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A study on the interaction between cisplatin and urease[1]

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Abstract

The present work gives the results of the interaction between cisplatin and urease. Microcalorimetry was used to measure enzymatic activity. The experimental results indicated that cisplatin could render urease inactive. Further spectral determinations showed that enzymatic 'circular dichroism' absorption peak decreased and its fluorescence was quenched by cisplatin. This proved that enzymatic secondary structure had been changed. Thermal denaturation experiments by DSC had given the same results that cisplatin resulted in the reduced denaturation enthalpy of urease, and the denaturation temperature of urease changed from 361.37 to 345.05 K under experimental conditions. It showed that cisplatin reduced enzymatic conformational stability, and the binding of cisplatin with urease was found to be a slow process. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Urease; Cisplatin; Microcalorimetry; Conformational stability

1. Introduction

Urease (EC 3.5.1.5) is the first enzyme isolated as a crystalline protein [1]. It is also the first example of a nickel metalloenzyme which includes six identical subunits and contains two nickel ions at the active site per subunit [2,3]. The biochemical significance of this enzyme is that it catalyzes the hydrolysis of urea to form ammonia and carbon dioxide at a rate which is 10^{14} times faster than that of the uncatalyzed reaction [4]. Recent research has revealed that its activity has been implicated in many diseases, such as infection-induced urinary stones, catheter encrustation, peptic ulcers and, possibly, stomach cancer [5,6]. Cisplatin is a square-planar Pt(II) complex which finds extensive use as an anticancer agent. Its use has been limited by toxicity to the kidney. Fremuth et al. [7] reported that treatment of Chinese hamsters with cisplatin (8 mg/kg, i.p.) produced chromosome aberrations in the bone-marrow cells. Pretreatment with urease (two doses of 4 and 5 units administered at four-day intervals) decreased the chromosomal damage caused by the mutagen. This work has carried out a study on the interaction between cisplatin and urease using microcalorimetry and conformational research methods, and contributed some basic experimental data for biochemical and medical study.

2. Experimental

2.1. Materials

Urease (*Canavalia ensiformis*, M.W.480KD) crystalline was from Boehringer Mannheim, and

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stored at 253 K until used. Fresh enzymatic solution was prepared by dialysis in HEPES buffer (pH 7.0) for a day and its concentration measured spectrometrically [8], using the extinction coefficient $E^{1\%}_{280} = 6.2\ \mathrm{cm}^{-1}$.

Urea (superpurity) was from Serva and HEPES from Merck. Cisplatin used as anti-cancer drug was produced by Qi-Lu Pharmaceutical Factory. Other chemicals were commercial products of analytical grade. All solutions were prepared in 5 mmol l^{-1} HEPES buffer (pH 7.0) using redistilled water. The mixing solutions of cisplatin and urease were kept at 277 K for 24 h before being used for the experimental measurements.

2.2. Apparatus and procedure

The calorimetric apparatus used to measure enzymatic activity was a Calvet MS-80 microcalorimeter [9]. An aliquot (60 mmol l^{-1}) of urea solution (2.5 ml) was added to the stainless steel cell, and 0.5 ml of urease or a mixed solution of urease and cisplatin was added to the glass cup in this experiment. In the control cell, urea was replaced by the buffer. The reaction was started by turning the microcalorimeter over five times ($5 \times 180^\circ$) after the machine had come to thermal equilibrium at 298.15 K. All concentrations in this part referred to the reagents in the calorimetric vessel before mixing.

Circular dichroism (CD) spectra were recorded by a Jasco J-500 automatic-recording spectropolarimeter in a quartz cell with a path length of 0.2 cm. Scanning wavelength was from 200 to 250 nm.

Fluorescence of urease was measured by F-4500 Hitachi fluorescence spectrophotometer. Voltage of the photomultiplier tube was 400 V with a slit of 5.0 nm. Excitation wavelengths were 280 and 295 nm, respectively. Emission spectrum was recorded from 305 to 500 nm.

Measurements of the thermal denaturation of urease were studied with a new high-sensitive differential scanning calorimeter (MicroDSC III, Setaram, France). Temperature correction and baseline correction had been done before proceeding with the experiment. The experimental temperature range was from 298.15 to 383.15 K. The volume of solution was 0.8 ml.

All the measurements performed at least twice.

