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Site-Directed Mutagenesis of Cytochrome *c*: Reactions with Respiratory Chain Components and Superoxide Radical

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Abstract—Three forms of horse heart cytochrome *c* with specific substitutions of heme cleft surface located amino acid residues involved in specific interactions with ubiquinol:cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) were constructed, and their reactions with superoxide radical produced by NADH:ubiquinone reductase (complex I) were studied. The proteins with six (K27E/E69K/K72E/K86E/K87E/E90K and K8E/E62K/E69K/K72E/K86E/K87E) and eight (K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) substitutions were inactive in the cytochrome *c* oxidase reaction, and their reduction rates by complex III were significantly lower than that seen with acetylated cytochrome *c*. The reduction of these modified cytochromes *c* under conditions where complex I generates superoxide was almost completely (about 90%) inhibited by superoxide dismutase. The genetically modified cytochromes *c* are useful analytical reagents for studies on superoxide generation by the mitochondrial respiratory chain. Quantitative comparison of superoxide-mediated cytochrome *c* reduction with hydrogen peroxide-mediated Amplex Red oxidation suggests that complex I within its native environment (submitochondrial particles) produces both superoxide (~50%) and hydrogen peroxide (~50%).

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Key words: cytochrome *c*, site-directed mutagenesis, complex I, respiratory chain, superoxide radical, hydrogen peroxide, mitochondria

Studies on the formation and decomposition of "reactive oxygen species" (ROS) are an extremely popular area of physiologically oriented modern biochemistry. It is hard to find a pathology including natural aging for which participation of ROS have not been suggested based on indirect data with different degrees of confidence. In addition to their toxic nature, ROS are also believed to be important regulatory species.

Molecular oxygen is relatively inert specie and its extremely high consumption rate (cellular respiration, the major source of aerobic ATP production) is due to O_2 activation by the oxidases catalyzing 4-electron reduction to H_2O ($O_2 + 4\ e^- + 4\ H^+ \rightarrow 2\ H_2O$) without release of any partially reduced species into the surrounding media. The intermediates of oxygen reduction – superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) – are conventionally called "reactive oxygen species". There are two ways of intracellular hydrogen peroxide formation. H_2O_2 is formed as the product of a number of flavin-dependent oxidases (monoamine oxidase, peroxisomal acyl-CoA oxidases, xanthine oxidase), which catalyze two-electron oxygen reduction (O_2 +

Abbreviations: Amplex Red, 10-acetyl-3,7-dihydroxyphenoxazine; $O_2^{\cdot-}$, superoxide radical; $OH^{\cdot}$, hydroxyl radical; ROS, reactive oxygen species; SMP, submitochondrial particles; SOD, superoxide dismutase.

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$2\ e^- + 2\ H^+ \rightarrow H_2O_2$). The other way of H_2O_2 production is spontaneous or specifically catalyzed by superoxide dismutases (SOD) reaction: $2\ O_2^{\cdot-} + 2\ H^+ \rightarrow O_2 + H_2O_2$, where one-electron reduced oxygen, superoxide anion (pK_a for the dissociation $O_2^{\cdot}H \rightarrow O_2^{\cdot-} + H^+$ in aqueous solutions is 4.8), is an immediate precursor of hydrogen peroxide [1]. The formation of H_2O_2 during respiration (up to 1.5% of the total oxygen consumption) has been known for more than 50 years [2]. Quantitative approximation suggests that about 30% of H_2O_2 formed in liver can be accounted for by mitochondria [3]. It is generally believed that mitochondria contain no H_2O_2 producing oxidases (except for relatively low activity monamine oxidases of the outer mitochondrial membrane) and the major source of hydrogen peroxide is superoxide radical originated from nonspecific one-electron leakage to oxygen from the matrix-located dehydrogenases [4-6] and from the low redox potential components of the respiratory chain such as FMN of complex I [7-11] and ubisemiquinone of complex III [12]. The proposals that explain the mitochondrial superoxide formation as nonspecific leakage do not seem attractive: evolution has had enough time and instruments to avoid the defects. The existence of the mitochondrially located SOD [13], catalase [14], and glutathione peroxidase [15, 16] points by itself to coordinated and *regulated* intramitochondrially produced hydrogen peroxide level. Indeed, it has been predicted [17] and recently directly demonstrated [18] that the mitochondrial matrix contains soluble enzyme capable of NAD(P)H-supported, ammonium-stimulated hydrogen peroxide formation [18].

