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Superoxide Biosensing with Engineered Cytochrome c

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Abstract

Several mutation positions have been chosen for introducing positively charged lysines in human cytochrome c (cyt c) with the aim of increasing the reaction rate with superoxide radicals (SO) and thus, the sensitivity of an electrochemical cyt c based SO biosensor. The impact of the mutations on structural and redox properties as well as on the reaction rate with SO are verified. Four mutants show a higher reaction rate with the radical compared to the wild type. These mutants are used for the construction of SO sensors based on thiol-modified gold electrodes and covalently fixed proteins. The E66K mutant electrode has a clearly higher sensitivity in comparison to the wildtype based sensor.

Keywords: biosensor; cytochrome c, superoxide, protein engineering

1. Introduction

As one of the reactive oxygen species the SO radical is involved in several patho-physiological situations such as cancer and reperfusion. Thus, especially in the medical field there is in interest in the online detection of this short-lived species. Electrochemical biosensors can meet these needs. Because of the robustness and possibility for in vivo studies cyt c is often used as recognition element for these sensor electrodes [1, 2, 3]. Several approaches have already been developed to enhance the sensitivity via increasing the amount of electro-active cyt c [4, 5]. Here we use mutant forms of cyt c to be applied as sensorial recognition element. This approach is based on the concept to enhance the interaction of the radical with the heme center of the protein through electrostatic guidance of the negatively charged SO with help of introduced positive charges. Thus neutral and negative amino acids are chosen to be replaced by lysines. These additional charges can increase the reaction rate of cyt c with superoxide and hereby also the sensitivity of the sensor.

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2. Results and discussion

2.1. Selection of mutations sites and expression in E.coli

Human cyt c is chosen to introduce positively charged lysines replacing negatively charged amino acids and hydrophobic amino acids near the heme group and on the surface of the protein. This approach shall support the electrostatic interaction of SO with cyt c as well as the guidance of the radical to the heme iron. The following 11 single mutations sites are selected in this first mutation round: Tyr46Lys, Ala50Lys, Ala51Lys, Glu61Lys, Asp62Lys, Glu66Lys, Glu69Lys, Tyr74Lys, Gly77Lys, Ile81Lys, and Phe82Lys. The selected sites are shown in Fig.2. Plasmids containing the site directed mutated gene of human cyt c are introduced into *E.coli* bacteria. Wild type cyt c and all 11 cyt c mutants can be expressed successfully and are then purified using cationic exchange and size exclusion chromatography.

2.2. Photometric determination of the rate constant between cyt c mutants and superoxide

The reaction rate constant for the reaction between the cyt c mutants and the SO radical is determined spectrophotometrically by measuring the cyt c reduction rate at the wavelength of 550 nm in the presence of SO. Different areas of mutational effects can be observed (Fig. 2). The first area is composed of three amino acids which have a higher reaction rate with SO (E66K, Y74K, and F82K: 12.5×10^{-4}, 10.7×10^{-4}, and 10.7×10^{-4} M^{-1} s^{-1} compared to the wildtype (8.3×10^{-4} M^{-1} s^{-1}) whereas the mutants E66K exhibits here the highest reaction rate. Here a possible access pathway for SO to the heme iron might be supported. However, for several mutants the reaction rate is not influenced, whereas three mutants (Y46K, A50K, A51K) show a reduced reaction with superoxide. In the latter case the mutation position might be too far from the SO entrance area and thereby mislead the radical which can slow down the reaction rate. These results give already an indication clue which mutants can be used to build an improved biosensor since the reaction rate of cyt c with superoxide limits the sensitivity of cyt c based SO sensor.

2.3. Electrochemical characterization

All mutants are first investigated electrochemically in solution on mercaptopropanol modified gold electrodes to get general thermodynamic and diffusional properties. The results are collected in Table 1. Regarding the redox potential no remarkable influence from the mutations has been observed despite for Y46K. The formal redox potential, E_f, of Y46K is -40 mV (±8 mV) versus Ag/ AgCl which is clearly below the range of the other proteins; hence, Y46K is more easily oxidized. The calculated diffusion coefficients from the voltammetric measurements vary slightly among the mutants. E61K shows a 1.7-fold increased diffusion coefficient, whereas F82K has a 4-fold decreased diffusion coefficient with respect to the wild type. These variations can be explained by small conformational changes.

