Effects of Platinum Complexes on Chymotrypsin

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A variety of platinum complexes were evaluated as inhibitors of α-chymotrypsin. A standard enzyme assay was used with benzoyl-L-tyrosine ethyl ester as the substrate. Rb_2PtBr_4, *trans*-$Pt(NH_3)_2Cl_2$, and K_2PtCl_4 were all effective enzyme inhibitors. $Pt(Gly\text{-}L\text{-}Met)Cl_2$, $Pt(Met)(NH_3)_2Cl$, *cis*-$Pt(NH_3)_2Cl_2$, and two ethylenediamminedichloroplatinum complexes were weak inhibitors of α-chymotrypsin. A surprising finding was the noninhibition of an immobilized preparation of α-chymotrypsin by the above inhibitors and by active-site-directed modifying reagents.

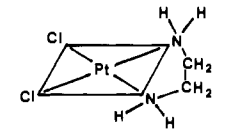
Platinum compounds are potential antitumor agents but have relatively high cytotoxicities. In the work reported here, the effect of various platinum complexes on α-chymotrypsin in solution and immobilized to glass beads was investigated in order to gain further information on how the platinum complexes may exert cytotoxic effects and to find ways of rationally reducing these toxic effects. α-Chymotrypsin is a digestive enzyme which functions physiologically as a protease, cleaving preferentially at the carboxyl side of the aromatic amino acids phenylalanine, tryptophan, and tyrosine.

The effect of platinum complexes on proteins and enzymes was reviewed by Melius and Friedman.[1] It had been demonstrated through X-ray crystallographic studies by Matthews et al.[2] that $PtCl_4^{2-}$ binds to the disulfide bridge (1–127) and methionine residue 92 in α-chymotrypsin. Volshstein[3] found that methionine reacts very rapidly with $PtCl_4^{2-}$ and both *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ bond through the $-SCH_3$ group. A wide variety of complexes were reported in which the $-NH_2$, $-SCH_3$ and $-CO_2^-$ groups were involved as ligands. The binding of platinum complexes in the three-dimensional structure of α-chymotrypsin has been described by Dickerson et al.[5]

Our focus of interest in the interactions of platinum complexes with the α-chymotrypsin enzyme system is the Met-192, which is situated at the entrance to the active-site pocket. Met-192 has an undelineated role in α-chymotrypsin catalysis, but it is known that photooxidation of this residue leads to a rapid inactivation of α-chymotrypsin with a concurrent decrease in the initial rate of the enzyme activity.

If the toxicity of platinum antitumor agents is due mainly to their interactions with enzymes, it would be very valuable to know how the platinum–protein interactions occur so that the platinum complexes could be modified in such a way as to eliminate the cytotoxic effects without affecting the properties responsible for antitumor activity. The $PtCl_4^{2-}$ has been shown by X-ray studies to bind to Met-192[5] of α-chymotrypsin in the crystalline state and may be expected to bind to Met in other proteins.

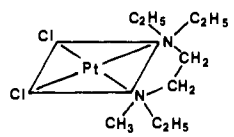
Chart I. Structures of Platinum Complexes Used in This Study



$Pt(NH_2CH_2CH_2NH_2)Cl_2$

Dichloro Ethylene Diamine Platinum (II)

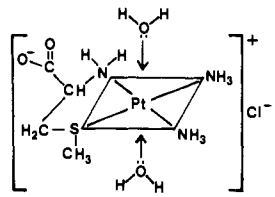
1



$Pt(Me\cdot Et\cdot NCH_2CH_2N\cdot Et_2)Cl_2$

Dichloro-N,N,N'-Triethyl-N'-Methyl Ethylene Diamine Platinum (II)

