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Inhibition and photo-deinhibition of glutathione (*S*)-transferase activity by an organometallic complex: (*S*)-[3-CpFe(CO)$_2$(η^1-*N*-succinimidato)]glutathione

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Abstract

The bioorganometallic complex (*S*)-[3-CpFe(CO)$_2$(η^1-*N*-succinimidato)]glutathione **1** resulting from the reaction of glutathione with (η^5-Cp)Fe(CO)$_2$(η^1-*N*-maleimidato) is shown to inhibit in a competitive manner the enzymatic activity of glutathione (*S*)-transferase. Moreover, when the Fp fragment is removed by photochemical means, the resulting compound **2** loses this property, which thus appears to be due to the presence of the organometallic moiety. © 1999 Elsevier Science S.A. All rights reserved.

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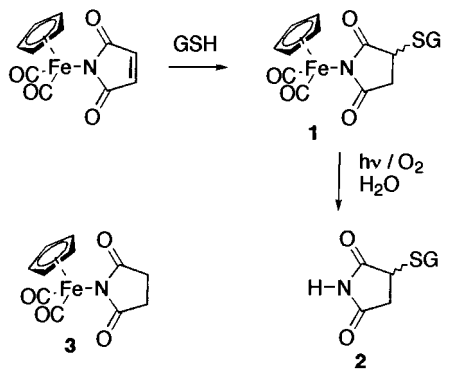
1. Introduction

Transition organometallic complexes containing bioligands (steroid hormones, drugs, proteins, DNA, etc.) have proven to be useful tools in hormone–receptor interaction studies and in non-isotopic immunoassays (i.e. antigen–antibody interactions) [1–3]. We set our interest in the use of bioorganometallics in the field of another biologically essential high affinity interaction, the enzyme–substrate interaction. Several examples of enzyme-catalysed enantio- and diastereoselective transformations of organometallic complexes have been reported so far, with the aim of obtaining homochiral materials [4], but to our knowledge, there is no example of a bioorganometallic complex able to inhibit enzyme–substrate systems to date.

We report herein that the enzymatic activity of glutathione-(*S*)-transferase (GST) can be reversibly inhibited by the organometallic complex **1** which contains an organometallic moiety bound to the sulphur atom of the tripeptide glutathione. Moreover, this inhibition can be suppressed by irradiation of the enzyme–inhibitor system with visible light.

Mammalian cytosolic GSTs are a family of enzymes (α, μ, π and θ) that catalyse the nucleophilic addition of the thiolate of glutathione (γ-glutamylcysteinylglycine, GSH) to a wide variety of electrophiles (either alkyl or aryl) [5,6]. As some GSTs are overexpressed in certain cancer tumours, sometimes as a result of the development of resistance to chemotherapeutic agents [7], an organometallic complex exhibiting high affinity towards these enzymes could be a potential marker for the detection of and/or as an adjuvant in the therapy of these cancers.



Scheme 1. Synthesis of **1** and **2**. Structure of **3**.

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2. Results and discussion

2.1. Synthesis of 1

We have briefly reported that complex $CpFe(CO)_2(\eta^1$-*N*-maleimidato) reacts with glutathione by addition of its thiol function to the double bond of the maleimide ring to give **1** (Scheme 1) [8].

Taking advantage of the solubility of $CpFe(CO)_2(\eta^1$-*N*-maleimidato) in water, this reaction was carried out in aqueous solution, using an excess of the organometallic maleimide, to ascertain that glutathione was fully conjugated. Excess maleimide was extracted with dichloromethane, while **1** remained in the aqueous layer. Reverse-phase HPLC analysis of the product revealed no traces of starting materials and formation of two species in virtually 1:1 ratio at 18.5 and 18.7 min. We assigned these products to the diastereoisomers of **1** resulting from the creation of a new centre of chirality at the succinimide ring. Their presence in 1:1 ratio indicates that the addition of GSH to $CpFe(CO)_2(\eta^1$-*N*-maleimidato) was not diastereoselective. As the chromatographic properties of both diastereomers were very similar, we did not attempt to separate them on a preparative scale. The molecular formula of **1** was also confirmed by high-resolution mass spectrometry.

