*Inorganica Chimica Acta, 91* (1984) 161–165



# Studies of Enzyme Inhibition. The Interaction of some Platinum(II) Complexes with Fumarase and Malate Dehydrogenase

JOANNE P. McGUIRE, MICHAEL E. FRIEDMAN*

*Department of Chemistry, Auburn University, Auburn, Ala. 36830, U.S.A.*

and CHARLES A. McAULIFFE

*Department of Chemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, U.K.*

Received July 30, 1983

*Both malate dehydrogenase and fumarase have been found to be able to distinguish between the* cis *and* trans *isomers of* $Pt(NH_3)_2Cl_2$. *A mode is proposed to account for this. Enzyme inhibition by* $PtCl_4^{2-}$ *is also reported. The effect of aquation on the mechanism of action of the platinum drugs is discussed.*

## Introduction

In 1965 Rosenberg, VanCamp and Krigas [1] accidently discovered the ability of certain platinum complexes to inhibit cell division in *E. coli*, although cell growth in the form of out-sized filaments was able to continue. Further studies and characterization of the platinum complexes showed that they were potent anti-tumor agents and could regress Sarcoma 120 tumors and the L1210 leukemia in mice [2, 3]. Since then clinical investigations have taken *cis*-$Pt(NH_3)_2Cl_2$, the most active of the complexes, through several stages of clinical testing. It is currently being used in trials on head and neck cancers [4, 5], cancers of the urinary tract [6], disseminated testicular cancer [7], ovarian cancer [8] and various others. Its effectiveness is enhanced in combination chemotherapy with other recognized anti-tumor drugs such as adriamycin, vinolastin and bleomycin [5, 8].

Whilst clinical testing continues there has been considerable activity aimed at elucidating the mechanism of action. The diverse types of cancer towards which *cis*-$Pt(NH_3)_2Cl_2$ is active suggests a very general mechanism. Studies have been aimed at elucidating changes in the platinum coordination sphere on aquation [9–11], interactions with DNA [12–22] and protein [23, 34]. In our laboratories we have paid particular attention to enzyme inhibition studies. For example we examined the effect of platinum complexes on malate dehydrogenase [25–28] and leucineaminopeptidase [30, 31]. Studies involving yeast or liver alcohol dehydrogenase with *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ show that the latter exhibits the enzyme 3.5 times more than the former. We have suggested why this may be so [31].

Our present study was undertaken to measure the activities of the enzymes malate dehydrogenase (EC 1.1.1.37) and fumarase (EC 4.2.1.2) in the presence of $PtCl_4^{2-}$, *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ and physiological saline solution, in an attempt to correlate the anti-tumor activity and toxicity of these species with their ability to inhibit these enzymes.

## Experimental

### *Assay of Fumarase*

Fumarase was assayed by a modification of the method of Racker [32]. A substrate solution of 0.1 *M* phosphate buffer and 0.025 *M* L-malic acid was adjusted to pH 7 ± 0.1. Ten μL aliquots of enzyme solution (approximately 0.5 μ*M*) were mixed with 3 ml of the substrate solution in a 1 cm-path cuvette, and the rate of formation of fumarate was measured at 240 nm.

### *Assay of Malate Dehydrogenase*

Malate dehydrogenase was assayed under conditions described by Friedman *et al.* [25]. The substrate solution was 0.1 *M* L-malic acid, 0.1 *M* glycine and 0.2 m*M* $NAD^+$, adjusted to pH 9.5 ±

*Author to whom correspondence should be addressed.

0020-1693/84/$3.00

0.1 The enzyme concentration in all experiments was approximately 0.5 $\mu M$.

All assays were done on a Gilford Model 250 recording spectrophotometer.

Protein concentrations were determined by the Lowry method [33] and by the absorptivities of the enzymes; $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 340 mm for malate dehydrogenase and $2.4 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 240 mm for fumarase.