One-electron reduction of hydrogen peroxide in model reactions, for example by superoxide radical or by transition metal cations (Fe^{2+}, Cu^+, Mn^{2+}) results in a formation of short-living hydroxyl radical ($H_2O_2 + e^- \rightarrow OH^- + OH^{\cdot}$) which is capable of nonspecific reactions with the protein amino acid residues, nucleic acids, carbohydrates, activated methylene groups of fatty acids, etc. at almost diffusion-controlled rates. It follows that the real "danger" for cells is the presence of hydrogen peroxide along with the one-electron reductants, particularly superoxide radical. The identification of the intracellular sources of superoxide radical and its quantitative determination thus seems to be important problems.

A number of instrumental difficulties in quantitative approach to this problem exist. Superoxide-radical is unstable: the bimolecular reaction rate constant of its spontaneous dismutation in aqueous media is of about 100 $M^{-1}\cdot sec^{-1}$ at $pH > pK_a$ (4.8) and this value significantly increases upon protonation at $pH \leq pK_a$ [1]. The "classic" methods for quantitative determination of superoxide concentration (quenching the reaction by deproteinization with subsequent sampling) are thus not acceptable. To determine the rate of $O_2^{\cdot-}$ formation several high molar extinction red-ox active dyes rapidly reacting with superoxide-anion are used [19]. Another approach is to use spin-traps that react with superoxide and to determine its concentration by quantification of ESR-signal of the free-radical probe [20-22]. No superoxide-specific red-ox active reagents are available. When applied for mitochondria or their membrane preparations those approaches are especially difficult to apply because of their high intrinsic oxido-reductase activities whereas the rates of superoxide production are of only very small fraction (~0.1%) of their major respiratory activities. To overcome these difficulties Azzi et al. introduced acetylated cytochrome *c* as a specific superoxide-sensitive reagent [23]. Acetylation of cytochrome *c* lysyl residues greatly decreases its reactivity in the cytochrome *c* reductase and cytochrome oxidase reactions, whereas the reduction of heme by superoxide is retained. This assay is not lacking of some shortcomings. First, chemical acetylation results in a heterogeneous mixture of cytochrome *c* with uncertain number of substituted lysyl residues and reactivity of the protein towards the natural reductants and oxidants, cytochrome *c* reductases and cytochrome oxidase, respectively, depends on degree of acetylation. Second, the acetylation decreases total positive charge of cytochrome *c* and the reactivity of modified protein towards negatively charged superoxide is also expected to decrease. An alternative way to modify cytochrome *c* is site-directed mutagenesis. An obvious advantage of this approach is two-fold: (i) a well defined analytical reagent for the detection of superoxide can be obtained and (ii) valuable information on the specific role of particular amino acid residues in cytochrome *c* interaction with complex III and cytochrome oxidase (complex IV) is expected.

In this paper, we describe preparative procedure for a number of mutated forms of horse heart cytochrome *c* and their characteristic properties in the cytochrome *c* reductase reactions as well as their potential application for specific detection of superoxide production.