2.2. Biochemical studies

The effect of compound **1** on the enzymatic activity of equine liver GST was tested using the conjugation of 1-chloro-2,4-dinitrobenzene (CdNB) to GSH as model reaction. The initial velocities V_0 in the presence of various concentrations of **1** were measured and compared to those measured in its absence. The Lineweaver–Burk (double reciprocal) plots of V_0 versus CdNB concentration are displayed Fig. 1(A). It reveals that compound **1** acts as a competitive inhibitor of CdNB with an inhibition constant K_i of 35 μM. The same results displayed in the Dixon convention (reciprocal V_0 versus concentration of **1**) confirm the competitive inhibition property of **1** with an inhibition constant K_i of 31 μM, i.e. very close to the value determined from the double reciprocal plot (Fig. 1(A), inset).

A second series of experiments was carried out, with GSH as the variable substrate. From the double reciprocal plot (Fig. 1(B)), we found that compound **1** was a mixed non-competitive inhibitor of GSH and calculated a competitive inhibition constant K_{i1} of 7 μM and a non-competitive inhibition constant K_{i2} of 57 μM.

It seemed interesting to compare the inhibition activity of **1** with that of known GST inhibitors, (*S*)-alkylglutathiones. This comparison was performed with (*S*)-methyl- and (*S*)-hexylglutathione, the latter known

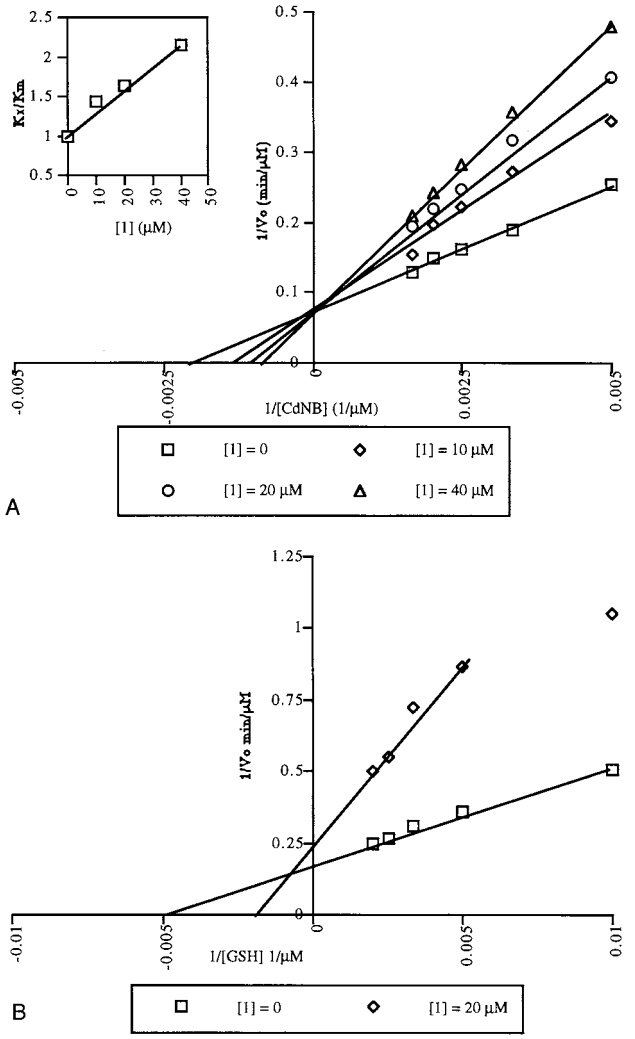

Fig. 1. (A) Competitive inhibition of GST (2 μg ml^{-1}) by **1** with CdNB as the variable substrate. [GSH] = 0.2 mM. (B) Mixed non-competitive inhibition of GST (2 μg ml^{-1}) by **1** with GSH as the variable substrate. [CdNB] = 0.2 mM.

for its strong inhibition activity [9]. Our experiments showed that the inhibition activity of (*S*)-methylglutathione was negligible in comparison to that of **1**, whilst (*S*)-hexylglutathione was a stronger competitive inhibitor of CdNB with $K_i = 1.1$ μM.

