KINETIC STUDY ON THE REACTION OF CISPLATIN WITH METALLOTHIONEIN

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ABSTRACT:

The binding of cisplatin to metallothionein (MT) was investigated at 37°C in 10 mM Tris-NO₃ (pH 7.4) and 4.62 mM NaCl. The conditions were chosen to mimic passage of clinical concentrations of cisplatin through the cytosol. The reactions were monitored by high-performance liquid chromatography (HPLC), atomic absorption spectroscopy, and ultraviolet (UV) absorption spectroscopy. The UV data showed that several reactions occurred, one of which does not affect the absorbance (no Pt-sulfur bond formation). They also suggested that if [cisplatin] is large compared with [MT], the rate of subsequent reaction is between first and second order in [cisplatin] and between zeroth and first order in [MT]. HPLC elutes with 24 < retention time (tᵣ) < 27 min contained undialyzable Pt, which increased with reaction time and corresponded to Pt-thionein product. Eluates with 3 < tᵣ < 7 min corresponded to unbound cisplatin and allowed determination of second-order rate constants (k), using the second-order rate equation. The k value for cisplatin reacting with apo-MT was ~0.14 M⁻¹ s⁻¹, Cd/Zn-MT ~0.75 M⁻¹ s⁻¹, Cd₄-MT ~0.53 M⁻¹ s⁻¹, and Zn₄-MT ~0.65 M⁻¹ s⁻¹. Thus, cisplatin displaced Cd and Zn equally well. Leukocyte MT concentration was ~1.0 mM, so that the kinetics of cisplatin binding to cellular MT is pseudo-first order (pseudo-first-order rate constant, ~0.63 x 10⁻¹⁰ s⁻¹; half-life, ~18 min). With [cisplatin] = 10 μM, the rate of cisplatin reaction with MT is ~6.3 μmol s⁻¹ cm⁻². We conclude that cellular MT can trap significant amounts of cisplatin and may efficiently contribute to cisplatin resistance.

Cisplatin [cis-diaminedichloroplatinum(II), CDDP] exerts its antitumor activity by binding to cellular DNA (Gelasco and Lippard, 1999). When the drug enters the cell, it passes through the cytosol and across the nuclear membrane, binds to nitrogen atoms on the bases of DNA, and promotes cell death by apoptosis (Demarcq et al., 1994). The cytotoxic activity of CDDP thus correlates with the amount of platinum (Pt) bound to DNA (Zwelling et al., 1979).

Cisplatin entering the cell also binds to endogenous thiols, e.g., MT and glutathione (GSH), which limits the amount of the drug available for binding to DNA (Dedon and Borch, 1987; Berners-Price and Kuchel, 1990a, b; Perez-Benito et al., 1995; Bose et al., 1997; Reedijk and Teuben, 1999; Volckova et al., 2002). Moreover, continued exposure to CDDP up-regulates the amount of MT, GSH, and other cellular thiols, which increases the cell’s resistance to CDDP (Schilder et al., 1990; Eastman 1991; Godwin et al., 1992; Pattanaik et al., 1992; Zhang and Tang, 1994), there is little kinetic data under clinically relevant concentrations of CDDP. We used HPLC, atomic absorption spectroscopy (AAS), and UV absorption to measure the rate of CDDP binding to MT. We also measured the concentration of MT in leukocytes. The data obtained are used to estimate how cellular MT influences DNA platination by CDDP.

Materials and Methods

Chemicals. CDDP (1 mg/ml, ~3.3 mM in 154 mM NaCl) was obtained from American Pharmaceutical Partners (Los Angeles, CA); cadmium (Cd)/zinc (Zn)-thioneine I (from rabbit liver; mol. wt. ~6000; 5.0 mg containing 3% Cd and 1% zinc), Cd atomic absorption standard (994 μg of Cd/ml in 1% HNO₃), Zn atomic absorption standard (994 μg of Zn/ml in 1% HNO₃), CdCl₂, dihexylamine, glacial acetic acid, NaCl, pH Test Strips, EDTA, and Tris were
purchased from Sigma-Aldrich (St. Louis, MO). Monobromobimane (mBBr) was purchased from Molecular Probes (Eugene, OR), methanesulfonic acid purchased from Fluka Chemical Corp. (Ronkonkoma, NY), and perchloric acid was purchased from Aldrich Chemical Co. (Milwaukee, WI). Highest purity dithiothreitol (DTT) was purchased from Calbiochem (San Diego, CA). SnakeSkin pleated dialysis tubing (mol. wt. cutoff, 3500) was purchased from Pierce Chemical (Rockford, IL), distilled deionized water (water for injection quality water, dH2O) was purchased from Mediataech (Herndon, VA), and Pt atomic spectroscopy standard (H2PtCl6, 1 mg/ml in 10% HCl) was purchased from PerkinElmer Instruments (Norwalk, CT). The chemicals were of reagent grade or better. All prepared solutions were stored in polyethylene tubes.