To check the suitability as a recognition element in a biosensor the mutants which reveal a higher radical reaction rate constant, the thermodynamic and kinetic properties of the cyt c mutants have been characterized electrochemically with the protein adsorbed on a promoter-modified gold electrode. The experiments demonstrate that all chosen mutants can be adsorbed on the negatively charged mercaptoundecanoic acid/mercaptoundecanol (MU/MUA) layer. Cyclic voltammograms of the mutants are very similar to those achieved with the wild type. Values of redox potential and the heterogeneous electron-transfer rate constants (k_s) are presented in Table 1. The rather similar redox properties and surface coverage of the proteins, in comparison with the values for the wild-type cyt c, are promising for sensor application. The mutants F82K and Y74K have a 2.2- and 1.8-fold increased electron-transfer rate.

Further, all four mutants selected have been covalently attached to the MU/MUA-modified electrode for sensor construction. Fig. 3 shows a typical current response with SO present in solution. Different superoxide concentrations are established by a variation of the xanthine oxidase activity in the enzymatic generation system in the presence of hypoxanthine. The current follows the steady-state SO concentration in a linear manner in the concentration range from 0.15 to 0.4 μM. In addition, the signal can be completely suppressed by the enzyme superoxide dismutase which effectively removes the radical from solution by dismutation. This leads to the

conclusion that the sensors responds selectively to superoxide. In the comparison of the current signals from wild-type and mutant E66K electrodes in Fig. 3 an increase of the sensor signal for E66K for the same SO concentration can be clearly noted. In fact mutant E66K shows the highest sensitivity for the oxygen radical (620 A m^{-2} M^{-1}), whereas for the other protein electrodes, no significant increase in the signal has been observed. This approximately 40% increase in sensitivity found for the mutant E66K is in good agreement with the photometrically determined rate constants for the cyt c - radical reaction. In both cases, the mutant E66K shows the highest values.

In contrast F82K exhibit a decreased sensitivity and could not fulfill the expectations from the reaction rate measurements with superoxide in solution. This can be attributed to the fact that with this mutant a lower surface coverage, is achieved compared to the other mutants. Y74K and E69K electrodes show only a slightly enhanced sensitivity, which correlates to the slight increase in reaction rates with superoxide found for these proteins in solution. From these sensitivity measurements the mutant E66K seems to be best suited for the construction of superoxide sensor electrodes.

Table 1. Electrochemical properties of cyt *c* in solution (electrode, mercaptopropanol-modified gold) and adsorbed on an MU/MUA-modified gold electrode determined from cyclic voltammetric experiments (formal potential E_f, diffusion coefficient D, surface coverage Γ, heterogeneous electron-transfer rate constant k_s). All values are determined as the mean of at least three electrodes; n.d. - value was not determined. Formal potentials were determined using a Ag/AgCl reference electrode with 1 M KCl (237 mV vs NHE).

Cyt c protein / mutant	E_f in solution (*mV*)	D (10^{-7} *cm²/s*)	E_f adsorbed (*mV*)	Γ adsorbed (*pmol/cm²*)	k_s adsorbed (s^{-1})
Human Wild type	15 ± 5	1.8 ± 0.4	-8 ± 5	10 ± 2	64 ± 6
Y46K	-40 ± 5	0.9 ± 0.3	n.d.	n.d.	n.d.
A50K	22 ± 5	1.3 ± 0.2	n.d.	n.d.	n.d.
A51K	14 ± 5	1.9 ± 0.7	n.d.	n.d.	n.d.
E61K	18 ± 5	3.1 ± 0.2	n.d.	n.d.	n.d.
D62K	13 ± 5	1.4 ± 0.5	n.d.	n.d.	n.d.
E66K	20 ± 5	1.6 ± 0.6	1 ± 5	12 ± 2	55 ± 6
E69K	11 ± 5	1.6 ± 0.8	2 ± 5	11 ± 3	47 ± 6
Y74K	15 ± 5	2.6 ± 0.8	0 ± 5	7 ± 2	113 ± 5
G77K	12 ± 5	1.5 ± 0.3	n.d.	n.d.	n.d.
I81K	18 ± 5	1.2 ± 0.3	n.d.	n.d.	n.d.
F82K	2 ± 5	0.4 ± 0.2	-19 ± 5	6 ± 2	139 ± 8