3. Results

3.1. Measurements of enzymatic activity

Enzymatic activity can be experimentally determined in several ways. Calorimetry is a very good method because it avoids most interferences. The microcalorimeter employed in this work is of the heat-conduction type. Its output is directly proportional to the heat-exchange rate during the reaction and, hence, proportional to enzymatic activity [10]:

$$\Phi = \Delta(q/\Delta t)$$

where Φ is the maximum heat-exchange rate $\Delta(q/\Delta t)$ from the experimental curve, therefore:

$$\Phi \propto E \times \Delta_\mathrm{r} H_\mathrm{m}$$

where E is the enzymatic activity, and $\Delta_\mathrm{r} H_\mathrm{m}$ the mole reaction enthalpy change of the hydrolysis reaction of urea under enzymatic catalysis. Result for $\Delta_\mathrm{r} H_\mathrm{m}$ was basically the same as that obtained by Oehlschlager [11], with the value of 42.0 kJ mol^{-1}. The urea concentration was chosen to approach the maximum reaction rate since Michaelis constant K_M of enzymatic catalysis reaction in HEPES (pH 7.0) is 3.0 mmol kg^{-1} [12]. The working curve was obtained by plotting the calorimetric value Φ vs. urease concentration (Fig. 1). Under the condition of ensuring enzymatic active sites being saturated by the substrate urea, enzymatic activity could be measured within the linear range since enzymatic activity is proportional to Φ within this range. As cisplatin was added to the urease solution whose concentration was 0.12 and 0.3 mg ml^{-1}, respectively, the enzymatic activity gradually decreased to zero with increasing cisplatin concentration (Fig. 2). The decreasing enzymatic activity was expressed by the relative activity Φ/Φ_0, where Φ_0 and Φ were the maximum heat-exchange rates in the absence, and presence, of cisplatin. From Fig. 2, it can be seen that the decreasing curve of relative activity was linear within the range of experimental measurements. For higher concentrations of urease, the decreasing rate of enzymatic activity was slower than for lower concentrations of urease. The binding of cisplatin with urease was a slow process, and the immediate determination after mixing them showed no decrease in enzymatic activity.

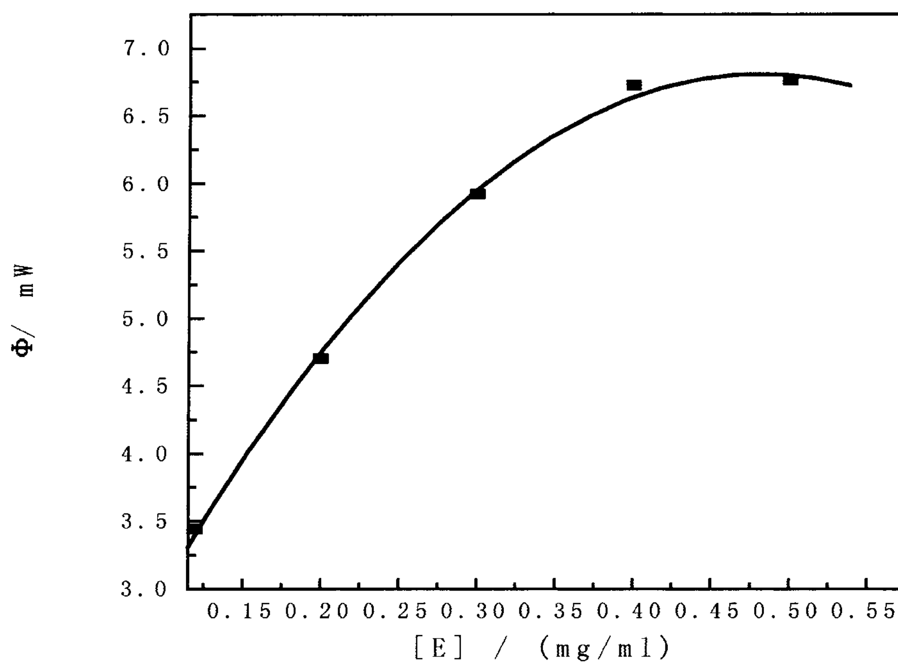

Fig. 1. The working curve of enzymatic activity.