The use of volunteer blood was approved by the institutional review board (State University of New York, Upstate Medical University) for the protection of human subjects. The cell count and volume were determined on the Coulter Z2 model (Beckman Coulter, Inc., Fullerton, CA).

Solutions. Cd/Zn-thionein (5.0 mg, from Sigma-Aldrich) was dissolved in 1.0 ml of dH2O (final concentration, ~0.83 mM) and stored at ~20°C. Diethylenetriaminetetraacetic acid (2.5 mM) HPLC solvent A was prepared in the hood by the addition of 590 μl of 4.24 M diethylenetriamine and 144 μl of 17.4 M glacial acetic acid to each liter of dH2O; the pH was adjusted to ~7.0 by small additions of diethylenetriamine or acetic acid (the solution was continuously stirred). The sodium methane sulfonate and mBBr solutions were prepared and stored as described (Souid et al., 2001).

Preparation of Apo-thionein. The mixture contained 100 μl of Cd/Zn-thionein (from the 5.0 mg/ml solution described above), 4.0 M HCl, and 5.0 mM EDTA (final pH ~2 and final volume, 200 μl). The solution was incubated at 37°C for ~16 h. DTT (from a 0.1 M solution freshly made in dH2O) was added to a final concentration of 10 mM, and the pH was adjusted to ~7.5 by the addition of ~450 μl of 1.0 M Tris-base (final volume, 1.0 ml with dH2O). The mixture was incubated at 37°C for ~5 h, and then placed in a SnakeSkin pleated dialysis tubing (mol. wt. cutoff, 3500) and dialyzed at 4°C for ~16 h against two changes of 2 liters of 10 mM Tris-NO3 (pH ~7.4). The control experiment contained 100 μl of Cd/Zn-thionein that had undergone the same incubations and dialysis, but without the additions of HCl, EDTA, Tris-base, and DTT. The UV absorbance spectrum of apo-thionein dialysate was compared with that of the control dialysate (Cd/Zn-thionein). The acid-treated MT lacked molecular absorbance at 240 to 300 nm, reflecting loss of the mercapto-complex. The apo-thionein concentration was determined by the absorbance at 220 nm (ε220, 48,200 M-1 cm-1) (Vasak, 1991; Pattanaik et al., 1992; Ye et al., 2000).

Preparation of Cd+, Zn2+, and Pt2+ Thionein. The mixtures contained 100 μl of Cd/Zn-MT (from the 5.0 mg/ml solution described above) and 1.0 mM CdCl2, 1.0 mM ZnSO4, or 1.0 mM CDDP (final volume, 1.0 ml). The reactions were incubated at 37°C for ~60 h and then placed in a SnakeSkin pleated dialysis tubing (mol. wt. cutoff, 3500) and dialyzed at 4°C for ~16 h against two changes of 2 liters of 10 mM Tris-NO3 (pH ~7.4). The metal contents of the dialysates were determined on the AAS. The MT concentration was estimated using a stoichiometry of 7 g-atoms of metal per mole.

Binding of CDDP to MT Monitored by HPLC-AAS. The reactions were carried out at 37 ± 0.1°C in a total volume of 1.0 ml. A typical mixture contained 970 μl of MT dialysate (containing ~50–83 μM of Cd/Zn-thionein, Cd-thionein, Zn2-thionein, Pt2-thionein, or apo-thionein as described above), 4.62 mM NaCl (the approximate concentration of Cl– in the cytosol), 10 mM Tris-NO3 (pH 7.4) and 33 μM CDDP. The control experiments contained the same mixture except that 970 μl of the dialysis solution was used instead of the MT dialysate. Fifty microliters of the reaction mixture was loaded onto the HPLC and analyzed as described below.

Preparation of Leukocyte Acid-Soluble Supernatant (Leu-AS). Five microliters of blood were collected from a volunteer into an EDTA Vacutainer tube (BD Biosciences, Franklin Lakes, NJ). The sample was diluted to 50 ml with ice-cold dH2O and incubated on ice for ~10 min. The leukocytes were collected by centrifugation (~3000g for 10 min), and the procedure was repeated twice. The final pellet was washed with 0.9% NaCl. Leu-AS was prepared by the addition of 0.6 ml of 2.5% perchloric acid, 2 M sodium methane sulfonate. The mixture was vigorously vortexed (with three passages of the pestle through a glass-Teflon homogenizer). The supernatant was recovered by centrifugation and suspended in 50 mM NaOH, 1.0 mM DTT, and 10 mM Tris-Cl, pH 8.0 (final volume, 1.0 ml). The solution was then placed in a SnakeSkin pleated dialysis tubing (mol. wt. cutoff, 3500) and dialyzed at 4°C for ~16 h against 1 liter of 10 mM Tris-Cl (pH 8.0) plus 0.1 μM CdCl2. This step was followed by ~16 h of dialysis against two changes of 1 liter of 10 mM Tris-Cl. This dialysis procedure removes unbound Cd and cellular GSH. The Cd content of the dialysate was determined on the AAS. The MT concentration of the dialysate was estimated using a stoichiometry of 7 g-atoms of Cd per mole.

mBBr Labeling. Labeling reaction contained ~1.0 ml of the Leu-AS plus 0.1 mM of mBBr (from 0.1 M stock in acetonitrile). The mixture was incubated in the dark at 37°C for 30 min and then dialyzed at 4°C against two changes of 1 liter of 10 mM Tris-Cl (pH 8.0) for ~6 h (to remove unbound mBBr). Fifty microliters of the dialysate was injected on HPLC and analyzed as described (Souid et al., 1998, 1999, 2001).

