Catalytic Activity of the Serine Proteases α-Chymotrypsin and α-Lytic Protease Tagged at the Active Site with a (Terpyridine)platinum(II) Chromophore†

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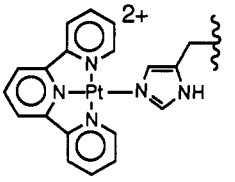
ABSTRACT: Incubation of α-chymotrypsin and α-lytic protease with chloro(2,2′:6′,2″-terpyridine)platinum(II), $[Pt(trpy)Cl]^+$, results in attachment of $Pt(trpy)^{2+}$ tags at both His 57 and His 40 in the former and His 57 in the latter. The $[Pt(trpy)His]^{2+}$ chromophores are readily detected and quantitated owing to their characteristic, strong UV absorption. Although the tagging of His 57 modifies the catalytic triad (Ser 195, His 57, and Asp 102) and disrupts the charge relay, the platinated enzymes retain significant esterase and amidase activity for both specific and nonspecific substrates. Unlike suicide inhibitors, which inactivate the enzymes by filling the active site and imitating the tetrahedral intermediate, $[Pt(trpy)Cl]^+$ reacts with a particular amino acid and permits binding of substrates. The kinetic constants for the following are reported: two esters and two amides with α-chymotrypsin and an amide with α-lytic protease. The k_{cat} values are between 1 and 25% of, and the K_m values are a little higher than, the corresponding values for the native enzymes. The catalytic activity is not due to the native enzymes, trypsin, or some zinc-containing protease. Activities of the native and of the platinated α-chymotrypsin depend similarly on pH although the pK_a of His 57 is raised to 9.7 upon platination. The platinated enzymes undergo autodigestion slower than do the native enzymes. Because the $Pt(trpy)^{2+}$ tags are noninvasive, stable, and yet easily removable by thiourea, $[Pt(trpy)Cl]^+$ may be used to retard autodigestion of stored proteolytic enzymes.

Serine proteases, and α-chymotrypsin in particular, have been much studied by various methods (Schowen, 1989; Matthews et al., 1967; Tulinsky & Blevins, 1987; Steitz et al., 1969; Kraut, 1977, 1988; Landis & Berliner, 1980; Wu & Hilvert, 1989; Smith et al., 1989). All of these enzymes contain a triad of Ser, His, and Asp at the active site. Their proteolytic action is commonly explained by the charge-relay mechanism, for which all these three residues are essential (Blow, 1976). But very recent theoretical calculations of electrostatic energies in proteins (Warshel & Russell, 1986; Warshel et al., 1989) and biochemical studies have cast doubt on this mechanism. Methylation of His 57 in α-chymotrypsin (Henderson, 1971) and even removal (by mutagenesis) of this residue and of the entire triad in subtilisin (Carter & Wells, 1987, 1988; Craik et al., 1987) do not completely inactivate these enzymes.

Histidine 57 of α-chymotrypsin has been modified irreversibly with various organic reagents (Henderson, 1971; Schoellmann & Shaw, 1962; Poulos et al., 1976; Nakagawa & Bender, 1969, 1970), but only the methylated α-chymotrypsin has been studied for its enzymatic activity (Henderson, 1971; West et al., 1988; Scholten et al., 1988; Byers & Koshland, 1978). We have recently shown that histidine residues in proteins can be labeled selectively and noninvasively with the new inorganic reagent, chloro(2,2′:6′,2″-terpyridine)platinum(II) or $[Pt(trpy)Cl]^+$ (Kostić, 1988; Ratilla et al., 1987; Brothers & Kostić, 1988). Displacement of the Cl^- ligand of the imidazole group yields the complex $[Pt(trpy)His]^{2+}$, which is easily detected and quantitated in the protein because of its strong, characteristic UV absorption spectrum. Although the complex is stable both kinetically and thermodynamically, the $Pt(trpy)^{2+}$ tag can easily be removed from the protein by treatment with ligands more nucleophilic than imidazole (Ratilla et al., 1987). This combination of noninvasiveness, detectability, stability, and removability makes $[Pt(trpy)Cl]^+$ well suited to investigation of enzymes. We used the new reagent with advantage to study the esterase and amidase activity of α-chymotrypsin and α-lytic protease.



$[Pt(trpy)His]^{2+}$

Experimental Procedures

Chemicals. Bovine α-chymotrypsin (type II), PMSF,[1] TPCK, BTEE, ATEE, BAPNA, AAPNA, *N*-succinyl-AAPF-*p*-nitroanilide, *N*-acetyl-AAPA-β-naphthylamide, proflavin, and lima bean trypsin inhibitor for affinity chromatography were obtained from Sigma Chemical Co. Pure α-lytic protease was donated by W. W. Bachovchin. Chloro(2,2′,6′,2″-terpyridine)platinum(II) chloride dihydrate, $[Pt(trpy)Cl]Cl\cdot 2H_2O$, was obtained from Strem Chemicals; it is easily synthesized (Howe-Grant & Lippard, 1980) and is now available from Aldrich Chemical Co. (*Aldrichimica*

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ATEE, *N*-acetyl-L-tryptophan ethyl ester; BAPNA, N^{α}-benzoyl-DL-arginine-*p*-nitroanilide; AAPNA, *N*-acetyl-L-alanine-*p*-nitroanilide; *N*-succinyl-AAPF-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; *N*-acetyl-AAPA-β-naphthylamide, *N*-acetyl-L-Ala-L-Ala-L-Pro-L-Ala-β-naphthylamide; Tris, tris(hydroxymethyl)aminomethane; *A*, absorbance; Im, imidazole; EDTA, ethylenediaminetetraacetic acid; DIFP, diisopropyl fluorophosphate.