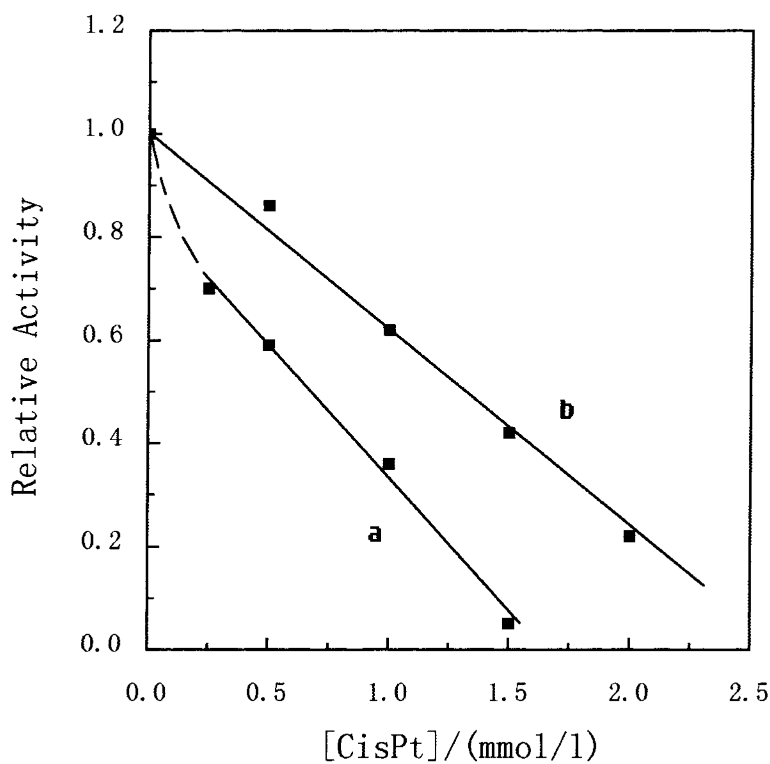

Fig. 2. The effect of cisplatin on enzymatic activity (urease): (a) 0.12 mg ml^{-1}; and (b) 0.3 mg ml^{-1}.

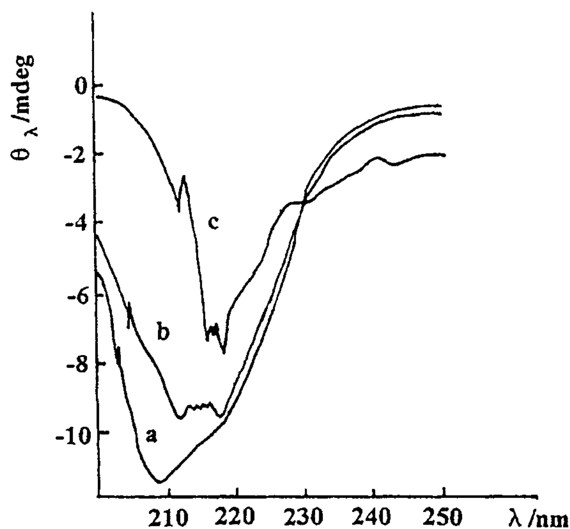

Fig. 3. The effect of cisplatin on urease CD spectra (cisPt): (a) 0. (b) 1.5 mmol l^{-1}; and (c) 4.0 mmol l^{-1}. (urease) = 0.12 mg ml^{-1}.

3.2. Measurements of urease 'circular dichroism' (CD) spectra

CD spectrum in the far-ultraviolet region has proved to be useful in the study of the secondary structure of proteins [13,14]. CD spectrum of urease showed a negative absorption peak at 208 nm and a shoulder at 220 nm (Fig. 3(a)). It corresponds to the kind of protein which contains greater amounts of α-helix and β-sheet [15]. With the addition of cisplatin, the negative absorption peak reduced. It meant that the secondary structure of urease had changed. Urease concentration used in this experiment was also 0.12 mg ml^{-1}. It should be noted from Fig. 3(b), that the change of CD spectrum was small, when 1.5 mmol $^{-1}$ cisplatin was added while the enzymatic relative activity reduced to 5% then (see Fig. 2(a)). It indicated that the rate of inactivation was faster than the rate of conformational change for urease by cisplatin.