*24 Hour Inhibition Studies*

The concentrations (0.3 m$M$ → 20 m$M$) of *cis* and *trans* platinum complexes which were needed to inhibit malate dehydrogenase and fumarase over a long period of time were noted. Enzyme solutions were prepared at pH 7 in phosphate buffer. Fumarase studies were done in 0.1 $M$ phosphate, but malate dehydrogenase activity was better maintained in 0.2 $M$ phosphate. Platinum complexes were prepared by dissolving a few mg in a few ml of the appropriate pH 7 phosphate buffer, with gentle heating to 70 °C. These solutions were then diluted to give the desired platinum protein ratio. All of the complexes were sufficiently soluble in water for the purpose of this study. The process of dissolving the platinum complexes took a few minutes only, and 0.50 ml of each diluted solution was immediately added to 0.50 ml of the enzyme solution and the initial enzyme activity of the system was at once determined. In the experiments involving the effects of chloride ions on platinum inhibition, the platinum complexes were dissolved in 0.2 $M$ NaCl solution made up in phosphate buffer at pH 7, so that the incubated systems were 0.1 $M$ $Cl^-$.

The enzyme–platinum systems were then incubated at 25 °C for 24 h. Appropriate blanks containing the enzyme in phosphate and phosphate-chloride buffer were always included. Assays were made after 24 h, and in one experiment the activity was measured after 48 h to ensure that no further significant inhibition had occurred.

The molar ratio of platinum complex to protein had been adjusted to give measurable inhibition, and it was usually quite high, between $6 \times 10^2$ and $4 \times 10^4$. The binding constants (Ke) were calculated as previously described [25]. All experiments were run in duplicate.

*Kinetic Experiments*

The inhibition of the enzymes by $K_2PtCl_4$ were studied kinetically. The platinum complex solutions were prepared as before but without heating and 0.5 ml was added rapidly to 0.5 ml of enzyme. The concentrations of the $K_2PtCl_4$ were between 0.1 and 0.2 m$M$ yielding ratios of platinum complex to enzyme of 200:1 → 400:1. This mixture was assayed as quickly as possible, within 40 s, and thereafter at intervals of 250 to 500 s until enzyme activity was negligible or had levelled off. A blank containing enzyme and buffer alone was always run, and this was used as the reference in calculation of the percent remaining activity. All experiments were run in duplicate.

*Preaquation Studies*

All complexes were preaquated for 24 h before addition to the enzymes.

*Calculation of Kinetic Rate Constants*

Kinetic data was recorded as activity remaining ($\Delta A/m$) after inhibition, at time t. To calculate rate constants it was assumed that the enzyme inhibition was pseudo first order for the reaction,

$$E + I \xrightarrow{k_1} EI \tag{1}$$

the rate equation is,

$$\frac{d[EI]}{dt} = k_1[e_o - EI][I] \tag{2}$$

where $k_1$ is the second order rate constant.

However [I] in these experiments is several thousand times greater than the $e_o$ concentration, and so may be considered effectively constant. This means that the reaction is pseudo-first order, the rate being dependent only on the concentration of free enzyme, [E]. Separation of the variables and integration of the rate equation yields:

$$\ln \frac{e_o}{e_o - [EI]} = k_1[I]t \tag{3}$$

Since $e_o$ is proportional to the initial activity at t = o, and $e_o - [EI]$ is the concentration of uninhibited enzyme, which is proportional to the activity remaining after the inhibition at time t, it can be written:

$$\ln \frac{\text{initial activity}}{\text{remaining activity}} = k_1[I]t \tag{4}$$

the plot of ln (initial activity/remaining activity) *versus* time gave linear plots with a slope of $k_1[I]$.

## Results

Inhibition of the enzymes by $PtCl_4^-$ was studied kinetically (Fig. 1). It was found that inhibition by the fresh complex solution occurred at a similar rate in the presence or absence of exogenous $Cl^-$, but it was considerably enhanced by preaquation of the complex. The calculated rate constants are reported in Table I, and it is observed that in all studies (fresh and aquated $PtCl_4^-$) fumarase and malate dehydrogenase yield essentially the same value.

TABLE I. Rate constants for the Inhibition of Fumarase and Malate Dehydrogenase by $K_2PtCl_4$. These values were computed from the curves in Fig. 1.

| Enzyme | Environment of $PtCl_4^-$ | $k_1$ ($s^{-1}$) |
|---|---|---|
| Fumarase | Fresh or 0.1 *M* $Cl^-$ | 2.6 |
| | Aquated | 4.8 |
| Malate Dehydrogenase | Fresh or 0.1 *M* $Cl^-$ | 3.0 |
| | Aquated | 5.5 |

TABLE II. Binding Constants for the Inhibition of Fumarase and Malate Dehydrogenase by *Cis*- and *Trans*-$Pt(NH_3)_2Cl_2$ under Various Conditions. Inhibition time was 24 h, and the aquation time (before mixing the complexes with the enzymes) was also 24 h.