MATERIALS AND METHODS

Mutation of horse heart cytochrome *c* gene within expression plasmid vector pBP(CYCS) was performed by site-directed mutagenesis as recommended using the QuikChange™ Mutagenesis Kit (Stratagene, USA). Oligonucleotide primers containing both substitutions were used to construct a gene bearing two mutations, K86E/K87E. Mutated gene K86E/K87E was then used for further mutagenesis with corresponding primers to obtain cytochrome *c* genes with four, six, and eight substitutions: K27E/K86E/K87E/E90K, E69K/K72E/K86E/K87E, K27E/E69K/K72E/K86E/K87E/E90K, K8E/E62K/E69K/K72E/K86E/K87E, and K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K.

The production of mutated DNA was analyzed electrophoretically in 1% agarose gel. The nucleotide

sequences of the mutated cytochrome *c* genes were determined using an ABI Prism 3100-Avant Genetic Analyzer automated sequencer (Applied Biosystems, USA). Selected mutant genes were cloned into expression vector pBP(CYC1) [24], modified for the expression of horse heart cytochrome *c* [25].

Mutated genes were expressed in *E. coli* strain JM109 grown at 37°C for 22-24 h in vigorously stirred liquid medium SB containing ampicillin (0.2 mg/ml) [26].

The cells were harvested by centrifugation at 4000*g* (4°C) for 20 min. The precipitate was resuspended in buffer (25 mM Na-P_i, pH 6.0) containing 1 mM NaN_3 and frozen at −20°C for 20-30 min. The suspension was thawed, and the cells were disrupted using a French press (Spectronic Instruments, Inc., USA). The membranes were precipitated by centrifugation at 100,000*g* for 20 min and the supernatants were collected.

The protein was purified using a BioLogic HR System liquid chromatography system (BioRad, USA) as described previously [26]. The cell extracts prepared as described above were applied on a Mono S HR 10/10 cation-exchange column (BioRad) equilibrated with the same buffer (25 mM Na-P_i, pH 6.0, 1 mM NaN_3). The extracts containing mutant proteins with four or more substitutions were dialyzed against the same buffer before chromatography. Cytochromes *c* were eluted by the same buffer with linear gradient of 1 M NaCl at the rate of 3 ml/min. The fractions eluted from Mono S were analyzed spectrophotometrically and by SDS-electrophoresis in 12% polyacrylamide gels. The fractions containing cytochromes *c* were dialyzed against the buffer for absorption chromatography (10 mM Na-P_i, pH 7.0, 1 mM NaN_3) and applied on a column with hydroxyapatite CHT-I (BioRad). Cytochromes *c* were eluted in a 0.5 M Na-P_i (pH 7.0) gradient at the rate of 2 ml/min. The mutated variants K8E/E62K/E69K/K72E/K86E/K87E, K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K were further purified by gel filtration on Superdex-200 10/300 column (GE Healthcare, USA) equilibrated with 50 mM Na-P_i (pH 7.2), 150 mM NaCl at the rate of 0.5 ml/min.

The purification degree of cytochromes *c* was estimated spectrophotometrically and by SDS-electrophoresis. The fractions containing 95%-pure proteins were combined, the cytochromes were oxidized by equimolar potassium ferricyanide, and the samples were dialyzed twice against 10 mM ammonium carbonate, pH 7.9. The proteins were lyophilized on ALPHA I-5 and stored at −20°C.

Rat liver mitochondria were prepared as described by Johnson and Lardy [27]. Cytochrome *c* deficient mitoplasts were prepared by washing mitochondria with high ionic strength medium after hypotonic treatment [28]. The mitoplasts thus obtained were suspended and stored in 0.25 M sucrose at liquid nitrogen temperature.

Inside-out bovine heart submitochondrial particles (SMP) were prepared by the standard procedure routinely used in our laboratory [29]. The particles were artificially coupled by oligomycin and their NADH oxidase was activated as described [30].