Low-oxidation-state organometallic complexes containing carbonyl ligands are generally light sensitive, light causing decoordination of CO and formation of coordinatively unsaturated intermediates. We thought to take advantage of this property in the hope that irradiation of the GST–**1** complex might lead to irreversible deactivation of the enzyme by covalent binding of the photo-activated organometallic moiety to the enzyme active site.

Irradiation with visible light of a mixture of GST and compound **1** in aerated buffered solution was carried out, followed by addition of the enzyme substrates and measurement of the enzymatic activity. Concurrently,

compound **1** was irradiated alone then GST, glutathione and CdNB were added and enzymatic activity measured under the same conditions. Results are reported in Table 1.

Irradiation of the mixture of GST and **1** led to a significant decrease of the inhibition activity of **1**. After 5 and 10 min irradiation, the enzyme recovered 73 and 89% of its original activity, respectively (entries 3 and 4). Interestingly, very similar results were obtained when **1** was irradiated prior to the addition of GST (entries 5 and 6). The above results clearly show that the irradiation of **1** by visible light, either in the absence or in the presence of GST, resulted in the almost complete loss of its inhibiting activity. However, the slightly lower enzymatic activity measured after 10 min irradiation of the GST–**1** complex as compared to that measured after irradiation of **1** alone (entries 4 and 6), might suggest the existence of some other mode of deactivation process occurring to some extent through irradiation of the GST–**1** system (maybe irreversible binding to GST of an intermediate formed from **1**). Entry 6 shows that the species formed by photolysis of **1** exhibits not more than 1% of the inhibiting activity of **1**.

To find out what happened when **1** was irradiated in aerated aqueous solution, we carried out this photolysis on a preparative scale. We observed the formation of an off-white water-soluble product **2**. Its IR spectrum did not display any absorption in the 2000–2100 cm^{-1} region and its ^{1}H-NMR spectrum any signal in the Cp protons area. The reverse-phase HPLC analysis of this species gave two peaks at 12.9 and 13.05 min. All these results suggest that compound **2** has the molecular formula depicted in Scheme 1. This structure was confirmed by mass spectrometry. The organometallic part of compound **1** presumably underwent a complete destruction as Fe(III) ions were detected in the photolyte. The effect of compound **2** on the catalytic activity of GST showed that it is an uncompetitive inhibitor of CdNB with $K_i = 56$ μM and a competitive inhibitor of GSH with an inhibition constant K_i of 37 μM (Fig. 2). Thus, compound **2** is shown to behave in a completely different manner from compound **1**.

Table 1
Photolysis of the GST–**1** system. [GST] = 2 μg ml^{-1}, [GSH] = [CdNB] = 0.2 mM, [**1**] = 0.02 mM

Entry	Enzymatic system	V_0 μM min^{-1} [a]
1	GST	3.7
2	**1**+GST	2.0
3	**1**+GST+*hv* (5 min)	2.7 (73%) [d]
4	**1**+GST+*hv* (10 min)	2.9 (89%) [d]
5	[**1**+*hv* (5 min)]+GST [b]	3.3 (78%) [d]
6	[**1**+*hv* (10 min)]+GST [c]	3.6 (99%) [d]

[a] Calculated by taking $\varepsilon_{340\ nm} = 9600$.
[b] **1** Irradiated for 5 min and then GST added.
[c] **1** Irradiated for 10 min and then GST added.
[d] (In parentheses) recovery of initial enzyme activity (entry 1).

