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Analysis of the binding of *p*-chlorophenyl-methoxybenzyl-ketoxime (CPMB-Oxime) to mitochondrial cytochrome *c* reductase

Ulrich Brandt and Gebhard von Jagow

Universität Frankfurt, Zentrum der Biologischen Chemie, Theodor-Stern-Kai 7, Haus 25B, D-6000 Frankfurt am Main 70, Germany

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Cytochrome *c* reductase is inhibited by *p*-chlorophenyl-methoxybenzyl-ketoxime (CPMB-oxime). CPMB-oxime induces a red-shift of the reduced spectrum of cytochrome *b*. The inhibitor blocks the oxidation of ubihydroquinone at the Q_P center of this enzyme in a non-competitive way. The binding stoichiometry equals one inhibitor molecule per Q_P center. The apparent K_d in a red-shift assay was 6.9 ± 0.6 μM. All binding characteristics analysed in this study were very similar to those of the E-β-methoxyacrylate inhibitors, although the chemical structure is different from these inhibitors. This result is interpreted as a support for the inhibitory mechanism based on the model of a 'catalytic switch' proposed recently for the E-β-methoxyacrylate inhibitors (MOA-inhibitors (Brandt and von Jagow, Eur. J. Biochem. (1991) 195, 163–170).

Cytochrome *c* reductase; Inhibitor; CPMB-oxime; Myxothiazol; Red-shift

1. INTRODUCTION

A large number of inhibitors has been described for mitochondrial cytochrome *c* reductase, which vary considerably in their chemical structure. Certain structural elements were found to be correlated to specific effects on the optical spectrum of reduced cytochrome *b* as well as the EPR spectrum and midpoint potential of the iron-sulfur cluster [1]. Following this line the inhibitors of the ubihydroquinone oxidation center as defined by the Q-cycle mechanism [2] can be classified into three groups: (i) the hydroxynaphthoquinone analogs (undecylhydroxynaphthoquinone, undecylhydroxybenzothiazol, etc.), which change the EPR spectrum and the redox potential of the 'Rieske' iron-sulfur cluster [3]; (ii) the chromone inhibitors (stigmatellin) which change the EPR spectrum and the redox potential of the 'Rieske' iron-sulfur cluster and induce a red-shift of the optical spectrum of the reduced cytochrome *b*; (iii) the E-β-methoxyacrylate (MOA) inhibitors (myxothiazol, strobilurin), which induce a red-shift of the reduced cytochrome *b*.

Abbreviations: CPMB-oxime, *p*-chlorophenyl-methoxybenzyl-ketoxime; MOA, E-β-methoxyacrylate; NBH, nonylubihydroquinone; Q_N center, inner (negative side of the membrane) ubiquinone reaction center of the mitochondrial cytochrome *c* reductase; Q_P center, outer (positive side of the membrane) ubiquinone reaction center of the mitochondrial cytochrome *c* reductase

Enzymes: Ubihydroquinone:cytochrome *c* oxidoreductase: EC 1.10.2.2

Correspondence address: U. Brandt, Universität Frankfurt, Zentrum der Biologischen Chemie, Theodor-Stern-Kai 7, Haus 25B, D-6000 Frankfurt am Main 70, Germany. Fax: (49) (69) 6301 6970.

Analysis of the binding characteristics of the latter class, revealed a non-competitive type of inhibition [4]. As this means that blockage of electron transfer by this type of inhibitor is not based on displacement of the substrate ubiquinol, the question of the inhibitory mechanism arises.

According to the recently proposed 'catalytic switch' the inhibitors block the ubihydroquinone oxidation at the Q_P center by stabilising one of two redox-dependent conformational states of the enzyme [5]. If this is the case, the distortion of the ligang field of haem b_{566}, which results in the observed red-shift of the spectrum, correlates to the 'b-state' conformation.

This assumption is supported by the data presented in this paper on the binding characteristics of a new inhibitor of the mitochondrial cytochrome *c* reductase, which is not related structurally to any of the inhibitors described so far, but which has binding characteristics almost identical to those of the E-β-methoxyacrylates.

2. MATERIALS AND METHODS

2.1. *Chemicals*

All inhibitors were used as ethanolic stock solutions. The concentrations were determined spectrophotometrically in ethanol using the following wavelengths: antimycin (Boehringer, Mannheim) at 336 nm ($\epsilon = 4.8\ mM^{-1} \cdot cm^{-1}$); strobilurin A (Prof. Anke, Kaiserslautern) at 292 nm ($\epsilon = 21.9\ mM^{-1} \cdot cm^{-1}$); CPMB-Oxime (Prof. Beechey, Aberysthwyth) at 250 nm ($\epsilon = 15.6\ mM^{-1} \cdot cm^{-1}$).

