Platinum Complexes and Pyruvate Kinase Activity

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Abstract. The interaction of platinum complexes with bovine heart pyruvate kinase (PK) was studied by absorption, CD, fluorescence spectroscopy and enzymic activity test Our results showed that activity of PK was reduced by cis-DDP and potassium tetrachloroplatinate in a time-and concentration dependent manner Cis-DDP was less effective than K_2PtCl_4 in reducing PK activity The native enzyme showed well defined negative Cotton effect at 222 and 208 nm indicating the presence of α-helical and β structure Platinum binding lowered the Cotton effect in this region by about 10–20% and 30–50% for the system with cis-DDP and K_2PtCl_4, respectively Fluorescence study showed that platinum binding quenched tryptophan fluorescence suggesting that binding occurs at the tryptophan residue or its proximity PK modifications induced by platinum binding would result in a greater resistance to denaturing agents

Key words: *cis*-Diamminedichloroplatinum(II) — Potassium tetrachloroplatinate — Pyruvate kinase — Conformational change — Fluorescence

Introduction

Pyruvate kinase (E C 2 7 1 40), is an important regulatory glycolytic enzyme, catalyzing the conversion of phosphoenolpyruvate to pyruvate with regeneration of ATP PK is generally found to be a tetrameric protein with a molecular mass of about 240 kDa (Warner 1958, Cottam et al 1969, Terlecki 1987) A number of isoenzymes of pyruvate kinase have been identified and classified into four types L, R, M_1 and M_2 (Ibsen 1977, Munday et al 1980, Noguchi et al 1987) Type M pyruvate kinase is apparently found in skeletal muscle, and is also the major isoenzyme of the heart and the brain The total number of cysteine residues in

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pyruvate kinase was previously estimated to be 36 (Cottam et al. 1969), the number of tryptophan residues being 12 (Cardenas et al. 1975).

Flashner et al. (1972) have shown that four sulfhydryl residues play a critical role in the activity of this enzyme. Pyruvate kinase is known to be inhibited by p-mercuribenzoate and certain other sulfhydryl-binding agents (Jacobson and Black 1971; Bloxham et al. 1978; Annamalai and Colman 1981; Vollmer et al. 1994). It seemed very interesting for us to study the interaction of pyruvate kinase with other metal ions and complexes which are used in the treatment of human diseases. We considered that some platinum complexes would be a suitable subject of investigation, because they are known as anticancer drugs and because they have a high affinity to sulfhydryl groups (Aull et al. 1979). Since the discovery of cis-DDP as an antitumor drug the research on the mechanism of action has mainly focused on the interactions with DNA (Reedijk 1996). Although such interactions are generally accepted to be ultimately responsible for the antitumor activity, there are many important biomolecules that can react with platinum amine compounds as well.

In general, interactions of platinum antitumor compounds with sulfur-containing biomolecules are considered to have an overall negative effect on the antitumor activity. Such an interaction can be responsible for the inactivation of Pt(II) species, for the development of resistance, and for toxic side effects such as nephrotoxicity. Numerous in vitro studies have reported cis-DDP to bind to enzymes and proteins (Hannemann and Bauman 1988; Yotsuyangi et al. 1991; Zou et al. 1995). The concentration of chloride ion in the plasma (103 mmol/l) is sufficiently high to maintain cis-DDP in the neutral, dichloro form. After diffusion across the lipid membrane, however, the chloride ion concentration decreases sharply (to 4 mmol/l), promoting hydrolysis of the labile chloride ligands. The aquated species subsequently react with a variety of intracellular components. It has been postulated that significant aspects of nephrotoxicity, bone marrow damage and gastrointestinal toxicity induced by platinum agents may involve ligand exchange reactions of complexes by sulfhydryl groups with subsequent inactivation of essential enzymes.

Undertaking the study of enzyme-platinum complexes interaction, we tried to estimate the influence of the applied drug cis-DDP and potassium tetrachloroplatinate (as a model), on the structure and activity of pyruvate kinase. In this work the studies on Pt(II) binding to PK were performed using UV-VIS spectroscopy, circular dichroism, protein fluorescence and enzymic activity measurements.

