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THE BLOCKING OF THE TETRACHLOROPLATINATE (II) INHIBITION OF MALATE DEHYDROGENASE BY SULFUR-CONTAINING AMINO ACIDS

M. E. FRIEDMAN[a] and J. E. TEGGINS[b]

[a]*Department of Chemistry, Auburn University, Auburn, Ala. and* [b]*Auburn University at Montgomery, Montgomery, Ala. (U.S.A.)*

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SUMMARY

The inhibition of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) by the tetrachloroplatinate (II) complex, $PtCl_4^{2-}$, in the presence of various concentrations of the amino acids D,L-methionine and L-cysteine was measured. The relative concentrations of $PtCl_4^{2-}$ to malate dehydrogenase was 100:1.

From the data, the half-life and the relative rate of inhibition in the presence and absence of the amino acids were calculated. Using these values it was possible to calculate a stability constant for each system. The stability constants for the malate dehydrogenase–$PtCl_4^{2-}$–L-cysteine (K_c), malate dehydrogenase–$PtCl_4^{2-}$–D,L-methionine (K_m) and, malate dehydrogenase–$PtCl_4^{2-}$(K_E) were 56 M^{-1}, 917 M^{-1} and $8 \cdot 10^5$ M^{-1}, respectively.

The reversibility of the malate dehydrogenase–$PtCl_4^{2-}$ complex was also demonstrated, by addition of D,L-methionine to the completely inhibited enzyme. About 40% of the enzyme activity was regenerated. Using the stability constants it was calculated that 27% of the enzyme's activity should be regenerated.

From the results it is suggested that the free platinum complexes may be maintained in solution for a longer period of time if some or all of the halide ligands were replaced by sulfur groups from molecules like cysteine or methionine.

INTRODUCTION

Dickerson et al. [1] have shown that a methionine molecule can coordinate to a platinum(II) ion. Mogilievkina et al. [2] have demonstrated further that methionine acts as a bidentate ligand binding through sulfur and nitrogen atoms to the platinum. Other examples of sulfur-containing platinum complexes have also been reported [3, 4]. In previous work in this laboratory [5] it was demonstrated that several platinum(II) complexes inhibit malate dehydrogenase. Because it is known that d^8–d^{10} metal ions similar to Pt^{2+} (e.g. Hg^{2+}, Ag^+, and Pb^{2+}) inhibit enzymes via attachment to sulfhydral groups [6, 7], it is possible that the observed malate dehydrogenase inhibition by platinum complexes also could involve reaction with sulfur atoms in the

enzyme. Consequently, this study reports the results of a series of experiments which were performed to compare the affinities of malate dehydrogenase and several sulfur-containing amino acids for $PtCl_4^{2-}$ in aqueous solution.

The biological activity of chloro–platinum(II) complexes has been of great interest since Rosenberg et al. showed that these compounds can be potent tumor inhibitors [8]. In further work [9], Rosenberg et al. have found that *cis*-$Pt(NH_3)_2Cl_2$ binds to nucleoside derivatives, and Roberts and Pascoe [10] have shown that the same complex can cross-link complimentary chains of DNA. It is believed that the described studies could help elucidate the above observations.

MATERIALS AND METHODS

Materials

Unless specified, all biological materials were purchased from the Sigma Chemical Co. Pig heart malate dehydrogenase (Lot No. 40C-4700) was purified on carboxymethyl cellulose [11] and recrystallized from a saturated $(NH_4)_2SO_4$ solution. NAD^+ and NADH Lot No. 12C-7400 and Lot No. 69B-6300, respectively, were used without further purification. DL-Methionine, L-histidine, L-aspartic acid, L-lysine, and glutathione were purchased from the Eastman Organic Chemical Co. K_2PtCl_4 was prepared from metallic platinum obtained from J. Bishop Co. [12]. All other chemicals were of reagent grade.

Enzyme assay

The method as described in a previous paper [13] was utilized to assay malate dehydrogenase.

