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Inhibition of Succinate-cytochrome C Reductase by a Ferromacrocyclic Complex*

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Succinate-cytochrome c reductase (SCR) from mouse liver was inhibited strongly and reversibly by an iron (II) macrocyclic complex 3. The inhibition was observed for the enzyme toward the reduction of both 2,6-dichlorophenol indophenol (DCIP) and cytochrome c (cyt c). The inhibition was a mixed type and noncompetitive with respect to the reduction of DCIP and cyt c, respectively. Values of the inhibition constant ranged from 6.6 to 8.3 μM. The IC_{50} for the complex 3 was found to be 16.6 ± 0.8 and 12.1 ± 0.5 μM for the enzyme toward DCIP and cyt c, respectively. The reduced form of complex 3 also exhibited enzyme inhibition but to a less extent. Complex 3, at a lower level, equal to 25% of its LD_{50} showed about 50% inhibition of the enzyme through *in vivo* dose-dependent effect. These findings suggested that the structure of the equatorial benzoquinoid macrocyclic ligand of the Fe(II) complex is involved in the enzyme inhibition.

Keywords: Reductase; Inhibition; Kinetic parameters; Macrocycles

Abbreviations: SCR, succinate-cytochrome c reductase; DCIP, 2,6-dichlorophenol indophenol; cyt c, cytochrome c

INTRODUCTION

Oxidation of succinate involves succinate dehydrogenase and different types of cytochromes including cytochrome c (cyt c).[1] Succinate-cyt c reductase complex (SCR) is a part of the mitochondrial electron transport system, which catalyses electron transfer from succinate to cyt c.[2] The enzyme is composed of succinate-ubiquinone reductase (SQR) and ubiquinol-cyt c reductase (QCR).[3] It is fractionated into succinate dehydrogenase, ubiquinone-binding protein and QCR.[4] It has been reported previously that the SCR preparation catalyses the reduction of cyt c, 2,6-dichlorophenol indophenol (DCIP) and phenazine methosulfate under appropriate conditions.[2] Moreover, reduction of DCIP by succinate, which is catalyzed by SQR in the absence of exogenous ubiquinone was found

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to be highly dependent upon DCIP concentration.[4]

Macrocyclic ligands are a class of organic molecules closely related to porphyrins and phthalocyanines. Remarkably, macrocycles have some fascinating features such as stabilization of unusual oxidation of their metal complexes,[5–10] corrosion inhibition[11,12] and chelation therapy.[13] The biological importance of these compounds has also been reported. For example, antibiotic macrocycles can act as ionophores of monovalent cations and are also involved in the catalytic oxidation of NADH and ascorbic acid by oxygen.[14,15] Several biological aspects have also been reported for these systems such as electron transfer within proteins and nucleic acids,[16] superoxide-scavenging activity,[17] selective recognition of consecutive G sequence in double-stranded DNA[18] and as bifunctional chelators for radiotherapeutic studies.[19] The present study was designed to explore the similarities and differences between various Fe (II) macrocyclic complexes which mimic the heme group, in order to determine the features of the macrocyclic ligands that are related to the biologically important porphyrin system.

MATERIALS AND METHODS

Chemicals and Reagents

Macrocyclic complexes (**1–4**) were prepared and characterised as described previously.[10] The cobalt complex (**5**) was prepared and characterised as reported previously[6] as was the copper complex (**6**).[5] All the investigated complexes contain quadridentate tetraazamacrocyclic ligands and were chosen to show variations in the type and oxidation state of the central metal ion, nature of the in-plane macrocyclic ligand and the type of axial ligation. The investigated complexes are shown in Fig. 1. Complexes **1–4** are iron (II) macrocyclic complexes. In these complexes, Fe (II) as a central metal ion is coordinated to the macrocyclic ligand in the equatorial plane and the two methyl imidazole or pyridine groups are axially coordinated to the central metal ion. Complex **5** is a cobalt (III) macrocyclic complex in which the cobalt ion is axially coordinated to two chloride ions. The cobalt atom of this complex lies in the N_4 donor plane atom of the macrocycle and is coordinated to two amino and two imine nitrogen atoms. Alternatively, complex **6** is a copper (II) macrocyclic complex in which the copper atom is coordinated to four imine nitrogen atoms. The complexes were dissolved either in absolute ethanol or in deionised water for determination of their absorption spectra.