Materials and Methods

Materials

Bovine heart pyruvate kinase was isolated and crystallized by the modification of Parkinson and Easterby method (Parkinson and Easterby 1977). The preparation steps were: water extraction, ammonium sulphate fractionation (to 0.4 saturation),

the heat treatment (at 69 °C for 3 min), Sephadex G-25 filtration, phospho-cellulose chromatography, dialysis to saturated ammonium sulphate, and crystallization. The purity of the enzymes was confirmed by sodium dodecyl sulphate (SDS) gel electrophoresis in 7.5% gels. The preparation had a specific activity of 260 units/mg and gave a single band on polyacrylamide gel electrophoresis.

Cis-DDP and K_2PtCl_4 were obtained from Sigma Chemical Co. (St. Louis, USA).

Methods

Absorption and difference spectra were obtained with a Specord-M-42 spectrophotometer. Circular dichroism spectra were recorded on a JASCO J-600 spectropolarimeter. The solutions of the complexes for CD measurements were prepared by mixing the buffered solutions of pyruvate kinase (2.4×10^{-7} mol/l) and Pt(II)-complexes (2.4×10^{-4} mol/l – 2.4×10^{-6} mol/l) and incubating the reaction mixture for 24 h at 25 °C. All experiments were done at 25 °C in phosphate buffer pH 7.4. The data were expresed as mean residue ellipticity (deg cm^2/dmol). Fluorescence measurements were made on a SLM AMINCO SPF-500 spectrofluorometer. The protein was excited at 292 nm, and fluorescence was observed between 300 and 350 nm.

Assays of activity. The coupled lactate dehydrogenase assay was used (Bergmeyer et al. 1970). The reaction mixture at 25 °C contained 87.3 mmol/l TEA buffer pH 7.6; 10 mmol/l KCl; 2.5 mmol/l $MgSO_4$; 4.7 mmol/l ADP; 0.53 mol/l PEP; 0.2 mmol/l NADH; and 10 units of LDH. The oxidation of NADPH was followed by a decrease in absorbance at 340 nm. Protein concentration was determined spectrophotometrically with an extinction coefficient ($E_{280nm}^{0.1\%}$) of 0.55 (Cardenas and Dyson 1973).

Determination of enzyme inactivation by platinum complexes. The bovine heart PK-platinum complexes systems were allowed to incubate at 25 °C in the water bath during three days. Final enzyme concentration in the incubation mixture was 0.01 μmol/l and the molar ratios of the enzyme to Pt(II)-complexes were 10; 100; 1000 and 10,000. At one hour period intervals (5 hours), 50 μl aliquots of the mixtures were removed for the assay, and the remaining activities were measured. Enzyme activity was then determined after 24, 48 and 72 hours. All determinations were corrected for the loss of activity as measured in the control samples.

Results and Discussion

Absorption spectra of the aqueous solutions of K_2PtCl_4 consist of a single band at about 400 nm which is assigned as $^1A_{1g} \rightarrow {}^1A_{2g}$ ($d_{xy} \rightarrow d_{x^2-y^2}$) transition. The other $d - d$ bands occur at higher energies and they overlap with the protein transitions. The addition of protein into the solution of tetrachloroplatinate and
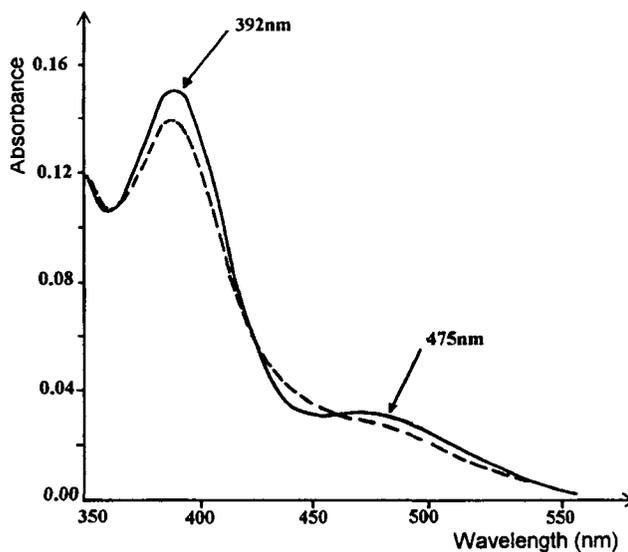
Figure 1. Absorption spectra of K_2PtCl_4 (———) and K_2PtCl_4 – PK (– – –) at the molar ratio 10 1, in phosphate buffer pH 7 4, after 24 h of the reaction running at 25 °C Concentrations $K_2PtCl_4 = 2 \times 10^{-4}$ mol/l, PK = 2×10^{-5} mol/l