3.3. Measurement of urease fluorescence

Protein fluorescence arises from tryosine (Tyr) and tryptophane (Try) residues mainly. The quantum yield of phenylalanine (Phe) residue is too small to be detected in urease fluorescence. The emission spectrum under excitation wavelength 295 nm is from Try residue only and the emission spectrum under excitation wavelength 280 nm is from both, Tyr and Try residues [16]. One of the major areas of application of fluorescence quenching involves the study of proteins, enzymes and their structures [17,18]. Measurements of urease fluorescence indicated that, in the HEPES buffer, urease produced a strong fluorescence at 334 nm. Excitation with different wavelengths produced the same wavelength emission with different fluorescence intensities. Cisplatin caused quenching of urease fluorescence (Fig. 4). The quenching curve was obtained by plotting (F_0/F) vs. the concentration of cisplatin, where F_0 and F are the fluorescence intensities of urease in the absence, and presence, of cisplatin, respectively. It showed a linear relationship between F_0/F and the concentration of cisplatin. Under excitation with different wavelengths, the effect of cisplatin on urease fluorescence was basically the same. Under the condition of 0.12 mg ml^{-1} urease solution 1.7 mmol l^{-1}, cisplatin led to a quenching of fluorescence intensity to 55%. However, the solution of 0.12 mg ml^{-1} urease with 1.7 mmol l^{-1} cisplatin showed zero enzymatic activity (Fig. 2(a)). Also, 5.2 mmol l^{-1} cisplatin quenched fluorescence to 18% and the emission wavelength shifted to 338 nm. This indicated that the binding of cisplatin with urease had influenced the microenvironment of residues with aromatic group and the overall conformation of the macromolecule.

3.4. Thermal denaturation of urease

The DSC method provided fundamental information about the thermodynamic properties of protein molecules, such as the enthalpies of thermal denaturation processes, the influence of molecular interactions on the stability of proteins, and the correlations between thermodynamic and structural features of proteins [19]. The denaturation experiment of urease by DSC started from 298.15 to 383.15 K and returned from 383.15 to 298.15 K. Urease showed an irreversible denaturation process. The results are listed in Table 1. There was an exothermic peak at 361.37 K. The molar enthalpy change of denaturation of $>10^4$ kJ mol^{-1} was too large for conformational change and possibly due to protein aggregation. The process could be observed even under 0.2 mg ml^{-1} urease concentration. Cisplatin resulted

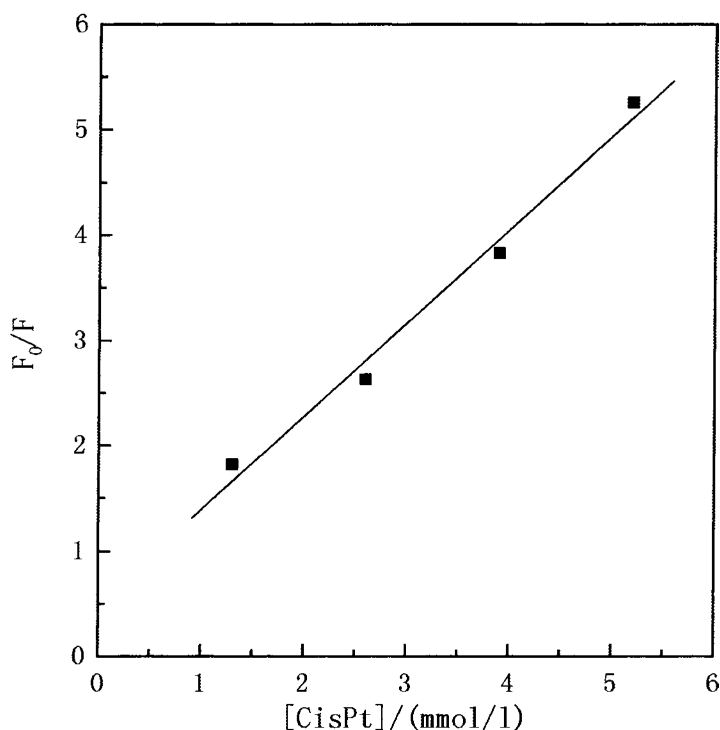

Fig. 4. The quenching curve of urease fluorescence by cisplatin. Excitation wavelength, 295 nm.

in the reduction of enthalpy change and denaturation temperature. Moreover, the lower concentration of cisplatin rapidly reduced ΔH.

4. Discussion

Experimental results indicated that cisplatin interacted with urease. According to microcalorimetric measurements, different enzymatic concentrations showed different decreasing rates of the relative activity by cisplatin. The same concentration of cisplatin made lower-concentration enzymatic relative activity smaller. This was possible because cisplatin could easily enter the binding site in lower concentrations of enzyme due to its relatively larger quantity in solution. Although the enzymatic activity was lowered, the $\Delta_r H_m$ of enzymatic catalysis reaction of urea hydrolysis was the same as that of pure enzymatic catalysis reaction. It may be suggested that cisplatin binded only to urease and not to urea, and that no ternary complex were formed.