| Enzyme | $Pt(NH_3)_2Cl_2$ Complex | Ke, $M^{-1}$ | Ke (aquated, $M^{-1}$) | Ke (in 0.1 *M* $Cl^-$) $M^{-1}$ |
|---|---|---|---|---|
| Fumarase | *Cis* | $3.1 \times 10^3$ * | $5.8 \times 10^2$ | $3.1 \times 10^3$ |
| | *Trans* | $2.5 \times 10^3$ | $9.4 \times 10^2$ | $1.7 \times 10^4$ |
| Malate | *Cis* | $2.8 \times 10^2$ | $2.2 \times 10^2$ | $1.8 \times 10^2$ |
| Dehydrogenase | *Trans* | $3.1 \times 10^2$ | $9.0 \times 10^1$ | $5.3 \times 10^3$ |

*The average of duplicate experiments were all within 25% error, which was within 0.005 Å units in the activity.

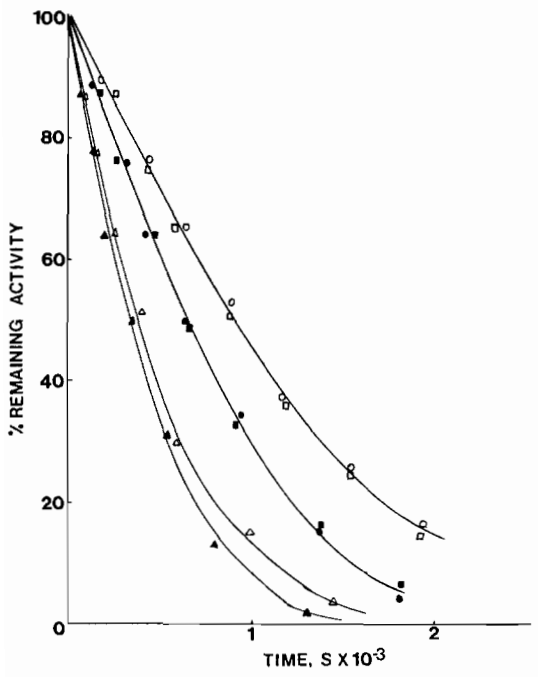


Fig. 1. Inhibition of Fumarase and Malate Dehydrogenase by High Concentrations of $K_2PtCl_4$. Inhibition of fumarase; –●–■– (fresh –●–, 0.1 *M* $Cl^-$ –■–) and –▲– (24 h aquated complex). Inhibition of malate dehydrogenase; –○–□– (fresh –○–, 0.1 *M* $Cl^-$ –□–) and –△– (24 h aquated complex).

Fumarase is fairly strongly inhibited by both the *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ in fresh solution, with binding constants of the order of $3 \times 10^3$ $M^{-1}$, while the aquated species are somewhat less inhibitory being of the order of 5 to $10 \times 10^2$ $M^{-1}$ (Table II). In the presence of exogenous $Cl^-$ the inhibition by the *cis*- complex is not significantly changed while the *trans* inhibition is enhanced by a factor of 7. The data for the *cis*- and *trans*- complex inhibition of malate dehydrogenase is also presented in Table II, and it is similar to the results using fumarase except that the binding constants are approximately an order of magnitude less. One small difference is that the *trans*-complex inhibition in the presence of chloride ion shows a 17-fold enhancement compared with the same experiments in the absence of chloride.

## Discussion

The sensitivity of the two enzymes toward the platinum complexes may be generally explained in terms of the known essential side-chain groups and their nucleophilicity and their affinity for platinum. Fumarase has at its active site both an essential methionine and histidine [34, 35], the $-SCH_3$ of methionine has a high affinity for platinum [36], while histidine may have some affinity. Fumarase has 12 thiol groups, but they all seem to be too well buried in the hydrophobic regions of the mole-

cule to be of importance except under perturbing conditions. Thus, fumarase is probably inhibited by platinum complexes largely by their complexation with the essential methionine and/or histidine. The interaction of the complexes with mitochondrial-malate dehydrogenase can be described by a different mechanism. This enzyme has two essential thiol groups, and its activity is very sensitive to sulfhydryl modifiers. These thiols appear to be near the coenzyme binding sites [37]. There is also a histidine side-chain which can be modified to produce an inactivated enzyme, and that also appears to be involved in the catalytic mechanism [38]. There is no evidence of sensitivity to methionine reagents.