Crystalline oxidized horse cyt c type III was purchased from Sigma, St. Louis, MO, U.S.A. The concentrations of cyt c were determined spectrophotometrically using the millimolar extinction coefficient 18.5 at 550 nm (difference spectrum of reduced minus oxidized forms).[20] DCIP was obtained from (BDH, Alexandria, Egypt). Other chemicals of the highest purity available were obtained commercially.

Animals and Treatments

Swiss albino mice were obtained from the animal house of the Medical Research Institute, Alexandria University. The animals were 8-weeks old with an approximate body weight of 20 g, and were housed in wire cages in groups of 2–3 mice per cage. They were kept healthy under conventional conditions of temperature, humidity and were subjected to a 12 h photoperiod. The mice were supplied with a diet consisting principally of whole milk and bread with minerals and vitamins added from time to time. Food and water were provided *ad lib.*

A group of mice were given different oral doses of the macrocyclic complexes (10–2000 μg)

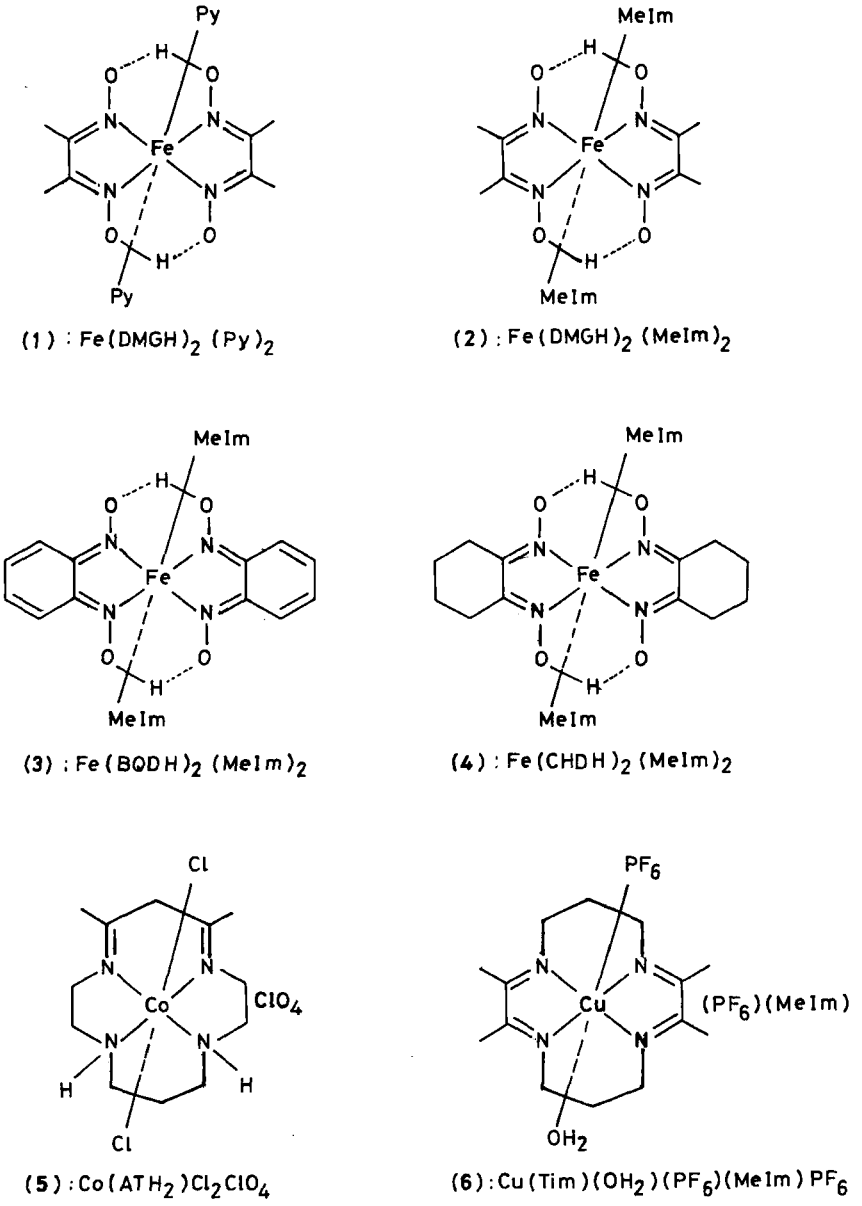

FIGURE 1 Macrocyclic complexes (1–6) used in the present study. DMGH, dimethylglyoximato; py, pyridine; MeIm, *N*-methylimidazol; BQDH, benzo-quinonedioximato; CHDH, cyclohexane-dioximato.

dissolved in the least amount of absolute ethanol. All dose administration occurred for 24 h and was repeated again over the same period. Triplicate treatment was done for each dose. Control mice were given only the same volume of ethanol.

Calculation of the LD_{50} of Complexes

Groups of mice (6–10) were individually caged, given various doses of each complex (10–2000 μg) as mentioned above and observed for mortality. The tested compounds were

administered at chosen dose levels sufficient to achieve test groups with sufficient mortality rates to permit calculation of the LD_{50}.

Isolation of SCR