UV Absorbance. The absorbance at 260 nm (which reflects the presence of metal-S and disulfide bonds) as a function of time was measured using a single-beam spectrophotometer (model DU 640B; Beckman Coulter, Inc.). Samples were in a 1-cm path length quartz cuvet with a Teflon stopper, which was thermostatted at 37 ± 0.1°C. The spectrophotometer was “zeroed” immediately after adding MT, which initiated the reaction. The reaction mixture contained variable concentrations of MT and CDDP in 10 mM Tris-NO3 (pH 7.4) and 4.62 mM NaCl. The control experiment for measuring the rate of disulfide formation contained MT (~50 μM), buffer, and NaCl but no CDDP. The other control experiment contained CDDP (33 μM), buffer, and NaCl but no MT. In these experiments, the noise was negligible. The signal drift of 20-h absorbance readings was at most ~0.002 to ~0.007.

HPLC-UV. Analysis was performed on a Beckman Coulter reversed-phase HPLC system, which consisted of an automated injector (model 507e), a pump (model 125), and a UV detector (model 166). The detection at 260 nm was used. Solvent A was 2.5 mM diethylenetriaminetetraacetic acid in dH2O (continuously stirred), and solvent B was HPLC-grade methanol. The column, 4.6 x 250 mm Beckman Coulter Ultrasphere IP, was operated at room temperature at a flow rate of 0.5 ml/min. The chromatography procedure employed linear gradients as follows: 0 min, 10% B; 5 min, 10% B; 20 min, 75% B; 40 min, 100% B; 45 min, 100% B; 46 min, 10% B; 60 min, re-inject (Dabrowiak et al., 2002). The injection volume was 50 μl. HPLC eluates were collected for Pt determination as AAS was described below.

AAS. Cd, Zn, and Pt analyses were performed using the graphite furnace of a Shimadzu AAS (model AA-6800), with an argon (Imaging and Sensing Technology, Horsesheds, NY) hollow cathode Pt lamp, deuterium arc background correction, and pyrolytically coated graphite tubes (Dabrowiak et al., 2002). Calibration curves were generated prior to each measurement and proved to be linear from 0 to 10 pmol (ε220 ~ 0.0276 μM−1 cm−1). A background reading of ~0.005 optical density (for dH2O) was subtracted from each of the determinations. The injection volume was 5 to 20 μl.

Kinetics of CDDP Binding to MT. The kinetics of CDDP binding to MT were analyzed using the second-order rate, eq. 1:

$$\ln(Q) = \ln\left[\frac{[\text{CDDP}]_0}{[\text{MT}]_0 - [\text{CDDP}]_0} \right] - k_t \cdot t = \ln\left[\frac{[\text{CDDP}]_0}{[\text{MT}]_0 \cdot [\text{CDDP}]_0} \right] - k_t \cdot t \quad (1)$$

Cisplatin concentrations were determined by AAS (calibrated with a standard) from Pt contents of the eluates with 3 < τg < 7 min. In eq. 1, [CDDP]0 and [MT]0 are the initial concentrations of CDDP and MT, respectively. [CDDP] and [MT] are the concentrations of CDDP and MT at time τg, respectively. The concentration of Pt-thionein product that was formed at time τg, [produced], was calculated as the difference between [CDDP]0 and [CDDP] at time τ.
three cases, confirming that one molecule of MT reacts with several thionein or CDDP alone. This is confirmed by noting that the change in absorption with time of solutions containing either Cd/Zn-thionein or CDDP is the limiting reagent, even though [CDDP]₀ is the smallest of the two reactants. The increase in absorption results predominantly from the reaction of MT and CDDP alone subtracted, is shown in Fig. 1. The increase in absorption (at 260 nm) as a function of time of several solutions, with [MT]₀, the absorption at 260 nm increases with time with no delay, indicating that reaction 1 has been speeded up so as to make the formation of Pt-S bonds rate-determining (e.g., Fig. 1, [MT]₀ / [CDDP]₀). This second reaction is referred to as reaction 2, although it is likely a series of reactions.