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Acta, 1988). Amicon YM-5 membranes for ultrafiltration had a cutoff limit at the molecular mass of 5 kDa. Tris buffer was 50 mM and was adjusted with H_2SO_4, usually to pH of 8.0. Sodium acetate buffer was 20 mM and had a pH of 5.0. Sodium phosphate buffer usually was 85 mM and usually had a pH of 7.0. Different concentrations and pH values are specified in particular procedures. The Fisher Accumet 805 pH meter was calibrated at 4.00 and 10.00.

α-Chymotrypsin. Ultrafiltration of the commercial preparation with 20 mM sodium acetate buffer of pH 5.0 and spectrophotometric measurements (with IBM 9430) at 280 nm, where the enzyme absorptivity is 50 000 M^{-1} cm^{-1} (van Iersel et al. 1985), showed that α-chymotrypsin constituted 49% of the original dry weight. Because two different commercial lots have nearly identical activities per milligram of solid, only one lot was analyzed for the protein content. One milligram of the commercial preparation was chromatographed on a 1 × 10 cm column of lima bean trypsin inhibitor (Ako et al., 1972) previously equilibrated with Tris buffer. Peptides and salts were eluted in the void volume with this buffer, and the enzyme was eluted with 100 mM solution of Na_2SO_4 adjusted to pH 3.0 with H_2SO_4; both solvents flowed at 20 mL h^{-1}.

α-Lytic Protease. The enzyme was used without further purification. Its concentration was determined by the absorbance at 280 nm, where the absorptivity is 19 400 M^{-1} cm^{-1} (Whitaker, 1970).

Enzyme Labeling and Purification. The platinum reagent was always used as a 30 mM aqueous solution, containing 16.1 mg of [Pt(trpy)Cl]Cl·$2H_2O$/mL. In order to retard autodigestion of α-chymotrypsin, its labeling was done at pH 5.0. To a solution containing 25 mg of the commercial preparation in 25 mL of acetate buffer was added 1.60 mL of the stock solution of [Pt(trpy)Cl]Cl. After 1 day at room temperature the reaction was stopped, and the small molecules were removed, by ultrafiltration into Tris buffer. The platinated enzyme was purified by affinity chromatography as explained above, except the second eluent had a pH of 3.5. The solution of the purified platinated enzyme was frozen at −5 °C and thawed at 4 °C immediately before use in other experiments.

Labeling of α-lytic protease was done in 50 mM Tris buffer of pH 8.0. All other incubation conditions were the same as for α-chymotrypsin. To a solution containing 1.2 mg of α-lytic protease in 1.0 mL of Tris buffer was added 100 μL of the stock solution of [Pt(trpy)Cl]Cl. The reaction was stopped after 1 day, and the platinated enzyme was frozen at −5 °C.

To inactivate α-chymotrypsin, 750 μL of a 2.50 mM solution of TPCK in methanol was added to a solution containing 2.50 mg of the commercial preparation in 25.0 mL of phosphate buffer of pH 6.1. To block the active site, 2.50 mL of a 10 mM solution of BTEE (a substrate) or 100 mL of a 50 mM solution of indole (an inhibitor) in methanol was added to 25.0 mL of a 40 μM solution of the commercial preparation in phosphate buffer of pH 7.5 for the substrate and in acetate buffer of pH 5.0 for the inhibitor. The inactivated, substrate-bound, or inhibited α-chymotrypsin was then incubated with 160 μL (for TPCK) or 1.67 mL (for BTEE and for indole) of the stock solution of [Pt(trpy)Cl]Cl. In the case of TPCK and indole, this incubation lasted for 1 day. In the case of BTEE, it lasted for 2 h, and fresh 2.00-mL portions of the substrate solution were added every 30 min. In another attempt to block the active site, 1.25 mL of a 2 mM solution of proflavin in water was added to 20 mL of a 50 μM solution of α-chymotrypsin in 20 mM acetate buffer of pH 5.0. The inhibited enzyme was then incubated with 1.67 mL of the stock solution of [Pt(trpy)Cl]Cl for 18 h.

Quantitation and Stability of the [Pt(trpy)His]$^{2+}$ Tags. The enzymes, TPCK, and the [Pt(trpy)His]$^{2+}$ chromophore all absorb at 280 nm, but only the last chromophore absorbs at 342 nm. Since the absorptivities of all three are known (Ratilla et al., 1987; van Iersel et al., 1985; Whitaker, 1970), the number of tags, n, could be calculated from the absorbances (A) at these two wavelengths; see eq 1. The absorptivities ϵ_{280} (in M^{-1} cm^{-1}) are as follows: 50 000 for α-chymotrypsin, 52 800 for α-chymotrypsin first modified with TPCK, and 19 400 for α-lytic protease. Incubation, ultrafiltration, and spectrophotometry showed that the Pt(trpy)$^{2+}$ tags remain attached to α-chymotrypsin in the pH range 1.5–12, in buffers containing 3% of methanol or 10% of DMSO, and after purification by affinity chromatography.

$$n = \frac{\epsilon_{280} A_{342}}{14000 A_{280} - 19000 A_{342}} \quad (1)$$

The strong metal-to-ligand charge-transfer bands of the [Pt(trpy)His]$^{2+}$ chromophore are at 342 and 328 nm; the enzymes do not absorb at either wavelength. These absorption maxima permit spectrophotometric quantitation of the Pt(trpy)$^{2+}$ tag attached to the proteins. The A_{342}/A_{328} quotients for 10 μM solutions of [Pt(trpy)Im]Cl_2 in 20%, 40%, 60%, and 80% mixtures by volume of dioxane and water were 1.72, 1.64, 1.39, and 0.68, respectively.