The rate of superoxide generation was measured as the SOD-sensitive (3 units per ml of the assay mixture) reduction of acetylated horse heart cytochrome *c* [23] or its mutated forms by following an increase of absorption at 550 nm ($\varepsilon_{mM} = 20$) at 30°C in the mixture comprised of 0.25 M sucrose, 50 mM Tris-HCl, 100 mM potassium phosphate, pH 8.0, 0.2 mM EDTA, and 5 mM succinate or 50 μM NADH as the substrates. When NADH was used as the substrate, the mixture was supplemented with 5 μM rotenone. The protein content in the assay samples was 70 μg/ml. The spectra of all mutated forms of cytochrome *c* were identical to that of the native protein. All the activities were expressed as nmol of reduced cytochrome *c*/min per mg of SMP protein.

The rate of hydrogen peroxide formation was measured as stoichiometric oxidation of 10 μM Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine) by following absorption increase at 572 nm ($\varepsilon_{mM} = 54$) [31] at 30°C in the mixture comprised of 0.25 M sucrose, 50 mM Tris-HCl, 100 mM potassium phosphate, pH 8.0, 0.2 mM EDTA, horseradish peroxidase (2 units/ml), SOD (3 units/ml), and 5 mM succinate or 50 μM NADH as the substrates. The protein content in the assay samples was 70 μg/ml. The activities were expressed as nmol of hydrogen peroxide formed/min per mg of SMP protein.

The succinate:cytochrome *c* reductase activity of the cytochrome *c*-deficient mitoplasts (10 μg/ml) was followed by cytochrome *c* reduction at 550 nm at 30°C in the mixture containing of 0.15 M sucrose, 20 mM KCl, 20 mM Tris-HCl, pH 7.4, 5 mM NaN_3, and oxidized cytochrome *c*. The activities were expressed as μmol of cytochrome *c* reduced/min per mg of mitoplast protein.

The cytochrome *c* oxidase activities of mitoplasts (65 μg/ml) were measured amperometrically (coated platinum electrode) at 20°C in the mixture comprised of 0.15 M sucrose, 20 mM KCl, 20 mM Tris-HCl, pH 7.4, 10 mM sodium ascorbate, 0.2 mM tetramethyl-*p*-phenylenediamine, and cytochrome *c*. The activities were expressed as μg-atom of oxygen consumed/min per mg of mitoplast protein.

The protein content was determined by the biuret reaction. The concentrations of cytochromes *c* were measured spectrophotometrically.

The components of growth media and buffers used for chromatography and electrophoresis were from AppliChem (Germany); ampicillin, horse heart cytochrome *c*, NADH, succinic acid, EDTA, rotenone, SOD from bovine erythrocytes, horseradish peroxidase, and bovine liver catalase were from Sigma (USA); Amplex Red was from AnaSpec (USA), and hypoxanthine was from Merck (Germany). The restriction endonucleases were as follows: *Dpn* I from Fermentas (Lithuania), *Xho* I from Promega (USA), *Bam*H I from

New England Biolabs Inc. (USA). *Pfu* DNA-polymerase and T4 DNA ligase were from Fermentas. Acetylated cytochrome *c* was prepared as described [23]. Other chemicals were highest purity grade available from local sources.

RESULTS

The major aim of this study was to engineer the mutated form of cytochrome *c* that would rapidly react with superoxide-anion, whereas the reactivity towards the respiratory chain components would be lost. Figure 1 depicts the structure of horse heart cytochrome *c* and shows the amino acid residues, which were subjected to mutagenic substitutions. Obviously, at least three types of cytochrome *c* interaction with the inner mitochondrial membrane within intermembrane space exist: (i) more or less specific sorption by the phospholipids that form the outer surface of the inner mitochondrial membrane; (ii) the specific binding to cytochromes c_1 of dimeric complex III, and (iii) the specific binding to cytochrome oxidase (complex IV). Because the rate of superoxide production is evaluated as the rate of cytochrome *c* reduction, the major source of possible assay artifacts originates from the natural cytochrome *c* reductase reactions, not from the cytochrome oxidase activity, providing that the *initial* rates are to be measured. Cytochrome *c* is an alkaline protein bearing (at physiological pH values) twelve uncompensated positive charges distributed on the protein surface. The binding (dissociation) of cytochrome *c* to the inner membrane is strongly ionic strength-dependent and its heme one-electron red-ox transformation should not significantly influence the total protein charge. Nineteen lysine residues are present in the molecule. The conserved residues at the positions 8, 13, 72, 73, 86, and 87 are the major contributors in the interaction with negatively charged residues of cytochrome *c* partners, whereas the residues at the positions 5, 7, 25, 27, 79, and 88 located at the periphery of the contact area contribute less to the specific binding [32-35]. Based on these considerations, we decided to start the engineering from the substitutions of lysine and glutamic acid (to keep the total positive charge as constant as possible) residues. We constructed series of mutated genes of cytochrome *c* where positively charged lysines (K) at the positions 8, 27, 72, 86, and 87 were changed for glutamic acid (E).