When reaction 2 is rate-determining, one can attempt to establish its order with respect to CDDP and MT. The absorption versus time curves (not including 27 μM/33 μM) were fitted, using the Solver least-squares routine, to a constant plus two exponentials; i.e., Abs(t) = A + Be⁻ⁿ⁻¹ + De⁻ⁿ⁻¹.

The initial slope was calculated as -(BC + DE), with results given in Table 1. If the rate of reaction 2 is proportional to [CDDP]ⁿ[MT]ᵐ, one can determine m and n by comparing initial rates with different initial concentrations of the reactants. From the last two rows of Table 1, one obtains 2.1 μM/33 μM = 2.32 (ratio of rates), or n = 0.905 ± 0.096 (errors calculated from slope errors, Table 1), i.e., close to the expected value of 1. From the first two rows of Table 1, one obtains 198 μM/33 μM = 13.9, or m = 1.47 ± 0.13, suggesting the reaction is first or second order in CDDP, or that several reactions occur rapidly after the first.

Binding of CDDP to MT As Monitored by HPLC-AAS. A typical chromatogram of the reaction of CDDP with MT is shown in Fig. 2. The mixture (at 37°C) contained 33 μM CDDP, ~77.5 μM apo-thionein, 10 mM Tris-NO₃ (pH 7.4) and 4.62 mM NaCl (final volume, 1.0 ml). Pt (determined by AAS) was present in eluates with 3 < tₖ < 7 min (corresponding to unbound CDDP), 24 < tₖ < 27 min (corresponding to Pt-thionein product), and, at a lesser extent, 27 < tₖ < 30 min (corresponding mostly to CDDP hydrolysis products). Otherwise, the remaining chromatogram was free of Pt.

Kinetics of CDDP Binding to MT As Monitored by HPLC-AAS. The Pt contents of eluates with 3 < tₖ < 7 min (containing unbound CDDP) diminished with reaction time and allowed determination of the k values for CDDP binding to MT (using eq. 1). Two experiments are shown in Fig. 3. For Fig. 3a, the mixture (at 37°C) contained 970 μl of Cd/Zn-thionein dialysate (~50 μM MT), 33 μM CDDP, and 4.62 mM NaCl. Pt AAS intensities after various reaction times for this mixture are shown as squares. The control mixture contained 970 μl of the dialysis solution, 33 μM CDDP, and 4.62 mM NaCl. Pt AAS intensities after various reaction times for the control mixture are shown as triangles. The solid curve is the average exponential fit to the control intensities from all experiments.

For all experiments, the CDDP recovery from the eluates with 3 < tₖ < 7 min at reaction time 0 was (mean ± S.D.) 86 ± 18%. As the reaction proceeded, the concentration of unbound CDDP diminished. At 8 h, the concentration of unbound CDDP decreased to 29% of its value at 0 h and at 26 h decreased to 9% (Fig. 3a, squares). In the control mixture, at 27 h, the concentration of CDDP was 84% of its value at 0 h, reflecting the slow rate of CDDP hydrolysis in the absence of MT (Fig. 3a, triangles). The solid curve in Fig. 3a (the average decrease for the control mixture) was used to correct the measured AAS reaction intensities. The results of correction are shown as circles in Fig. 3a.

The experiment shown in Fig. 3b, using Cd/Zn-thionein, involved more points at shorter reaction times, to see whether the delay observed in some of the UV absorption measurements (see above) could be observed in the HPLC-AAS measurements. Original CDDP and MT concentrations were 33 and 6.4 μM, respectively. The AAS intensities have been converted to concentrations of CDDP in micromolar concentration. The squares are measured intensities for the reaction mixture, and the triangles are measured intensities for the control mixture. The curves are exponential fits (R² ~0.5) to the data. There seems to be no evidence for a delay in the observed reaction.

For the control-corrected data of Fig. 3a (circles), the quantity ln(Q), eq. 1, was calculated. Divided by [CDDP]₀ = [MT]₀ (labeled as: [C0 - M0]), it is plotted versus time in Fig. 4a. The linear fit shown (R² = 0.953) is 0.0062 + 0.00214t, corresponding to a second-order rate constant k = 0.00214 μM⁻¹·h⁻¹ or 0.59 M⁻¹·s⁻¹. The data of Fig. 3b, treated similarly, lead to a linear fit (R² = 0.453) of 0.000147 + 0.0000243t/min. This gives k = (0.41 ± 0.13) M⁻¹·s⁻¹.
The dashed line is a chromatogram of a 50-μl injection on the HPLC of the reaction containing 77.5 μM apo-thionein (dialysate), 33 μM CDDP, and 4.62 mM NaCl (1-h incubation at 37°C). The MT peak (arrow) appears with 25 < tR < 27 min. The solid lines are chromatograms of a 50-μl injection on the HPLC of the control reaction (containing 970 μl of the dialysis solution, 33 μM CDDP, and 4.62 mM NaCl) and of dH2O. Unbound CDDP (arrow) is found in eluates with 3 < tR < 7 min. The Pt-thionein product is found in eluates with 24 < tR < 27 min. Eluates with 27 < tR < 30 min also contain Pt, mostly representing hydrolyzed CDDP. Otherwise, the remaining chromatogram is free of Pt.

