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PROTECTION OF THE ACTIVE SITE OF MITOCHONDRIAL MALATE DEHYDROGENASE FROM INHIBITION BY POTASSIUM TETRACHLOROPLATINATE

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Summary

Mitochondrial malate dehydrogenase (L-malate : NAD^+ oxido-reductase, EC 1.1.1.37) was inhibited by potassium tetrachloro platinum (II), K_2PtCl_4, in the presence of varying concentrations of NADH, NAD^+ and L-malate and mixtures of NAD^+ and L-malate. It was observed that NADH is an effective protector of the enzyme from inhibition while both NAD^+ and L-malate are poor protectors. Spectral studies have suggested that the protection afforded by the substrates are accomplished by reaction with specific groups on the enzyme rather than by complexation of the substrates with $PtCl_4^{2-}$.

From the above data it has been concluded that the tetrachloroplatinate ion binds only at the active site and that this site which is effectively protected by NADH, and moderately protected by a NAD^+-L-malate complex probably contains one or more sulfur containing amino acid side chains. It is also proposed that when the tetrachloroplatinate complexes with the enzyme there is some effect, possibly a conformational change, which causes the release of NADH at the allosteric site.

Introduction

Previous work [1—5] in the field of protein-platinum complex interaction centered on the X-ray crystallographic studies of general interactions between the two molecules. Most of these studies simply suggested bonding sites on the protein without determining the actual stability of the complex. The amino acid most often involved in the interaction was methionine.

Friedman and Teggins [6] recently showed that only the sulfur-containing amino acids, L-cysteine and L-methionine, prevented the complex ion $PtCl_4^{2-}$

from inhibiting L-malate: NAD^+ oxidoreductase (malate dehydrogenase) EC 1.1.1.37. It was also shown by these same workers [7,8] that both the cis and trans isomers of $Pt(NH_3)_2Cl_2$ inhibit the enzymes L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27 liver and yeast alcohol : NAD^+ oxidoreductase, EC 1.1.1.1 and mitochondrial malate dehydrogenase, by cross-linking at some site on each enzyme molecule. Since only one molecule of platinum per molecule of enzyme binds tightly enough to form a stable complex [9], the present work was performed in order to locate the specific site of binding. Mitochondrial malate dehydrogenase was used as the model, even though the only X-ray crystallographic studies were performed on the cytoplasmic form [4].

Materials and Methods

Pig heart malate dehydrogenase (Lot No. 40C-4700) was purchased from the Sigma Chemical Co., as were NAD^+ (Lot No. 120C-7400) and NADH (Lot No. 69B-6300). L-Malate (Lot No. 11.257-7) was obtained from the Aldrich Chemical Co. and K_2PtCl_4 was prepared from metallic platinum purchased from J. Bishop and Company. All other chemicals were of reagent grade.

Enzyme purification

The enzyme was further purified on carboxymethyl cellulose [10] and was recrystallized from saturated $(NH_4)_2SO_4$. Determination of the purity by ultracentrifugation, disc gel electrophoresis and cellulose-acetate strip electrophoresis has already been reported by Friedman et al. [6]. The results showed that the enzyme was in the mitochondrial form and better than 95% pure.

Enzyme assay

Mitochondrial malate dehydrogenase was assayed as described by Melius et al. [11]. The final assay system contained 0.1 M glycine, 0.01 M L-malate and $2 \cdot 10^{-4}$ M NAD^+ buffered at pH 9.5. 3-ml assay samples were used and 5 to 10 μl aliquots of enzyme were added to this assay solution for the activity measurements. Readings were made in a 1 cm cuvette, temperature controlled at 25° C in a Hitachi Model 139 Spectrophotometer.

Platinum preparation

Potassium tetrachloroplatinate(II) (K_2PtCl_4) was prepared and analyzed as described previously [12,13].