This was done as described previously.[21] Briefly, mice were sacrificed by deep anaesthesia and the liver was removed, minced and washed in distilled water. Efficient stirring was carried out for 20 min and usually six washings gave the resulting mince as a light yellow, but not pink preparation. The mince was then used either directly or further mixed with 0.1 M potassium phosphate buffer, pH 7.4, stirred and finally washed with deionised water. The mince thus obtained was ground in a mortar at 4°C with 0.02 M potassium phosphate buffer, pH 7.4 containing 0.5% sodium cholate and 1 mM EDTA. The mixture was diluted with the same buffer, centrifuged at 800*g* for 30 min and the turbid supernatant fluid cooled to approximately 2°C by addition of crushed ice and brought to pH 5.5 with 1 M acetic acid. The mixture was then centrifuged at 4000–5000*g* for 15 min. The clear supernatant fraction was discarded and the precipitate was washed with 0.01 M KH_2PO_4 at 4°C and then again centrifuged at 4000–5000*g* for 15 min. The residue was finally suspended in borate–phosphate buffer, pH 7.4 containing 0.5% sodium cholate, using a Potter homogenizer and then centrifuged for 30 min at 4000–5000*g*. The enzyme was then prepared from the supernatant according to previously reported methods.[4]

Kinetic Assay of SCR

Assay of SCR in catalysing the oxidation by cyt c was determined as described previously.[22] The reaction mixture (3 ml) contained 20 mM potassium succinate, 5.0–50 μM oxidized cyt c unless indicated otherwise, 1 mM sodium azide, and 0.2 mM EDTA. All enzyme assays were carried out at 25 ± 0.1°C in 0.1 M KH_2PO_4 buffer, pH 7.4 in cells of 10 mm pathlength at wavelength 550 nm. The increase in absorbance (ΔA/s) was measured in a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan). The reaction was started by the addition of cyt c[23] and the appropriate enzyme source concentrations were chosen to give initial rates for the first 1–2 min of the reaction time.

For the assay of the reductase in catalysing the oxidation of succinate by DCIP, the decrease of absorbance at 600 nm (ΔA/s) was estimated as described previously.[23] The reaction mixture is the same as that described above for cyt c except that 5.0–50 μM DCIP was used instead of cyt c, unless indicated otherwise. For *in vitro* studies, all activities were followed in the presence or absence of macrocyclic complexes at a concentration of 0–50 μM, unless indicated otherwise.