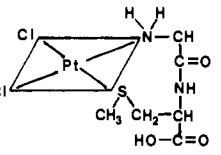
2



$[Pt(MET)(NH_3)_2]Cl\cdot 2H_2O$

Diamine Methionine Platinum (II) Chloride

3



$[Pt(GLY\text{-}L\text{-}MET)Cl_2]$

Dichloro-L-Glycyl-Methionine Platinum (II)

4

A series of platinum complexes were prepared by C. A. McAuliffe at the University of Manchester, England, which contain a variety of ligands coordinated to the platinum, in order to explore both size and charge interaction factors.[1,3,7,8]

Experimental Section

α-Chymotrypsin (bovine pancreas; three times crystallized and freeze-dried; type II) and *N*-benzoyltyrosine ethyl ester were obtained from Sigma Chemical Co., St. Louis, Mo., and were used without further purification.

The α-chymotrypsin assays[4] were carried out in tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7–8, 0.08 M (0.1 M in $CaCl_2$), using *N*-benzyl-L-tryosine ethyl ester (BTEE), 1×10^{-3} M, dissolved in 50% methanol ($MeOH/H_2O$). The conversion of BTEE to benzoyltyrosine was measured by recording the absorbance at 256 nm. Control samples were utilized for measurement of untreated enzyme activity.

The α-chymotrypsin was immobilized to glass beads obtained from H. H. Weetall, Corning Glass Works, by the procedure of Lee et al.[6] The activated beads were mixed with 2.5% aqueous glutaraldehyde and then rinsed with a few volumes of buffer. The

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(3) L. M. Volshtein and M. F. Mogilevkina, *Russ. J. Inorg. Chem. (Engl. Transl.)*, 10, 293 (1965); 832 (1967).
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(5) R. E. Dickerson, D. E. Eisenberg, J. Varnum, and J. Kopka, *Mol. Biol.*, 45, 77 (1969).
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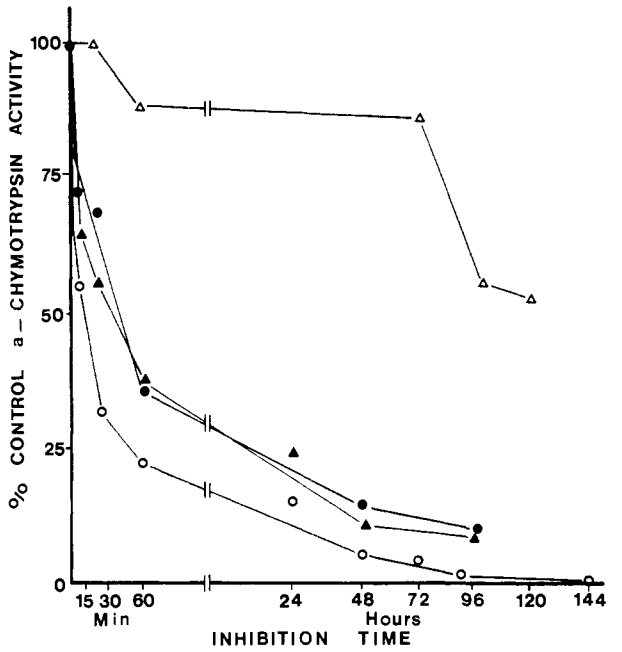

Figure 1. Inhibition of "free" α-chymotrypsin by Pt complexes. The incubation mixture consisted of 0.75 mg/mL α-chymotrypsin, 5×10^{-3} M for all Pt complexes except *cis*-$Pt(NH_3)_2Cl_2$ (△) which was 9×10^{-4} M, and Tris buffer, pH 7.8, 0.08 M (0.1 M in $CaCl_2$). The enzyme assays are described under Experimental Section: (○) Rb_2PtBr_4; (▲) *trans*-$Pt(NH_3)_2Cl_2$; (●) K_2PtCl_4.

α-chymotrypsin solution (1 mg/mL Tris buffer, pH 7.8, 0.08 M) was then added to 1 mL of activated glass beads and stored in the refrigerator. The beads were then washed with buffer three times and the glass-immobilized α-chymotrypsin was ready to be used. Protein and enzyme analysis indicated that about 63% of the enzyme activity was retained on the glass beads.