The substrate-analogue nonylubiquinone ($\epsilon_{red,290\ nm} = 4.2\ mM^{-1} \cdot cm^{-1}$) was synthesized as described previously [4].

All other chemicals were of the highest purity available.

2.2. *Isolation of cytochrome c reductase*

Cytochrome *c* reductase was prepared according to Schägger et al. [6] from beef heart mitochondria and stored in 55% glycerol at

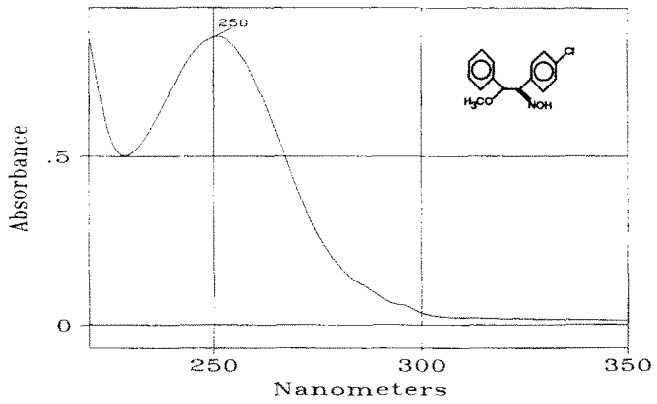

Fig. 1. Molecular structure and UV-spectrum of CPMB-oxime. The spectrum of a 50 μM solution of CPMB-oxime was recorded.

−20°C. The concentration was determined by the reduced minus oxidized haem *b* spectrum at 562–575 nm ($\epsilon = 28.5\ mM^{-1} \cdot cm^{-1}$).

2.3. *Determination of apparent IC_{50} values for mitochondrial respiration*

The apparent IC_{50} values as inhibitor concentration resulting in half-maximal O_2 consumption rate of uncoupled bovine heart mitochondria respiring on succinate were determined as described previously [4].

2.4. *Measurement of red-shifts*

Red-shift spectra were recorded in a Shimadzu UV-300 double-beam spectrophotometer as the spectral difference of the dithionite-reduced enzyme with and without a saturating amount of inhibitor.

Titration of binding was followed as the spectral shift of the dithionite-reduced enzyme at 568–558 nm in the dual-wavelength mode. Either ethanol or nonylubiquinone in the same volume of ethanol were added. Cytochrome *c* reductase and quinone were reduced by adding a few grains of sodium dithionite. The data were analysed as described previously [4].

2.5. *Measurement of cytochrome* b *and cytochrome* c_1 *reduction*

The relative extent of reduction of the cytochromes of cytochrome *c* reductase was determined under varying conditions using a stirred cuvette in a Shimadzu UV-300 double-beam spectrophotometer in the dual wavelength mode, at 562–575 nm for cytochrome *b* and at 553–540 nm for cytochrome c_1. To abolish the influence of pre-reduced cytochrome c_1 a stoichiometric amount of $K_3[Fe(CN)_6]$ was added before adding the inhibitors, dissolved in ethanol in at least 20-fold excess, and NBH in about 5- to 10-fold molar excess over the concentration of cytochrome *c* reductase.

3. RESULTS

3.1. *General properties*

The UV spectrum of CPMB-oxime in ethanol has a featureless maximum at 250 nm (Fig. 1). The extinction coefficient at this wavelength was determined as 15.6 $mM^{-1} \cdot cm^{-1}$. Besides the content of aromatic and methoxy residues, the structural formula shows no similarities to any MOA inhibitor or the substrate ubihydroquinone.

3.2. *Inhibition of electron transfer*

CPMB-oxime is a potent inhibitor of the steady state activity of cytochrome *c* reductase. The apparent IC_{50} value was 0.2 μM (data not shown), which is comparable to the weakest MOA inhibitor Oudemansin A [4]. In order to determine, whether CPMB-oxime is a Q_P or a Q_N center inhibitor, we tested its effect on the reduction of cytochromes *b* and c_1. Fig. 2 shows that neither the Q_N center inhibitor antimycin, (Fig. 2B) nor CPMB-oxime (Fig. 2C) alone slowed down cytochrome *b* reduction significantly. The extent of cytochrome *b* reduction was increased from about 50% to approximately 80% by antimycin, since equilibration via the Q_N site is blocked by this inhibitor. The extent of cytochrome *b* reduction was decreased to 30% by CPMB-oxime, which could be referred to a shift of the midpoint potential of cytochrome *b*. When both inhibitors were added prior to substrate, the rate of reduction was slowed down drastically.