The maximum velocity was expressed as μmoles/min/mg protein of SCR in catalysing the reduction of both cyt c and DCIP. ΔA/s was divided by the mmolar extinction of cyt c and DCIP, respectively. All assays were performed in triplicate and average values were calculated.

Determination of Kinetic Parameters

Initial velocities as ΔA/s were measured from the time scanning of the reactions either at 550 nm for cyt c or at 600 nm for DCIP. Lineweaver–Burk plots[24] were used to determine the kinetic parameters of SCR. K_i value was determined by both Lineweaver–Burk and Dixon plots.[25]

Protein Assay

Protein was assayed in the homogenates and enzyme preparations as described previously using bovine serum albumin as standard.[26]

RESULTS

In Vitro Inhibition of SCR by Complex 3

All reactions of SCR in the presence of complex **3** obeyed Michaelis–Menten kinetics using the assay method described. As observed in Table I, the reduction of DCIP is completely inhibited by complex **3** at 50 μM for the SCR catalysed oxidation of succinate in its presence. When V_{max} of the enzyme was measured using cyt c instead of DCIP, only 19.3% of V_{max} remained as a result of the inhibition by 50 μM of complex **3**, i.e. 80.7% inhibition (Table I). The IC_{50} values (concentration inhibiting V_{max} by 50%) of complex **3** calculated from the data in Table I were 16.6 ± 0.8 and 21.1 ± 0.5 μM with DCIP and cyt c, respectively. Furthermore, preincubation of the enzyme with complex **3** is not necessary for inhibition of SCR activity. Thus, the inhibition of the enzyme is dependent on the presence of complex **3** in the reaction.

TABLE I Effect of different concentrations of complex **3** (0–50 μM) on the V_{max} of the *in vitro* treated SCR in catalysing the oxidation of succinate by DCIP and cyt c

[Complex **3**] (μM)	V_{max}%	
	DCIP	cyt c
0	100.0	100.0
5	80.3	85.0
10	60.7	74.0
20	44.4	53.2
50	00.0	19.3

V_{max} was determined at each concentration of complex **3** as described under "Materials and Methods" without preincubation and V_{max}% was calculated.

TABLE II Effect of dialysis on the *in vitro* inhibition of SCR by complex **3** in catalysing the oxidation of succinate by DCIP

[Complex **3**] (μM)	Relative V_{max} (V_{max}% of control)	
	Before dialysis	After dialysis
5	66.4	108
20	22.4	106
50	50.0	90

SCR (6.65 μg/ml) was mixed with complex **3** in 2 ml of borate phosphate buffer, pH 7.4 for 60 min at room temperature. Enzymatic activities of the mixture were measured before and after the dialysis against 1 l of the buffer, at 4°C for 16 h with three changes.

Dissociation of Complex 3 from the *In Vitro*-treated Enzyme

In order to examine whether or not the inhibition of intact SCR was reversible, the enzyme preincubated at room temperature with complex **3** (5, 20 and 50 μM) for 60 min was dialysed at 4°C for 16 h. Dialysis resulted in recovery of full enzyme activity (Table II) and indicated reversible inhibition of the enzyme by **3**. Increasing the time of incubation to 12 h followed by dialysis gave a similar result.

Inhibition Type

On studying the Lineweaver–Burk plot for the intact enzyme towards DCIP inhibition by increasing concentrations of **3** (0–20 μM), a pattern characteristic of a mixed-type inhibition was obtained. A K_i value of 7.6×10^{-6} M was observed indicating that complex **3** was a very effective inhibitor. The kinetic constants were calculated from a double reciprocal plot. The K_m value for DCIP was found to be 48 μM, while the value of V_{max} was 0.12 μmoles/min/mg protein in the control. At 20 μM concentration of **3**, the V_{max} value decreased to 0.056 μmoles/min/mg protein, which is about 46% of that of the control. The apparent K_i value for **3** for the enzyme towards DCIP was 7.3×10^{-6} M calculated from the Dixon plot, which is nearly equal to that obtained from the reciprocal plots.