Kinetic studies

A solution containing a compound used to block the inhibition of malate dehydrogenase by K_2PtCl_4 was added to the enzyme preparation which was obtained as described in a previous publication [5]. The enzyme inhibition reaction was initiated by addition of an aliquot of the aged platinum solution to the mixture of malate dehydrogenase and the particular sulfur-containing compound being tested. The enzyme and platinum concentrations were maintained at $1.5 \cdot 10^{-6}$ and $1.5 \cdot 10^{-4}$ M, respectively, and the concentration of the sulfur-containing compound was varied as indicated in Table I. Potassium hydrogen phosphate was incorporated in reaction solutions in order to control H^+ concentrations and ionic strength. The reaction cell was maintained at 25 °C. 5-μl aliquots of the reaction mixture were removed for assay at various time intervals as described in a previous publication [5].

Equilibrium study

In order to test the proposed equilibria (Eqns 1 and 2), it was decided to completely inhibit malate dehydrogenase with K_2PtCl_4, add a relatively large concentration of methionine, and allow the composite solution to equilibrate. Finally, the enzyme activity was assayed as indicated in Table II. All experiments were run in a 0.1 M phosphate buffer (pH 7.0) and 25 °C. A solution containing $1.5 \cdot 10^{-6}$ M enzyme and a 100-fold excess of K_2PtCl_4 was allowed to stand for 4 h. After this period of time, enzyme inhibition was essentially complete (as shown by the measured half-life in

TABLE I

EFFECT OF L-CYSTEINE ON THE INHIBITION OF MALATE DEHYDROGENASE BY K_2PtCl_4 AT 25 °C

pH = 7.0, [malate dehydrogenase] = $1.5 \cdot 10^{-6}$ M, [K_2PtCl_4] = $1.5 \cdot 10^{-4}$ M, enzyme activity measured in $M \cdot ml^{-1} \cdot min^{-1} \times 10^4$.

Time (min)	Activity ($M \cdot ml^{-1} \cdot min^{-1}$) $\times 10^4$ at given L-cysteine concn			
	0 M	0.005 M	0.01 M	0.05 M
0*	195	188	192	188
1	193	186	190	186
20	132	129	138	145
40	76	90	103	129
60	40	55**	68**	113**

* By extrapolation.
** Some ppt. of L-cysteine was noticed.

Table II). An aliquot of a concentrated methionine solution was added to the reaction mixture with a resultant overall methionine concentration of 0.05 M. The activity of this solution was assayed after approx. 1- and 2-day time intervals to detect any possible enzyme reactivation.

TABLE II

CALCULATED PARAMETERS FROM TABLE I

L-Cysteine concn (M)	0	0.005	0.01	0.005
Half-life (min)*	30	38	47	118
Relative rate of inhibition	1.00	0.79	0.64	0.26
Stability constant for Pt–cysteine (M^{-1})**	—	55	57	57

* Half-life for blank from first-order plot. When cysteine present first-order treatment for 20–40 min employed to calculate half-life.
** As defined in Eqn 2.

RESULTS AND DISCUSSION

Several amino acids, with functional side chains, were used to study their effects on the inhibition of malate dehydrogenase by K_2PtCl_4. Even at concentration levels of 0.05 M (compared to 10^{-6} M malate dehydrogenase and 10^{-4} M K_2PtCl_4), L-histidine, L-lysine, and L-aspartic acid did not decrease the rate of inhibition of the enzyme by the platinum complex to any significant extent. On the other hand, both D,L-methonine and L-cysteine decreased the rate of inhibition at relatively low concentrations compared to the other amino acids.

Data for the reactions containing L-cysteine are given in Tables I and II. It can be seen that the presence of cysteine decreases the rate of inhibition of the enzyme by the platinum complex. In the absence of cysteine, the rate of enzyme inhibition approximates a first-order kinetic reaction with a half-life of 30 min. (This value can be computed by considering the change in activity between 1 and 40 min). When

cysteine was present, the half-life was calculated from the change of activity between 20 and 40 min in order to allow time for any reaction between cysteine and the platinum complex to reach equilibrium before the half-life was calculated. Because the half-life of a first-order reaction is independent of concentration this procedure should give a reasonable estimate of the half-life.

