Interaction of Carbonyl Cyanide 3-chlorophenylhydrazone with Cytochrome c Oxidase.

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Abstract. Cytochrome c oxidase binds protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) with high affinity. There are 1.46 high-affinity binding sites per cytochrome c oxidase for CCCP with dissociation constant $2.7 \cdot 10^{-7}$ mol/l. The bond between the CCCP and cytochrome c oxidase accomplishes through the group on cytochrome c oxidase with pK_a 6.64 and is based on the electrostatic interaction. Interaction of CCCP with low-affinity binding sites of cytochrome c oxidase induces the shift of the anion CCCP spectrum to UV-region. The similar effect is characteristic for CCCP interaction with protons. Lipophilic non-dissociated derivative NCH_3CCP is not binding to cytochrome c oxidase.

Key words: Cytochrome c oxidase — Carbonyl cyanide phenylhydrazone

Introduction

Carbonyl cyanide phenylhydrazones belong to the weak acids and are effective uncouplers of oxidative phosphorylation. They are commonly used in the studies of various membrane transport processes (Heytler and Prichard 1962, Hanstein 1976, Terada et al. 1981, 1988, Yagi and Hatefi 1989). There are some important differences between the effects of various ionophores in model phospholipid bilayer and in natural membrane systems containing proteins which cannot be solely explained by the differences in the above mentioned properties.

In two papers (Katre and Wilson 1977, 1978) it was shown that CCP deriva-

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tives might interact with mitochondrial proteins (cytochrome c oxidase, bc_1 complex, H^+ ATPase), but mechanisms of their interaction are still poorly understood It has been assumed by Hanstein and Hatefi (1974) that interaction of different protonophores (dinitrophenols) with mitochondrial membrane proteins is an electrostatic one and occurred between the negatively charged protonophore molecules and the positively charged groups in the proteins

Electrostatic interaction are characteristic for the cytochrome c oxidase, an enzyme of the mitochondrial respiratory chain The accessible information mostly concerns the interaction with cytochrome c, where positively charged lysine groups of the cytochrome c are binding with the negatively charged carboxyl groups of the cytochrome c oxidase (Osheroff et al 1980) This type of interaction has been assumed to be the bond between the valinomycine – K complex and cytochrome c oxidase (Steverding and Kadenbach 1989) Not much information concerns the interaction of anions with cytochrome c oxidase The problem consists in the presence of the electrostatic barrier for anions because the total charge of the cytochrome c oxidase is negative at a neutral pH

This paper reports on the interaction of the two derivatives, CCCP and NCH_3CCP, with the cytochrome c oxidase We point out that CCCP (at concentrations usual for the study of its transporting properties) is bound to this enzyme and electrostatic interactions between positively charged groups of cytochrome c oxidase and negatively charged CCCP occur in this process

Abbreviations CCCP, carbonyl cyanide 3-chlorophenylhydrazone, CCP, carbonyl cyanide phenylhydrazone, NCH_3CCP, 4 chlorophenyl-N methylhydrazonopropanedinitril, FCCP, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone, HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, MES, 2-(N-morpholino)ethane sulphonic acid

Materials and Methods

The bovine mitochondrial cytochrome c oxidase (obtained from Dr Musatov) was prepared according to Yonetani (1960) and stored at the temperature of liquid nitrogen in 0 1% Tween 80 at the pH 7 4 NCH_3CCP was prepared as described previously (Antalik et al 1988) CCCP, HEPES, MES and Tween 80 were obtained from Sigma and laurylmaltozid from Calbiochem

The binding of CCCP and NCH3CCP to cytochrome c oxidase was studied as follows 0 1–0 2 mmol/l stock solution of cytochrome c oxidase was dissolved in the mixture of 0 1% Tween 80 and 50 mmol/l HEPES, pH 7 4 The mixture was added to buffers of different pH values containing known concentrations of CCCP and NCH_3CCP In about five minutes, the release of the detergent bound to the cytochrome c oxidase caused the aggregation of the cytochrome c oxidase and, subsequently, the binding of the studied ligands The concentration of free ligands was obtained spectrophotometrically after the centrifugation of this aggregate cytochrome c oxidase with the binding ligand (7 min, 3000

x g). The concentration of cytochrome c oxidase was determined by using the differential extinction coefficient for reduced minus oxidized oxidase $\Delta\varepsilon = 25.3$ mmol^{-1}.l. cm^{-1} at 605 nm (Vanneste 1966).