The reduction of DCIP by intact SCR was also investigated at various concentrations of succinate and increasing concentrations of **3** (0–20 μM). The Lineweaver–Burk plot was characteristic of noncompetitive inhibition and gave a K_i value of 6.6×10^{-6} M. Lineweaver–Burk plot for the inhibition of SCR mediated cyt c reduction by **3** showed a noncompetitive pattern. The observed K_i was 8.3×10^{-6} M, which is

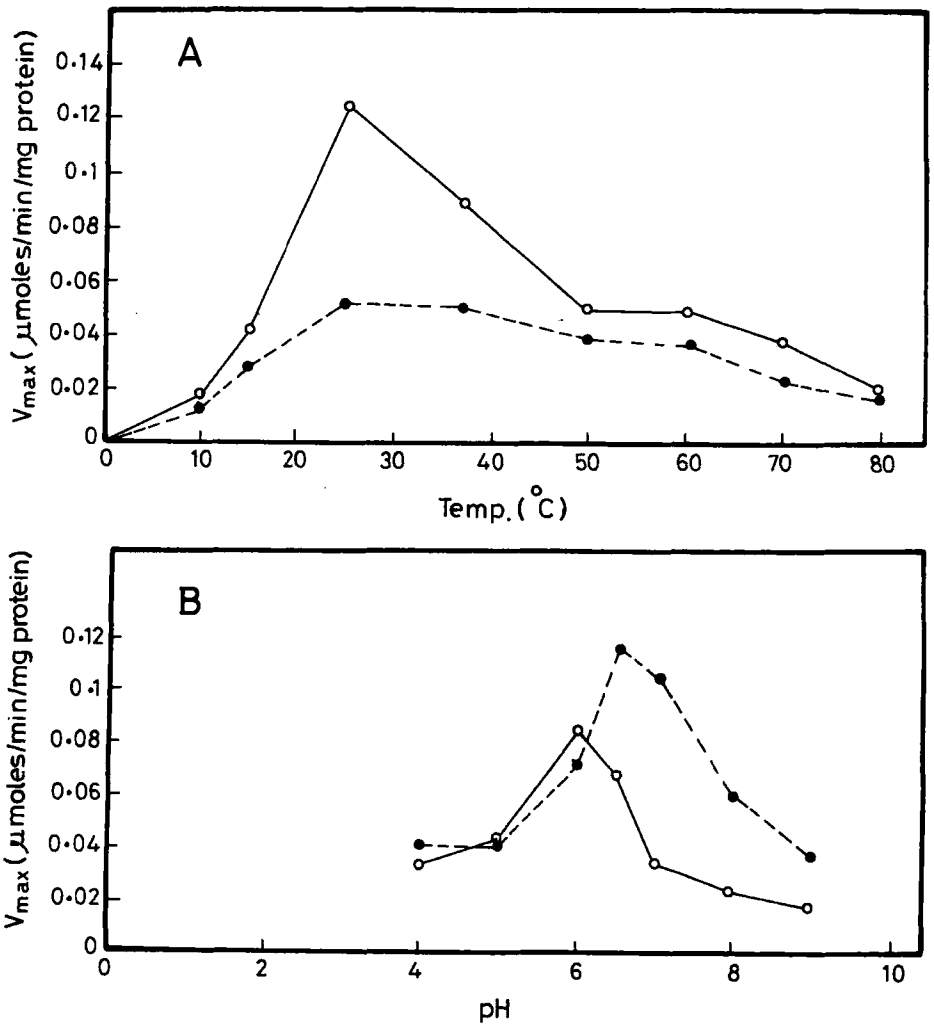

FIGURE 2 Heat stability and pH-dependence of SCR. (A) SCR was heated at different temperatures (10–80°C) for 10 min and V_{max} was determined under standard conditions as described for other data in absence (○) or presence (●) of complex **3**. (B) Effect of pH on V_{max} of SCR in absence (○) or presence (●) of complex **3**. Values are means of $n = 6$. The reaction mixtures contained 5.6 µg/ml of SCR, 50 µM DCIP and 20 mM of potassium succinate in 0.1 M potassium phosphate buffer. Concentration of complex **3** was 20 µM.