An obvious possibility to explain the effect of cysteine in these reactions would be to postulate that $PtCl_4^{2-}$ and cysteine form a complex according to Eqn 1, where K_c would be the stability constant.

$$PtCl_4^{2-} + \text{cysteine} \rightleftharpoons \text{cysteine–}PtCl_4^{2-} \quad (1)$$

$$K_c = \frac{[\text{cysteine–}PtCl_4^{2-}]}{[\text{cysteine}]\,[PtCl_4^{2-}]} \quad (2)$$

The complex formation would reduce the free platinum concentration in solution which would, in turn, reduce the rate of inhibition of malate dehydrogenase. If it is assumed that the observed rate of inhibition, which would be inversely proportional to the half-life for a particular reaction, is directly proportional to the concentration of $PtCl_4^{2-}$ in solution, it is possible to calculate the latter value. Because the malate dehydrogenase concentration is so low, any reduction in free $PtCl_4^{2-}$ concentration would be caused by the reaction shown in Eqn 1. By simple substitution in the equilibrium expression, the values of K_c in Table II can be calculated. Although, in view of experimental errors, the very close agreements between the calculated constants are probably fortuitous (55–57 M^{-1}), it is obvious that the previous assumptions concerning the nature of the reaction do result in consistent values for the equilibrium constant.

TABLE III

EFFECT OF D,L-METHIONINE ON THE INHIBITION OF MALATE DEHYDROGENASE BY K_2PtCl_4 AT 25 °C

pH 7.0, [malate dehydrogenase] = $1.5 \cdot 10^{-6}$ M, [K_2PtCl_4] = $1.5 \cdot 10^{-4}$ M, enzyme activity measured in $M \cdot ml^{-1} \cdot min^{-1} \times 10^4$.

Time (min)	Activity ($M \cdot ml^{-1} \cdot min^{-1}$) $\times 10^4$ at given DL-methionine concn				
	0 M	0.001 M	0.002 M	0.005 M	0.02 M
0*	195	188	190	191	193
1	193	186	188	190	193
20	132	141	151	167	193
40	76	111	127	145	186
60	40	76	105	141	196

* By extrapolation.

It was possible to use a similar treatment to explain the effect of D,L-methionine in reducing the rate of inhibition of malate dehydrogenase by $PtCl_4^{2-}$. As can be seen from Tables III and IV, however, methionine blocks inhibition of the enzyme much more efficiently than similar concentrations of cysteine. This would correspond to the higher value for the stability constant (K_m) in Eqn 3.

$$PtCl_4^{2-} + \text{methionine} \rightleftharpoons PtCl_4^{2-}\text{–methionine} \quad (3)$$

$$K_m = \frac{[\text{methionine–}PtCl_4^{2-}]}{[PtCl_4^{2-}]\,[\text{methionine}]} \quad (4)$$

TABLE IV

CALCULATED PARAMETERS FROM TABLE III

D,L-Methionine concn (M)	0	0.001	0.002
Half-life (min)*	30	58	80
Relative rate of inhibition	1.0	0.52	0.37
Stability constant** for Pt–methionine (M^{-1})	—	985	848

* Same method employed in Table I.
** As defined in Eqn 4.

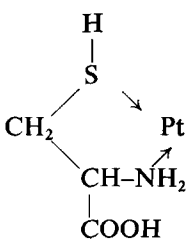
Because the free platinum complex was removed so efficiently by reaction with methionine, the enzyme never became completely inhibited when the methionine concentration was greater than 10^{-3} M. Consequently, the kinetic treatment used to estimate values of K_c was only used to calculate the value of K_m for the lowest methionine concentration. These data are shown in Table IV.

The stability constant for the complex formed between malate dehydrogenase and $PtCl_4^{2-}$ has been reported in a previous paper [5]. This constant (K_c) is defined by the equilibrium reaction shown in Eqn 3.