Complementary results were obtained, when the kinetics of cytochrome c_1 reduction were monitored: with antimycin only the extent of fast cytochrome c_1

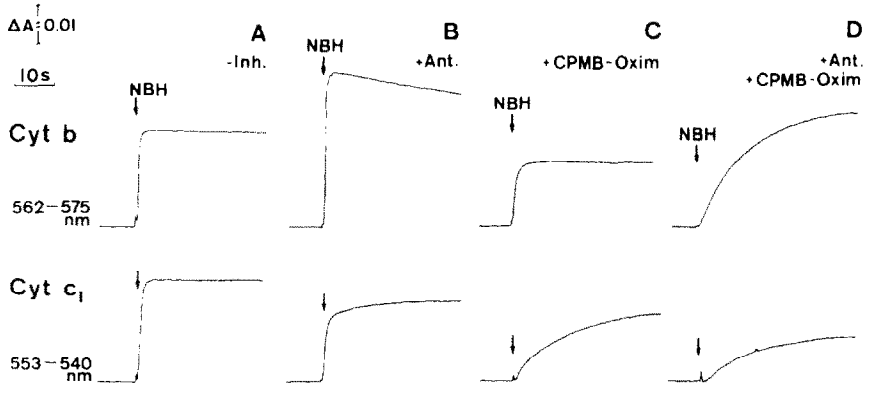

Fig. 2. Reduction kinetics of the intrinsic heme centres of cytochrome *c* reductase. The reduction of cytochrome *b* and cytochrome c_1 was determined at a reductase concentration of 1 μM in cytochrome c_1. Buffer: 20 mM K^+/MOPS, 0.05% Triton X-100, 100 mM NaCl, 2 mM NaN_3, pH 7.2. After preincubation with inhibitor, 7 μM of NBH was added. The maximal amount of reducible cytochrome was determined by adding a few grains of dithionite after the experiment. (A) no inhibitor; (B) 15 μM antimycin; (C) 37 μM CPMB-oxime; (D) 15 μM antimycin, 37 μM CPMB-oxime.

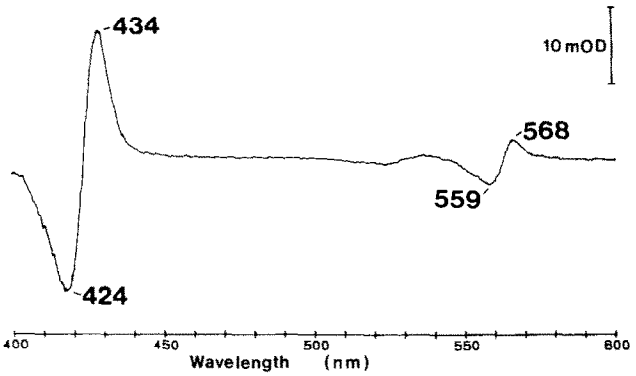

Fig. 3. Red-shift spectrum of CPMB-oxime. The spectrum was recorded at a reductase concentration of 3 μM in cytochrome c_1. Inhibitor was added to a final concentration of 70 μM. Buffer: 100 mM K^+/MOPS, 0.05% Triton X-100, 100 mM NaCl, 2 mM NaN_3, pH 7.2.

reduction decreased (Fig. 2B, [4]), but in the presence of CPMB-oxime much slower reduction-kinetics were observed (Fig. 2C,D)

Taken into account that complete inhibition cannot be obtained with such a low affinity inhibitor in this type of experiment, the results identify CPMB-oxime unequivocally as a Q_P site inhibitor, conferring an incomplete 'double kill'.

This observation [7] is known to be specific for the combined action of a Q_N and a Q_P center inhibitor and therefore demonstrated that CPMB-oxime is a Q_P center inhibitor.

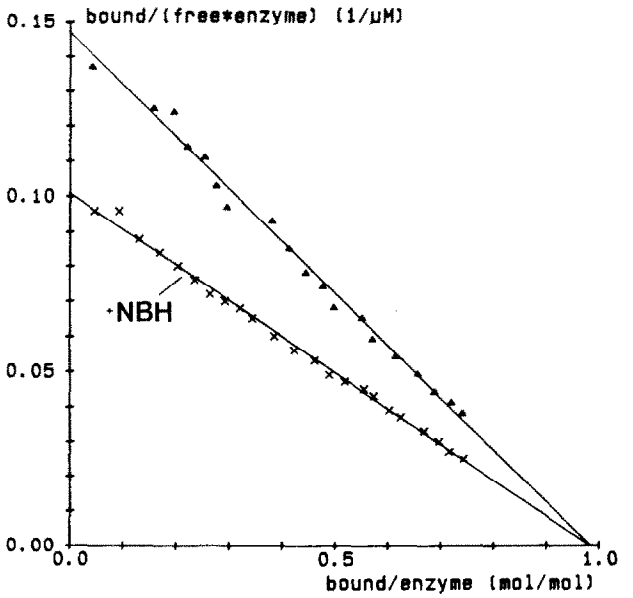

Fig. 4. Scatchard plot of red-shift tritrations. (▲—▲) no additions; (×—×), 0.8 mM NBH added. Experimental conditions and concentrations as described in Fig. 3. Enzyme refers to the monomer of cytochrome *c* reductase.