slightly higher than that obtained for the inhibition of the enzyme towards DCIP and succinate. However, the V_{max} value of the enzyme towards cyt c in controls was 0.34 µmoles/min/mg protein. Thus, about 81% of V_{max} was seen (Table I), while in the presence of 50 µM of complex **3**, it decreased to 0.06 µmoles/min/mg protein.

Effect of Temperature and pH

The variation of intact SCR activity catalysing the reduction of DCIP over the temperature range 10–80°C, in the presence and absence of **3** was investigated. SCR was incubated at the given temperatures and then assayed as mentioned under "Materials and Methods". The optimum

temperature in the absence and presence of complex **3** was 25°C, but in its presence, a broad optimum peak was obtained (25–37°C). Above 37°C no temperature-dependence was exhibited (Fig. 2, panel A). A similar study of the enzyme over the pH range (4–9) was made in absence and presence of complex **3**. The general bell-shape of the curve was the same for the two cases, but the reduction of DCIP was maximal at pH 6.0 and 6.5 in the absence and presence of the complex, respectively (Fig. 2, panel B).

In Vivo Effect of Complex 3

The LD_{50} of complex **3** for mice was determined as 80 μg/g body weight/48 h. Complex **3** treatment occurred in a range of doses from 5 μg/g body weight/48 h (less than 10% of LD_{50}) up to the value of LD_{50}. As shown in Table III, this complex has a clear inhibitory effect on the enzyme in a dose-dependent manner. The V_{max} value of the enzyme towards DCIP or cyt c at 25°C showed about 50% of its control value at a dose of 20 μg/g body weight/48 h, which is about 25% of the LD_{50}. At a dose equal to the LD_{50}, V_{max} was equal to 5.79; 26.58% for the enzyme towards DCIP and cyt c, respectively (Table III). At increasing doses of **3**, the K_m value of the enzyme towards DCIP decreased gradually from 15 to 1 μM whereas only slight variations in K_m were observed towards cyt c (Table III).

Comparable Kinetic Parameters of SCR from *In Vitro* and *In Vivo* Studies in the Presence of Various Macrocyclic Complexes

The effect of *in vitro* treatment of the complexes at 50 μM on the relative V_{max} of the enzyme towards DCIP (V_{max} in presence of the complex compared to control) is shown in Table IV. Relative V_{max} values for complexes **1** and **2** are near to that of the control whereas in the presence of **3** complete inhibition takes place as the V_{max} values reaches zero. However, in the presence of the cobalt complex (**5**) the enzyme exhibited a sigmoidal relationship and did not obey Michaelis–Menten kinetics. Overall, the value of K_m did not have a large variation like the value of V_{max}, and ranged from 28 ± 5 to 34 ± 7 μM (Table IV). On the other hand, *in vivo* treatments of SCR at a dose of 20 μg/g body weight/48 h (25% of LD_{50}) showed that **3** is the most potent inhibitor in the series (Table IV). Complexes **1** and **2** had no effect while **4** and **6** had only a slight inhibitory effect. The relative V_{max} towards DCIP was 80.7 and 96.3 for the inhibition by **4** and **6**, respectively (Table IV). Treatment with **5** showed a sigmoidal relationship in the plot of DCIP or cyt c versus the initial velocity of the enzyme (data not shown).