The spectral differences were performed by spectrophotometer Shimadzu UV 3000. First, the spectrum of CCCP in buffers of different pH and containing 0.1% laurylmaltozid were registered and stored into the memory of the spectrophotometer. Then, cytochrome c oxidase was added to the sample and reference cuvettes and after 1 minute the difference spectrum was recorded. At the end of the first minute after mixing the first fast change of the CCCP spectrum was observed. The next very slow changes were not studied. If the influence of the pH on the spectrum of anion CCCP should be observed, the spectrum of anion CCCP at pH 7.4 had to be taken into the spectrophotometer memory and the difference spectra caused by the change of pH were recorded. The pH was decreased by small additions of concentrated HCl (2 μl). All the measurements were performed at the 25 °C.

Associative curve of CCCP binding on cytochrome c oxidase was fitted as a linear product of the two dissociative curves (dissociation of the CCCP and the cytochrome c oxidase) by the program FUMILI from the CERN LIBRARY. The other experimental conditions are described in the legends to the figures.

Results

Addition of concentrated cytochrome c oxidase solution to the water medium without detergents induces aggregation of the enzyme and causes turbidity of the medium. When CCCP is present, its binding to cytochrome c oxidase results in forming a complex with cytochrome c oxidase. After centrifugation at 3000 × g (30 minutes, 20 °C) it is possible to determine the concentration of the free CCCP in the supernatant. The difference between free and total CCCP concentration corresponds to protonophore bound to enzyme. Fig. 1 presents the Scatchard plot dependence of CCCP binding to cytochrome c oxidase. From the non-linear dependence, the existence of binding sites with different affinities can be deduced. 1.46 binding sites per cytochrome aa_3 were found for the high-affinity binding with a dis-

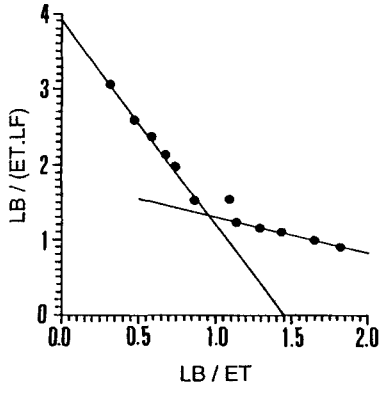

Figure 1. Scatchard plot of the binding of the CCCP to cytochrome c oxidase determined by centrifugation. LB – CCCP bound, LF – CCCP free, ET – cytochrome c oxidase total; 50 mmol/l HEPES, pH 7.4, 0.002% Tween 80, 5 mmol/l $MgCl_2$.

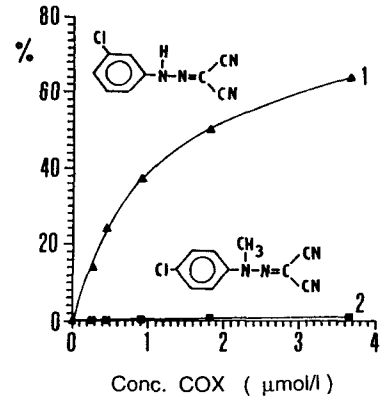

Figure 2. The relation between the amount of bound CCP and concentration of cytochrome c oxidase. (1) $2.4 \cdot 10^{-6}$ mol/l CCCP (100%), (2) $2.4 \cdot 10^{-6}$ mol/l NCH_3CCP (100%); 50 mmol/lHEPES, pH 7.4, 0.002% Tween 80.

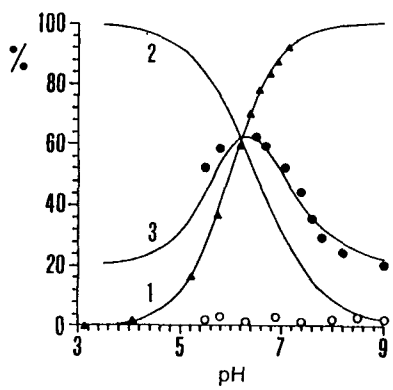

Fig. 3. The dependence of the amounts of bound CCCP and NCH_3CCP (full and empty circles) on pH. (1) the dissociative curve of the CCCP; (2) the calculated associative curve of the group which interacts with CCCP; (3) curve used for the simulation of the theoretical course of interaction of CCCP and cytochrome c oxidase calculated by equation $(x^a/x^a + pK_1)(pK_2/x^c + pK_2)e + f$, where pK_1 is pK of the CCCP, pK_2 is pK of the group of the cytochrome c oxidase, a and c are the slopes of the increasing and decreasing curves, e and f are coefficients. Values calculated for this curve are: $pK_1 = 5.98$, correction factor (c.f.) = 1.2526, $pK_2 = 6.64$, c.f. = 3.3, $a = 12.796$, c.f. = 1.2526, $c = 14.088$, c.f. = 1.6, $e = 100$, c.f. = not given, $f = 22.7$, c.f. = 3.4; 50 mmol/l HEPES, 50 mmol/l MES, 0.1% laurylmaltozid.