Results

The structures of some new platinum complexes which are used in this work are indicated in Chart I. The completely alkylated ethylenediamine ligand of **2** introduces a large bulky group into the platinum complex while retaining the cis configuration of the chlorine ligands. Structures **3** and **4** introduce two methionine complexes in order to use more biologically compatible ligands and to determine whether halogens on platinum are essential for enzymatic inhibition.

The rubidium platinum tetrabromide, *trans*-diamminedichloroplatinum(II) and, potassium platinum tetrachloride all at 5×10^{-3} M were very effective inhibitors of free α-chymotrypsin, as can be seen in Figure 1. Within 1 h at room temperature, these compounds produced 60–75% inhibition over the controls and almost complete inhibition after 48–96 h. The *cis*-diamminedichloroplatinum(II) complex, in contrast, required 96 h to produce 48% inhibition. It is interesting to note that the bromo compound is a more effective inhibitor as compared to the chloro compound, and the apparent biphasic inhibition pattern may be indicative of titration of both Met-192 and Met-204 residues in the α-chymotrypsin. Figure 2 summarizes the results of the ethylenediammineplatinum complexes which produce only a 20–25% inhibition in the first hour. Even after 72 h, the best inhibition of this group of compounds only achieved 75% inhibition, as compared to 95% inhibition of Rb_2PtBr_4. The relative ineffectiveness of these bulky platinum complexes may require a considerable amount of conformational change in the α-chymotrypsin to get at the Met-192. However, over a long time the enzyme does undergo sufficient deformation to allow significant inhibition to occur. It is of interest here that

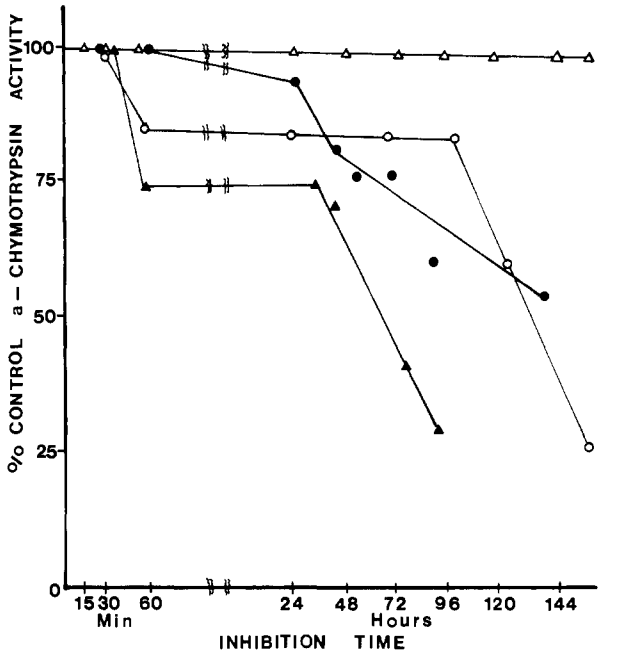

Figure 2. Effect of bulky ligands on Pt complexes. Similar conditions were used here as in Figure 1 with (○) Pt(Gly-L-Met)Cl_2, 6×10^{-4} M; (▲) $[Pt(Met)(NH_3)_2]\cdot Cl\cdot 2H_2O$, 5×10^{-3} M; (●) $Pt(NH_2(CH_2)_2NH_2)Cl_2$, 5×10^{-3} M; and (△) Pt-$(MeEtNCH_2CH_2CH_2NEt_2)Cl_2$, 5×10^{-3} M.