TABLE III *In vivo* effect of complex **3** on SCR

Complex **3** dose (μg/g body weight/48 h)	DCIP		Cyt c	
	Relative V_{max} (V_{max}% of control)	K_m (μM)	Relative V_{max} (V_{max}% of control)	K_m (μM)
0	100	15	100	17
5	86.36	14	90	15
10	72.72	14	77	15
20	52.23	13	50	10
40	38.46	6	41.6	10
60	18.34	4	31.9	15
80	5.79	1	26.58	15

The effect of complex **3** on SCR in catalysing the oxidation of succinate by DCIP or cytochrome was determined as stated under "Materials and Methods" and the kinetic parameters were measured.

TABLE IV Effect of various macrocyclic complexes on intact SCR

Macrocyclic Complex	In vitro		In vivo	
	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)
None	100.0	32±7*	100.0	19±6*
1	100.0	31±4	100.0	10±5
2	96.4	32±6	103.0	10±4
3	00.0	nd†	52.2	16±6
4‡	35.6	28±5	80.7	20±3
5	nd†	nd†	nd†	nd†
6	51.6	34±7	96.3	23±5

*Mean ± S.E, $n = 9$.
†Not determined.
‡Non-competitive inhibitor with $K_i = 10\,\mu M$.
The kinetic parameters of both *in vitro* and *in vivo*-treated SCR, in catalysing the oxidation of succinate by DCIP, were determined. The *in vitro*-treatments occurred in an assay mixture contained 50 μM of each complex as noted under "Materials and methods". *In vivo*-treatments were examined at a dose of 20 μg/g body weight/48 h as explained in the legend of Table III. Kinetic parameters were measured at 25°C and pH 7.0. Relative V_{max} is V_{max}% of control.

DISCUSSION

The $Fe(DMGH)_2(Py)_2$ and $Fe(DMGH)_2(MeIm)_2$ complexes **1** and **2** contain the same central metal ion Fe^{2+} and the equatorial dimethylglyoximate $(DMGH)_2$ macrocyclic ligand. However, these two complexes contain different axial ligands; complex **1** contains pyridine (Py) while complex **2** contains *N*-methylimidazole (MeIm). $Fe(BQDH)_2(MeIm)_2$ and $Fe(CHDH)_2(MeIm)_2$ (complexes **3** and **4**) contain the same central metal ions as complexes **1** and **2** and the same MeIm axial ligands as complex **2**, but have different macrocyclic equatorial ligands. In complex **3**, there is an unsaturated benzoquinone-dioximato $(BQDH)_2$, while in complex **4** there is a cyclohexane-dioximato $(CHDH)_2$ ring, which is a fully reduced analogue of the benzoquinone-dioximato ring (Fig. 1).

Interestingly, **3** completely inhibited the reduction of DCIP and most of the reduction of cyt c (Table I) *in vitro* of the enzyme-catalysed oxidation of succinate, while **1** and **2** had no effect on the reduction of DCIP and cyt c. Complexes **4** and **6** gave 35.6 and 51.6% of the V_{max} value for the enzyme, respectively (Table IV). This indicates that the reduction of DCIP is inhibited by the very stable benzoquinoid structure of the macrocyclic complex **3**. However, the different values of K_i for the inhibitor complex **3** are 7.6×10^{-6}, 6.57×10^{-6} and 8.25×10^{-6} M indicate that it is a potent inhibitor compared to malonate, ($K_i = 5 \times 10^{-6}$ M).[23] Also, the complex acts as a mixed type inhibitor for the reduction of DCIP (Fig. 2), indicating that it binds to some site other than the substrate binding site. This was confirmed by the noncompetitive pattern that was observed in the reduction of cyt c by the enzyme.

The effect of the inhibitor complex **3** and other complexes is reversible since the complex easily dissociates from the enzyme and enzyme activity is completely recovered after dialysis. Therefore, the formation of the enzyme–inhibitor complex is readily reversible.[27] However, some inhibitors have been reported to dissociate from enzyme–inhibitor complexes very slowly, i.e. as trehazolin with trehalase,[28] castanospermine with sucrase[29] and swainsonine with αmannosidase.[30]

In order to show whether SCR inhibition by complex **3** results in a possible change in its heat stability, a study of the effect of temperature was investigated. At high temperatures (50–80°C), the tested enzyme velocities followed the same pattern in the absence and presence of complex **3**

(Fig. 2, panel A). So it seems likely that SCR inhibition by this macrocycle does not induce a change in its heat stability. On the other hand, the change of the pH-rate profile of the enzyme during inhibition by complex **3** (Fig. 2, panel B) implies that it is possible that protonation of the complex may occur and in addition, ionisable group(s) of some polar amino acid residues near the active site on SCR might influence the inhibition.

