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Electrochemical study of the interaction between cytochrome P450sccK201E and cholesterol

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Abstract

Cytochrome P450sccK201E, mutated form of cytochrome P450scc native recombinant (P450sccNR), was employed to study the enzyme–substrate interaction. The detection of the cholesterol was performed by electrochemical method using cyclic voltammetry (CV) and chronoamperometry measurements. The biochemical analysis was realized to observe the electrochemical responses of the engineerized enzyme to three different forms of cholesterol: free, low-density lipoprotein (LDL) and high-density lipoproteins (HDL). Compared to cytochrome P450sccNR, the cytochrome P450sccK201E displays a different behavior in the interaction with the substrate detection.

The results show that the engineerized enzyme can be utilized for the cholesterol detection in biosensor field.

Keywords: Cytochrome P450scc; Cyclic voltammetry; Chronoamperometry; Cholesterol; LDL; HDL

1. Introduction

It is now well recognized that electrochemical techniques are powerful tools for evidencing electron transfer properties of proteins and catalysis of enzymes. Electrochemical studies of proteins can in principle be employed to reveal the energetic and pathways of these electron transfer processes, and to investigate individual donor-acceptor electron transfer events. In general, the cyclic voltammetry (CV) is widely used to characterize the redox properties of transition metal complexes [1] and to provide information about the kinetics of electron transfer reactions of any coupled chemical reactions [2]. One group of most interesting and challenging metalloenzymes is the cytochrome P450s. Cytochromes P450 [3–7] are a class of enzymes which play an important role catalyzing several biochemical reactions in nature, and participating in oxidation of a wide range of compounds [8]. The Phase I of the biotransformations are mainly dependent to the monooxigenases belonging to the P450 superfamily [9] as the steroid oxidations [10] and the cellular detoxification of xenobiotic compounds [7,11,12] and the pharmaceutical drugs [13]. In particular, a cytochrome, namely P45011A1 or P450scc, catalyzes the key stereogenic reaction of cholesterol side cleavage by three sequential oxidative steps to form pregnenolone [14]. The main organ for cholesterol metabolism is the liver, in which the synthesis of cholesterol is regulated, its conversation into bile acids, its secretion into bile and the uptake and hydrolysis/metabolism of plasma cholesterol/esters in the form of high-density lipoproteins (HDL) and low-density lipoprotein (LDL) [15–17]. HDL and LDL steady-state levels in blood are reciprocally associated with the risk of heart coronary disease [18]: HDL particles are considered anti-atherogenic [19] and inversely correlate with the development of premature coronary heart disease [20], instead LDL particles are pro-atherogenic [21,22]. Therefore, the determination of HDL and LDL cholesterol concentrations is important in the clinical diagnosis of several diseases [23].

In this work, electrochemical analyses were finalized to explore possible employment of the cytochrome P450sccK201E as sensitive element in an amperometric biosensor. This new enzyme was produced by site-directed mutagenesis, following a prediction study of molecular modeling. The K201E mutation was previously identified by a theoretical approach, finalized to the optimization of the stability/ordering during the protein immobilization. In particular, the molecular modeling prediction was realized studying the surface of cytochrome P450scc native

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recombinant (P450scc NR) and identifying a hypothetical complex based on two P450scc molecules. The aim of this mutation was to increase the probability of formation of such a pattern by improving electrostatic interactions and simultaneously adding hydrophobic amino acid residues into the surface regions included in the pattern [24].

During biochemical analysis of cytochrome P450scc-K201E new important properties of catalytic activity came out.

In particular, the effect of the single mutation (Lys 201 was changed into negatively-charged glutamic acid residue) on the enzyme–substrate interaction was studied. Moreover, the HDL and LDL detection was made in order to verify the possible applications of cytochrome P450sccK201E.

2. Experimental

2.1. Materials

Reagents for bacterial growth were purchased Fluka (Buchs, Switzerland). Emulgen 913 was kindly provided by Kao Chemical (Tokio, Japan).

Protein molecular weight standards were obtained from Promega. Hydroxyapatite column from Bio-Rad (Milan, Italy). Oligonucleotides were synthesized by TIB-Mol. Biol. (Genoa, Italy).