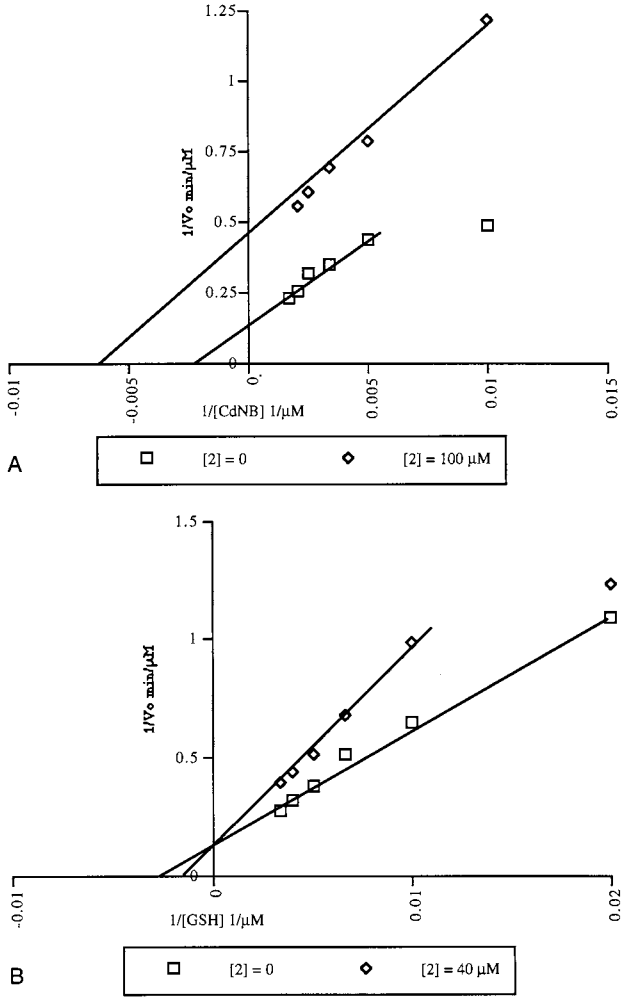

Fig. 2. (A) Uncompetitive inhibition of GST (2 μg ml^{-1}) by **2** with CdNB as the variable substrate. [GSH] = 0.2 mM. (B) Competitive inhibition of GST by **2** with GSH as the variable substrate. [CdNB] = 0.4 mM.

GST has been found to display a complex, biphasic mechanism of action, which depends on the concentration of glutathione. At low concentrations of GSH, a ping-pong mechanism predominates in which the electrophilic substrate enters first the binding site. At high concentrations of GSH, an ordered sequential mechanism operates in which GSH binds first [5].

Our results indicate that **1** is a competitive inhibitor of the electrophilic substrate and a mixed non-competitive inhibitor of GSH. This suggests that **1** is able to enter the electrophilic-substrate binding site of GST (H-site) and also the glutathione binding site (G-site), at least partially. To check this, we have studied the biochemical behaviour of compound **3** (Scheme 1), whose structure closely resembles that of **1** except that the glutathione part is missing. We found that com-

pound **3** indeed acted as a competitive inhibitor of CdNB with an inhibition constant K_i of 66 μM. Thus, the comparison of the inhibition activities of **1**, **2** and **3** clearly shows that the higher activity of the first could be attributed to the presence of the organometallic $CpFe(CO)_2$ moiety, presumably because of its non-polar, aromatic character. Compound **1** *is therefore not only the first competitive organometallic enzyme inhibitor, but also the first inhibitor whose activity is due to the presence of an organometallic moiety and virtually disappears after removal of this moiety by photochemical means.*

3. Experimental

(η^5-Cp)$Fe(CO)_2$(η^1-*N*-maleimidato) was synthesised according to the literature method [8]. (η^5-Cp)$Fe(CO)_2$(η^1-*N*-succinimidato) **3** was synthesised according to [10]. GSH, equine liver GST and 1-chloro-2,4-dinitrobenzene were purchased from Sigma. IR spectra were recorded on a Specord 75 IR spectrometer. ^{1}H-NMR spectra were recorded in D_2O solutions on a Varian Gemini 200BB apparatus (200 MHz for ^{1}H) and referenced to the HDO signal (δ = 4.75). Mass spectra were obtained on a Finnigan MAT 95 spectrometer. UV–vis spectra were recorded of a UV mc^2 spectrometer (Safas).