In Fig. 4b, ln(Q) divided by [CDDP]0 – [MT]0 (labeled as [C0 – M0]) was calculated from three other sets of measurements after correction for control, is plotted versus time t. The triangles are for the reaction containing 77.5 μM of the dialysis solution, 33 μM CDDP, and 4.62 mM NaCl (dialysate) and of dH2O. Unbound CDDP (arrow) is found in eluates with 3 < tR < 7 min. The Pt-thionein product is found in eluates with 24 < tR < 27 min. Eluates with 27 < tR < 30 min also contain Pt, mostly representing hydrolyzed CDDP. Otherwise, the remaining chromatogram is free of Pt.

HPLC-AAS experiments were also performed on the apo-thionein. After subtraction of the water background, uncertainties were corrected for the control reactions and used to calculate ln(Q), eq. 1. Then, ln(Q) was divided by [CDDP]0 – [apo-thionein]0 (labeled as [C0 – M0]) and plotted versus time t, as shown in Fig. 5a. From the linear fit (R2 = 0.766), one gets the results in the last row of Table 2. As noted, the rate constants were very similar for all forms of MT, but lower for apo-thionein. In contrast, CDDP binding to Pt-thionein was negligible (see below).

Kinetics of Pt-Thionein Product Formation. The amount of Pt in eluates with 24 < tR < 27 min (representing Pt-thionein products) increased with reaction time. A typical example is shown in Fig. 5b. The reaction mixture (at 37°C) contained 970 μl of apo-thionein dialysate (~77.5 μM MT), 33 μM CDDP, and 4.62 mM NaCl. The control mixture contained 970 μl of the dialysis solution, 33 μM CDDP, and 4.62 mM NaCl. In this experiment, the recovery of Pt-thionein product was ~20%, reflecting retention of the bulk of the product on the column.

The 24 < tR < 27 min eluates of the apo-thionein reaction (Fig. 5b, circles) were placed in the dialysis tubing (mol. wt. cutoff, 3500) and dialyzed at 4°C for ~16 h against two changes of 2 liters of 10 mM Tris-NO3 (pH ~7.4). The Pt was fully (100%) retained in the dialy-sates, confirming that it was bound to MT. Otherwise, the Pt in eluates with 24 < tR < 27 of the control mixture (Fig. 5b, circles) was fully dialyzable.

In Fig. 6, the reaction mixture (at 37°C) contained 970 μl of Pt-thionein dialysate (~55 μM MT), 33 μM CDDP, and 4.62 mM NaCl. The control mixture contained 970 μl of the dialysis solution, 33 μM CDDP, and 4.62 mM NaCl. Figure 6a shows the amount of CDDP in the reaction mixture (squares) and the amount in the control mixture (circles) as functions of reaction time. These were measured by HPLC-AAS and converted to Pt concentration (micromolar). The slopes of the exponential fits, shown as lines, are the same within the experimental error. Figure 6b shows the measured concentration of Pt-thionein product, in micromolar concentration, as a function of reaction time, for the reaction mixture (squares) and the control mixture (circles). Again, the slopes (linear fits are shown) are very
substantially influence the amount of Pt drugs available for binding to DNA (Dabrowiak et al., 2002; Sadowitz et al., 2002), and hence they affect the outcome of cells exposed to Pt compounds (Mistry et al., 1991). We recently showed that the Pt-DNA adducts increase by ~8-fold when cellular thiols are blocked by N-ethylmaleimide (Sadowitz et al., 2002). This finding reflects the capacity of cellular thiols to trap CDDP.

The precise role of MT in trapping CDDP in the cytosol during a treatment course is unknown. We employ here clinically relevant conditions of CDDP to estimate its reaction rate with MT. Platinol, the formulation of CDDP given to patients, is studied at concentrations that are close to the therapeutic range (Corden et al., 1985; Soud et al., 2003). The reactions with MT are investigated at 37 ± 0.1°C and a pH of 7.4 with 10 mM Tris-NaNO₃; the nitrate ion is chosen because it binds to Pt²⁺ only weakly.

The UV absorptions of solutions of CDDP and Cd/Zn-thionein were monitored as functions of time, and the absorptions of MT and CDDP alone were subtracted. Some of these results are shown in Fig. 1. The increase in absorption is primarily due to the formation of Pt-S bonds. Unless [CDDP] was much bigger than [MT], the initial slope of the absorption-versus-time curve was close to zero (sometimes negative), indicating that the first reaction occurring (reaction 1), which was rate-determining, did not involve Pt-S bond formation. The subsequent reaction (reaction 2), which involves Pt-S bond formation, is shown by the increase of absorption after a delay of ~1 to 2 h.