incubation of both species for 24 h at 25 °C diminishes the $d - d$ band of Pt(II) (Fig 1) Interesting information is obtained from difference spectra recorded between 200–250 nm (Fig 2) New bands appear in this region after incubation with the enzyme The change in the UV region may indicate S → Pt(II) charge-transfer transitions, usually observed in this energy range (Dedock-LeReverend and Kozlowski 1985) Very similar changes are observed when Cys or Met are added to a Pt(II) salt solution Although this result is not very informative it clearly indicates the involvement of the protein coordinating to the Pt(II) species

Conformational changes in pyruvate kinase Pyruvate kinase is a tetrameric protein, the subunits of which being folded into three distinct domains Two of them consist of an α-helical and a β-structure and form intersubunit contact areas The third domain, which is not in contact with neighbouring subunits, consists of a β-structure only (Levine et al 1978) The conformational properties of the platinum- modified pyruvate kinase were studied by CD and fluorescence measurements CD studies confirmed the presence of conformational differences between native and platinum modified enzyme (Fig 3) Interaction of Pt(II) complexes with PK results in a decrease of the secondary structure as compared with the native protein The native enzyme shows well defined negative Cotton effects at 222 nm and 208 nm indicating the presence of the α-helical and β-
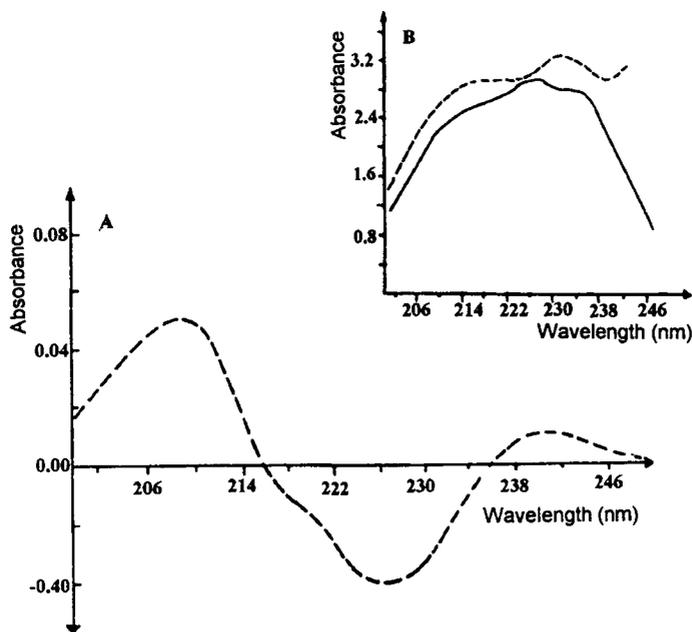
Figure 2. UV difference spectrum of K_2PtCl_4 PK, recorded against both unmixed solutions Conditions as described in legend to Fig 1 Concentrations $K_2PtCl_4 = 3 \times 10^{-5}$ mol/l, PK $= 3 \times 10^{-6}$ mol/l (*A*) Absorption spectra of K_2PtCl_4 (——) and K_2PtCl_4-cys(SH) (–   ) (*B*)