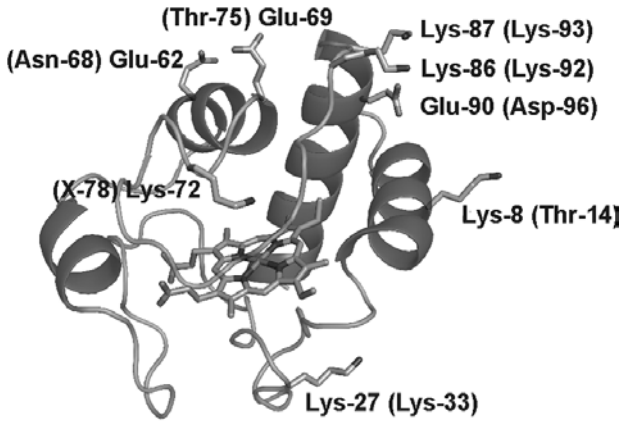

Fig. 1. Structure of horse heart cytochrome *c*. Amino acid residues subjected to substitution are indicated. Homologous amino acids in yeast cytochrome *c* are indicated in parentheses.

We took into consideration that a decrease of total positive charge is expected to decrease the rate of cytochrome *c*–superoxide anion interaction. Also, the preliminary experiments showed that insertion of only two K → E substitutions (a decrease of total charge from +12 to +8) resulted in technical difficulties in purification of recombinant proteins due to their weaker binding on a cation-exchanger column. To compensate negative charges the E at the positions 62, 69, and 90 were changed for K. Thus a number of mutated variants containing substitutions K8E, K27E, E62K, E69K, K72E, K86E, K87E, E90K in different combinations were prepared.

The succinate:cytochrome *c* reductase and cytochrome *c* oxidase activities of mitoplasts assayed with cytochrome *c* containing two (K86E/K87E) or four (K27E/K86E/K87E/E90K or E69K/K72E/K86E/K87E) substitutions were higher than those measured with acetylated cytochrome *c*, the "classical" superoxide detector [23] (the results are not shown). Figure 2 depicts the data on relative reactivities of cytochromes *c* containing six (K27E/E69K/K72E/K86E/K87E/E90K and K8E/E62K/E69K/K72E/K86E/K87E) and eight (K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) substitutions in the cytochrome *c* oxidase (a) and cytochrome *c* reductase (b) reactions. The reactivity of acetylated cytochrome *c* is also shown for comparison (Fig. 2). The direct correlation between the reactivity changes in both reactions is evident for either modified proteins.

