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INHIBITION OF LEUCINE AMINOPEPTIDASE AND MALATE DEHYDROGENASE BY AQUOPLATINUM (II) COMPLEXES

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SUMMARY

Halide complexes of platinum have been previously shown to inhibit tumors and cell growth as well as to possess immunosuppresive activity. Evidence is presented here that the active species in Rb_2PtBr_4 solutions which inhibits both leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1) and malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) enzymes is $PtBr_3(H_2O)^-$. The aquo complex earlier has been shown to be in equilibrium with $PtBr_4^{2-}$ in solution and the rate of formation is known. This is in accord with the rate of inhibition of enzyme activity by fresh solutions of Rb_2PtBr_4. This reagent should provide another method for studying the active site and function of enzymes.

INTRODUCTION

In the past few years halide complexes of platinum (II) and (IV) have been found to possess tumor-inhibiting properties, immunosuppresive activity and cell growth and division inhibiting activity. For example, in the initial study ROSENBERG *et al.*[1], reported that several halide complexes of platinum inhibited sarcoma 180 and leukemia L 1210 in mice. More recently[2–4], a very large number of different tumors, such as sarcoma 180 and Ehrlich ascites, have been reported to be inhibited by platinum complexes containing at least two halide ligands which also possess immunosuppresive activity in mouse-spleen cells. GUTHRIE *et al.*[5], have observed that bromo complexes of platinum (II) inhibit leucine aminopeptidase (L-leucyl-peptide hydrolase, EC 3.4.1.1). Although no direct connection between this enzyme and tumor growth has been reported, elevated leucine aminopeptidase levels have been found in certain tumors[6].

Many characteristics of a simple inorganic substitution reaction were observed[5] in the inhibition of leucine aminopeptidase by $PtBr_4^{2-}$. It is known that substitution of a halide ligand in a platinum (II) complex often proceeds *via* an aquo intermediate[7]. This reaction scheme is illustrated in the following equations in which the slow aquation reaction is followed by the rapid displacement of an aquo ligand by the incoming group(s).

$$\text{Pt(X)halide} + H_2O \xrightarrow{\text{slow}} \text{Pt(X)}H_2O + \text{halide} \quad (1)$$

$$\text{Pt(X)}H_2O + S \xrightarrow{\text{fast}} \text{Pt(X)}S + H_2O \quad (2)$$

It is possible that the inhibition of leucine aminopeptidase involves the displacement of halide ligands bound to the platinum by nucleophilic groups on the enzyme with the formation of a stable platinum–enzyme complex. Consequently, if such a substitution reaction possessed similarities to related inorganic reactions an aquo complex of platinum could be involved in the inhibition reaction. Because the rate of aquation of $PtBr_4^{2-}$ has been reported[8], this possibility has been investigated in the present paper. Although malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) is not related to leucine aminopeptidase, preparations of this enzyme were readily available and could be treated by similar techniques. Conclusive experimental evidence is reported in this study to show that both enzymes are, in fact, inhibited much more rapidly by $PtBr_3(H_2O)^-$ than by $PtBr_4^{2-}$.

EXPERIMENTAL PROCEDURE

Leucine aminopeptidase. The method of MOSELEY AND MELIUS[9] was employed to prepare aqueous enzyme solutions. Assays of enzyme activity were performed by titrating NH_3 liberated by the hydrolysis of L-leucine amide using the equipment described in a previous publication[5]. All assays were carried out at pH 8.0 and 37°. Reaction mixtures contained approx. $4 \cdot 10^{-7}$ M enzyme and had a total volume of 50 ml. The enzyme activity was estimated separately by reaction with the substrate in the absence of $PtBr_4^{2-}$, in the presence of freshly dissolved $PtBr_4^{2-}$, and in the presence of an aqueous solution of $PtBr_4^{2-}$ which had been aged overnight. Prior to addition the total platinum concentrations were $5 \cdot 10^{-3}$ M. It is known that $PtBr_4^{2-}$ equilibrated with $PtBr_3(H_2O)^-$, according to Eqn. 3, in the time allowed for ageing[8].

$$H_2O + PtBr_4^{-2} \rightleftharpoons PtBr_3(H_2O)^- + Br^- \quad (3)$$

Malate dehydrogenase. Pig heart malate dehydrogenase was purchased from the Sigma Chemical Company. The enzyme was purified according to a previously reported procedure[10]. Rubidium tetrabromoplatinate (II) was prepared and analyzed as previously described[8].