structure in the enzyme molecule Platinum binding lowers the Cotton effects in this region by about 10 20% and 30–50%, for the system with cis-Pt and potassium tetrachloroplatinate, respectively In all cases, the unfolding of the native enzyme secondary structure depends on the concentration of the platinum complexes The strongest changes are observed in the α-helical region of the protein structure These conformational changes can result from platinum interaction with thiol groups of cysteine It is known (Li et al 1992) that about half of cysteine residues in proteins are located in domains of the α-helical secondary structure, and play a very important role in the stabilization of the native protein structure

Fluorescence Tryptophan fluorescence is most frequently examined among aminoacid residues in protein molecules to obtain information about conformational changes When pyruvate kinase is excited at 292 nm the resulting fluorescence efficiency reflects changes of the microenvironment of tryptophan residue Fig 4 shows typical changes of fluorescence intensity of the reaction mixture in which
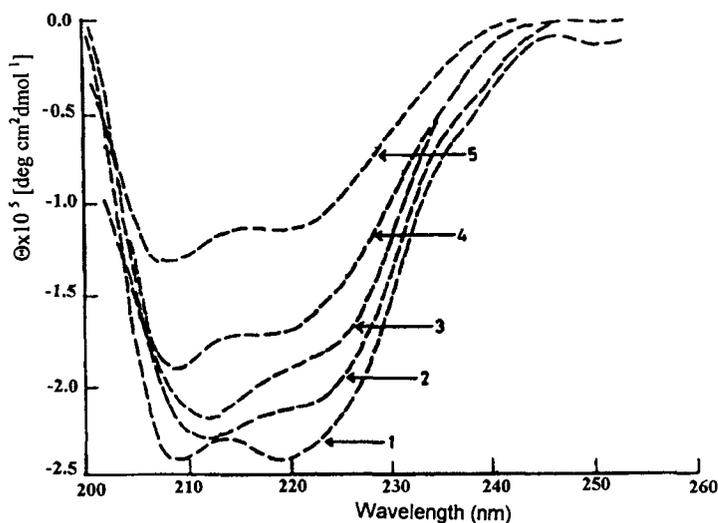
Figure 3. UV CD spectra in the 260–200 nm region of PK native (1), cis-DDP-PK at molar ratio 10 1 (2), cis-DDP-PK at molar ratio 1000 1 (3), K_2PtCl_4-PK at molar ratio 10 1 (4) and K_2PtCl_4 – PK at molar ratio 1000 1 (5) in phosphate buffer pH 7 4, after 24 h reaction running at 25 °C Concentration of PK $= 2$ 4 $\times$ 10^{-7} mol/l

platinum complexes were incubated with pyruvate kinase at various molar ratios The fluorescence intensity at 340 nm decreased with increasing molar ratio of the platinum complexes to the enzyme. According to empirical rules for fluorescent spectra of proteins (Freifelder 1976), the tryptophan residue is considered to be brought to a more hydrophilic environment as a result of Pt(II) binding. It is very probable that the multiple bindings of platinum result in tryptophan exposure to a polar environment, if the bindings are responsible for massive unfolding of the protein.