Activity and Kinetics. The α-chymotrypsin activity at a single concentration of BTEE was expressed as the number of micromoles of this substrate hydrolyzed by 1.0 mg of enzyme/min at pH 8.0 at room temperature. The α-lytic protease activity was measured at a single concentration of *N*-acetyl-AAPA-β-naphthylamide and was expressed in the same manner as the α-chymotrypsin activity. The Lineweaver–Burk kinetic assays for α-chymotrypsin were done with BTEE, ATEE, BAPNA, and *N*-succinyl-AAPF-*p*-nitroanilide, whose hydrolyses in buffered solutions, at 25.0 ± 0.1 °C, were monitored at 256, 300, 410, and 410 nm, respectively. In the assays with BTEE and ATEE, the α-chymotrypsin concentration was 0.5 μM, the substrate concentrations varied between 55 and 500 μM, and the buffer contained 1.5% by volume of methanol. In the assays with BAPNA, the enzyme concentration was 1 μM, the concentration of the L-BAPNA (half of the racemic amide) varied between 180 μM and 1.25 mM, and the buffer contained 5% by volume of DMSO. In the assays with the tetrapeptide, the enzyme concentration was 0.1 μM, the substrate concentration varied between 55 and 500 μM, and the buffer contained 2.5% by volume of DMSO. The Lineweaver–Burk kinetic assays for α-lytic protease were done with AAPNA and *N*-acetyl-AAPA-β-naphthylamide. In the assays with AAPNA, the enzyme concentration was 1.5 μM, the substrate concentrations varied between 278 μM and 2.5 mM, and the buffer contained 2.5% by volume of DMSO. In the assays with the tetrapeptide, the enzyme concentration was 0.2 μM, the substrate concentration varied between 55 and 500 μM, and the buffer contained 2.5% by volume of DMSO. The data were fitted to a hyperbolic curve (Cleland, 1979). The error bounds reported for the k_{cat} and K_m values are those from individual Lineweaver–Burk plots; the error bounds for the averages from different plots, obtained with different batches of the enzyme, were greater.

Before all assays the platinated enzymes were incubated with a 3-fold molar excess of PMSF for 3 h at 4 °C, and PMSF was present throughout the measurements. The platinated enzymes for some assays were also incubated with a 50-fold molar excess of [Pt(trpy)Cl]Cl for 18 h at 4 °C, and this

compound was present (together with PMSF) throughout the measurements. The reaction mixtures for some assays were made 1 mM in EDTA.

Assays with BTEE were done in acetate buffer of pH 5.0, in phosphate buffers of pH 6.0–7.5, in Tris buffers of pH 8.0 and 8.5, and in carbonate buffer of pH 9.0. The ionic strength always was 50 mM.

Binding of proflavin to the native and to the platinated α-chymotrypsin was monitored spectrophotometrically in a solution that was 22 μM in this dye and 32 μM in the enzyme (Bernhard et al., 1966). Mixtures that were 100 μM in proflavin and 10 μM in [Pt(trpy)Cl]Cl or in [Pt(trpy)His]Cl_2, with Tris buffer as a solvent, were monitored spectrophotometrically for 1 day. Some assays for the ATEE hydrolysis by the platinated α-chymotrypsin were done in the absence and presence of 50 and 100 μM proflavin.

Acid–Base Titrations. The titrations were followed by measuring both pH and the UV spectra. All solutions were in water. A 10 μM solution of platinated α-chymotrypsin at pH 7.50 was taken to pH 1.90 with 50.0 mM H_2SO_4 and to pH 12.5 with 100.0 mM NaOH. A 15.0 mM solution of [Pt(trpy)Im]Cl_2, whose ionic strength was kept at 200 mM with Na_2SO_4, was taken to pH 11.32 with 100.0 mM NaOH, to pH 3.24 with 100.0 mM HCl, and to pH 1.0 with 50.0 mM H_2SO_4. The last two titrations yielded identical UV spectra. All the absorbance changes proved reversible.

Removal of the Pt(trpy)$^{2+}$ Tags. A 40 μM solution of the platinated α-chymotrypsin in Tris buffer was made 20 mM in thiourea, kept for 30 min at 4 °C, ultrafiltered, and examined spectrophotometrically.

Computer Graphics. Steric interactions between the Pt(trpy)$^{2+}$ tag, whose platinum atom is placed at 2.30 Å from the $N^{\epsilon 2}$ atom of His 57, and its environment in α-chymotrypsin were examined with the program FRODO 6.3 and an Evans and Sutherland PS 300 terminal. The van der Waals radii of H, C, N, O, and Pt were set at 1.2, 1.7, 1.6, 1.5, and 1.7 Å, respectively. The overlap between the van der Waals spheres was minimized visually.

