Biochimica et Biophysica Acta, 350 (1974) 263–272

BBA 67227

THE REACTIVITIES OF ISOMERS OF DICHLORODIAMMINE–PLATINUM (II) WITH DEHYDROGENASE ENZYMES

EVIDENCE FOR INHIBITION VIA CROSS-LINKAGE

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(Received November 26th, 1973)

SUMMARY

The reversible inhibition of malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37), lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27) and horse liver alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) by both *cis*- and *trans*-dichlorodiammine–platinum (II) ($Pt(NH_3)_2Cl_2$) were carried out at pH 7.1 and 25 °C. Inhibition of both liver and yeast alcohol dehydrogenase were measured at 4 °C due to the latter's instability at higher temperature for long periods of time under the experimental conditions used in this study. The equilibrium constant (K_e) was calculated for each enzyme–platinum complex system.

It was shown that inhibition of both malate dehydrogenase and liver alcohol dehydrogenase was independent of the particular platinum isomer, while the trans isomer was a significantly better inhibitor than the cis form when either yeast alcohol dehydrogenase or lactate dehydrogenase was used. Thus, it has been proposed that the two former enzymes are being inhibited by a monodentate chelation with the platinum derivatives while the latter enzymes are being inhibited by a bidendate chelation. It has also been proposed that absolute differences in inhibition of various enzymes by a specific platinum inhibitor is due to different geometries about the inhibitor site while similar inhibition values are caused by similar geometries.

INTRODUCTION

Since Rosenberg et al. [1] showed that chloro–platinum complexes can be effective tumor inhibitors, the biological activities of platinum complexes have been of great interest to many workers [2]. Further work by Rosenberg [3] has shown that *cis*–$Pt(NH_3)_2Cl_2$, which has proved to be a superior tumor inhibitor, binds to nucleoside derivatives, while Roberts and Pascoe [4] have shown that the same complex can cross-link complementary chains of DNA. Several studies involving enzyme inhibition by platinum complexes have been reported [5–7]. Consequently, this study was undertaken to compare the inhibitive properties of *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ with a series

of related enzymes. Reactions involving chloro–platinum complexes invariably involve substitution of chloride rather than ammonia ligands [2]. Because of the differences in the halide–platinum–halide bond angles for the cis isomer (90°) and the trans isomer (180°), the steric requirements for cross-linkage with a protein chain would be vastly different for the isomers. On the other hand, apart from steric differences, the chemical properties of both isomers are quite similar. Very different inhibition of a particular enzyme by one of the isomers compared to the other would indicate that geometrical considerations were important in the inhibition mechanism. Reaction of both chloride ligands in a $Pt(NH_3)_2Cl_2$ molecule, which would amount to cross-linkage, would be suspected in such a case.

It has been known for some time that compounds of elements related to platinum (e.g. mercury, silver, and lead) inhibit enzymes by coordination to sulfhydryl groups [8,9]. Dickerson et al. [10] and Mogilievkina et al. [11] have demonstrated that sulfur-containing compounds can bind to platinum via sulfur atoms. Recently, work completed in our laboratory showed that only sulfur-containing amino acids such as cysteine and methionine can compete with malate dehydrogenase for platinum (II) complexes [12]. Because of these facts it was decided to begin investigations into possible cross-linking by platinum complexes with three enzymes containing reactive sulfhydryl groups related to malate dehydrogenase, namely beef heart lactate dehydrogenase [13,14] and both horse liver and yeast alcohol dehydrogenase [15–18].

MATERIALS AND METHODS

Materials

Beef heart lactate dehydrogenase (lot No. L-2625), horse liver (lot No. 12C-8120) and yeast alcohol dehydrogenase (lot No. 340-26) were purchased from the Sigma Chemical Co. Nicotinamide adenine dinucleotide (NAD^+), lot No. 12C-7400, and reduced nicotinamide adenine dinucleotide (NADH), lot No. 69B-6300, were also purchased from the same company. *Cis*- and *trans*-dichlorodiammine platinum (II) [19] were prepared from metallic platinum obtained from the J. Bishop Co. All other chemicals were of reagent grade.

Enzyme purity

A few samples of each enzyme were passed through a carboxymethyl-cellulose column [20] and recrystallized from saturated $(NH_4)_2SO_4$ solution. The specific activity of these enzymes as well as the physical properties did not significantly change from the original sample so further purification by this method was omitted.