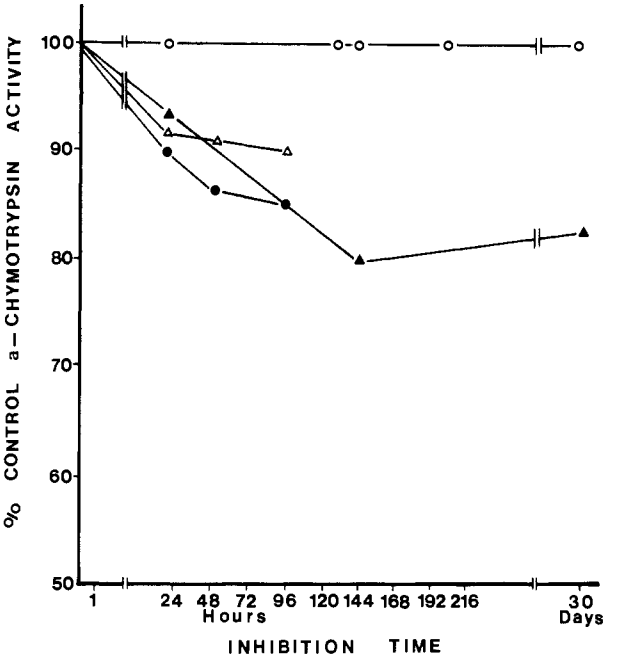

Figure 3. Inhibition of immobilized enzyme. Conditions described under Experimental Section for enzyme immobilization and assay. Each incubation mixture contained about 3 mg of enzyme and (○) Rb_2PtBr_4, 3×10^{-3} M; (●) Pt(Gly-L-Met)Cl_2, 3×10^{-3} M; (▲) *cis*-$Pt(NH_3)_2Cl$, 5×10^{-3} M; and (△) *trans*-Pt-$(NH_3)_3Cl_2$, 5×10^{-3}, all in Tris, pH 7.8, 0.10 M buffer.

the Met complex with only one chloro ligand is an effective inhibitor of α-chymotrypsin.

When the Rb_2PtBr_4 was added to the immobilized α-chymotrypsin, the results were most unexpected, as this potent inhibitor of the free α-chymotrypsin had no effect on the enzyme even after 30 days. The *trans*- and *cis*-platinum complexes and the glycyl-L-methionine (IV) complex all gave very mild inhibition of the immobilized enzyme (Figure 3). However, in contrast to free enzyme, the latter compounds produced a greater inhibition than

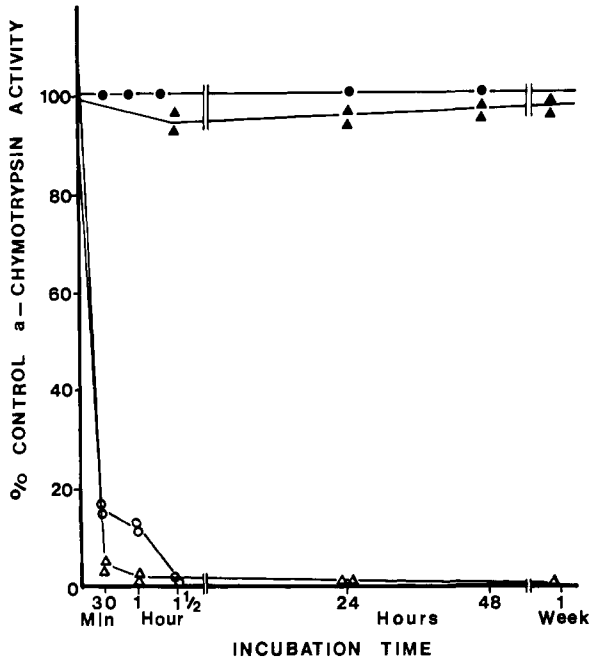

Figure 4. Effect of active-site reagents on "free" and immobilized α-chymotrypsin. The concentration of α-chymotrypsin was 2×10^{-4} M, TPCK 3×10^{-3} M, and DFP 2.5×10^{-7} M: (○) TPCK with α-Chy; (●) TPCK with immobilized enzyme; (△) DFP with α-Chy; (▲) DFP with immobilized enzyme.

the $PtBr_4^{2-}$. The immobilized enzyme in our hands has been found to retain its activity for at least 18 months. The controls in this experiment did not lose any activity over the 30-day period under these specific conditions.