Results and Discussion

Binding Sites and Labeling. Our new reagent, [Pt(trpy)Cl]$^+$, reacts only with the cysteine and histidine side chains (Kostič, 1988; Ratilla et al., 1987; Brothers & Kostič, 1988). Since all the cysteine residues in both α-chymotrypsin and α-lytic protease form disulfides (Birktoft & Blow, 1972; Brayer et al., 1979), toward which [Pt(trpy)Cl]$^+$ is unreactive, the only potential binding sites are His 40 and His 57 in α-chymotrypsin and His 57 in α-lytic protease. All of these residues are on the protein surface, and His 57 belongs to the catalytic triad at the active site.

The labeling was achieved simply by incubation under mild conditions. The progress of the reaction over time was followed by occasional measurements of the enzymatic activity toward BTEE as a substrate for α-chymotrypsin and toward *N*-acetyl-AAPA-β-naphthylamide as a substrate for α-lytic protease. The activity of α-chymotrypsin decreased to ca. 2% of the native value already after 5 h, and the activity of α-lytic protease decreased to ca. 40% of the native value after 1 day. The incubation was prolonged to 1 day for α-chymotrypsin and to 2 days for α-lytic protease to ensure complete labeling.

Since both enzymes are transparent at wavelengths greater than ca. 300 nm (Figure 1A), the Pt(trpy)$^{2+}$ chromophore is easily detected by UV spectrophotometry. Comparison between panels B and C of Figure 1 shows the presence of [Pt(trpy)His]$^{2+}$ in labeled α-chymotrypsin. The model complex in Figure 1C and several other complexes [Pt(trpy)L]$^{2+}$

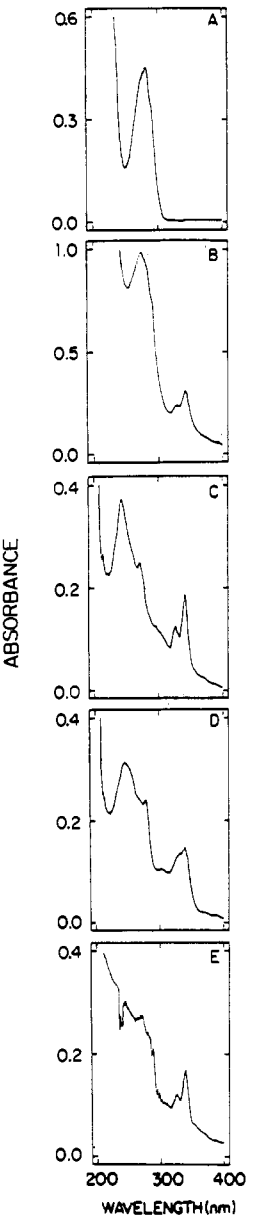

FIGURE 1: Absorption spectra of 10 μM solutions in 50 mM Tris buffer of pH 8.0. (A) α-Chymotrypsin. (The spectrum of α-lytic protease is similar.) (B) α-Chymotrypsin tagged with Pt(trpy)$^{2+}$ at His 40 and His 57. (C) [Pt(trpy)(Gly-His-Gly)]Cl_2, in which the tripeptide is coordinated to the Pt atom via the imidazole ring. (D) [Pt(trpy)Cl]Cl. (E) Difference between (B) and the spectrum of α-chymotrypsin modified with TPCK and tagged with Pt(trpy)$^{2+}$ only at His 40.

in which L represents imidazole-containing ligands (Ratilla et al., 1987) have identical λ_{max} values. Comparison between panels C and D in Figure 1 shows that the absorption pattern in the range 300–350 nm depends markedly on the ligand L (Ratilla et al., 1987; Brothers & Kostič, 1988). Since the ratio of [Pt(trpy)His]$^{2+}$ to enzyme is 2.00 ± 0.08 for α-chymotrypsin and 1.00 ± 0.10 for α-lytic protease, both His 40 and His 57 are modified in the former, and only His 57 is modified in the latter. Since the ratio of [Pt(trpy)His]$^{2+}$ to α-chymotrypsin is unaffected by long incubation of the platinated enzyme (in buffers at 4 °C), by changes of pH in the range 1.5–12, by repeated ultrafiltrations, and by affinity chromatography, the tags are stable. Indeed, coordination complexes of Pt(II) are known for both thermodynamic and kinetic stability (Hartley, 1973). The Pt(trpy)$^{2+}$ tag, once attached to α-lytic protease, also is stable, but this enzyme at pH 5.0 is less reactive than α-chymotrypsin toward [Pt(trpy)Cl]$^+$

Table I: Activity at pH 8.0 and 25 °C of Native α-Chymotrypsin and of the Derivative Tagged with $Pt(trpy)^{2+}$ at His 40 and His 57

	native		platinated[a]		platinated[b]	
substrate	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})
BTEE[c]	140 ± 10[d]	100 ± 1.0[d]	165 ± 65	1.66 ± 0.12	269 ± 11	2.21 ± 0.04
ATEE[e]	93 ± 10[f]	52 ± 1.0[f]	426 ± 150	0.51 ± 0.14	886 ± 150	0.76 ± 0.14
BAPNA[g]	2100 ± 920	(7 ± 3) × 10^{-3}	8340 ± 400	(8 ± 2) × 10^{-4}	15700 ± 12700	(3 ± 2) × 10^{-4}
AAPF[h]	59 ± 11	19 ± 1.1	73 ± 20	5.10 ± 0.50	47 ± 14	4.40 ± 0.40

[a] With 3-fold excess of PMSF over total enzyme. [b] With 3-fold excess of PMSF and 50-fold excess of [Pt(trpy)Cl]Cl over total enzyme. [c] *N*-Benzoyl-L-tyrosine ethyl ester. [d] From Gutfreund and Hammond (1959). [e] *N*-Acetyl-L-tryptophan ethyl ester. [f] From Zerner et al. (1964). [g] N^{α}-Benzoyl-DL-arginine-*p*-nitroanilide. [h] *N*-Succinyl-L-alanine-L-alanine-L-proline-L-phenylalanine-*p*-nitroanilide.