The cholesterol (5-cholesten-3-βol), low-density lipoproteins (LDL), high-density lipoprotein (HDL) and other chemicals were from SIGMA Chemical Comp.

2.2. Site-directed mutagenesis, expression and enzyme purification

cDNA gene for mature form of P450scc was cloned in the pTrc99A vector [25] to obtain bacterial expression of bovine P450scc (the product of CYPA11A gene) [26,27].

The correct orientation of the insert was checked by restriction mapping. The sequence was confirmed by the sequencing using a Automatic Sequencer (“Applied Biosystems” Model 373A 1.2.0 version).

P450scc mutant was generated by Quick-Change site-directed mutagenesis kit (Stratagene; La Jolla, CA). Complementary mutagenic oligonucleotides purified by fast polynucleotide liquid choromatography (FPLC) were utilized.The sequences of the oligonucleotides containing the appropriate mutation are the following:

5′GATGCCGTCTACGAGATGTTCCACACC 3′;
5′GGTGTGGAACATCTCGTAGACGGCCATC 3′.

The mutant was realized by mutagenesis reaction containing: 5 μl reaction buffer 10×, 10 ng of pTrc99A-P450scc plasmid, 125 ng for each oligonucleotide, 1 μl of dNTPmix (100 mM) and 2.5 unit of Pfu DNA polymerase in a final volume of 50 μl. Parameters and cycles of reaction were summarized in Table 1.

Table 1
Parameters and PCR cycles of mutagenesis reaction

Cycles	Temperature (°C)	Time (min)
1	95	0.5
16	95	0.5
	55	1
	68	12

After reaction, each sample was treated with a restriction enzyme, Dpn I, and it was used a transformation reaction with JM109 strain. The mutation was verified by DNA sequencing.

The oligonucleotides used for sequencing are as follows:

5′ TGTGTGGAATTGTGAGCG 3′ (scc1f);
5′ CTAGCTGGATTGGTGGAA 3′ (scc1r);
5′ AGTGTCTCAGGACTTCGT 3′ (scc2f);
5′ CTTTCAGGGTATCTCTGC 3′ (scc2r).

E. coli strain JM109 were cultured from P450scc (native and mutant) expression as described in Wada et al. [26] with the following modification: isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration 1 mM) and δ-aminolevulinic acid (1 mM) were added when the culture achieved the optical density of 0.6–0.8 at $\lambda = 600$ nm.

Extraction and purification were performed according to Wada and Waterman [28]. Cytochrome P450scc integrity was verified by reduced CO-difference spectra. The concentration was measured according to Omura and Sato [29], using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the absorbance difference between 450 and 490 nm. SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli [30].

2.3. Electrochemical measurements

The electrochemical measurements were made by a Potentiostat/Galvanostat (EG& G PARC, model 263A) supplied with its own software (M270).

A three-electrodes standard configuration was employed where a platinum wire acted as counter electrode and a screen printed electrode (s.p.e.) was used for reference as well as working electrode. The s.p.e. was here employed to minimize the working cell dimensions (a quartz cuvette with a working volume of 3.5 ml). The paste tracks reference electrode was made by Ag/AgCl, and the working electrode was produced using a modified protocol according to [31,32].

The area of the working electrode was 2 mm × 10 mm.

CV experiments were carried out in 2 ml of 10 mM K-phosphate buffer, pH 7.4, at room temperature. The working electrode was cycled between initial and switch potentials of 500 and −500 mV, respectively, after holding the electrochemical system for 10 s at the initial potential. A scan rate of 20 $mV\,s^{-1}$ was used to optimize the signal to noise ratio. All measurements were repeated three times to verify their reproducibility [33,34].

Chronoamperometric experiments, under convective conditions, were performed as a function of substrate concentration. The cytochrome was completely reduced at a fixed potential of −600 mV vs. Ag/AgCl. The cholesterol (2 mg ml^{-1}) was dissolved in Na-cholate 30%, LDL (5.7 mg ml^{-1}), and HDL (11.6 mg ml^{-1}) were dissolved in 0.15 M NaCl containing 0.01% EDTA.

The electrochemical responses were obtained adding the substrate every 5 min, in 2 ml of a working solution (10 mM K-phosphate buffer, pH 7.4). Background current was allowed the decay of steady-state current.