Kinetic studies

The kinetic studies were similar to those described by Friedman et al. [9]. The concentrations of mitochondrial malate dehydrogenase and K_2PtCl_4 were $1.5 \cdot 10^{-6}$ and $1.5 \cdot 10^{-4}$ M, respectively. In order to prevent inhibition, NAD^+, NADH and L-malate were added to the enzyme solution in either the solid form (if a high concentration of blocking agent was desired) or in solution (if a low concentration of blocking agent was needed). The blocking agent was allowed to equilibrate with the enzyme for about 1 to 2 h before the inhibitor (K_2PtCl_4) was added, although it was later observed that these equilibration stages were unnecessary since the enzyme and the blocking agent equilibrated

instantaneously. The initial time of reaction was recorded upon addition of K_2PtCl_4, 5 to 10 μl of solution were removed for assay at various intervals.

Equilibrium studies

Equilibrium studies were performed as previously described by Friedman et al. [9]. The experiments were temperature controlled at 4° C in a cold room, which had fluctuations of no more than ±0.5° C over the 24 h allowed for equilibration. The solutions were preapred by adding enough stock solution of K_2PtCl_4 (prepared in 0.1 M phosphate buffer) to make the concentration between $7.5 \cdot 10^{-7}$ M and $7.5 \cdot 10^{-6}$ M. Th final mitochondrial malate dehydrogenase concentration was $1.5 \cdot 10^{-6}$ M. After equilibration, 5 to 10 μl were removed for assaying. All quoted results are average values obtained from triplicate experiments.

Fluorometric studies

Fluorometric spectra were obtained using a Farrand Fluorimeter, Model No. Mark I. 1 cm quartz cuvettes containing solutions of $7.5 \cdot 10^{-5}$ M NADH, $1.5 \cdot 10^{-4}$ M K_2PtCl_4, or $1.5 \cdot 10^{-4}$ M $HgCl_2$ or combinations of the NADH with each of the other two were employed in the studies. All solutions were buffered in 0.1 M phosphate at pH 7.0. The excitation wavelength used in the measurements was 340 nm (the absorbance peak of NADH), and the spectra were scanned from 200 nm to 700 nm.

Spectrophotometric studies

Difference spectra were performed on a Perkin-Elmer Double Beam Recording Spectrophotometer, Model No. 124, with recorder attachment, Model No. 165. The same concentrations and combinations of NADH, K_2PtCl_4 and $HgCl_2$ that were used in the fluorescence studies were also utilized in the ultraviolet studies. The reference samples contained only 0.1 M phosphate buffer at pH 7.0 when each of the above components was scanned; however, when combinations of NADH and K_2PtCl_4 or NADH and $HgCl_2$ were measured, then the reference cell contained the same concentration of metal complex in 0.1 M phosphate buffer at pH 7.0.

3-ml samples were run in one cm quartz cuvette cells, and the spectra were recorded over the range of 300 nm to 220 nm at a recording speed of 10 cm per min.

Results

Kinetic studies

As indicated in a previous section, mitochondrial malate dehydrogenase was allowed to equilibrate with a given concentration of NADH prior to the addition of K_2PtCl_4. All conditions for each of the experiments summarized in Table I were identical except for the variation in the concentration of NADH. The activity which was measured within 1 min of the addition of the platinum complex was given the value of 100% enzyme activity for that particular kinetic run. The actual values of absolute initial activities for all of the experiments at different NADH concentrations did not vary by more than 5%.

TABLE I

INHIBITION OF MALATE DEHYDROGENASE BY K_2PtCl_4 AS A FUNCTION OF NADH CONCENTRATION AT pH 7.0 AND 25°C

The concentrations of K_2PtCl_4 and enzyme were $1.5 \cdot 10^{-4}$ M and $1.5 \cdot 10^{-6}$ M, respectively. The mixture was assayed in a 0.1 M glycine, 0.01 M L-malate and $2 \cdot 10^{-4}$ M NAD^+ mixture which was buffered at pH 9.5.