Table II: Activity at pH 8.0 and 25 °C of Native α-Lytic Protease and of the Derivative Tagged with $Pt(trpy)^{2+}$ at His 57 toward *N*-Acetyl-L-Ala-L-Ala-L-Pro-L-Ala-β-naphthylamide

enzyme	k_{cat} (s^{-1})	K_m (μM)
native	15.6 ± 2.0	420 ± 94
platinated[a]	0.23 ± 0.02	495 ± 73

[a] With 50-fold excess of $[Pt(trpy)Cl]^+$ over total enzyme.

probably because of electrostatic and steric factors. First, α-lytic protease has a charge of +9 at pH 5.0 and an appreciable positive charge even at pH 9.0 (Whitaker, 1970). Second, α-lytic protease (a bacterial enzyme) and α-chymotrypsin (a mammalian one) have similar active sites but different tertiary structures around them (Fastrez & Houyet, 1977; Brayer et al., 1979).

Labeling of the Active Site. When α-chymotrypsin is first modified at the active site with TPCK (Schoellmann & Shaw, 1962) and is then platinated, the diagnostic bands (Figure 1E) match those in the model complex (Figure 1C), and the ratio of $[Pt(trpy)His]^{2+}$ to the enzyme is 1.01 ± 0.15. The single tag evidently is attached to His 40. Attempts at selective platination of His 40 without the use of TPCK failed. Neither indole, a competitive inhibitor, nor BTEE, a substrate, could shield His 57 from attack by $[Pt(trpy)Cl]^+$, and the doubly platinated derivative was obtained in both cases. The competitive inhibitor proflavin did lower the yield of doubly platinated enzyme but did not eliminate this product. The imidazole ring of His 57 evidently remains accessible to $[Pt(trpy)Cl]^+$. Indeed, this residue is known to move upon substrate binding and upon enzyme modification with reagents that mimic the tetrahedral intermediate (Bachovchin, 1986; Bachovchin et al., 1988).

Catalytic Activity. The platinated α-chymotrypsin is retained by the affinity column. Since its residual esterase activity for amino acid substrates (ca. 2% of the native value) is low, the platination must involve the active site (Chakravarty et al., 1982; Daniels et al., 1983); chemical modification elsewhere would not produce such a large effect (Landis & Berliner, 1980; Mozhaev et al., 1988). The platinated α-chymotrypsin hydrolyzes *N*-succinyl-AAPF-*p*-nitroanilide, a specific substrate for the native enzyme, at approximately 25% of the rate with which the native enzyme does it. Again, the large retardation indicates labeling at the active site. Similarly, platination of α-lytic protease substantially reduces the activity toward *N*-acetyl-AAPA-β-naphthylamide. Since, however, the residual activities of both enzymes are appreciable, a $Pt(trpy)^{2+}$ tag at His 57 must not completely inactivate them.

As Tables I and II show, the K_m values for the platinated enzymes are, on the whole, only a little higher than the corresponding K_m values for the native enzymes. Similar findings have been reported for α-chymotrypsin modified at $N^{\epsilon 2}$ of His 57 with small reagents (Byers & Koshland, 1978; Ryan & Feeney, 1975; Maehler & Whitaker, 1982) and for a mutant subtilisin in which His 57 was replaced with alanine (Carter & Wells, 1988). It is surprising, however, that the bulky $Pt(trpy)^{2+}$ tag so little impedes substrate binding. This finding may reveal something about enzyme–substrate interactions, but its proper analysis must await a crystallographic study of our platinated enzyme.

Until then, a preliminary examination of platinated α-chymotrypsin by computer graphics will suffice. When the platinum atom sits at approximately the bonding distance from His 57, the $Pt(trpy)^{2+}$ tag seems not to crowd the other two members of the catalytic triad. The tag appears to sit in the so-called primary-specificity pocket, defined by the residues Tyr 146, Ser 190, Cys 191, Met 192, Val 213, Ser 214, Trp 215, and Tyr 228, which interact with substrates (Blow, 1976). The tag does not much affect the K_m values because it is much smaller than the overall substrate-binding region.

The turnover numbers, k_{cat}, for the platinated α-chymotrypsin are the following fractions of the corresponding native values: ca. 1–2% for amino acid esters, as much as ca. 10% for amino acid amides, and as much as 25% for a peptide toward which the native enzyme is specific. These values are considerably higher than the turnover numbers of α-chymotrypsin methylated at the $N^{\epsilon 2}$ atom of His 57; the methylated enzyme proved unreactive toward amides (West et al., 1988; Scholten et al., 1988; West & Wong, 1986). This difference in catalytic activity may result from the difference between the electrostatic or steric properties of the $Pt(trpy)^{2+}$ and CH_3^+ tags.