The inhibition of the reduction of cyt c by complex **3** can be explained by the delocalisation of the equatorial $(BQDH)_2$ ligand of the complex which would result in mixing of the metal t_2g orbitals with the π^* orbitals of the benzoquinone ligands. In fact, $(BQDH)_2$ is a ligand with a delocalised conjugated π-system. This delocalisation would result in lowering of the π^* level of the dioxime moiety as a result of conjugation with the hexadiene group.[9,10] This would facilitate the electron transfer to the $(BQDH)_2$ ring and explains the behaviour of complex **3** as being the best electron acceptor among the other complexes in the series. This is consistent with the electrochemistry and kinetic results reported previously by Kalifa *et al.*[8,9] Dolphin *et al.*[31] also suggested that π-cation radical ligand states mediate electron transfer reaction of some cytochromes.

$$Fe^{II}\ \text{cytochrome} \rightleftarrows (Fe^{II}\ \text{cytochrome})^{+} \rightleftarrows (Fe^{III}\ \text{cytochrome})^{+} \quad (1)$$

However, the cytochrome $b-c_1$ particle contains less than 1 μmole of coenzyme Q per g protein[22] so that, in the present study, the reduction of the dye DCIP was less affected by coenzyme Q and complex **3** acts clearly as an electron carrier owing to the high delocalisation of the suggested π-system of the benzoquinoid structure.

In **5**, the Co^{3+} ion is surrounded by a macrocylic ligand in the equatorial plane containing a β-diiminato linkage that is a structural element similar to the linkage between the pyrrole rings of porphyrins. This system provides us here with a simple class of molecule with two sites, ligand or metal, for electron transfer.[7] The preferred site may be manipulated by protonating or deprotonating the ligand or by changing the metal. In these $Co^{3+/2+}$ couples, the donor and acceptor orbitals have σ^* symmetry, being thereby shielded from good spacial overlap by ligands of the 6-co-ordinate complexes. Furthermore, the reactions are often complicated by changes in spin multiplicity. As a consequence, some features of the reactivity patterns of such system have been attributed to variations in an electronic factor. This factor is assumed to be unitary in the usual Franck–Condon analyses.[32,33] Therefore, it could be suggested that these electronic factor variations result in the sigmoid nature of the enzyme activity curves, which did not obey Michaelis–Menten kinetics (Table IV).

The copper (II) macrocyclic complex (**6**) inhibited the enzyme *in vitro* (Table IV). This complex is easily reduced to Cu (I) and even to Cu (0) as reported previously.[5] Therefore, it could be suggested that the inhibitory effect of reduction of Cu (II) complex rather than DCIP or cyt c, is attributable to the ease by which this complex is reduced to form Cu (I) complex.

The kinetic assay of SCR were done *in vitro* on macrocycle-treated and vehicle-treated enzyme as well as *in vivo*. The inhibition of the enzyme by complex **3** was clearly detected in both studies (Table IV). Accordingly, we suggest that there may be a direct interaction between complex **3** and SCR. Also, *in vivo* inhibition supports our explanation that complex **3** may act as an electron carrier that interferes with the role of SCR.

To clarify other mechanisms of inhibition, subsequent work should aim at the inhibition of the separated SCR to study both the SQR and the QCR. Study of the conformation of each enzyme after inhibition would be of interest. In conclusion, the macrocyclic complexes through either the effect of the equatorial ligands

and/or the central metal ion, inhibit SCR for electron transfer.

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