The degree of purity was determined by the following methods. Ultracentrifugation studies were performed in a Beckman-Spinco Model E ultracentrifuge using Schlieren optics, and only a single peak was observed at 59 780 rev./min for each enzyme. The concentrations of the enzyme solutions were between 7.0 and 7.5 mg/ml and the solvent was 0.1 M phosphate buffer, at pH 7.1. Disc gel electrophoresis was run at pH 8.5 in a polyacrylamide gel using a Canalco Analytical Disc Gel apparatus. The gel was a 7.5% cross-linked polymer prepared as described by Gabriel [21], and the electrophoresis was run for 45 min. The gels were stained with amido black for protein concentration while lactate dehydrogenase gels were also developed using a phenozine methosulfate–nitroblue tetrazolium stain for enzymatic activity. In the

experiments using both stains the major bands appeared at the same positions indicating that the major protein bands were identified with the enzyme and not with impurities. However, there was a faint minor band which appeared when using the amido black stain, and which was tetrazolium negative, indicating the presence of a minor component in the lactate dehydrogenase sample.

Comparison with previous work, using conventional nomenclature [22], indicated that the two bands are H_4 and H_3M which corresponds with the source of enzyme (beef heart). The small fast-moving band was possibly some non-enzymatic impurity. Since this impurity was not detected in ultracentrifuge patterns, it was probably present in low concentrations.

Liver alcohol dehydrogenase gels showed one broad peak indicating the absence of isoenzymes while yeast alcohol dehydrogenase yielded two bands which supports previous evidence of two isoenzymes [23]. No impurities were observed in the liver alcohol dehydrogenase preparation. However, a small minor band, which moved more rapidly than either of the two enzyme bands, was observed in the yeast preparation. This impurity was not observed in the Schlieren patterns from the ultracentrifugal studies [13].

Determination of protein concentration

The protein concentrations were measured by two methods: the Lowry method [24] which utilizes the Folin–Ciocalteu reagent, and calculation of the concentration using the known extinction coefficients $E^{1\%}_{280\ nm}$. The results obtained by both methods were consistent to within 5 % of one another. The extinction coefficients which were used in these studies were: 14.9 for beef heart lactate dehydrogenase [22], 4.55 for liver alcohol dehydrogenase [25] and 12.6 for yeast alcohol dehydrogenase [26].

Enzyme assays

The optimum conditions for assaying each of these enzymes were determined as follows:

(a) Lactate dehydrogenase. The enzyme was diluted to various concentrations in 0.1 M phosphate buffer at pH 7.0, and a 10-μl aliquot was removed and added to a 3-ml assay sample. The enzyme concentration which yielded the most favorable change in absorbance per min was 3.75×10^{-7} M. The optimum nucleotide concentration (NADH) which was found to be 2×10^{-4} M was similar to previously reported values [7, 22].

The activity was then studied as a function of substrate (pyruvate) concentration. The maximum activity was obtained at a pyruvate concentration of approx. 5×10^{-4} M. As the substrate concentration was further increased there was a slight decrease in activity suggesting substrate inhibition. Variation of the activity with phosphate buffer concentration and pH showed that over the pH range 7.0–8.0 and concentration range 0.3–0.01 M, the conditions for which the enzyme activity was the maximum was 0.03 M and pH 7.5. These were the buffer conditions used for all further experimentation.

The assay of lactate dehydrogenase was carried out in a Hitachi Model 139 spectrophotometer, temperature controlled at 25 °C. 1-cm cuvettes were used, and 3 ml of the assay sample, containing the above concentrations of buffer, substrate, and cofactor were introduced. 5 or 10 μl of 3.75×10^{-7} M enzyme solution were added to the cuvette and a decrease in absorbance of NADH, starting from 0.300, was

read at 340 nm. Changes in absorbance were utilized to calculate NADH concentrations using the known molar extinction coefficient of NADH (6.22×10^3).

(b) Liver alcohol dehydrogenase. Variation of all the components in the assay system: pyrophosphate buffer, ethanol substrate, NAD^+ cofactor and enzyme concentration, were studied in a manner similar to that for lactate dehydrogenase. The optimum concentrations obtained were: 1.5×10^{-5} M enzyme, 0.33 M ethanol, 0.015 M pyrophosphate buffer at pH 8.9 and 1.5×10^{-4} M NAD^+. The actual assay procedure was similar to the one used for lactate dehydrogenase except an increase in the concentration of NADH per min was measured and 25-μl samples of enzyme were used in each assay.