Time (min)	% activity remaining at given μM of NADH						
	0	1.5	15	20	25	30	150
0	100	100	100	100	100	100	100
20	70	75	80	86	91	95	100
30	50	52	70	77	82	87	100
40	42	35	60	71	76	80	98
60	20	22	55	60	66	73	98
90	—	9	38	47	54	65	98
120	6	5	26	33	41	49	—
24 h	0	0	0	—	—	3	98
I.I.R.[a]	100	96	60	46	36	26	≈1

[a] Initial inhibition rate in % per h based upon the first 30 min of reaction.

Under all circumstances the enzyme activity decreased after the addition of the platinum complex. This inhibition rate, however, decreased when the enzyme had been allowed to pre-equilibrate with higher concentrations of NADH. The magnitude of this effect is indicated by the initial inhibition rates (I.I.R.) shown in Table I.

An attempt was made to explain these data in terms of a mechanism in which NADH blocked the site of attachment of the platinum complex on the enzyme. In order to explain the actual kinetic results, it was necessary to assume that two molecules of NADH formed a complex with the enzyme. The association constant is indicated in the following equation:

$$E + 2\ NADH = E(NADH)_2 \ldots\ldots K_e = 3 \cdot 10^9 M^{-2} \quad (1)$$

The above association constant was employed to calculate "free" equilibrium enzyme concentrations for each NADH concentration. As can be seen from Table II, an almost perfect correlation is obtained between the concentra-

TABLE II

DEPENDENCE OF THE ENZYME OF INHIBITION RATE ON THE FREE ENZYME CONCENTRATION

Experimental conditions similar to Table I.

NADH (μM)	% free enzyme[a]	% I.I.R.[b]
0	100	100
1.5	99	96
15.0	60	60
20.0	46	46
25.0	35	36
30.0	27	26
150.0	≈1	≈1

[a] Using association constant of $3 \cdot 10^9\ M^{-2}$.

[b] Initial inhibition rate (I.I.R.) as defined in Table I.

TABLE III

PERCENT OF INITIAL MITOCHONDRIAL MALATE DEHYDROGENASE ACTIVITY 1 h AFTER ADDITION OF K_2PtCl_4 FOLLOWING PRIOR EQUILIBRATION WITH L-MALATE AND/OR NAD^+.

pH was 7.0, and temperature was maintained at 25°C. Assay conditions were similar to those in Table I.

Equilibration mixture (L-malate and/or NAD^+)	Residual activity (%)	Inhibition rate[b] (%)
0	18	100
$5 \cdot 10^{-4}$M L-malate	17	101
$5 \cdot 10^{-3}$M L-malate	37	77
$3 \cdot 10^{-5}$M NAD^+	16	102
$1.5 \cdot 10^{-4}$M NAD^+	38	76
$1.5 \cdot 10^{-3}$M NAD^+	53	58
$1.5 \cdot 10^{-4}$M NAD^+ + $5.0 \cdot 10^{-4}$M L-malate	44	69
$1.5 \cdot 10^{-4}$M NAD^+ + $5.0 \cdot 10^{-3}$M L-malate	68	39
$1.5 \cdot 10^{-3}$M NAD^+ + $5.0 \cdot 10^{-3}$M L-malate	98	2
$1.5 \cdot 10^{-5}$M NADH[a]	55	55
$1.5 \cdot 10^{-4}$M NADH[a]	98	2

[a] Included from Table I for comparison.

[b] Considering the inhibition rate in the absence of L-malate, NAD^+, or NADH to be 100%. Zero percent corresponds to complete protection.

tion of free enzyme and the rate of inhibition of the enzyme by the platinum complex. The data for the highest NADH concentration are only approximate since significant denaturation of the enzyme preparation occurred before appreciable inhibition by the metal complex could take place.