Thus, platinum complex inhibition of the two enzymes is probably dominated by methionyl–platinum reactions in fumarase and cysteyl–platinum interactions in malate dehydrogenase. We generally find that fumarase is more strongly inhibited than malate dehydrogenase by the individual complexes. This is in compliance with earlier observations concerning the greater stability of the methionyl complexes of platinum [27].

The effect of aquation on the three complexes, $PtCl_4^-$, *cis* and *trans*-$Pt(NH_3)_2Cl_2$ brings forth the following suggestion. The tetrahalo complex will aquate to the diaquo species upon solvation of the complex (eqn. 5).

$$PtCl_4^- + 2H_2O \rightleftharpoons Pt(H_2O)_2Cl_2 + 2Cl^- \quad (5)$$

The neutral complex now reacts rapidly with the enzyme via displacement of the halide or water groups. The *cis*- and *trans*-complexes will also aquate more slowly, but in this case a positive mono or diaquo complex is produced (eqn. 6).

$$Pt(NH_3)_2Cl_2 \overset{+H_2O}{\rightleftharpoons} Pt(NH_3)_2(H_2O)Cl^+ \overset{+H_2O}{\rightleftharpoons} Pt(NH_3)_2(H_2O)_2^{2+} \quad (6)$$

The positive complexes are inactive towards the enzymes, as had been previously shown for $Pt(NH_3)_3Cl^+$ and $Pt(NH_3)_4^{2+}$ [26]. Thus, the most active species will be the neutral forms of the molecules.

The effect of addition of $Cl^-$ on the kinetics of inhibition by $PtCl_4^-$ is not significant due to the thermodynamic favorability of chloride displacement by the water. However, the $Cl^-$ enhancement of inhibition by the *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ towards both enzymes as well as the differentiation of inhibition between the two complexes can be explained as follows. Both isomers initially bind to the enzyme monofunctionally, with the more halogenated species (more neutral species) producing the more favorable protein–platinum complex interaction. Therefore, binding should be increased by exogenous chloride. The complex, now in contact with the protein, has a good chance of interacting bi-functionally with a second, near-by nucleophile, to yield a still more stable interaction and therefore an increase in the inhibition. The *trans* isomer could be sterically more favored by a specific, single binding site. Or, and this seems more likely, the group on the enzyme which binds the complex mono-functionally, be it $-SCH_3$, sulfhydryl or imidazole, is a relatively soft ligand, and exerts a strong *trans* effect on the remaining labile ligands – but only in the *trans* isomer. In the *cis* isomer there is an amine, which is unlikely to become reactive in the position *trans* to this protein–platinum bond. In this way, the *trans* complex may have a much greater tendency to bind bi-functionally, and thus inhibit any enzyme more strongly, than the *cis* one. It would be necessary to extend enzyme inhibition studies to a wider range of *trans* complexes to substantiate this idea. This type of effect may also account for the strong inhibitions observed with $PtCl_4^{2-}$.

A model such as this seems preferable to the idea that there is simply a specific binding site on the enzyme that 'fits' the *trans* isomer better than the *cis*. Several cases have now been reported where the *trans*-isomer of $Pt(NH_3)_2Cl_2$ is a stronger inhibitor than the *cis*: the two in this study, yeast alcohol dehydrogenase [31] (*trans* is 3.5 times better), LDH [31], (*trans* is twenty times better), and thymidylate synthase [39]. Rather than postulate a stereospecific binding site on each of these enzymes, which implies a series of coincidences in favor of the *trans* isomer, it is suggested that an intrinsic property of the *trans* complex itself is involved. This is then possible in any enzyme that can bind platinum through an activating group; the second, enhanced reaction can occur with any convenient nucleophilic group, and the resulting bi-functional binding should restrict and inhibit the enzyme strongly.