(c) Yeast alcohol dehydrogenase. The conditions were the same as those used in the liver enzyme experiments except that the enzyme concentration was considerably less (3×10^{-7} M) and only 10-μl aliquots were used for the assaying procedure.

The inhibitions proceeded in the absence of coenzymes and substrates. The only time in which free platinum complex, substrates and coenzymes were in solution together was during the 1-min interval needed for the measurement of activity. Since it has been shown in previous work [7] that the inhibition proceeds very slowly it is unlikely that the coenzyme or substrate could be displaced by the almost insignificant free inhibitor concentration (approx. 10^{-9} M) present in assay solutions.

Platinum complex preparations

cis-$Pt(NH_3)_2Cl_2$ was prepared and analyzed according to the method of Ramberg [27] as modified by Reishus and Martin [28]. The analysis calculated for the cis isomer is, Pt (63.03%), Cl (23.62%), while the measured values were, Pt (62.2%), Cl (23.7%). In addition aqueous complex solutions exhibited a maximum absorption at 300 nm and minimum at 247 nm in accordance with the reported values [28].

Trans-$Pt(NH_3)_2Cl_2$ was prepared by heating tetrammine platinum (II) chloride by the method of Drew [29] and modified by Bauer [30]. Chemical analysis showed Pt (64.9%), Cl (23.5%), while absorption spectra exhibited a maximum at 317 nm as had been previously observed [30].

From the elemental analysis, absorption spectra, as well as physical characteristics of the crystals, no detectable tetrahalide was observed; this compound is the most biologically active. The weak trans effect of Cl^- might produce some cis isomer in the trans compound, but from the absorption measurements this impurity could not exceed 1%.

Equilibrium studies

The equilibrium experiments were performed in a manner similar to the previously reported work of Friedman et al. [7]. In these studies a sample of either the *cis*- or *trans*-$Pt(NH_3)_2Cl_2$ isomer was dissolved in 0.1 or 0.3 M phosphate buffer at pH 7.0 depending upon the buffer concentration of the enzyme being investigated. An aliquot was removed and added to a stock solution of enzyme, and then the platinum complex–enzyme solution was diluted to the desired concentration with the phosphate buffer. In some of the experiments, a high concentration of the trans isomer was used and it was found to be necessary to warm the solution in order to solubilize the platinum complex. In those experiments in which the trans isomer solution was warmed a similar heating period was carried out for the cis isomer solution. The ultraviolet–

visible spectra of $Pt(NH_3)_2Cl_2$ isomer solutions at 25 °C does not change after heating and re-cooling to 25 °C in this manner. Equivalent experiments were performed in which the cis isomer solutions were and were not heated and no differences in inhibitions were observed. Lactate dehydrogenase equilibrium experiments were carried out in a 0.3 M phosphate buffer at pH 7.1 and 25 ± 0.1 °C. The phosphate buffer concentration was varied from 0.1 to 0.3 M and although no change in the activity was observed after 24 h, longer incubation times (48–72 h) did produce losses of activity at the lower salt concentrations. After 48 h a 30–40% loss of activity was measured using the 0.1 M buffer while no more than a 5% loss was measured using the 0.3 M buffer.

Yeast alcohol dehydrogenase is quite unstable at room temperature, and, therefore, all experiments were run at 4 °C. The enzyme solution was buffered using 0.1 M phosphate at pH 7.1. The enzyme activity was found to be independent of the phosphate concentration over a range of 0.05–0.5 M.

Liver alcohol dehydrogenase experiments were identical to those used for yeast alcohol dehydrogenase except that for the former enzyme source it was possible to employ reaction temperatures at both 4 and 25 °C.

The inhibition of malate dehydrogenase by a series of chloro–platinum complexes will be reported in detail in a later paper. Because of the direct relevance of the inhibition of malate dehydrogenase by *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ to this paper, equilibrium data for these inhibitions have been reported in Table III.

In all of the above studies the association inhibition constant (K_e), as defined in Eqn 2, has been employed.

$$E + I \rightleftarrows EI \tag{1}$$

$$K_e = \frac{[EI]}{[E]\,[I]} \tag{2}$$

where E is the enzyme and I the inhibitor.