Sulebele and Silverstein [14] have shown that an ordered complex binding between cofactor, substrate and enzyme is the probable mode of catalysis. Consequently, it was decided to investigate the protective powers of separate and combined solutions of NAD^+ and L-malate. From the data in Table III it can be seen that relatively high concentrations of L-malate or NAD^+ do provide protection for the enzyme against platinum inhibition. It should be noted that NAD^+ was the better protector. However, neither of these compounds protected the enzyme as well as NADH. It is interesting to note that mixtures of L-malate and NAD^+ possesses superior protective properties compared to either of the constituents, thus supporting the contention that a ternary complex is formed. Again, it should be noted, that the protective effect was much weaker than that produced by similar concentrations of NADH. It was also observed that only a negligible amount of NADH was produced by the simultaneous addition of NAD^+ and L-malate to the enzyme. For example, at pH 7.0 and 25°C, the $\Delta G^{\circ\prime}$ for the conversion of L-malate to oxalacetate is +7.1 kcal/mol [15], and if $5 \cdot 10^{-3}$ M L-malate is added to $1.5 \cdot 10^{-4}$ M NAD^+ the amount of NADH produced is $2 \cdot 10^{-6}$ M. The combination of L-malate and NAD^+ concentrations afford about 60% protection of the enzyme, while the NADH concentration produced in the reaction does not protect the enzyme to any appreciable extent.

Fluorometric and spectrophotometric studies

Since NADH is such an effective protector against K_2PtCl_4 inhibition of malate dehydrogenase, it was necessary to show that there was no specific

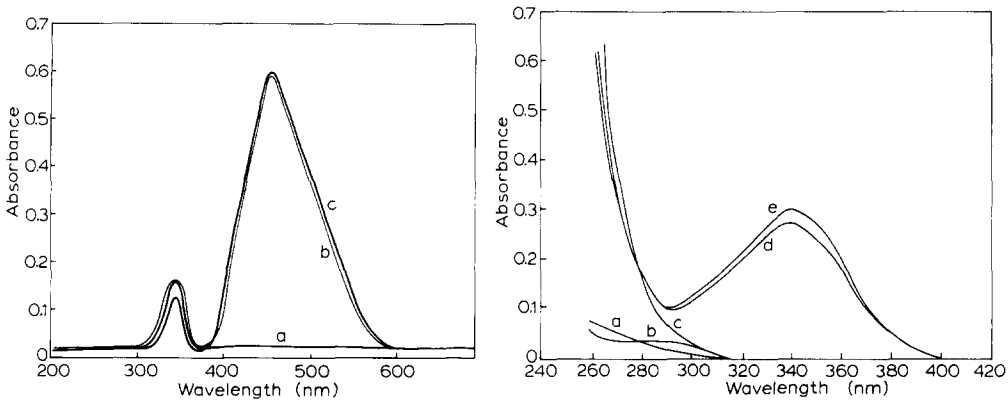

Fig. 1. Fluorescence spectra of NADH in the presence and absence of $HgCl_2$ and K_2PtCl_4 at pH 7.0 and 25° C. The concentrations of NADH ($7.5 \cdot 10^{-5}$ M), K_2PtCl_4 ($1.5 \cdot 10^{-4}$ M) and $HgCl_2$ ($1.5 \cdot 10^{-4}$ M) were all prepared in a 0.1 M phosphate buffer at pH 7.0. All spectra were made using 1 cm quartz cells. Curve a, NADH and $HgCl_2$; b, NADH; c, NADH and K_2PtCl_4. The excitation wavelength was 340 nm (the absorbance max. of NADH). The small peaks at 340 nm were due to scatter.

Fig. 2. Ultraviolet spectra of NADH in the presence and absence of $HgCl_2$ and K_2PtCl_4 at pH 7.0 and 25° C. The conditions were the same as those given in Fig. 1. Curve a, K_2PtCl_4; b, $HgCl_2$; c, NADH and $HgCl_2$; d, NADH and K_2PtCl_4; e, NADH. Curves a and b were made to show that there was no anomolous absorption from the K_2PtCl_4 and $HgCl_2$ in the NADH absorbance range.

complex formed between the tetrachloroplatinum(II) ion and the cofactor. It has previously been shown [16] that $HgCl_2$ forms a complex with NADH, and therefore that complex was used as a means of contrast with the reaction of NADH and the tetrachloroplatinate ion. These results are shown in the fluorescence spectra of Fig. 1 and the spectrophotometric spectra of Fig. 2. From Fig. 1 it is observed that in only the system in which $HgCl_2$ and NADH are mixed together is the emission peak missing. This is caused by a complex between the Hg^{2+} and the reduced nicotinamide ring. It has been postulated that the site of reaction is the double bond between carbons 5 and 6 in the ring [17,18]. The $PtCl_4^{2-}$-NADH curves do not show this effect, thus the tetrachloroplatinate ion probably does not react with the reduced nicotinamide ring. The same results can be seen in the spectrophotometric curves in Fig. 2.