The concentration of enzyme for the test was $1.5 \cdot 10^{-6}$ M, while the concentration of Rb_2PtBr_4 was $3.75 \cdot 10^{-5}$ M. The Rb_2PtBr_4 solution was prepared by dissolving 10.6 mg of $Rb_2PtBr_4 \cdot H_2O$ in 8 ml of buffer, and immediately removing a 5-μl aliquot and adding it to 0.25 ml of enzyme. The concentration of enzyme was determined by the method of LOWRY *et al.*[11]. In some experiments the Rb_2PtBr_4 complex was allowed to aquate with the buffer before being added to the enzyme. An enzyme control, without Rb_2PtBr_4, was also run along with the aquated and non-aquated Rb_2PtBr_4–malate dehydrogenase systems. All the experiments were buffered in a 0.1 M phosphate buffer (pH 7.0) in a 25° water bath.

The enzyme was assayed as follows: 5 μl were removed at various times and added to 3.0 ml of the following solution: 0.1 M glycine, $2 \cdot 10^{-4}$ M oxidized nicotinamide adenine dinucleotide (NAD^+) and 0.01 M malic acid buffered at pH 9.5. The

final concentration of malate dehydrogenase in this solution was $2.5 \cdot 10^{-9}$ M. The formation of NADH (reduced form of NAD^+) was followed on a Hitachi Spectrophotometer, water cooled at 25°, at 340 nm, and the activity was expressed as μmoles of NADH formed per min of assay per ml of solution. The percent of remaining activity was evaluated by dividing the activity of the inhibited enzyme solutions by that of the control, (whose activity did not vary, within experimental error, over the entire experiment).

RESULTS AND DISCUSSION

The experimental data for the reactions involving leucine aminopeptidase is best explained in terms of Fig. 1. The upper curve (Curve a) indicates the quantity

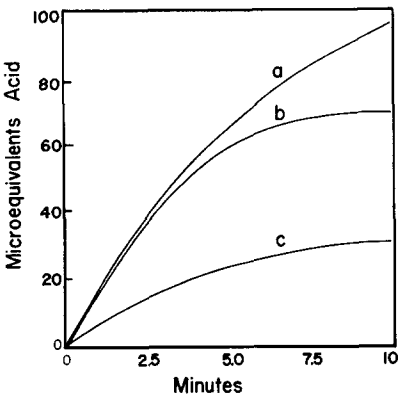

Fig. 1. Inhibition of leucine aminopeptidase activity by the platinum tetrabromide complex ion and its hydrolysis product $PtBr_3(H_2O)^-$. The reaction mixtures contained $4 \cdot 10^{-7}$ M leucine aminopeptidase in a total volume of 50 ml and were incubated at 37° and pH 8.0 (a) The enzyme was incubated with 0.01 M KBr before addition to 6 ml of 0.0125 M L-leucine amide solution. (b) The enzyme was incubated with $5 \cdot 10^{-3}$ M Rb_2PtBr_4 which had been freshly prepared before it was added to the 0.0125 M L-leucine amide solution. (c) The enzyme was incubated with a $5 \cdot 10^{-3}$ M Rb_2PtBr_4 solution which had previously been aged overnight. Then 5 ml of the enzyme solution were added to the 0.0125 M L-leucine amide substrate solution.