sociation constant $K_D = 2.7 \cdot 10^{-7}$ mol/l. Additional binding of the protonophore to other sites on cytochrome c oxidase takes place at higher CCCP concentrations. The dependence of the amounts of CCCP (dissociated derivative) and NCH_3CCP (non-dissociated derivative) bound when increasing the total concentration of enzyme are given in Fig. 2. At pH 7.4 increase in concentration of cytochrome c oxidase caused an increase of the amount of CCCP bound to cytochrome c oxidase and no binding of non-dissociating NCH_3CCP was observed. From Fig. 2 it is evident, considering the fact that lipophilicity of NCH3CCP (log $P_{7.4} = 2.62$) is higher than that of CCCP (log $P_{7.4} = 2.06$), that interactions of CCCP with cytochrome c oxidase are probably of electrostatic nature.

Experiments to find the dependence of CCCP binding to cytochrome c oxidase

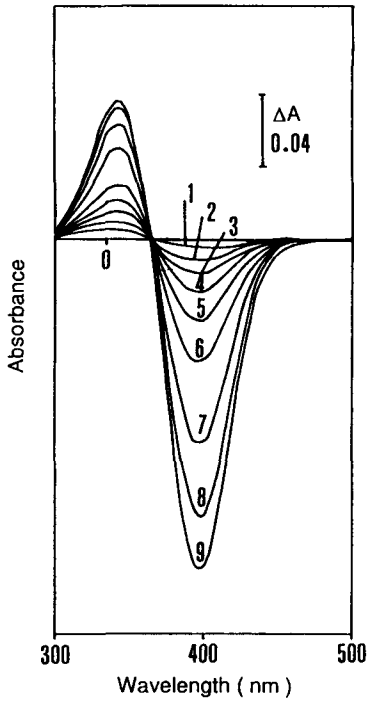

Figure 4. Difference spectra of CCCP after addition of H^+ ions. CCCP concentration $1.2 \cdot 10^{-5}$ mol/l, 2.5 mmol/l phosphate buffer; (0) pH 7.4, spectrum stored into the memory of the spectrophotometer; (1) pH 7.35; (2) pH 7.13; (3) pH 6.98; (4) pH 6.7; (5) pH 6.6; (6) pH 6.2; (7) pH 5.7; (8) pH 5.4; (9) pH 4.3 and pH 3.3.

on increasing pH values were undertaken to characterize the groups of cytochrome c oxidase which are participating in the interaction. From Fig. 3 it follows that by decreasing pH from 9 to 6 the amount of bound CCCP to cytochrome c oxidase increases. However, below pH 6 binding of the protonophore tends to decrease. The comparison of this dependence with the dissociation curve of CCCP (see Fig. 3) allows to assume that this decrease is caused by decreased concentration of dissociated CCCP. From the comparison between the pH dependence of the dissociation curve of CCCP and the quantity of the CCCP bound we can suggest that CCCP interacts with a protonated charged group of cytochrome c oxidase with *p*K of about 6.64. This is very close to a value found for histidine residues. The small binding of the non-dissociating derivative (NCH_3CCP) to cytochrome c oxidase in the pH range studied refers to the electrostatic origin of the interaction between them. Since there is a relatively high value of binding CCCP at pH higher than pH 9, the lipophilicity of CCCP takes place in the interaction, too.