Lastly, it was also suggested by Sulebele and Silverstein [20] that there is an allosteric site on mitochondrial malate dehydrogenase which has a reactive sulfhydral group. This site can be activated by *p*-chloromercuribenzoate, while the activation can be prevented by NADH. The maximum activation comes when 3—4 mol of *p*-chloromercuribenzoate per mol of malate dehydrogenase is added, and this activation is more pronounced at low temperatures (4° C). Thus, it was necessary to see if a similar effect would be observed with K_2PtCl_4. This effect was not seen at 25° C [9], but for conclusive evidence the experiments were repeated at a lower temperature. The results at both temperatures are given in Fig. 3. It is seen that there is a continued decrease in activity and thus no activation over the indicated concentration range of $PtCl_4^{2-}$ at either temperature. Further increase in K_2PtCl_4 (not included in Fig. 3) caused a continued decrease in activity suggesting that the platinate ion probably does not react at this site.

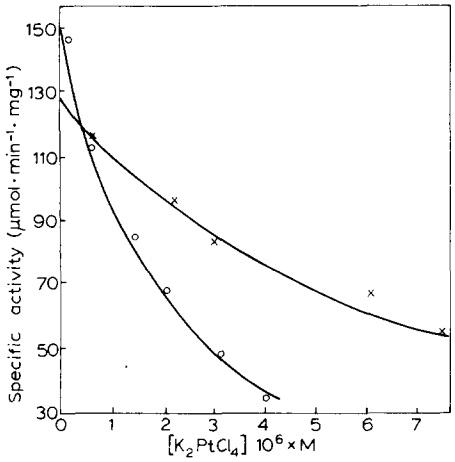

Fig. 3. Temperature dependence on the inhibition of mitochondrial malate dehydrogenase by K_2PtCl_4. $1.5 \cdot 10^{-6}$ M concentration of malate dehydrogenase was used in both experiments. The assay conditions are given in Table I. ○, 25° C and ×, 4° C. Each sample was allowed to equilibrate for 24 h before being assayed. Equilibration for longer times produced no change in activity.

Discussion

The results show that NADH effectively protects mitochondrial malate dehydrogenase from inhibition by K_2PtCl_4. For example, in the experiments in which equal ($1.5 \cdot 10^{-4}$ M) concentrations of K_2PtCl_4 and NADH were employed, 98% of the original enzyme activity remained after 24 h at 25° C. This protection can be explained by the formation of a complex containing one molecule of enzyme and two molecules of NADH, which is in agreement with other reports [19,20] which have shown that two molecules of NADH bond per molecule of enzyme. The assumption that this complex shields the enzyme from platinum attack is in excellent agreement with the inhibition data obtained for a wide range of NADH concentrations in Table II. Any other assumed complex stoichiometry (such as 1 : 1 or 1 : 3 enzyme/NADH) would require very different initial inhibition rates.

Previous reports [6—9] suggest that platinum complexes inhibit mitochondrial malate dehydrogenase, and possibly other enzymes, by cross-linking side chains. The obvious interpretation of the NADH shielding experiments would involve the shielding of both reactive sites in the enzyme by separate NADH molecules preventing initial attack by the tetrachloroplatinate ion. It is probable that in any solution containing significantly less than 100% of the 1 : 2 enzyme/NADH complex considerable concentrations of a 1 : 1 complex exist. Because of the excellent agreemente in Table II between free enzyme concentrations and initial inhibition rates, the platinum complex probably attacks the free enzyme rather than either the 1 : 1 or 1 : 2 NADH-enzyme complexes. It appears that once one strong platinum to enzyme bond has been formed the NADH remaining at the second site is displaced possibly by conformational changes in the tertiary structure.