When [CDDP] was much bigger than [MT] (Fig. 1, [MT]/[CDDP] = 2.1 μM/198 μM), the initial slope of the absorption-versus-time curve was positive, and its value depended on the concentrations of the reactants (Table 1). The rate of Pt binding to MT is proportional to the product of [CDDP] (calculations performed with UV data show an order of ~1.47 with respect to CDDP) and [MT]. With increased concentration of CDDP, the time taken to reach plateau decreases. This implies that reaction 2 has become rate-determining, since the rate of reaction 2 is now smaller than that of reaction 1. Thus, the rate of reaction 1 increases rapidly with [CDDP]₀. From the dependence of initial slopes on initial CDDP and MT concentrations, we estimate that reaction 2 is approximately first order in [MT] and approximately second order in [CDDP]. The latter probably means that reaction 2 actually involves several reactions, with sequential formations of Pt-S bonds. Reaction 1 may dominate in cells with excess MT and low CDDP concentration, allowing effective DNA platination by the drug.

Pattanaik et al. (1992), studying the reaction of CDDP with MT by UV absorption, HPLC-AAS, extended X-ray absorption fine structure spectroscopy, and other techniques, also concluded that at least two reactions occurred. They proposed that, in the first reaction, four Pt atoms became attached to MT, but without formation of Pt-S bonds or expulsion of Zn or Cd. This reaction did not increase the UV absorption. This reaction was found to be first-order in CDDP and first-order in MT. The second-order rate constant at 25°C was ~0.22 ± 0.072 M⁻¹ s⁻¹ (average of rate constants calculated

**Discussion**

There is a growing concern about cancer cells becoming resistant to Pt-based therapy (Richon et al., 1987; Eastman, 1991). Cellular thiols

*a.* The quantity \( \ln(Q) \) for the control-corrected data of Fig. 3a (circles) was calculated using eq. 1. Divided by \((\text{[CDDP]}_0 - \text{[MT]}_0) \), it is plotted versus time \( t \) (see text). b, the reactions (at 37°C) contained 970 μl of dialysate of Cd/Zn-thionein (~83 μM), Cd-thionein (~74 μM), or Zn-thionein (~80 μM), 33 μM CDDP, and 4.62 mM NaCl (final volume, 1.0 ml). The control experiments contained 970 μl of the dialysis solutions, 33 μM CDDP, and 4.62 mM NaCl. At the end of each incubation period, 50 μl of the reaction were injected on the HPLC. Eluates representing unbound CDDP (3.0 < \( f_a < 7.0 \) min) were collected, and the Pt contents were determined on the AAS. \( \ln(Q) \) divided by \((\text{[CDDP]}_0 - \text{[MT]}_0) \), calculated after correction for control, is plotted versus time \( t \). The triangles are for the reaction of CDDP with Cd/Zn-thionein, the diamonds for reaction with Zn-thionein, and the stars for Cd-thionein. The \( k \) values are summarized in Table 2.

**Accumulation of Cd in Leu-AS.** Leu-AS (Fig. 7, solid line) and Cd/Zn-thionein (Fig. 7, dashed line) were labeled with mBBr, separated on HPLC, and detected by fluorescence. The predominant thiols in the Leu-AS were the two MT isoforms (peaks 5 and 6).

The Cd accumulation was associated with increased molecular absorbance at 240 to 300 nm (Fig. 8, solid line), reflecting formation of the Cd-thionein (mercaptide) complex. Using a stoichiometry of 7 g-atoms of Cd per mole, the amount of MT was ~3.95 nmol per ~3.46 \times 10⁷ leukocytes or ~0.1 fmol per leukocyte. Giving the measured mean leukocyte volume of ~128 ± 33 nl, cellular concentration of MT was thus ~1.0 mM.

**Discussion**

There is a growing concern about cancer cells becoming resistant to Pt-based therapy (Richon et al., 1987; Eastman, 1991). Cellular thiols...
from their Table 1), somewhat smaller than our average $k$ for MT at $37^\circ C$. Zhang and Tang (1994) also studied the reaction of CDDP with MT, using excess concentrations of CDDP and monitoring the reactions by UV absorption. They propose stepwise binding of Pt. They resolve their data into three kinetic steps, each pseudo-first order in CDDP and each leading to an increase in UV absorption. However, they report that only 7, and not 10, moles of Pt bind per mole of thionein.