Figure 3 shows the actual tracings during coupled succinate oxidation-supported generation of superoxide radical by inside-out submitochondrial particles as assayed with mutated (eight substitutions K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) cytochrome *c*. Under the assay conditions complex I catalyzing the energy-dependent, rotenone-sensitive succinate:oxygen oxidoreduction (reverse electron transfer) is the major contributor to the overall rate of superoxide production [8, 17] with significantly lower contribution of complex III [36-38]. It might be thought that because of structural arrangements of SMP (inside-out orientation of the inner mitochondrial membrane, cytochrome c_1 is located inside of closed vesicles) the reaction of cytochrome *c* with complex III could be neglected. However, it should be emphasized that even very small contaminations by right-side out particles and/or "open" membrane fragments can significantly contribute to the observed rates

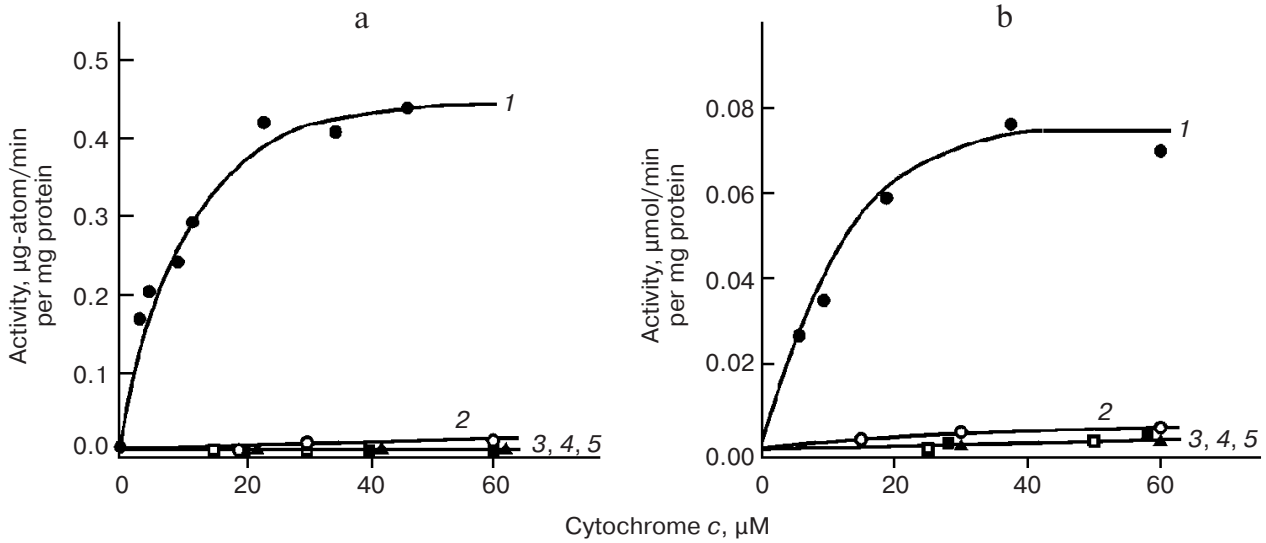

Fig. 2. Cytochrome oxidase (a) and succinate:cytochrome *c* reductase (b) activities of cytochrome *c*-deficient rat liver mitoplasts as a function of added cytochromes *c*. The activities were measured as described in the "Materials and Methods" section in the presence of horse heart cytochrome *c* (*1*), acetylated cytochrome *c* (*2*), mutant variants K8E/E62K/E69K/K72E/K86E/K87E (*3*), K27E/E69K/K72E/K86E/K87E/E90K (*4*), and K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K (*5*).

because of quantitative difference between the "normal" electron flow within the respiratory chain and one-electron "leakage" to oxygen. The maximal rates of superoxide generation by SMP are not higher than 0.1% of the total oxygen consumption rates in the succinate- or NADH-oxidase reactions [8]. Thus, negligibly small for the "normal" oxidase activities contaminating right-side out or open fragments can catalyze "natural" cytochrome *c* reduction at rates comparable or even exceeding those resulting from superoxide generation. This was confirmed when the sensitivities of mutant protein (and acetylated cytochrome *c*, not shown) reduction to SOD were checked at low and high (100 mM potassium phosphate) ionic strength (Fig. 3, a and b, respectively). In the absence of potassium phosphate, about 15% of observed cytochrome *c* reduction rate was not due to superoxide generation, whereas at high ionic strength the reduction was almost completely inhibited by SOD. It should be noted that we were not able to differentiate between complex III-dependent and direct reduction of cytochrome *c* by some component of complex I as the contributors to SOD-insensitive reaction. Whatever the origin of SOD-insensitive reaction was, the data presented in Fig. 3 show that mutated cytochrome *c* with eight substitutions (K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) is well suited for the registration of superoxide generation by the respiratory chain. This was found to be also true for the mutated forms with six substitutions (K27E/E69K/K72E/K86E/K87E/E90K or K8E/E62K/ E69K/K72E/K86E/K87E, see the data shown in the table).