of acid required to maintain a pH value of 8.0 after enzyme preparation was mixed with a substrate solution in absence of any platinum complex. A similar curve was obtained in the presence of 10^{-2} M RbBr; indicating that free bromide ion does not inhibit the enzyme. Curve b corresponds to a similar experiment in which aliquots of enzyme preparation and substrate solution were mixed to initiate the reaction in a beaker containing a small amount of Rb_2PtBr_4. This rubidium salt is soluble and passes into solution within a few seconds. For the first 5 min Curve b approximates Curve a indicating that substrate hydrolysis is not strongly inhibited. In the later stages of the reaction obvious inhibition occurs in the platinum-containing solution. An obvious explanation for this effect could involve the aquation of the initially dissolved $PtBr_4^{2-}$ according to Eqn. 1, to produce the $PtBr_3(H_2O)^-$. If the aquo complex is a stronger inhibitor than $PtBr_4^{2-}$ significant inhibition would occur after appreciable concentrations of $PtBr_3(H_2O)^-$ had built up in solution. At 37° the

aquation of $PtBr_4^{2-}$ is a first-order reaction as shown in Eqn. 4 (ref. 8). Consequently, after 5 min, approx. 17% of the complex would be

$$\text{Rate} = 10^{-3}\ \text{sec}^{-1} \cdot [PtBr_4^{2-}] \quad (4)$$

aquated. The resultant concentration of $Pt(H_2O)Br_3^-$ ($8.5 \cdot 10^{-4}$ M) would be in excess of the estimated enzyme concentration ($4 \cdot 10^{-7}$ M). The final experiment corresponding to Curve c involved ageing Rb_2PtBr_4 in water for 1 h prior to addition to the reaction mixture. The equilibrated Rb_2PtBr_4 solution has been shown[8] to contain approx. 60% $PtBr_3(H_2O)^-$ and 40% $PtBr_4^{2-}$. Curve c was obtained by mixing separate aliquots of enzyme preparation, substrate solution, and aged $PtBr_4^{2-}$ solution to initiate the reaction at zero time. Apart from the initial presence of $PtBr_3(H_2O)^-$ the compositions of reaction mixtures c and b were identical. It can be seen that considerable inhibition of leucine amide hydrolysis occured at all times when $PtBr_3(H_2O)^-$ was present.

Malate dehydrogenase was also inhibited by platinum complexes. The actual assay procedure required 30 sec after the enzyme had been added to the substrate at 25°. As can be seen for Table I, enzyme inhibition occured with 50% of the initial

TABLE I

INHIBITION OF MALATE DEHYDROGENASE BY PLATINUM COMPLEXES AT 25°

Malate dehydrogenase = $1.5 \cdot 10^{-6}$ M. $PtBr_4^{2-}$ added at zero time.

Time (min)	*% Initial activity remaining**		
	No $PtBr_4^{2-}$	*Solid $PtBr_4^{2-}$*	*Aged $PtBr_4^{2-}$*
1	100	100	43
5	100	82	25
10	100	66	15
15	100	55	10
20	100	42	6
25	100	31	0
30	100	22	0
40	100	9	0

* 100% activity = 148 μmoles/ml per min of NADH formed.

enzyme activity being destroyed in approx. 17 min. The reaction mixture, prepared by ageing Rb_2PtBr_4 for 1 h in phosphate buffer prior to addition of the complex to the enzyme preparation at zero time, lost enzyme activity much more rapidly than had been the case when the fresh complex solution had been dissolved in the enzyme preparation.

It is interesting to note that despite dissimilar structures both leucine aminopeptidase and malate dehydrogenase are inhibited by $PtBr_4^{2-}$ after at least a few minutes of reaction time. Also, in both cases, aged solutions of $PtBr_4^{2-}$, which contain approx. 50% aquo complex, are much stronger inhibitors. This suggests that reactions in which nucleophilic groups on the enzymes displace an aquo ligand in the platinum complex could result in inhibition of the enzyme. This type of mechanism has been observed for a large number of inorganic substitution reactions[7] and any resultant bond between platinum and a nucleophilic atom in the enzyme would be kinetically

inert[12]. Although we do not possess any evidence for specific mechanisms for inhibition of either enzyme, it is quite possible that binding a platinum complex to an enzyme could directly or indirectly modify the active site of the enzyme. This may provide another method of labelling the active site of an enzyme for structure and function studies.

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