## References

1 B. Rosenberg, L. VanCamp and T. Krigas, *Nature, 205,* 698 (1965).
2 B. Rosenberg and L. VanCamp, *Cancer Res., 30,* 1799 (1970).
3 B. Rosenberg, L. VanCamp, J. E. Traska and V. H. Mansour, *Nature, 222,* 385 (1969).
4 C. Jacobs, J. R. Bedino, D. R. Coffinet, W. E. Fee and R. L. Goode, *Cancer (Phila.), 42 (5),* 2135 (1978).
5 R. E. Wittes, E. Cvitkovic, I. H. Drakoff and E. W. Strong, *J. Clin. Hematol. and Oncol., 7 (2),* 711 (1977).
6 J. J. Sternberg, R. B. Bracken, P. B. Handel and D. E. Johnson, *J. Am. Med. Assoc., 238 (21),* 2282 (1977).
7 L. H. Einhorn and J. Donohue, *Ann. Intern. Med. 87 (3),* 293 (1977).
8 H. W. Bruckner, C. C. Cohen, G. Deppe, B. Kabakow, R. C. Wallach, E. M. Greenspan, S. B. Gusberg and J. F. Holland, *J. Clin. Hematol. and Oncol., 7 (2),* 619 (1977).
9 B. Rosenberg, *Biochimie, 60,* 859 (1978).

10 R. Faggiani, B. Lippert, C. J. L. Lock and B. Rosenberg, *J. Am. Chem. Soc., 99,* 777 (1977).
11 M. A. Tucker, C. B. Colbin and D. S. Martin, *Inorg. Chem., 3,* 1373 (1963).
12 H. C. Harder and B. Rosenberg, *Int. J. Cancer, 6,* 207 (1970).
13 J. A. Howle and G. R. Gale, *Biochem. Pharmacol., 19,* 2757 (1970).
14 E. Heinen and R. Bassleer, *Biochem. Pharmacol., 25 (16),* 1871 (1976).
15 J. M. Pascoe and J. J. Roberts, *Biochem. Pharmacol., 23,* 1345 (1974).
16 J. M. Pascoe and J. J. Roberts, *Biochem. Pharmacol., 23,* 1359 (1974).
17 P. J. Stone, A. D. Kelman and F. M. Sinex, *Nature, 251,* 736 (1974).
18 D. M. L. Goodgame, I. Jeeves, F. L. Phillips and A. C. Skapski, *Biochim. Biophys. Acta, 378,* 153 (1975).
19 J. Dehand and J. Jordanov, *J. Chem. Soc. Chem. Commun.,* 598 (1976).
20 R. C. Harrison and C. A. McAuliffe, *Inorg. Perspec. in Biol. and Med., 1 (4),* 261 (1978).
21 N. H. A. Fravel and J. J. Roberts, *Chem. Biol. Interact., 12 (3/4),* 375 (1976).
22 H. W. VanDenBerg and J. J. Roberts, *Chem. Biol. Interact., 11,* 493 (1975).
23 R. E. Dickerson, D. E. Eisenberg, J. Varnum and M. L. Kopka, *J. Mol. Biol., 45,* 77 (1969).
24 H. Kohl, S. Haghighi and C. A. McAuliffe, *Chem. Biol. Interact., 29,* 327 (1980).
25 M. E. Friedman, B. Musgrove, K. Lee and J. E. Teggins, *Biochim. Biophys. Acta, 250,* 286 (1971).
26 J. E. Teggins and M. E. Friedman, *Biochim. Biophys. Acta, 350,* 273 (1974).
27 M. E. Friedman, J. E. Teggins, *Biochim. Biophys. Acta, 341,* 277 (1974).
28 M, E. Friedman and P. Melius, *J. Clin. Hematol. and Oncol., 7 (2),* 503 (1971).
29 P Melius, J. E. Teggins, M. E. Friedman and R. W. Guthrie, *Biochim. Biophys. Acta, 268,* 194 (1972).
30 P. Melius, R. W. Guthrie and J. E. Teggins, *J. Med. Chem., 14,* 75 (1971).
31 M. E. Friedman and J. E. Teggins, *Biochim. Biophys. Acta, 350,* 264 (1974).
32 E. Racker, *Biochim. Biophys. Acta, 7,* 20 (1950).
33 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem., 193,* 265 (1951).
34 S. S. Taylor, S. S. Oxley, W. S. Allison and N. O. Kaplan, *Proc. Natl. Acad. Sci. U.S., 70,* 1790 (1973).
35 E. M. Gregory and J. H. Harrison, *Biochem. Biophys. Res. Commun., 40,* 995 (1970).
36 C. A. McAuliffe, *J. Chem. Soc. (A),* 641 (1967).
37 G. A. Rogers, N. Shaltiel and P. D. Boyer, *J. Biol. Chem., 251,* 5711 (1976).
38 J. J. Holbrook, A Lodola and N. P. Illsley, *Biochem. J., 139,* 797 (1974).
39 J. L. Aull, C. A. Rice and L. A. Tebbets, *Biochemistry, 16,* 672 (1977).