RESULTS AND DISCUSSION

The results for both *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ inhibition of liver alcohol dehydrogenase, beef heart lactate dehydrogenase and pig heart malate dehydrogenase are reported in Tables I–III, respectively. The molar ratios of platinum complex to enzyme were studied over approximately a 10-fold range for both liver alcohol dehydrogenase and lactate dehydrogenase while the malate dehydrogenase study was conducted over a 5-fold range of platinum complex. The calculated K_e values are consistent over the entire platinum complex concentration range, and it was observed that these values did not change when equilibration was allowed to proceed for 72 h.

Errors in individual measurements of enzyme activities were of the order of 3–5%. Such errors would result in values of K_e obtained for experiments involving approx. 50% enzyme deactivation being accurate to within 10%. In each set of experiments the association inhibition constants obtained over a wide range of concentrations were sufficiently consistent to justify the employed mathematical treatment. For example, all of the individual constants calculated for the inhibition of liver alcohol dehydrogenase by the cis isomer at 4 °C are within 30% of the average value. This

TABLE I

INHIBITION OF LIVER ALCOHOL DEHYDROGENASE AS A FUNCTION OF DICHLORODIAMMINE-PLATINUM (II) CONCENTRATION AT pH 7.1

Enzyme concentration, $1.5 \cdot 10^{-5}$ M; incubation time, 48 h.

Molar ratio of Pt to enzyme	Enzyme activity $\times 10^4$ ($\mu M \cdot ml^{-1} \cdot min^{-1}$)		$K_e \times 10^{-2}$ (M^{-1}) (from Eqn 2)	
	4 °C	25 °C	4 °C	25 °C
(A) *Cis*-$Pt(NH_3)_2Cl_2$				
0	190	185	—	—
3.2	159	153	42	44
8.0	135	117	32	49
16	101	88	37	45
24	—	64	—	53
40	74	35	26	71
80	51	—	23	—
Average values for K_e			32	52
(B) *Trans*-$Pt(NH_3)_2Cl_2$				
0	190	185	—	—
3.2	170	160	25	33
8.0	143	132	27	34
16	109	90	31	44
24	80	71	38	45
36	60	40	40	49
Average values for K_e			32	41

TABLE II

INHIBITION OF BEEF HEART LACTATE DEHYDROGENASE AT pH 7.1 AND 25 °C

Enzyme concentration, $3.75 \cdot 10^{-7}$ M; incubation time, 48 h.

Molar ratio of Pt to enzyme	Enzyme activity $\times 10^{-2}$ ($\mu M \cdot ml^{-1} \cdot min^{-1}$)	$K_e \times 10^{-2}$ (M^{-1}) (from Eqn 2)
(A) *Cis*-$Pt(NH_3)_2Cl_2$		
0	180	—
800	147	7.5
2000	118	7.0
2800	104	7.0
4000	90	6.7
6000	67	7.5
Average value for K_e		7.1
(B) *Trans*-$Pt(NH_3)_2Cl_2$		
0	200	—
80	145	126
160	117	118
400	55	175
800	37	145
Average value for K_e		141

TABLE III

INHIBITION OF PIG HEART MALATE DEHYDROGENASE AT pH 7.1 AND 25 °C

Enzyme concentration, $1.5 \cdot 10^{-6}$ M; incubation time, 48 h.

Molar ratio of Pt to enzyme	Enzyme activity $\times 10^4$ ($\mu M \cdot ml^{-1} \cdot min^{-1}$)	$K_e \times 10^{-2}$ (M^{-1}) (from Eqn 2)
(A) *Cis*-$Pt(NH_3)_2Cl_2$		
0	163	—
500	134	3.0
1000	118	2.5
2500	72	3.4
Average value of K_e		3.0
(B) *Trans*-$Pt(NH_3)_2Cl_2$		
0	166	—
500	132	3.4
1000	115	3.0
2500	76	3.2
Average value of K_e		3.2

variation is within tolerable limits considering that the platinum concentration was varied by 2500%.

Calculated inhibition constants are summarized in Table IV. In the cases of malate dehydrogenase and liver alcohol dehydrogenase inhibition data for the cis and trans isomers are essentially identical. In view of the previously discussed experimental errors, however, the exact agreement between the data in one instance must be merely fortuitous. Because the geometries and dipole moments of the two isomers are quite different, it is unlikely that the platinum complexes can play a part in the rate-determining reaction which results in enzyme deactivation. The most simple explanation to explain these data would involve the formation of a single bond from the platinum complex to a reactive group in the enzyme. Possibly, the exposure of this group at a regular rate followed by rapid reaction with the platinum complex present in solution could account for the forward reaction in the equilibrium ($E + I \rightarrow EI$).