Previous work [6—9] has shown that only one tetrachloroplatinate ion reacts strongly with one mitochondrial malate dehydrogenase molecule and

that the sulfur-containing amino acids, cysteine and methionine, react very strongly with the metal complexes compared with other amino acids. In view of the fact that no spectrophotometric evidence for the formation of a $PtCl_4^{2-}$-NADH complex could be obtained, it appears most probable that NADH protects the enzyme by shielding one or more sulfur-containing side chains from attack and from consequent cross-linkage by a tetrachloroplatinate ion at a specific site.

The fact that mixtures of NAD^+ and L-malate protect the enzyme more effectively than either individual constituent strongly suggests that this protection occurs at the active site since this is the only obvious location for the formation of a NAD^+-L-malate-enzyme complex. Although NADH is a more effective prorector than mixtures of NAD^+ and L-malate at equivalent concentrations, platinate inhibition of the enzyme almost certainly must occur at the same specific location on the enzyme in view of the previously mentioned observation that only one platinate ion reacts strongly with one enzyme molecule, Friedman et al. [9].

References

1 Wyckhoff, H.W., Hardman, K.D., Allewell, N.M., Inagami, T., Tsernoglou, D., Johnson, L.N. and Richards, F.M. (1967) J. Biol. Chem. 242, 3749—3753; 3984—3988
2 Sigler, P.B., Blow, D.M., Matthews, B.W. and Henderson, R. (1968) J. Mol. Biol. 35, 143—164
3 Lipscomb, W.N., Hartsuck, J.A., Reeke, G.N., Quicho, F.A., Bethge, P.H., Ludwig, M.L., Sterty, T.A., Muirhead, H. and Coppola, J.C. (1968) Brookhaven Symp. Biol. 21, 24—34
4 Dickerson, R.E., Eisenberg, D., Varnem, J. and Kopka, M.L. (1969) J. Mol. Biol. 45, 77—84
5 Wright, C.S., Alden, R.A. and Kraut, J. (1968) Nature 221, 235—238
6 Friedman, M.E. and Teggins, J.E. (1974) Biochim. Biophys. Acta 341, 277—283
7 Teggins, J.E. and Friedman, M.E. (1974) Biochim. Biophys. Acta 350, 263—272
8 Friedman, M.E. and Teggins, J.E. (1974) Biochim. Biophys. Acta 350, 273—276
9 Friedman, M.E., Musgrove, B., Lee, K. and Teggins, J.E. (1971) Biochim. Biophys. Acta 250, 286—296
10 Kitto, G.B. and Kaplan, N.O. (1966) Biochemistry 5, 3966—3980
11 Melius, P., Friedman, M.E., Teggins, J.E. and Guthrie, R.W. (1972) Biochim. Biophys. Acta 268, 194—198
12 Teggins, J.E., Gano, D.R., Tucker, M.A. and Martin, D.S. (1967) Inorg. Chem. 6, 69—75
13 Smith, E.D., McCann, J.A. and Teggins, J.E. (1969) Inorg. Chem. 8, 1872—1875
14 Sulebele, G. and Silverstein, E. (1970) Biochemistry 9, 274—283 and 283—290
15 Lehninger, A.C. (1970) Biochemistry, Worth Pub. Inc., New York pp. 353
16 Kaplan, N.O. (1960) The Enzymes, Vol. 3, 2nd edn., Acad. Press, New York, pp. 127—128
17 Wright, G.F. (1957) Ann. N.Y. Acad. Sci. 65, 436—453
18 Cotton, F.A. and Leto, J.R. (1958) J. Am. Chem. Soc. 80, 4823—4826
19 Cassman, M. and King, R.C. (1972) Biochemistry 11, 4937—4944
20 Silverstein, E. and Sulebele, G. (1969) Biochemistry 8, 2543—2550