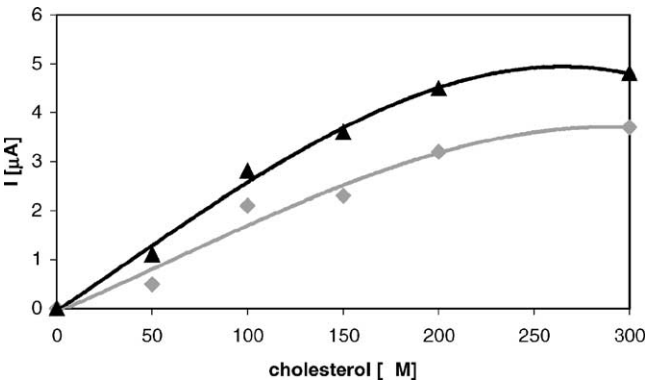

Fig. 1. Steady-state calibration curve of the cytochome P450sccNR (grey line and rhombus) and cytochrome P450K201E (black line and triangle) by chronoamperometry analysis, in function of the concentration of cholesterol.

3. Results and discussion

3.1. Cyclic voltammetric study

It was found that the electrochemical techniques have provided insights into the functional properties of redox-active centers of proteins [35–39]. Redox potential of proteins are one of the most fundamental physicochemical parameters for better understanding of physiological redox process. To test the functionality of enzymes some preliminary measurements of cyclic voltammetry was performed. In fact the characteristic cathodic and anodic peaks are indicative to the active enzymes. The buffer solution was previously degassed to prevent the oxygen reduction and, at the same time, few electrochemical high rate scans was performed to consume the oxygen of diffusion. Results of voltammograms are reasumed in Table 2; where are reported the formal potentials ($E^{0'}$), determined as the average of the anodic (E_a) and cathodic peak (E_c) potentials [$E^{0'} = (1/2)(E_a + E_c)$]. Usually, $E^{0'}$ and the ratio between electric intensity of cathodic and anodic peaks (i_{pa}/i_{pc}) of a redox compound suggest information about the reversibility of the electrochemical process (Nernstian equation) [40–42].

In Table 2, the values of $E^{0'}$ (−208 mV for P450scc NR and −220 mV for P450sccK201E) are summarized. According to the Nernstian equation value, calculated for a monoelectronic redox process in controlled diffusion mechanism (∼−59 mV), the data relative to the cytochromes suggest the irreversibility of the process. The anodic and cathodic peak ratio (i_{pa}/i_{pc}) approaches to 1 in all two cases.

Compared with cytochrome P450sccNR the mutant shows $\Delta E^{0'}$ equal to 12 mV. In literature similar little variations can be found for single aminoacid mutation for other metallo-proteins and for active electrochemical groups [43,44]. Smaller variations in the redox potential can be attributed to the change in the following factors: the hydrophobicy of the interior of the protein, the solvent accessibility of the active site and the electrostatic and hydrogen bonding interactions between the amino acid residue and the iron center [45,46]. All these condictions do not show alterations to the axial coordination of iron center [47–50].

Moreover, the formal potential value for the mutant is shifted to negative values. This behavior is probably due to the effect of mutation that could cause some changes in the heme environment and consequently on the electric response [51,52].

3.1.1. Chronoamperometry: free cholesterol

Chronoamperometry method was used to detect the cholesterol conversion to pregnenolone and isocapraldehyde of the P450 enzyme side chain cleavage [53].

In particular, this technique can investigate about the cytochrome-substrate interaction. In Fig. 1, the chronoamperometric data and the best fit, obtained with a second-order polynomial function, are shown. Both cytochromes show an amperometric response that increases in fuction of the cholesterol concentration. The current intensity is major for the mutant compared to the NR enzyme and reaches saturation point at first. Catalytic current study, in function of the substrate concentration, was carried out to estimate the kinetic parameters. The chronoamperometry permits to study enzyme–substrate interaction and to estimate the apparent Michaelis–Menten constant (K_M^{app}). The Michaelis–Menten

Table 2
Cytochrome P450sccNR and cytochrome P450K201E electrochemical parameters extracted by the cyclic voltammograms at 20 mV s^{-1}

	E_a (mV)	E_c (mV)	$E^{0'}$ (mV)	i_{pa} (μA)	i_{pc} (μA)	i_{pa}/i_{pc}
Cytochrome P450sccNR	−146 ± 3	−354 ± 3	−208 ± 6	8.89 ± 0.43	11.01 ± 1.10	0.81 ± 0.13
Cytochrome P450sccK201E	−106 ± 1	−326 ± 4	−220 ± 5	9.72 ± 0.58	8.33 ± 0.63	1.17 ± 0.17

The standard deviations were calculated from data of three independent determinations.