3.1. Synthesis of *1*

A deaerated solution of $CpFe(CO)_2$(η^1-*N*-maleimidato) (207 mg, 0.76 mmol) and glutathione (160 mg, 0.52 mmol) in water (11 ml) was kept at 35°C for 3 days. The excess of organometallic starting material was removed by extraction with dichloromethane, and the water layer was evaporated under vacuum to dryness to yield **1** (260 mg, 86%) as a yellow foam. HPLC analysis of this material was performed on a Kromasil Si-C8 column (5 μm, 4.6 × 250 mm^2). A linear gradient from 1 to 50% of 0.1% TFA–MeCN in 0.1% TFA–H_2O in 25 min was applied (flow rate = 1 ml min^{-1}, detection at 220 nm). IR (KBr, ν in cm^{-1}) 3350 (br, NH and OH) 2050, 1995 (Fe–CO) 1710, 1695 (CO acid and amide) 1630 (imidato). ^{1}H-NMR (D_2O pD = 7.0, δ) 5.03 (s, 5H, Cp) 4.45 (m, 1H, Hα of Cys) 3.83 (s, 2H, Hα of Gly) 3.73 (t, 1H, Hα of Glu) 3.06 (m, 2H, Hβ of Cys) 2.80 (m, 1H, CH succinimide) 2.50 (m, 1H, succinimide) 2.43 (m, 2H, Hβ of Glu) 2.03 (m, 2H, Hγ of Glu).

HR-FABMS (glycerin matrix, positive ions): *m*/*e* 581.06439; Calc. for $C_{21}H_{25}N_4O_{10}SFe$ (M + H) 581.064076.

3.2. Synthesis of *2*

An aerated solution of **1** (130 mg, 0.22 mmol) in water (5 ml) was photolysed (4 × 150 W tungsten lamps, external cooling with ice–water) until complete disappearance of the yellow colour. The small amount of a brownish precipitate was filtered off and the filtrate evaporated under vacuum to dryness to yield **2** (70 mg, 79%) as an off-white solid. Reverse-phase HPLC analysis of this material was performed under the same conditions as described above in synthesis of **1**.

IR (KBr, ν in cm^{-1}) 3350 (v br, NH and OH) 1780, 1715 (CO succinimide) 1710, 1650 (CO acid and amide). ^{1}H-NMR (D_2O pD = 7.0, δ) 4.54 (m, 1H, Hα of Cys) 3.94 (m, 1H, succinimide) 3.74 (s, 2H, Hα of Gly) 3.70 (m, 2H, Hα of Glu) unresolved, very complex pattern at 2.5–3.2, 2.39 (m, 2H Hβ of Glu) 2.02 (m, 2H, Hγ of Glu).

HR FABMS (*m*-nitrobenzyl alcohol matrix, positive ions) *m*/*e* 405 (M + H).

3.3. Inhibition assays

Absorption at 340 nm of 1 ml solutions containing GST (2 μg ml^{-1}), GSH and CdNB in phosphate buffer 0.1 M pH 6.5 containing 1 mM EDTA was monitored for 10 min at 22 ± 0.5°C, in the presence or not of variable concentrations of inhibitor.

3.4. Photolysis experiments

A mixture of enzyme (2 μg ml^{-1}) and **1** (20 μM) in phosphate buffer pH 6.5 was irradiated for 5 or 10 min with a 250 W tungsten lamp in an ice bath. GSH (0.2 mM) and CdNB (0.2 mM) were added (total volume = 1 ml) and kinetic measurement performed at 340 nm. Concurrently, **1** (20 μM) was photolysed alone for 5 or 10 min, then the two substrates and the enzyme were added (total volume = 1 ml) and kinetic measurements carried out.

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