Denaturation measurements. The solvent denaturation study of pyruvate kinase reveals three reversible structural transitions (Doster and Hees 1981). With the increasing concentrations of denaturant the native tetramer is first transformed into a dimer with a partial loss of the secondary structure. The final state is a completely disordered monomer. In our experiment, the low concentration of denaturant (2 mol/l urea) probably induced dissociation of the tetramer-dimer. This dissociation step was accompanied by a partial unfolds of the secondary structure of the protein (Fig. 5). Denaturation measurements of native enzyme and enzyme in the presence of platinum with urea, suggested that platinum, which by itself partly unfolds the secondary structure of the protein, seemed to protect further denaturation caused by urea. This may suggest that platinum binding to the enzyme caused a
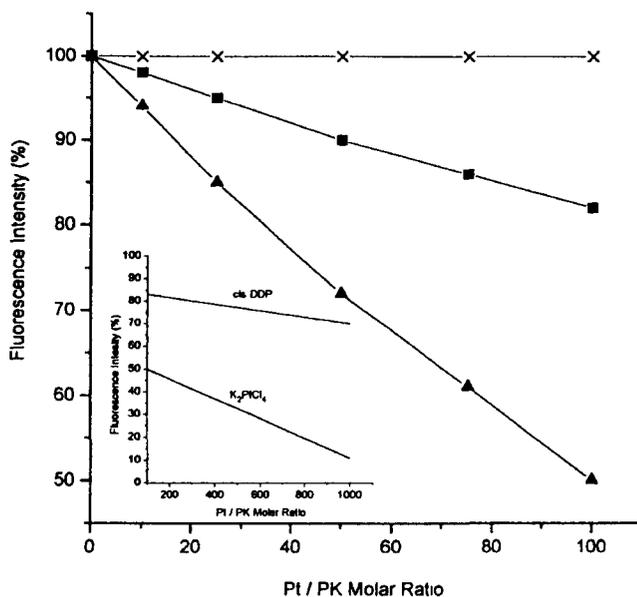
Figure 4. Relative fluorescence changes of PK incubated with K_2PtCl_4 (▼) and cis-DDP (■) at various molar ratios for 24h at 25 °C Concentration of PK 0 5 μmol/l Excitation at 290 nm and emission at 340 nm The relative fluorescence intensity of native PK (×) was set as 100%

significant destruction of its native structure but simultaneously made it more rigid, more stable and resistant against denaturing agents The change of the secondary structure can be related to the changes of the molecular weight

Inactivation of PK by Pt(II) complexes The inhibition reactions were carried out at 25 °C and pH 7 4 with a platinum to enzyme molar ratios, of 10, 100, 1000, and 10,000 The control samples were incubated in the same conditions without platinum At one hour time intervals, sample aliquots were taken and their activity determined The results obtained are shown in Fig 6 The activity of pyruvate kinase was reduced by cis-DDP and tetrachloroplatinate in a time-and concentration-dependent manner Cis-DDP was less effective in reducing the activity of the enzyme as compared to K_2PtCl_4 From Fig 6 it can be seen that for cis-DDP, approx 40% of the initial enzyme activity was observed in the incubated solution (with an inhibitor to enzyme molar ratio of 1000), which had been allowed to stand for 1 3 days In the same conditions, the enzyme was completely inactivated by K_2PtCl_4