The electric dipoles around the so-called oxyanion hole stabilize the anionic tetrahedral intermediate (designated t^-), and the anionic Asp 102 residue stabilizes the cationic His 57 residue in the intermediate (designated His^+t^-). Although the presence of either tag lowers the turnover number, the dipositive $Pt(trpy)^{2+}$ may be more effective than the formally monopositive CH_3^+ in stabilizing t^- in the oxyanion hole. (The tagged His 57 residue probably still is stabilized by the Asp 102 residue.) Unlike mutagenesis, which removes His 57 and disrupts the electrostatic network of the active site, platination of this residue does not preclude the crucial electrostatic interactions during catalysis. Alternatively, the bulkier $Pt(trpy)^{2+}$ tag at the $N^{\epsilon 2}$ atom may cause a greater reorientation of the His 57 side chain, so that the $N^{\delta 1}$ atom facilitates the proton transfer from Ser 195 to His 57. Similar rotation of the imidazole ring has already been invoked in enzymatic mechanisms (Pocker & Janjič, 1989).

The rate of hydrolysis by α-chymotrypsin depends on the number and spatial distribution of enzyme–substrate interactions (Kraut, 1971). The platinated α-chymotrypsin is more efficient toward the specific substrate (a peptide) than toward the nonspecific ones (an amide, and even esters, of amino acids) probably because the scissile bond in the larger substrate is better oriented for catalysis than are these bonds in the smaller substrates. Orientation seems to be more important than the intrinsic reactivity of the scissile bond (amide vs ester).

Platination lowers the catalytic activity of α-lytic protease more than the activity of α-chymotrypsin perhaps because the substrate fit is even more important with the former enzyme

than with the latter one (Whitaker et al., 1965). Neither native nor platinated α-lytic protease can hydrolyze the amino acid derivative AAPNA even at a high concentration of this substrate. The platinated enzyme hydrolyzes the specific substrate *N*-acetyl-AAPA-β-naphthylamide with a turnover number that is only ca. 1% of the native value.

The activation energies were calculated with eq 2 (Warshel & Russell, 1986). Replacement of His 57 with Ala in subtilisin

$$k_{cat} = (k_b T/h) \exp(-\Delta G^{\ddagger}_{cat}/k_b T) \quad (2)$$

raises $\Delta G^{\ddagger}$ by ca. 6 kcal·mol^{-1} (Carter & Wells, 1988). Platination of α-chymotrypsin, however, raises $\Delta G^{\ddagger}$ only by 1–3 kcal·mole^{-1}. This smaller effect is similar in magnitude to van der Waals interaction between a substrate and an active site (Warshel et al., 1989). The Pt(trpy)$^{2+}$ tag may also alter the effective dielectric constant at the active site.

Conventional organic reagents, such as phenylethaneboronic acid (Tulinsky & Blevins, 1987) or TPCK (Schoellmann & Shaw, 1962), inactivate α-chymotrypsin presumably because they occupy the active site and produce tetrahedral adducts that resemble the intermediates in the hydrolysis reactions. Unlike them, the inorganic tag Pt(trpy)$^{2+}$ does not completely fill the active site, is not a substrate analogue, and therefore does not completely inactivate the enzyme. The substrate apparently can still enter the hydrophobic crevice, and its functional groups can still interact in the acylamido and oxyanion-hole regions. Although these interactions may be weaker than in the case of the native enzyme, they are sufficient to permit hydrolysis of the substrate.

Several unlikely mechanisms may be hypothesized about. The hydrolysis may be catalyzed by other nucleophilic residues, such as Lys or Ser (Zemel, 1987), rather than by the so-called catalytic triad. The Pt(trpy)$^{2+}$ tag perhaps disrupts the arrangement of water molecules at the active site (Pocker & Janjič, 1989) and thus lowers the enzyme activity. Finally, the Pt(II) atom may be directly involved in polarizing the substrate or in activating the residues at the active site.

Precautions about Contaminants. Various control experiments showed that the hydrolysis reactions are indeed catalyzed by the platinated enzyme and not by other agents. Chromatography of the platinated α-chymotrypsin on lima bean trypsin inhibitor removed all salts, peptides, and any other enzymes unlike trypsin. Since the treatment with TPCK completely inactivated the native enzyme toward BTEE, trypsin is absent (Titani et al., 1981). Since addition to the assay mixture of a large excess of EDTA did not quench the hydrolysis, this reaction is not caused by some zinc-containing protease.

Because the absorptivity of the [Pt(trpy)His]$^{2+}$ chromophore depends somewhat on its location in the protein, the error in its spectrophotometric quantitation could be as high as ca. 5%. Although the α-chymotrypsin after the affinity chromatography showed 2.0 chromophores, a small amount of the native enzyme could not be ruled out. To fully platinate any trace of it, a 50-fold excess of [Pt(trpy)Cl]Cl was added to the kinetic assays with both α-chymotrypsin and α-lytic protease; to inactivate any residual native enzyme, a 3-fold excess of PMSF was added to them. Since the native enzyme could, at worst, be ca. 5% of the total enzyme, the two reagents were in at least a 1000-fold and a 60-fold excess, respectively, over the possible contaminant. These precautions proved unnecessary because assays with just the platinated enzymes and those with added [Pt(trpy)Cl]Cl or PMSF, or both reagents, yielded k_{cat} and K_m values that differed only slightly from one another. The Pt(trpy)$^{2+}$ tags in α-chymotrypsin and in α-lytic protease indeed are stable, and the catalytic activity indeed is due to the platinated enzymes.

