porphyrin seems to be the better inhibitor. A remaining problem is the poor recovery of ChT activity when FeP8M is detached by TMe-γ-CD. Improved inhibitors might be designed on the basis of the present results.

Experimental Section

Materials

FeTPPS[14] and ZnTPPS[15] were prepared and purified according to procedures described elsewhere. ChT (type II from bovine pancreas, Sigma, C4129), SPNA (Sigma), TMe-β-CD (Nacalai), and 5,10,15,20-tetrakis(*p*-sulfonatophenyl)porphyrinato manganese(III) chloride in acidic form (Frontier Scientific) were purchased and used as received. TMe-γ-CD was prepared according to procedures in the literature that describe the preparation of TMe-α-CD.[16] Water was purified by using Millipore Simpak 1.

Instruments

UV/Vis spectra were recorded on a Shimadzu UV-2100 spectrophotometer with a thermostatic cell holder. CD spectra were recorded on a Jasco J-820 spectropolarimeter with a 1-cm optical path. pH values were measured with a Horiba M-12 pH meter. ^{1}H NMR spectra were recorded on a JEOL JNM-A400 spectrometer (400 MHz). Elemental analysis was performed at the Center for Organic Elemental Microanalysis, Kyoto University.

Syntheses

Tetrakis[4-(3,5-diethoxycarbonylphenylmethoxy)phenyl]porphyrin (P8ester): A mixture of 5-bromomethyldiethylisophthalate[17] (1 g, 3.2 mmol), 5,10,15,20,-tetrakis(4-hydroxyphenyl)porphyrin (100 mg, 0.15 mmol), and K_2CO_3 (1 g) in dry *N*,*N*-dimethylformamide (DMF; 20 mL) was stirred at 75 °C for 15 h under N_2. After the reaction, K_2CO_3 was removed by filtration. Excess methanol was added to the reaction mixture, and the purple precipitates were collected and purified by silica-gel column chromatography with $CHCl_3$. Yield: 72 %. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = -2.83$ (s, 2H, NH), 1.41 (t, $J = 4$ Hz, 24H, $CO_2CH_2CH_3$), 4.42 (q, $J = 4$ Hz, 16H, $CO_2CH_2CH_3$), 5.36 (s, 8H, OCH_2), 7.31 (d, $J = 8$ Hz, 8H, phenyl), 8.08 (d, $J = 8$ Hz, 8H, phenyl), 8.44 (s, 8H, diethoxycarbonylphenyl), 8.67 ppm (s, 8H, diethoxycarbonylphenyl), 8.80 (s, 8H, β-pyrrole).

Tetrakis[4-(3,5-diethoxycarbonylphenylmethoxy)phenyl]porphyrinato iron(III) (FeP8ester): A mixture of P8ester (70 mg) and $FeCl_2$ (400 mg) in DMF (35 mL) and CH_2Cl_2 (25 mL) was stirred at 80 °C for 2 h in the dark under N_2. The progress of the reaction was monitored by fluorescence spectroscopy. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (250 mL). The solution was washed with saturated aqueous NaCl (3 × 200 mL) and then dried over Na_2SO_4. A dark-purple solid was obtained after evaporation of the solvent. Yield: 97 %.

FeP8M: A solution of FeP8ester (70 mg) and KOH (200 mg) in water (5 mL), methanol (10 mL), and CH_2Cl_2 (5 mL) was stirred at 80 °C for 5 h. The organic solvents were evaporated, and the aqueous residue was acidified with aqueous HCl to pH 1. The resulting solution was cooled by ice, and the precipitates of FeP8M were collected by filtration. Yield: 98 %. Elemental analysis: calcd (%) for $C_{80}H_{51}FeN_4O_{20}\cdot 5H_2O$: C 62.63, H 4.01, N 3.65; found: C 62.48, H 4.12, N 3.57.

Enzymatic Activity Assay

The ChT-catalyzed hydrolysis of SPNA was carried out by adding a stock solution (90 μL) of SPNA in ethanol to a solution of ChT/FeTPPS (3 mL). The final concentration of SPNA was 4×10^{-4} M. The time courses of the reactions were monitored by following the absorbance at 410 nm due to *p*-nitroaniline ($\varepsilon = 10800\ M^{-1}cm^{-1}$). The concentration of ChT was determined by using the extinction coefficient of $5.1 \times 10^4\ M^{-1}cm^{-1}$ at 280 nm.[18]

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