Table 3
Kinetic parameters of the cytochrome P450s-cholesterol interaction extracted by chronoamperometry (Fig. 1 and Table 3)

	I_{max} (μA)	K_M^{app} (μM)	I_{max}/K_M^{app} (μA mM^{-1})
Cytochrome P450sccNR	3.67 ± 0.12	111.23 ± 0.22	33.00 ± 1.14
Cytochrome P450sccK201E	4.87 ± 0.19	94.11 ± 0.18	51.74 ± 2.13

The standard deviations were calculated from data of three independent determinations.

equation in electrochemical experiments can be transformed in [54,55]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_M^{app}}{I_{max}}[S]$$

where I_{ss} is the steady-state current after addition of the substrate, [S] the bulk concentration of the substrate and I_{max} is the maximum current measured. To evaluate the affinity of both cytochromes with the cholesterol, I_{max}/K_M^{app} ratio was estimated (Table 3). The ratio values were found higher for the P450sccK201E than for P450sccNR probably because of the better effectiveness of enzyme–substrate interaction. The Lineweaver–Burk plot of the 1/I versus the reciprocal of the cholesterol concentrations is shown in Fig. 2 [56].

3.1.2. Chronoamperometry: LDL and HDL

In order to verify the possible employment of the cytochrome P450scc in diagnostic field [57], several measurements using the physiological cholesterol forms were performed. The results are shown in Figs. 3 and 4 and underlined in Tables 4 and 5. Compared with the free cholesterol, the LDL and the HDL kinetics of each cytochrome do not show dramatic changes. However, for the three substrates, the I_{max}/K_M^{app} ratio was found higher for to the mutant than for the P450sccNR. This result suggests that the single-site mutation could cause a faster and higher affinity of the mutant to its substrates.

For both cytochromes the amperometric response emphasizes the highest I_{max}/K_M^{app} ratio value for the free

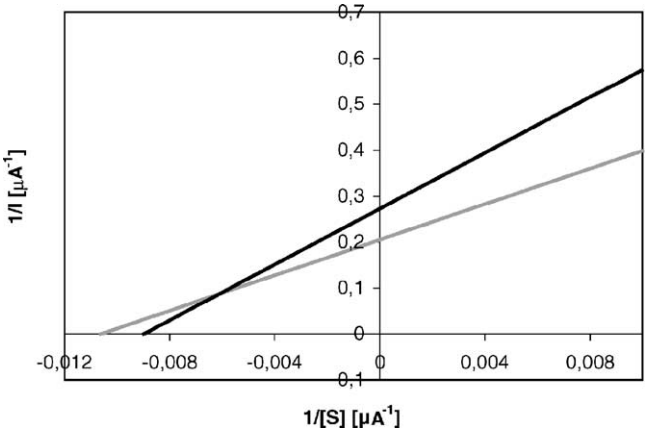

Fig. 2. Lineweaver–Burk plot for cytochrome P450sccNR (grey line) and K201E (black line). These parameters are extracted by the chronoamperometry and briefed in the Table 2.

Table 4
Kinetic parameters of the cytochrome P450s-LDL interaction extracted by chronoamperometry (Fig. 3 and Table 4)

	I_{max} (μA)	K_M^{app} (μM)	I_{max}/K_M^{app} (μA mM^{-1})
Cytochrome P450sccNR	1.19 ± 0.22	111.8 ± 1.02	10.64 ± 2.09
Cytochrome P450sccK201E	1.70 ± 0.32	84.22 ± 1.31	20.19 ± 4.17

The standard deviations were calculated from data of three independent determinations.