Semilogarithmic plots of residual activity versus time of pyruvate kinase inactivation by K_2PtCl_4 were linear at inhibitor PK molar ratio of 1 100, but became
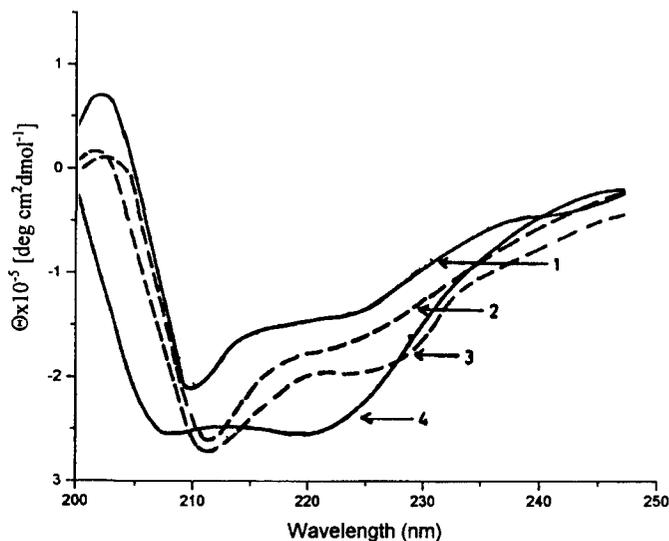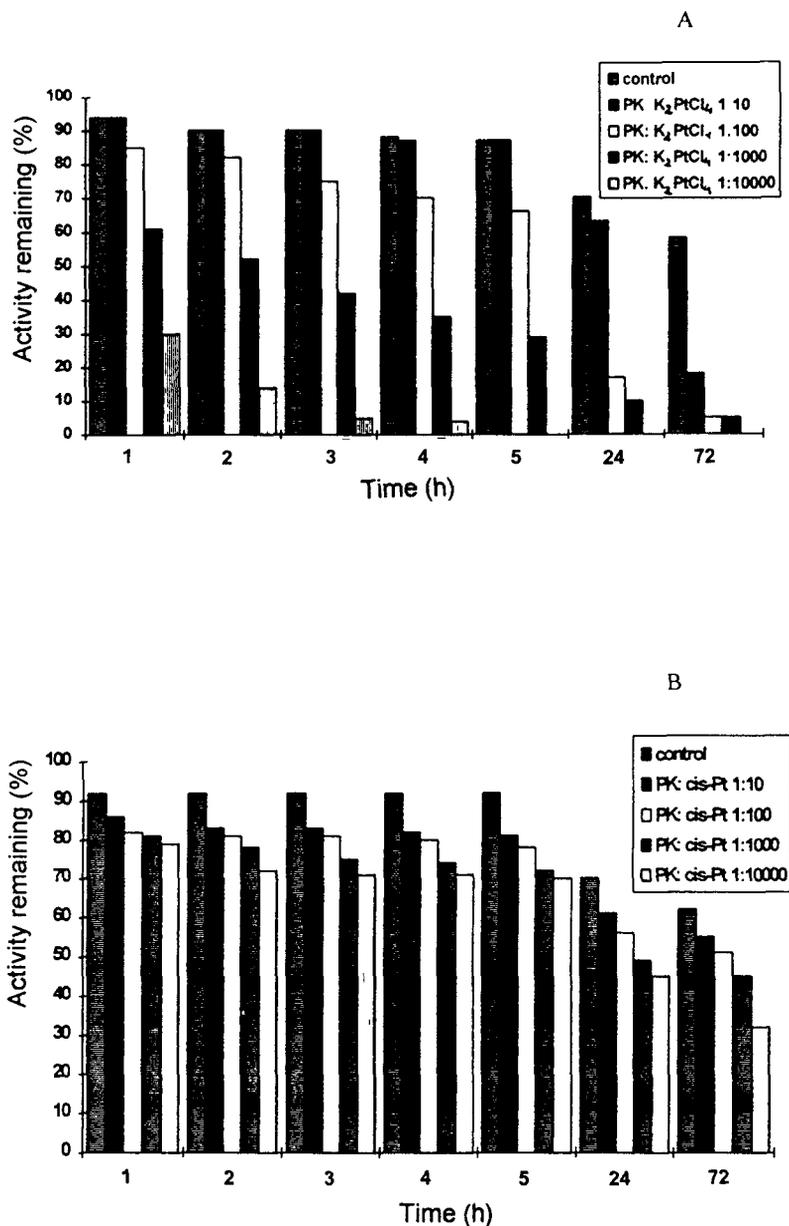
Figure 5. Effect of 2 mol/l urea on CD spectra of PK (1), K_2PtCl_4 – PK (2) and cisPt-PK (3) at the molar ratio of platinum complexes to enzyme of 1000 1 CD spectrum of native enzyme (4)

non-linear at the two higher inhibitor concentrations (Fig. 7). At high K_2PtCl_4 concentrations, pyruvate kinase became completely inactivated. The inhibition plots for four different concentrations of cis-DDP became non-linear. The pyruvate kinase retained more than 40% of its original activity after 24 h incubation with cis-DDP. The inhibition of the enzyme with high concentrations of K_2PtCl_4 and cis-DDP is not a simple first-order process. The non-linear characteristic of these plots could result from conformational changes induced by the modification of sulfhydryl groups, not only at the active site of the enzyme.