Fig. 4 shows an effect of the pH on the absorption spectra of CCCP. The shift of the spectrum to the shorter wave-lengths is observed on acidification. The presence of cytochrome c oxidase dissolved in 0.1% laurylmaltozide (at pH 7.4) brought about similar spectral changes of CCCP (Fig. 5). We suppose that the low-affinity binding sites of the CCCP on cytochrome c oxidase can be characterized by these changes. Despite the existence of several binding sites on cytochrome c oxidase,

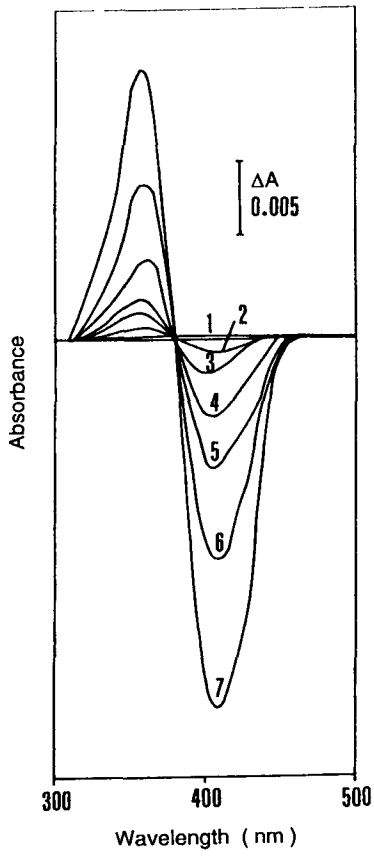

Figure 5. Difference spectra of CCCP after addition of the cytochrome c oxidase; $0.73 \cdot 10^{-6}$ mol/l cytochrome c oxidase, 50 mmol/l HEPES, pH 7.4, 0.1% laurylmaltozid; CCCP concentration (1) $0.8 \cdot 10^{-6}$ mol/l; (2) $2 \cdot 10^{-6}$ mol/l; (3) $4 \cdot 10^{-6}$ mol/l; (4) $8 \cdot 10^{-6}$ mol/l; (5) $16 \cdot 10^{-6}$ mol/l; (6) $3.2 \cdot 10^{-5}$ mol/l; (7) $6.4 \cdot 10^{-5}$ mol/l.

the maximum and minimum of the difference spectra at various concentrations of the uncoupler hold their positions as well as isobestic points.

The dependence of the amplitude of absorption changes of CCCP on the CCCP concentration is non-linear (Fig. 6). At a constant ratio of CCCP to cytochrome c oxidase the pH dependece of these differences suggests a character of their interaction – for low-affinity sites (Fig. 7). The difference increases by decreasing the pH value until pH 6. Below this pH a steep decrease in the difference spectrum is observed. Also in this case, cytochrome c oxidase addition to NCH_3CCP does not bring about any significant changes in the absorption spectrum of the compound in agreement with the assumption that there is no interaction between the compound and the cytochrome c oxidase. These data, as well as those for high affinity binding sites, are in support of the suggestion that the interaction of CCCP with the histidine residues of cytochrome c oxidase may be of electrostatic character. The existence of some small optical changes as observed at pH 9 may indicate that

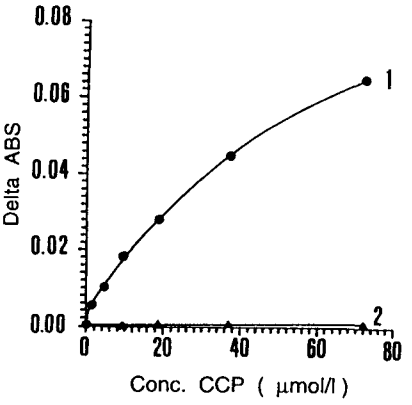

Figure 6. The spectral change (Delta ABS = $ABS_{max} - ABS_{min}$) of the CCP derivatives in the presence of cytochrome c oxidase; (1) CCCP, (2) NCH_3CCP; $0.73 \cdot 10^{-6}$ mol/l cytochrome c oxidase, 50 mmol/l HEPES, pH 7.4, 0.1% laurylmaltozid.

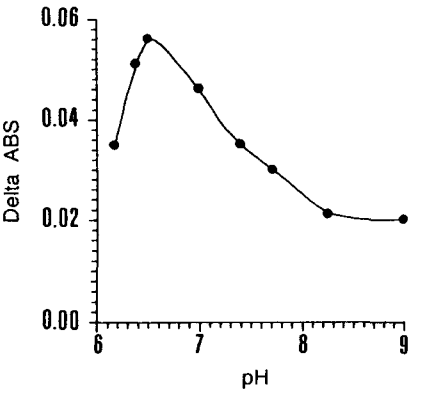

Figure 7. The spectral change (Delta ABS = $ABS_{max} - ABS_{min}$) of the CCCP in the presence of cytochrome c oxidase on the pH; $0.73 \cdot 10^{-6}$ mol/l cytochrome c oxidase, 50 mmol/l HEPES, 50 mmol/l MES, 0.1% laurylmaltozid.