The pseudo-first-order rate constants at $15^\circ C$ were reported (Zhang and Tang, 1994) as $8.5 \times 10^{-3} \text{min}^{-1}$, $1.3 \times 10^{-3} \text{min}^{-1}$, and $0.28 \times 10^{-3} \text{min}^{-1}$ when [CDDP] was 0.124 mM. The third rate constant

### Table 2

Rates of reactions of CDDP with MT obtained from HPLC-AAS data

<table>
<thead>
<tr>
<th>MT A</th>
<th>B</th>
<th>$R^2$</th>
<th>$k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd/Zn thionein</td>
<td>$0.0062$</td>
<td>$0.00214 \pm 0.00020$</td>
<td>$0.953$</td>
</tr>
<tr>
<td>Cd thionein</td>
<td>$-0.00279$</td>
<td>$0.00269 \pm 0.00009$</td>
<td>$0.997$</td>
</tr>
<tr>
<td>Cd$_2$-thionein</td>
<td>$0.00158$</td>
<td>$0.00192 \pm 0.00012$</td>
<td>$0.989$</td>
</tr>
<tr>
<td>Zn$_2$-thionein</td>
<td>$-0.00128$</td>
<td>$0.00233 \pm 0.00012$</td>
<td>$0.992$</td>
</tr>
<tr>
<td>Apo-thionein</td>
<td>$-0.000021$</td>
<td>$0.00049 \pm 0.00016$</td>
<td>$0.766$</td>
</tr>
</tbody>
</table>

*Undialyzed Cd/Zn-thionein (from Sigma-Aldrich).*

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**FIG. 5.** Kinetics of CDDP binding to apo-thionein and product formation by HPLC-AAS.

- **a**, second-order plot of the reaction of apo-thionein with CDDP. The reaction (at $37^\circ C$) contained 970 $\mu$l of apo-thionein dialysate ($\sim 77.5$ mM), 33 $\mu$M CDDP, and 4.62 mM NaCl. The control experiment contained 970 $\mu$l of the dialysis solution, 33 $\mu$M CDDP, and 4.62 mM NaCl. At the end of each incubation period, 50 $\mu$l of the reactions were injected on the HPLC. Eluates representing unbound CDDP ($3 < t_R < 7 \text{ min}$) were collected, and the Pt contents were determined on the AAS. The second-order plot is shown. The $k$ value is summarized in Table 2. **b**, kinetics of the Pt-thionein product formation. Eluates representing Pt-thionein products ($24 < t_R < 27 \text{ min}$) were collected, and the Pt contents were determined on the AAS (squares). The line is the best linear fit. The Pt contents in eluates with ($24 < t_R < 27 \text{ min}$) of the control experiment are also shown (circles).

**FIG. 6.** Kinetics of CDDP binding to Pt$_2$-thionein by HPLC-AAS.

- **a**, the reaction (at $37^\circ C$) contained 970 $\mu$l of Pt$_2$-thionein dialysate ($\sim 55$ mM), 33 $\mu$M CDDP, and 4.62 mM NaCl (squares). The control experiment contained 970 $\mu$l of the dialysis solution, 33 $\mu$M CDDP, and 4.62 mM NaCl (circles). At the end of each incubation period, 50 $\mu$l of the reaction were injected on the HPLC. Eluates representing unbound CDDP ($3 < t_R < 7 \text{ min}$) were collected, and their Pt contents were determined on the AAS. Exponential fits are shown: within experimental error, the slopes are the same. **b**, eluates representing Pt-thionein products ($24 < t_R < 30 \text{ min}$) were collected, and their Pt contents were determined on the AAS (squares). The Pt contents in eluates with ($24 < t_R < 30 \text{ min}$) of the control experiment are also shown (circles). The lines are linear fits, the slopes of which are the same and zero within experimental error.
increased to $0.35 \times 10^{-3} \text{ min}^{-1}$ at 37°C. The four values cited correspond to second-order rate constants of 1.14, 0.17, 0.038, and 0.047 M$^{-1}$ s$^{-1}$. Although the authors state that the first of the three steps is first-order in [CDDP] (and the others zero-order), a plot of $\ln(k)_{1}$ versus $\ln([CDDP])$ (data from their Table 5) has a slope of $0.15 \pm 0.03$, showing that it is zero-order, as found by Pattanaik et al. (1992).

In contrast to carboplatin and oxaliplatin, unbound cisplatin does not produce significant UV absorption at 260 nm. Therefore, the reaction was followed by Pt contents (by AAS) in the HPLC eluates, representing unbound cisplatin ($3 < t_{R} < 7$) as a function of reaction or incubation time. It appeared that the Pt concentration decreased smoothly with reaction time, with no evidence of a delay like the one that sometimes appeared in the UV absorption. Since we are measuring the disappearance of free CDDP, this is not surprising: we are measuring the rate of the first reaction, in which CDDP binds to MT, whether or not it involves formation of Pt-S bonds.

The reaction was analyzed according to mixed second-order kinetics. From the measured initial concentrations of CDDP and MT, we calculated $[MT]$ for each reaction time $t$, and then calculated $\ln(Q)$ (eq. 1). Plots of $\ln(Q)$ versus $t$, such as those shown in Fig. 4, a and b, and Fig. 5a, were almost always highly linear. From the slope of such a plot one obtains the second-order rate constant $k$. Values of $k$ (at 37°C) are given in Table 2. All four MTs have the same value of $k$ within experimental error: $0.63 \pm 0.08 \text{ M}^{-1} \text{s}^{-1}$. For apo-thionein, $k$ is smaller, $0.14 \text{ M}^{-1} \text{s}^{-1}$.