In order to investigate further the very unexpected effect of the platinum complexes on the immobilized α-chymotrypsin, we utilized the two active-site reagents diisopropyl fluorophosphate (DFP), which is specific for active site Ser, and L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK), which is specific for the active-site His-56 of α-chymotrypsin.

Figure 4 indicates that DFP, under the conditions used here, produced 95% inhibition of the free α-chymotrypsin in 30 min at 0.25 μM concentration, but only 5% inhibition of the immobilized α-chymotrypsin after a period of 1 week. When TPCK was used, an 85% inhibition of the free α-chymotrypsin was obtained within 30 min, while the immobilized enzyme was not affected at all, even after 48 h. An aliquot of supernatant over the beads was removed to test the inhibitors for retention of their potency against the free enzyme, and they were unchanged, that is, still capable of inhibiting the free α-chymotrypsin even after exposure to the plain beads or beads with immobilized enzyme.

Up to this point all of our measurements of α-chymotrypsin activity used BTEE, which measures esterase activity. In order to detect the effect on protease activity, we measured the effect of the immobilized enzyme on cytochrome *c* to detect any changes in proteolytic activity. The free α-chymotrypsin incubated with cytochrome *c* did produce one small fast-moving spot that was not obtained with the immobilized enzyme; however, the major peptide fragments from free α-chymotryptic and immobilized α-chymotryptic digestion were the same.

Discussion

A variety of platinum complexes have been shown to be good antitumor agents by Rosenberg et al.[1] However, they have certain cytotoxic effects which decrease their usefulness in human medicine. Of course, it would greatly facilitate the use of these compounds if their chemical sites of action in the cell were known well enough to be controlled and directed to the site of the lesion and not to adversely affect healthy cells. In this work we have shown that many of the platinum complexes, such as the dichloroethylenediamines and the methionines, are much milder inhibitors of α-chymotrypsin than the tetrahalo- and diamminedihaloplatinum complexes. Melius and McAuliffe[8] demonstrated that a variety of ethylenediamminehalo complexes of platinum were much weaker inhibitors than the diamminedihalo complexes. Also, various methionine complexes varied in their inhibitory effects from weak to no inhibition of the peptidase.[1] Thus, the results in this paper and those previously reported suggest that some of the Pt complexes may have much milder effects than the diamminedihalo complexes on sensitive enzyme systems required for viability of the living cell. Unfortunately, there is not much information on the antitumor potency of many of these compounds as yet from animal tests.

The other rather unusual finding that the platinum complexes did not inhibit the immobilized α-chymotrypsin led us to study the effects of active-site inhibitors on the immobilized enzyme. Even more surprising is the fact that the Rb_2PtBr_4, which in other studies has always been the most potent enzyme inhibitor, did not inhibit the immobilized α-chymotrypsin even after 30 days exposure. There is a possibility that the platinum tetrabromide is actually still complexing the Met-192 without causing any effect on the active site, whereas the other platinum complexes (with more bulky ligands) are complexing at Met-192 and perturbing the active site slightly, thus accounting for the 15–20% inhibitions observed. It may be possible that the hydrophobicity of the Met and ethylenediammine complexes allows them to get to the active site of the immobilized enzyme, whereas the Rb_2PtBr_4 can not.

It appears obvious that in the immobilized enzyme the active-site Ser-195 and His-57 are not available to attack by either the DFP or TPCK reagents, and yet not only can the small BTEE get to the active-site region for catalytic esterase action to occur but the immobilized α-chymotrypsin can cause proteolysis of cytochrome *c*.

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