For this protein electrode important sensor properties, such as stability and the influence of interferences, have been investigated. H_2O_2 is a possible interfering compound since it is known that that cyt c immobilized on electrodes can exhibit pseudoperoxidase activity. However in comparison to the wildtype no enhancement in the bioelectrocatalytic H_2O_2 reduction can be observed at a potential of 0 V vs. Ag/AgCl (5 mM H_2O_2). Furthermore all amperometric sensor measurements were done at +150 mV versus Ag/AgCl which is high enough to prevent hydrogen peroxide reduction. This was verified in the presence of 200 µM H_2O_2. For stability tests the surface coverage and the sensor response to a fixed superoxide concentration have been investigated for different time periods after the preparation while storing the prepared electrodes dry at 4 °C. The results lead to the conclusion that these mutant sensor electrodes can be used at least for 1 month after preparation without significant loss of sensitivity.

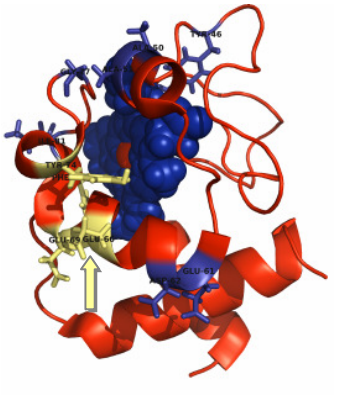

Fig. 2. NMR structure of human cyt c with mutation sites [light grey/yellow: higher reaction rate with superoxide (E66, Y74, F82, E69), dark grey/blue: lower reaction rate (Y46, A50, A51) and reaction rate not affected (E61, D62; G77, I81)]. The mutant E66K with the highest reaction rate with superoxide is marked with an arrow. Image was built with the software Pymol (pymol.sourceforge.net) using the pdb file 1J3S

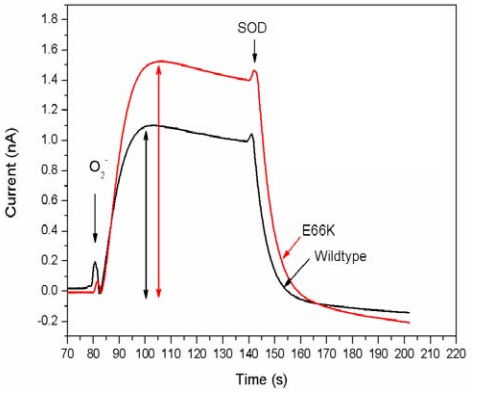

Fig. 3. Amperometric measurement of superoxide using cyt c based sensor electrodes (Au|MUA/MU|cyt c). Comparison of current responses of wild type cyt c with the variant E66K after starting the production of superoxide with xanthine oxidase in the presence of hypoxanthine and later scavenging all superoxide radicals with superoxide dismutase (50 mM sodium phosphate buffer pH 7.5 at +150 mV). Inset: Linear dependency of current signal on superoxide concentration.

3. Conclusion

Site directed mutagenesis and recombinant expression results in 11 single mutated forms of human cyt c. UV-vis, CD and ^{1}H-NMR-spectroscopy reveal that the structural integrity is maintained. Electrochemical investigations of the mutants in solution demonstrate that all mutants are electroactive and despite one exception (Y46K) all mutants have rather similar redox potentials and only slightly variable diffusion coefficients. The photometrically determined reaction rate constants with SO reveal four mutants (E66K, E69K, Y74K, and F82K) with increased reaction rates. Further studies of the cyt c variants adsorbed on modified gold electrodes can show that these selected mutants are suitable for sensor construction since k_s values and the formal potential as well as the surface coverage are found to be rather high. We have investigated the current response of biosensor electrodes built with four covalently fixed cyt c mutants towards superoxide. Two mutants exhibit only a slightly increased sensitivity (E69K and Y74K) whereas one mutant (F82K) reveals no enhancement. One protein electrode (E66K) show a significantly higher sensitivity compared to wild type electrodes while retaining linearity, and specificity.

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