Table 5
Kinetic parameters of the cytochrome P450s-HDL interaction extracted by chronoamperometry (Fig. 4 and Table 5)

	I_{max} (μA)	K_M^{app} (μM)	I_{max}/K_M^{app} (μA mM^{-1})
Cytochrome P450sccNR	2.62 ± 0.33	103.7 ± 0.44	25.26 ± 3.30
Cytochrome P450sccK201E	3.37 ± 0.12	115.1 ± 0.98	29.27 ± 1.31

The standard deviations were calculated from data of three independent determinations.

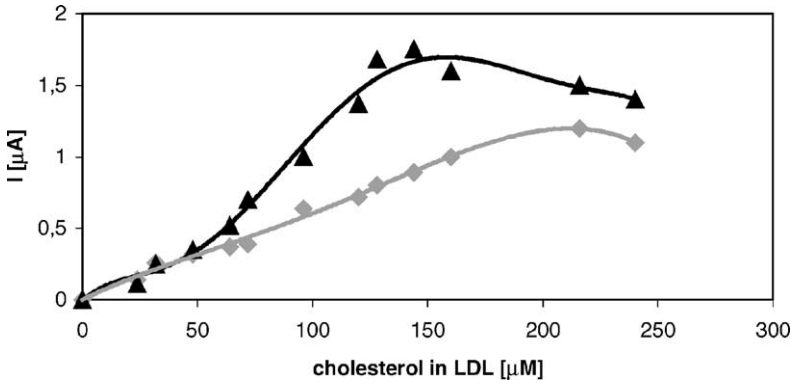

Fig. 3. Steady-state calibration curve of the cytochome P450sccNR (grey line and rhombus) and cytochrome P450K201E (black line and triangle) by chronoamperometry analysis, in function of the concentration of LDL.

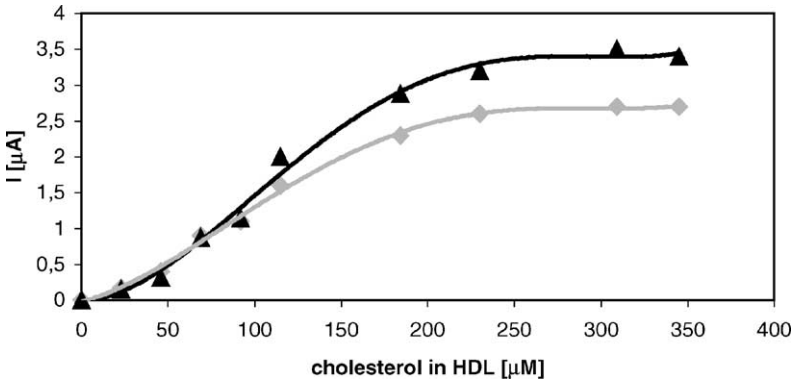

Fig. 4. Steady-state calibration curve of the cytochome P450sccNR (grey line and rhombus) and cytochrome P450K201E (black line and triangle) by chronoamperometry analysis, in function of the concentration of HDL.

cholesterol-enzyme interaction. In fact the cholesterol in HDL and LDL is connected with a lot of different compounds that limit the interaction. LDL and HDL are the two major lipoprotein subfractions responsible for cholesterol transport [58] and are composed of lipids, apoproteins, enzymes, and lipids transfer proteins. The difference between HDL and LDL is firstly the ratio between protein and fats that determines their size and density. The second difference is the type of the predominant lipids that are phospholids and cholesterol for HDL and esters of cholesterol for LDL [59].

Chronoamperometric responses of cytochrome P450scc-K201E and P450sccNR in function of the cholesterol concentration in HDL are displayed in Fig. 4. In these assays the trend of the kinetics does not show a Michaelis–Menten behavior. Probably the low apo-proteins disrup the interaction between the enzyme and the cholesterol in LDL.

4. Conclusions

This work describes the electrochemical behavior of native recombinant and engineerized cytochrome P450scc. Several differences were found in the electrochemical responses of the two enzymes. The possibility to detect LDL and HDL by chronoamperometry is strategic in clinical diagnostics. The experimental data have shown that the mutation has improved the affinity between the cytochrome P450 and the substrates; in particular, the best responses were found in the detection of cholesterol in HDL. Finally, the results suggest the possibility to employ the cytochrome P450sccK201E for the cholesterol monitoring. For this reason, this mutant can be used as sensitive element of an amperometric biosensor, combining the sensitivity and the versatility of electrical measurements with the recognition selectivity of enzyme.

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