3.3. *Red-shift spectrum*

The red-shift spectrum of reduced cytochrome *b* in the presence of CPMB-oxime (Fig. 3) shows maxima at 434 nm and 568 nm and minima at 424 nm and 559 nm. These values and the overall shape of the spectrum are almost identical to those reported for MOA inhibitors [4]. The only difference was a somewhat broader trough in the α-region of the shift spectrum. The maximum-minimum absorbance difference was 4 mOD/μM in the α-region and 22 mOD/μM in the Soret-region of the spectrum. The value for the α-region is slightly lower and the value for the Soret-region is twice as high when compared to the E-β-methoxyacrylates. However, the Soret/α-ratio shows also considerable variation, when different E-β-methoxyacrylates are compared [4].

3.4. *Binding analysis*

From quantitative red-shift titrations (Fig. 4) we determined an apparent K_d of 6.9 ± 0.6 μM ($n = 6$). The binding stoichiometry was 0.99 ± 0.01 per Q_P centre in these experiments. In the presence of saturating amounts of nonylubihydroquinone the apparent K_d was raised to 8.8 ± 0.7 μM ($n = 3$), which is about 30% higher than without the substrate; i.e. the inhibitor was not displaced by the substrate, but a shift of the K_d similar to that observed for the E-β-methoxyacrylates occurred.

4. DISCUSSION

CPMB-oxime has a relatively low affinity to cytochrome *c* reductase. However, it inhibits effectively the Q_P center of mitochondrial cytochrome *c* reductase. The red-shift spectrum is almost identical to those reported for the MOA inhibitors [4]. Like these inhibitors CPMB-oxime is non-competitive to the substrate ubihydroquinone but its binding is influenced by simultaneous binding of substrate.

The analysis presented in this paper reveals that CPMB-oxime represents another type of a specific, non-competitive inhibitor of the Q_P center of the mitochondrial cytochrome *c* reductase. Although there are essentially no structural similarities, we have found binding characteristics of CPMB-oxime which are almost identical to those of the E-β-methoxyacrylates. Of particular interest is that both types of inhibitors induce nearly the same red-shift of reduced cytochrome *b*.

The observed differences in the red shift spectra are not likely to be in conflict with this conclusion, not only because similar variations occur when different E-β-methoxyacrylates are compared [4], but also because the lipidation and thereby the structural rigidity of the multiprotein complex affects the absolute and relative sizes of the Soret/ α-shift spectra of E-β-methoxyacrylate inhibitors (manuscript in preparation). Therefore, the shift amplitude should be indicative for the

'elasticity' of the conformational block, which is effected by the inhibitor molecule, and which is consequently dependent on its structure.

If the red-shift spectrum is interpreted as simply reflecting a structural distortion of the ligand-fields of the two haem *b* centres imposed by the binding of the inhibitor molecule, the conclusion would be that two inhibitors binding and interacting differently in the Q_P pocket happen to have a similar spectral effect. A more likely explanation is the inhibitory mechanism we proposed recently for the E-β-methoxyacrylates [5]: if both types of inhibitor would trap the same conformational state of a 'catalytic switch' of the Q_P center, the red-shift would be directly correlated to a structural property of the protein allowing some variability of the structure of the inhibitor molecules.

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REFERENCES

[1] Von Jagow, G. and Link, T.A. (1986) Methods Enzymol. 126, 253–271.
[2] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[3] Matsuura, K., Bowyer, J.R., Ohnishi, T. and Dutton, P.L. (1983) J. Biol. Chem. 258, 1571–1579.
[4] Brandt, U., Schägger, H. and Von Jagow, G. (1988) Eur. J. Biochem. 173, 499–506.
[5] Brandt, U. and Von Jagow, G. (1991) Eur. J. Biochem. 195, 163–170.
[6] Schägger, H., Link, T.A., Engel, W.D. and Von Jagow, G. (1986) Methods Enzymol. 126, 224–237.
[7] Von Jagow, G. and Engel, W.D. (1981) FEBS Lett. 136, 19–24.