The results presented above clearly indicate that potassium tetrachloroplatinate and cis-DDP bind effectively to pyruvate kinase, considerably changing its structure and activity. Drastic changes in the structure and activity were observed for $PtCl_4^{2-}$. Cis-DDP with only two labile chloride ligands was a less effective inhibitor. Although the interactions with enzymes are not responsible for the antitumor activity of cis-DDP, they can modulate it, and they probably contribute to some of the mentioned overall negative effects.

References

Annamalai A E , Colman R F (1981) Reaction of the adenine nucleotide analogue 5'-p-
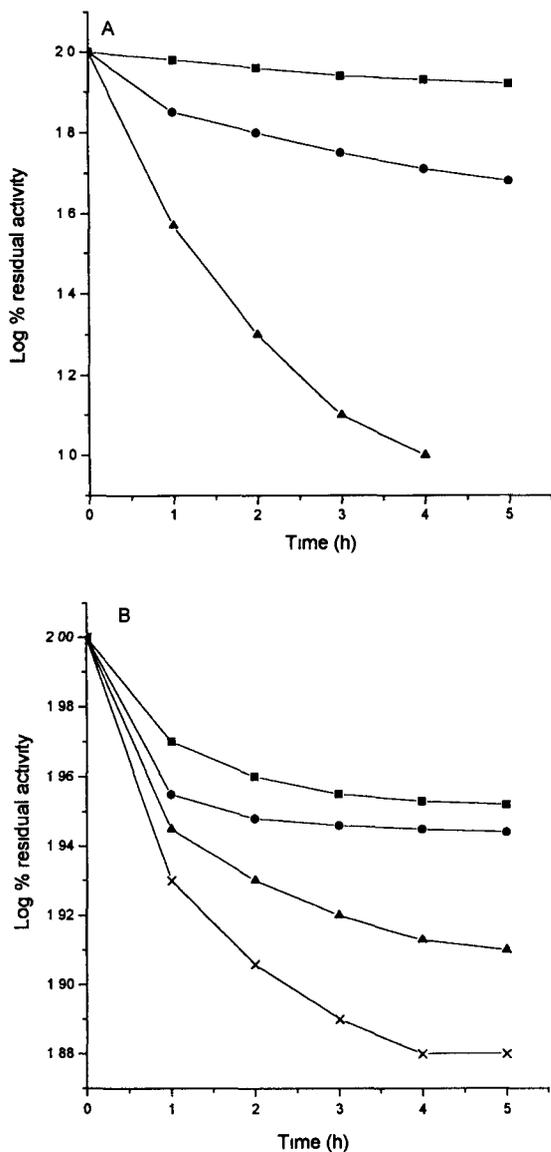
A

B

Figure 6. Time-dependent effect of K_2PtCl_4 (A) and cis-DDP (B) on the activity of PK. The molar ratios are indicated.

Figure 7. Semilogarithmic plots of the effect of K_2PtCl_4 (A) and cis-DDP (B) on pyruvate kinase inhibition at 25 °C The molar ratios of platinum complexes to the enzyme 10 1 (■), 100 1 (●), 1000 1 (▼) and 10 000 1 (×) Activity means residual activity remaining after the inhibition times indicated, and is expressed as percentages of the enzyme activity in reaction mixtures in the absence of Pt compounds

fluorosulfonylbenzoyl adenosine at distinct tyrosine and cysteine residues of rabbit muscle pyruvate kinase J Biol Chem **256**, 10276—10283

Aull J L , Allen R L , Bapat A R , Daron H H , Friedman M E , Wilson J F (1979) The effects of platinum complexes on seven enzymes Biochim Biophys Acta **571**, 352—358

Bergmeyer H U , Gawehn K , Grassl M (1970) Methoden der Enzymatischen Analyse, Verlag Chemie, Weinheim

Bloxham D P , Coghlin S J , Sharma R P (1978) Use of methanethiolation to investigate the catalytic role of sulfhydryl groups in rabbit skeletal muscle pyruvate kinase Biochim Biophys Acta **525**, 61—73