In a previous experiment the malate dehydrogenase preparation was completely inhibited by the addition of a 5-fold excess of tetrachloroplatinate (II) [12]. This platinum-containing ion is a much more effective enzyme inhibitor than the dichlorodiammine isomers [12]. Addition of a very high concentration of methionine, which com-

TABLE IV

ASSOCIATION INHIBITION CONSTANTS FOR MALATE DEHYDROGENASE, LACTATE DEHYDROGENASE, AND LIVER ALCOHOL DEHYDROGENASE BY $Pt(NH_3)_2Cl_2$ ISOMERS

Enzyme	Measured value of $K_e \times 10^{-2}$ (M^{-1})	
	cis isomer	trans isomer
Malate dehydrogenase at 25 °C	3.0	3.2
Lactate dehydrogenase at 25 °C	7.1	141
Liver alcohol dehydrogenase at 4 °C	32	32
Liver alcohol dehydrogenase at 25 °C	52	41

plexes free platinum, regenerated approximately one-half of the original enzyme activity. For all of the reported enzyme inhibitions consistent values of calculated equilibrium association constants were obtained over a wide range of platinum concentrations with the exception of the yeast alcohol dehydrogenase experiments. These values are time independent between a period of from 24 to 48 h after initiation of the inhibition reaction. These data suggest that true equilibrium states have been reached. In any event the numerical values of these constants provide a valid measure of the relative inhibition reactivities of the isomers of the platinum complex, which was the primary purpose of this study. The fact that the actual absolute numerical values of the inhibition constants for malate dehydrogenase and liver alcohol dehydrogenase differ by a factor of approx. 10, indicates that the environments of the enzyme location bound to the platinum in the inhibited complex are not identical. Minor differences in enthalpies or entropies of reaction could account for this difference, however, and it is possible that the actual immediate reactive sites could be similar in both cases.

In the case of lactate dehydrogenase, the trans isomer inhibits the enzyme much more efficiently than the cis isomer. The obvious explanation for this phenomenon would involve the formation of two bonds between the *trans*-platinum complex and the enzyme. Because bonding must take place in the positions of the original chloride ligands [2] (because platinum–ammonia bonds are invariably stable) this necessitates an enzyme–platinum–enzyme bond angle of 180°. Such a geometric situation would not be possible using the cis isomer. The distance between the two active groups on the enzyme must lie within very narrow limits in order for both groups to bond with *trans*-$Pt(NH_3)_2Cl_2$ or the aquo complexes formed from the halide complex. This information could help elucidate the nature of the bonding sites.

Yeast alcohol dehydrogenase denatured very readily at room temperature,

TABLE V

INHIBITION CONSTANTS FOR YEAST ALCOHOL DEHYDROGENASE AT 4 °C AND pH 7.1

Enzyme concentration, $3.0 \cdot 10^{-7}$ M.

Molar ratio of Pt to enzyme	Effective value of $K_e \times 10^{-2}$ (M^{-1})		
	48 h	72 h	96 h
(A) *Cis*-$Pt(NH_3)_2Cl_2$			
60	45	110	150
80	39	130	200
200	68	130	280
400	54	120	300
1000	97	190	—
Average K_e	61	140	230
(B) *Trans*-$Pt(NH_3)_2Cl_2$			
8	350	—	—
20	320	430	—
40	430	670	800
60	510	620	730
80	520	670	900
Average K_e	420	600	810

consequently, it was necessary to work at 4 °C. Over 72 h at this lower temperature only 8% of the activity of the uninhibited enzyme was lost. Despite the fact that we were not dealing with a true equilibrium situation a comparison of the effective values for the equilibrium constants for inhibition by the cis and trans isomers of the platinum complex is of interest. The data are presented in Table V. Although it is apparent that calculated K_e values are still increasing steadily after 96 h the trans isomer is the more effective inhibitor under all circumstances. It is possible that true equilibrium values of K_e would result in very different relative inhibition constants for yeast alcohol dehydrogenase. Nevertheless, the trans isomer is a very effective inhibitor for this enzyme. A future kinetic rather than equilibrium study of these systems involving yeast alcohol dehydrogenase might be profitable.

ACKNOWLEDGEMENT

We would like to thank Mr Robert Smith for his technical assistance in carrying out the alcohol dehydrogenase studies.

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