Table 1
The effect of cisplatin on thermal denaturation of urease by DSC; (urease), 0.44 mg ml^{-1}

(Cisplatin)/(mmol l^{-1})	ΔH/J	T_M/K
0	−0.0676	361.37
0.8	−0.0345	361.25
1.3	−0.0327	358.99
1.7	−0.0286	351.25
2.2	−0.0261	347.78
3.3	−0.0247	345.05

CD spectrum is often used in investigating the conformational change of protein secondary structure. From the experimental results, it could be seen that cisplatin gave rise not only to the enzymatic inactivation but also to a further structural change. Before the enzymatic activity reduced to zero, CD spectral change was smaller (Fig. 3(b)) compared with fluorescence. However, correlating Fig. 4 with Fig. 2(a), one can easily see that fluorescence quenching had been up to 55%, when the relative activity was zero. It

showed that cisplatin influenced the aromatic amino acids microenvironment dramatically. These results coincided with the DSC results.

Thermal denaturation of urease produced a large exothermic peak in DSC thermogram which probably arose from protein aggregation. Cisplatin reduced the denaturation temperature of urease and, consequently, the enthalpy change decreased. Since the concentration of enzyme was 0.44 mg ml^{-1}, cisplatin of experimental concentration could not make urease fully inactive under such concentrations of urease. However, the enzymatic stability had decreased greatly, as the denaturation temperature had reduced from 361.37 to 345.05 K under experimental conditions.

On the other hand, microcalorimetric measurements had shown that 3.5 mmol l^{-1} cisplatin made urease fully inactive. Then the solution was dialyzed by a buffer, and enzymatic activity was measured again (the experiments were not mentioned above). The result showed that urease did not resume its activity. This indicated a possible irreversible binding process between cisplatin and urease. The binding process was found to be a slow process in all the experiments. Immediate determination after mixing cisplatin and urease showed no effect of cisplatin on the activity of urease.

Acknowledgements

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References

[1] J.B. Summer, J. Biol. Chem. 69 (1926) 435.
[2] N.E. Dixon, C. Gazzola, R.L. Blakeley, B. Zerner, J. Am. Chem. Soc. 97 (1975) 4131.
[3] N.E. Dixon, C. Gazzola, C.J. Asher, Can. J. Biochem. 58 (1980) 474.
[4] E. Jabri, M.B. Carr, R.P. Hausinger, P.A. Karplus, Science 268 (1995) 998.
[5] J. Alper, Science 260 (1993) 159.
[6] A. Lee, J. Fox, S. Hazell, Infect. Immun. 61 (1993) 1601.
[7] F. Fremuth, Z. Dienstbier, I. Bartaetal., Proc. Int. Congr. Chemother., 7th 1971 (Pub. 1972) 827.
[8] R.L. Blakeley, Zerner, E.C. Webb, Biochemistry, 8 (1969) 1984.
[9] X. Liu, L. Yen, B. Wang, Y. Zhang, Thermochim. Acta 253 (1995) 167.
[10] M.L. Antonelli, V. Carunchio, M. Luciant, G. Vinci, Thermochim. Acta 122 (1987) 95.
[11] K. Oehlschülager, R. Hüttl, G. Wolf, Thermochim. Acta 271 (1996) 41.
[12] R. Hüttl, K. Bohmhammel, G. Wolf, R. Oehmgen, Thermochim. Acta 250 (1995) 1.
[13] M.J. Gorbunoff, Arch. Biochem. Biophys. 138 (1970) 684.
[14] Z. Lu, T. Cui, Q. Shi, The Applications of Circular Dichroism and Optical Rotatory Dispersion in Molecular Biology, Beijing, Science Press, 1987, p. 92.
[15] Z. Lu, T. Cui, Q. Shi, The Applications of Circular Dichroism and Optical Rotatory Dispersion in Molecular Biology, Beijing, Science Press, 1987, p. 111.
[16] W. Tao, W. Li, Y. Jiang, G. Luo, Y. Lin, Molecular Basis of Protein, Beijing, Adv. Ed. Press, 1981, p. 229.
[17] C.N. Ho, G. Patonay, I.M. Warner, Trends Anal. Chem. 5(2) (1986) 37.
[18] A.R. Prasad, J.S. Nishimura, P.M. Horowitz, Biochem. 22 (1983) 4272.
[19] V.L. Shnyrov, J.M. Sanchez-Ruiz, B.N. Boiko, G.G. Zhadam, E.A. Permyakov, Thermochim. Acta 302 (1997) 165.