Cardenas J M , Dyson R D (1973) Bovine pyruvate kinase Purification of the liver isozyme and its hybridization with skeletal muscle pyruvate kinase J Biol Chem **248**, 6938—6944

Cardenas J M , Blachly E G , Cecotti P L , Dyson R D (1975) Properties of chicken skelet muscle pyruvate kinase and a proposal for its evolutionary relationship to other avian and mammalian isozymes Biochemistry USA **14**, 2247—2252

Cottam G L , Hollenberg P , Coon M J (1969) Subunit structure of rabbit muscle pyruvate kinase J Biol Chem **244**, 1481—1486

Dedock-LeReverend B , Kozlowski H (1985) Some aspects of the thioether donation in amino acid and peptide complexes J Chimie Phys **82**, 883—890

Doster W , Hees B (1981) Reversible solvent denaturation of rabbit muscle pyruvate kinase Biochemistry USA **20**, 772—780

Flashner M , Hollenberg P F , Coon M J (1972) Mechanism of action of pyruvate kinase J Biol Chem **249**, 8114—8121

Freifelder D (1976) Physical Biochemistry Applications to Biochemistry and Molecular Biology pp 410—443, W H Freeman and Co , San Francisco

Hannemann J , Bauman K (1988) Inhibition of lactate-dehydrogenase by cisplatin and other platinum-compounds Res Commun Chem Pathol Pharmacol **60**, 371—379

Ibsen H (1977) Interrelationships and functions of the pyruvate kinase isozymes and their variant forms a review Cancer Res **37**, 341—353

Jacobson K W , Black J A (1971) Conformational differences in the active sites of muscle and erythrocyte pyruvate kinase J Biol Chem **246**, 5504—5509

Levine M , Muirhead H , Stammers D K , Stuard D J (1978) Structure of pyruvate kinase and similarities with other enzymes possible implications for protein taxonomy and evolution Nature **271**, 626—630

Li H , Wurrey C J , Thomas G J jr (1992) Cysteine conformation and sulfhydryl interaction in proteins and viruses Normal coordinate analysis of the cysteine side in model compounds J Amer Chem Soc **114**, 7463—7469

Munday K A , Giles I G , Poat P C (1980) Review of the comparative biochemistry of pyruvate kinase Comp Biochem Physiol 67-B, 403—413

Noguchi T , Yamada K I , Inoue H , Matsuda T , Tanaka T (1987) L-type and R-type isozymes of rat pyruvate kinase are produced from a single gene by use of different promoters J Biol Chem **262**, 14366—14371

Parkinson J , Easterby J S (1977) Purification and molecular properties of bovine heart pyruvate kinase Biochim Biophys Acta **481**, 471—480

Reedijk J (1996) Improved understanding in platinum antitumour chemistry J Chem Soc , Chem Commun 801—806

Terlecki G (1987) Purification and properties of pyruvate kinase Type M_1 from bovine brain Int J Biochem **21,** 1053—1060

Vollmer S H , Walner M B , Tarbell K V , Colman R F (1994) Guanosine 5'-O-[S-(4- bromo-2,3-dioxobutyl)] thiophosphate and adenosine 5'-O-[S-(4-bromo-2,3-dioxybutyl)] thiophosphate New nucleotide affinity labels which react with rabbit muscle pyruvate kinase J Biol Chem **269,** 8082—8090

Warner R C (1958) Physical properties of crystalline "fluorokinase" Arch Biochem Biophys **78,** 494—496

Yotsuyangi T , Ohta N , Futo T , Ito S , Chen D , Ikeda K (1991) Multiple and irreversible binding of cis-diamminedichloroplatinum (II) to human serum albumin and its effect on warfarin binding Chem Pharm Bull (Tokyo) **39,** 3003—3006

Zou J , An F , Lui G , Wang K (1995) The binding of platinum (II) complexes to rabbit skeletal muscle G-actin induces conformational changes Metal-Based Drugs **2,** 233—241