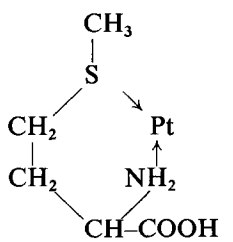
$$MDH + PtCl_4^{2-} \rightleftharpoons MDH\text{–}PtCl_4^{2-} \quad (5)$$

$$K_E = \frac{[MDH\text{–}PtCl_4^{2-}]}{[MDH]\,[PtCl_4^{2-}]} \quad (6)$$

The stability constants for the complexes formed by cysteine, methionine, or malate dehydrogenase (MDH) under identical conditions at 25 °C are 56 M^{-1}, 917 M^{-1}, and $8 \cdot 10^5$ M^{-1}, respectively. The greater stability of the methionine complex compared to the cysteine complex can be explained in a conventional manner. For example, the sulfur atom in methionine should be a stronger Lewis base than the similar atom in cysteine because of electron donation by the methyl group in the former compound.



cysteine complex



methionine complex

Also, a bidentate methionine complex would contain a six-membered ring rather than the five-membered ring which would be present in the cysteine complex. Possible complexes are shown above.

TABLE V

PARTIAL REACTIVATION OF K_2PtCl_4-INHIBITED MALATE DEHYDROGENASE BY D,L-METHIONINE AT 25 °C

pH = 7.0, [malate dehydrogenase] = $1.5 \cdot 10^{-6}$ M.

Time (h)	Enzyme activity $\times 10^4$ ($\mu M \cdot ml^{-1} \cdot min^{-1}$)	
	Uninhibited*	Inhibited**
0	193	190
4	189	0***
24	188	73
53	180	79

* No methionine or K_2PtCl_4 present.
** Solution was made $1.5 \cdot 10^{-4}$ M in $PtCl_4^{2-}$.
*** Solution was made $5 \cdot 10^{-2}$ M in D,L-methionine after 4 h.

The stability of the malate dehydrogenase complex, however, is enormously greater than those for the complexes formed with the simple amino acids. This would require an exceptionally favorable steric situation, which would suggest that the platinum complex reacts at a specific site on the enzyme.

An experiment was devised to test the reversibility of the reactions proposed in this paper. This involved completely inhibiting a malate dehydrogenase sample with $PtCl_4^{2-}$ and then allowing the reaction mixture of equilibrate with a relatively high concentration of methionine. Methionine was employed because of its greater affinity for $PtCl_4^{2-}$ compared to cysteine. As can be seen in Table III, partial reactivation of the enzyme was achieved. These data show that the inhibition of the enzyme is reversible, because 38% of the initial activity was regenerated after complete inhibition by $PtCl_4^{2-}$. The results are also consistent with competition between malate dehydrogenase and methionine for the platinum complex. The added methionine reduces the free platinum concentration in solution causing the breakdown of the malate dehydrogenase–$PtCl_4^{2-}$ in a mixture obtained by equilibrating a solution which was initially 0.05 M in methionine and $1.5 \cdot 10^{-4}$ M in $PtCl_4^{2-}$ would be $3.3 \cdot 10^{-6}$ M. Using the value of K_E in Eqn 3 ($8 \cdot 10^5$ M^{-1}), the concentration of free enzyme in an equilibrated solution which initially contained $3.3 \cdot 10^{-6}$ M $PtCl_4^{2-}$ and $1.5 \cdot 10^{-6}$ M enzyme would be $4.1 \cdot 10^{-7}$ M. (It is to be noted that the relatively large concentration of the methionine–$PtCl_4^{2-}$ complex present in solution would hold the free $PtCl_4^{2-}$ concentration constant despite the reaction of the latter with the enzyme.) The above calculation predicts that 27% of the initial enzyme activity should remain in the described equilibrated solution. From Table III it can be seen that approx. 40% of the initial enzyme activity was observed in the reactivated solution which had been allowed to stand for 1–2 days. In view of the experimental errors involved, these values are in excellent agreement. It does appear that cysteine, methionine, and malate dehydrogenase all compete for $PtCl_4^{2-}$ in solution by means of reversible equilibria, although the malate dehydrogenase–$PtCl_4^{2-}$ complex is by far the most stable of the group.

Considerable current work is being performed on the use of chloro–platinum(II) complexes as tumor-inhibiting agents. The equilibria studied in this publication suggests that almost constant concentrations of free platinum complexes could be main-

tained in animals by injecting complexes in which the platinum complex is bound to a sulfur-containing amino acid. These chelates would slowly decompose to replace consumed free platinum. It should also be possible to purge an animal of free platinum by the injection of suitable chelating agents.

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