We also measured the concentration of Pt in the HPLC peak corresponding to the product. Only a fraction of the Pt lost from the parent peak was found in the product peak because significant amounts of Pt-thionein product were retained in the column. In general, the concentration of Pt in the product peak increases linearly with reaction time for $t$ up to 9 h (Fig. 5b).

For the reaction of Pt-thionein with CDDP, Pt concentrations in the product and parent peaks were measured as functions of reaction time. Linear fits of the data were performed (Fig. 6). The slopes were small and, within experimental error, the same for the reaction mixture as for the control mixture. This indicates that the Pt-thionein is saturated with Pt and unable to react with additional CDDP, and that no unreacted thionein was present. On the time scale of our experiments, the Pt is thus irreversibly bound.

We adapt the mBBr labeling method to detect MT in Leu-AS (Fig. 7); mBBr rapidly reacts with biologic thiols (Soud et al., 2001; Tacka et al., 2002). The resulting bimane (thioether) derivatives are highly fluorescent and can be detected with great sensitivity. The cellular concentration of MT in human leukocytes is estimated here to be $\sim 1.0$ mM, which is similar to the values reported for human liver (0.017–2.5 μmol/g wet weight) and mononuclear blood cells (0.022 fmol/cell) (Vasak, 1991). Our estimation is based on the capacity of Leu-AS to accumulate Cd (Fig. 8). Nevertheless, since MT can be induced, cells may increase their MT content substantially.

It seems clear that the HPLC-AAS measurements on the $3 < t_{R} < 7$ min eluate are detecting the initial reaction of MT with CDDP. This reaction cannot be seen in our UV time studies because it does not lead to a change in the extinction coefficient. It is followed by other reactions in which Pt-S bonds form, increasing the extinction coefficient. The first reaction has been reported (Pattanaik et al., 1992; Zhang and Tang, 1994) to be zero-order in CDDP and first-order in MT, but this is probably because the studies were done with millimolar CDDP concentrations and micromolar MT concentrations. We analyzed the HPLC-AAS measurements according to mixed second-order kinetics.

The value of the second-order rate constant $k$ was essentially the same for all the MT we measured, $0.63 \pm 0.08 \text{ M}^{-1} \text{s}^{-1}$ (Table 2). It was much lower, $0.14 \text{ M}^{-1} \text{s}^{-1}$, for unmetallated apo-thionein, probably because the formation of disulfide bonds makes some of the sulfur hydrys unsuitable for Pt binding (Ejnik et al., 2002). The average value of $k$ for MT of 0.63 M$^{-1}$ s$^{-1}$ is ~50-fold higher than that for CDDP reaction with GSH, $k = 0.013 \text{ M}^{-1} \text{s}^{-1}$ (Dabrowiak et al., 2002). Thus, since the concentrations of GSH and MT are both in the millimolar range, the contribution of MT to trapping CDDP can be more than that of GSH.

An estimate of the rate at which CDDP binds to MT in the cytosol can be obtained from our estimated concentration of MT (~1.0 mM), clinical concentration of CDDP (~10 μM), and measured $k$ value for CDDP binding to MT (0.63 M$^{-1}$ s$^{-1}$). The result is $6.3 \times 10^{-6} \text{ mol}$. 

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**Fig. 7.** Representative HPLC chromatograms of the bimane derivatives of Leu-AS and Cd/Zn-thionein.

The dashed chromatogram is the analytical run of 50 μl of ~10 μM bimane derivative of Cd/Zn-thionein. The solid chromatogram is the analytical run of 50 μl of bimane derivative of Leu-AS. Peaks 1 to 4 were undetermined. Peaks 5 and 6 are MT isoforms.

**Fig. 8.** Absorption spectrum changes associated with Cd accumulation in Leu-AS.

The solid line is an absorption spectrum of Leu-AS that was placed in the dialysis tubing (mol. wt. cutoff, 3500) and dialyzed at 4°C for ~16 h against 1 liter of 10 mM Tris-Cl (pH 8.0) plus 0.1 mM CdCl$_2$, followed by ~16 h of dialysis against two changes of 1 liter of 10 mM Tris-Cl. The dashed line is an absorption spectrum of Leu-AS that was dialyzed as above but without the addition of CdCl$_2$. 

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of CDDP per cm$^3$ per s. Using the estimate of $\sim 0.022$ fmol of MT per cell, the estimated rate is $\sim 3 \times 10^{-6}$ fmol per min per cell.

The rate constant reported here for the reaction of CDDP with MT is important for accurate modeling of cellular DNA platination by CDDP (Sadowitz et al., 2002). We have constructed a simple model (describing complex processes with simple rate laws) that involves several undetermined kinetic parameters. With values for more parameters fixed by measurement, the model should lead to more reliable values for the others. This would enable a test of the model as a guide to clinical practice.

References
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