CCCP anion can react also with lysine residues of cytochrome c oxidase or interact with cytochrome c oxidase hydrophobically.

Discussion

There are some problems in the study of interaction between low-molecular lipophilic ligands and membrane proteins. At natural conditions the proteins are bound to membrane and addition of the lipophilic ligands causes a high ligand absorption by the lipidic part of the membrane. It complicates characterization of assumed fine effects due to interaction of ligands with proteins. For proteins isolated and dissolved in water by means of detergents, the presence of regions occupied by detergents at the protein surfaces increases the unspecific interaction of ligands with proteins, as well as in the case of membrane. The mentioned effects can be minimalized using the membrane proteins isolated with the lowest quantity of phospholipids and detergents. For aggregation of isolated membrane proteins after

their addition into medium without detergents, important is a fact that aggregates do not create closed spaces containing free ligands. Therefore it is necessary to use a comparative material with similar properties such as studied ligand, except one (for example a charge) which is crucial for a possible interaction. Further assumption is a minimal change of the protein structure during aggregation. Cytochrome c oxidase is a large protein complex whose dominant part of the surface is localized outside the membrane (Saraste 1990). It allows to observe the electron transport activity of cytochrome c oxidase without the presence of detergents (Ambe and Venkataraman 1959).

In water cytochrome c oxidase creates dimers in the presence of detergents as well as other aggregation states (Michel and Bossard 1989). They can only be separated from solution by ultracentrifugation (Garber and Margoliash 1990). The addition of the cytochrome c oxidase solution containing some detergent into water solution without detergents results in gradual loss of the detergent molecules from the enzyme surface. This brings about aggregation of the enzyme to larger formation which can be fully removed from the solution by low speed centrifugation. This fact is useful for the investigation of the interaction of ligands with cytochrome c oxidase in processes which are faster than the process of releasing detergent from the surface – i.e. electrostatic interactions. The formation of aggregates of cytochrome c oxidase apparently does not create a non-specific adsorption envelope of ligand molecules. This can be seen from the fact, that a lipophilic analog N-methyl carbonyl cyanide phenylhydrazone (NCH_3CCP) is not removed from solution by centrifugation. The ability of the CCCP binding to the cytochrome c oxidase is dependent on pH of the medium.

The interaction between CCCP and cytochrome c oxidase (dissolved in water with the aid of detergents) has been studied by means of following the changes in absorption of the ligand as well. The spectral changes of CCCP derivatives after reaction with biological systems have been followed using three different methods. First, the reaction of the CCCP with thiols is accompanied by a red-shift of the spectrum and is pH dependent (Šturdík et al. 1985). The rate of additional reaction of CCCP with thiols is rather slow in comparison with the momentary rate of interaction of the CCCP with the cytochrome c oxidase. The second system is a solvent effect due to the spectral changes of the CCCP derivative. The interaction of CCCP with liposome causes the red-shift of the spectrum typical for the anion form of the derivative as well as for the non-dissociated form (Bakker et al. 1975). Thirdly, the spectrum of the anion CCCP is changed by cations. Binding of protons causes a large shift of the spectrum to the shorter wave lengths for anion CCCP as well as for other CCP derivatives which enable determination of their dissociation constants (Šturdík et al. 1985). On the other hand, the red-shift of the spectrum of the FCCP derivative is induced by the complex of the valinomycine with K^+ (O'Brien et al. 1978). From the above mentioned data it is evident that the shift

of the CCCP spectrum cannot determine the type of interaction. That is why it is necessary to use an analog which is not to be dissociable and possesses a similar lipophilicity. Since the spectrum of the analog in the presence of cytochrome c oxidase does not change pH values at all, we conclude that the CCCP is bound to the cytochrome c oxidase electrostatically. The partners of the interaction may be the positively charged groups of histidine and some of lysine. The electrostatic basis of the interaction of the CCCP and its derivatives can be assumed also for other membrane proteins of the respiratory chain – e.g. for the H^+–ATPase. This type of interaction between the uncouplers and proteins of the inner mitochondrial membrane was assumed in the paper (Hanstein and Hatefi 1974). Our results suggest the importance of the CCCP and other uncouplers on the transport activity of the cytochrome c oxidase and other mitochondrial proteins. The activity is influenced by changing the properties of the positively charged groups on the protein surface and by decreasing the concentration of free uncoupler which is affected in the transfer of the protons across the membrane.

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