Table III: Catalytic Activity at 25 °C of α-Chymotrypsin Tagged with Pt(trpy)$^{2+}$ at His 40 and His 57 toward *N*-Benzoyl-L-tyrosine Ethyl Ester (BTEE)[a]

pH	k_{cat} (s^{-1})	K_m (μM)
5.0	0.19 ± 0.03	41 ± 36
6.0	1.16 ± 0.17	167 ± 62
7.0	2.40 ± 0.02	280 ± 5
7.5	2.15 ± 0.07	333 ± 21
8.0	2.21 ± 0.04	269 ± 11
8.5	1.11 ± 0.12	328 ± 67
9.0	0.54 ± 0.01	112 ± 2

[a] With 50-fold excess of [Pt(trpy)Cl]$^{+}$ over total enzyme.

Additional evidence for this claim comes from the finding that proflavin, a competitive inhibitor for native α-chymotrypsin, does not inhibit the reactions of the platinated enzyme. Spectrophotometric experiments showed that proflavin does not remove the Pt(trpy)$^{2+}$ tags from the protein. In control experiments, even a 10-fold excess of proflavin failed to displace the Cl^{-} ligand in [Pt(trpy)Cl]$^{+}$, let alone the more nucleophilic His ligand in [Pt(trpy)His]$^{2+}$. The nitrogen atom of proflavin and the platinum atom in the two complexes probably are too shielded to bind to each other (Ratilla et al., 1987). The proflavin absorption band at 445 nm shifts to greater wavelengths in the presence of the native (Bernhard et al., 1966) α-chymotrypsin, but not in the presence of its platinated derivative. Evidently, proflavin binds to the former, but not to the latter. Since the Lineweaver–Burk assays with the platinated enzyme and BTEE showed no competitive inhibition by proflavin, the catalytic activity is not due to a native contaminant. Although this finding does not prove that catalysis occurs at the platinated active site, it rules out catalysis by the native active site of α-chymotrypsin.

Finally, platination differently affects the activity of α-chymotrypsin toward different substrates (see Table I). If the hydrolysis were caused by the native enzyme, the k_{cat} value of the platinated enzyme would be the same fraction of this value for the native enzyme regardless of the substrate. With the same batch of the platinated enzyme, with different substrates, this fraction varies from ca. 1 to ca. 27%. The platinated and the native enzyme clearly are different catalysts.

Effects of pH on Catalysis. Native α-chymotrypsin (Bender et al., 1964) and the derivative methylated at His 57 (Henderson, 1971) have similar pH profiles—a bell-shaped curve with the maximum at 7.5 and two inflections on either side of it. The inflection on the basic side in both cases is attributed to the amino group at the N-terminus, Ile 16 (Kraut, 1971). The inflection on the acidic side is attributed to the N$^{\epsilon 2}$ atom of His 57 in the native enzyme, and to the N$^{\delta 1}$ atom of the same residue in the methyl derivative (Henderson, 1971; Byers & Koshland, 1978). Data for the platinated enzyme are shown in Table III. The maximum occurs at 7.0, and fitting shows inflections at 6.3 and 8.6. The inflection at 8.6 may be attributed to Ile 16, but the origin of the one at 6.3 is unclear. The pK_a of the N$^{\delta 1}$ atom, so-called pyrrole nitrogen, in the model complex [Pt(trpy)Im]$^{2+}$ is 9.7; this value is typical of imidazole complexes with divalent transition metals (Harrowfield et al., 1976; Sundberg & Martin, 1974). Although the hydrophobic environment at the active site (Simonsson & Lindskog, 1981) and hydrogen bonding to Ser 195 can lower the basicity of this atom, it is uncertain whether such lowering actually occurs (see the next section).

This work and a previous finding that replacement of His 57 with alanine in the catalytic triad of subtilisin does not abolish pH dependence of k_{cat} (Carter & Wells, 1988) indicate

that the pH profiles of serine proteases may be affected by more factors than are now recognized. The His 57 residue, although it enhances the nucleophilicity of Ser 195 and thus increases the turnover of the substrate, is not essential for catalytic activity, as shown by site-directed mutagenesis. Even the removal of the entire catalytic triad does not completely abolish the catalytic activity (Carter & Wells, 1987, 1988).

The Pt(trpy)$^{2+}$ Tag as a Spectroscopic Probe. The relative intensities (absorptivities), but not the positions (λ_{max} values), of the near-UV bands (300–350 nm) in the spectrum of [Pt(trpy)His]$^{2+}$ depend on the environment of the tag in proteins (Ratilla et al., 1987). Because these absorption bands arise from metal-to-ligand charge-transfer (MLCT) transitions, they are affected by the polarity of the medium and by the solvation of the chromophore. The platinum complex has absorption maxima at 342 and 328 nm. The quotient A_{342}/A_{328} is higher when the environment is hydrophilic than when it is hydrophobic. In this study the effect of the medium was amplified by a wide variation of the solvent hydrophobicity in studies of the [Pt(trpy)Im]$^{2+}$ complex. As the volume fraction of dioxane in water increased from 20 to 80%, the quotient A_{342}/A_{328} of the model complex decreased from 1.72 to 0.68.

For α-chymotrypsin, the composite quotient A_{342}/A_{328} for both chromophores at His 40 and His 57 is 1.38, and this quotient for the single chromophore at His 57 (from Figure 1E), is 1.41; therefore, the quotient for the chromophore at His 40 should be 1.35–1.40. Indeed, the quotient A_{342}/A_{328} for the [Pt(trpy)His]$^{2+}$ chromophore in α-lytic protease is 1.40. Both the tags at His 40 and His 57 seem to be located in hydrophilic environments. This spectroscopic evidence is consistent with the fact that platination causes only a small increase in the K_m values of the enzymes (Tables I and II). The active site evidently remains accessible to substrates despite tagging of His 57. Although computer graphics indicates that the Pt(trpy)$^{2+}$ tag sits in the active site, which is hydrophobic, the tag is exposed to the solvent outside.

The titration curve in Figure 2A represents an acid–base equilibrium involving the $N^{\delta 1}$ atom. In addition to this process, in the diplatinated (at His 40 and His 57) and monoplatinated (at His 40) derivatives of α-chymotrypsin there is also a process in the acidic region, which we did not investigate. The similarity between the two enzyme derivatives—only one curve is shown in Figure 2B—indicates that both His 40 and His 57 in the diplatinated derivative have similar pK_a values. Indeed, the corresponding [Pt(trpy)His]$^{2+}$ chromophores have similar A_{342}/A_{328} quotients. The inflection at pH 6.3 in the pH profile of the diplatinated enzyme perhaps is not due to the $N^{\delta 1}$ of His 57.

Autodigestion of α-chymotrypsin in solution causes turbidity and precipitation over time. After 1 week at pH 5.0 and 4 °C, a solution of the platinated enzyme exhibited less autolysis (no precipitation) than a comparable solution of the native enzyme (visible precipitation). The best way to keep the platinated enzyme is to freeze its solution in 100 mM Na_2SO_4 whose pH was adjusted to 3.5 with H_2SO_4. Platination of α-lytic protease similarly retards the autodigestion process. After 1 week at pH 8.0 and 25 °C, the platinated enzyme showed no visible denaturation, whereas the native enzyme gave a slight precipitate. Since α-lytic protease is less susceptible to autodigestion than α-chymotrypsin (Brayer et al., 1970), the protecting effect of platination is less noticeable with it.

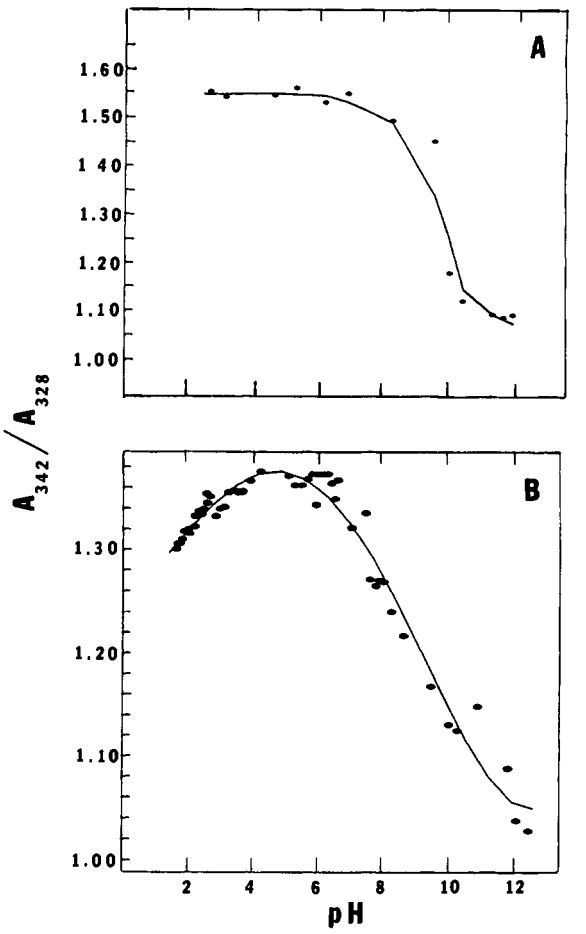

FIGURE 2: Spectrophotometric titrations of aqueous solutions. (A) [Pt(trpy)Im]Cl_2. (B) α-Chymotrypsin tagged with Pt(trpy)$^{2+}$ at both His 40 and His 57.

The Pt(trpy)$^{2+}$ tags can easily be removed, and the enzymatic activity restored, by incubation with an excess of thiourea, a sulfur nucleophile. The substitution reaction involved is shown in the simplified eq 3, in which His represents a residue in the protein.

$$[Pt(trpy)His]^{2+} + (NH_2)_2CS \rightarrow [Pt(trpy)(NH_2)_2CS]^{2+} + His \quad (3)$$

Advantages and Prospective Applications of the New Reagent. The strong bands in the region 300–350 nm, where proteins usually do not absorb, permit easy detection and quantitation of the [Pt(trpy)L]$^{n+}$ chromophores. The band positions (λ_{max} values) depend on the identity of the binding group L, and their relative intensities depend on the hydrophilicity or hydrophobicity of the environment.

Our reagent, [Pt(trpy)Cl]Cl, is particularly applicable to serine proteases, such as trypsin, chymotrypsin, subtilisin, elastase, thrombin, and lytic protease. Unlike suicide inhibitors, which recognize the entire active site and bind to it irreversibly, [Pt(trpy)Cl]$^+$ reacts with histidine and can be removed noninvasively. Because of its different affinity, it may prove complementary to the conventional reagents such as PMSF, TPCK, and DIFP. In particular, platination may be used as a temporary protection of proteolytic enzymes from autodigestion.

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