The reduction of cytochrome *c* by superoxide anion is a bimolecular reaction and its rate depends equally on the concentrations of both reactants. The observed rate of cytochrome *c* reduction is equal to the rate of superoxide generation only at "saturating" concentration of cytochrome *c*. The mutations change total charge of the protein and the bimolecular rate constant for the anion (superoxide) and cation (cytochrome *c*) is expected to depend on their particular charges. With this reasoning, we decided to measure the dependencies of observed SOD-sensitive reaction rates on cytochromes *c* concentration. Figure 4 shows the results for the best (most "impaired") mutated cytochrome. High ionic strength

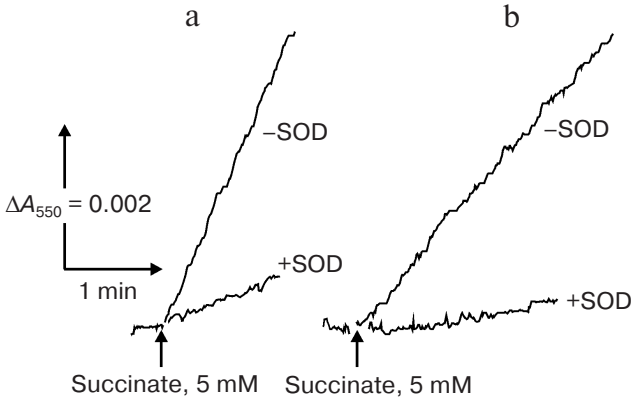

Fig. 3. Generation of superoxide radical by tightly coupled submitochondrial particles measured as reduction of 20 μM mutated (K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) variant of cytochrome *c*. The activity was measured as described in the "Materials and Methods" section in the absence (a) and in the presence (b) of 100 mM K-P_i.

Generation of superoxide and hydrogen peroxide by tightly coupled submitochondrial particles (pH 8.0, 30°C)

	Superoxide production (nmol/min per mg of SMP protein)			
	5 mM succinate		50 μM NADH	
	−K-P_i	+100 mM K-P_i	−K-P_i	+100 mM K-P_i
Acetylated cytochrome *c*	1.5 (20%)*	0.9 (40%)	2.0 (55%)	1.8 (65%)
K27E/E69K/K72E/K86E/K87E/E90K	1.4 (65%)	0.8 (80%)	2.0 (45%)	1.9 (70%)
K8E/E62K/E69K/K72E/K86E/K87E	1.4 (70%)	0.8 (85%)	2.3 (40%)	2.0 (55%)
K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K	1.4 (85%)	0.9 (90%)	2.0 (50%)	1.8 (75%)
	Hydrogen peroxide generation (nmol/min per mg of SMP protein)			
Amplex Red	–	1.1	–	1.8

* Initial rates of product formation. Contribution of SOD-sensitive reactions is given in brackets. The reactions were initiated by the addition of succinate (5 mM) or NADH (50 μM). Concentration of cytochromes *c* was 20 μM.

(100 mM K-P_i) decreased both apparent "affinity" of cytochrome *c* in the coupled registration system and the maximal reaction rate. This phenomenon was not further investigated. Most likely, high concentrations of K-P_i uncouple respiration and decrease the rate of reverse electron transfer from succinate to flavin of complex I. The direct effects of phosphate on complex I or on superoxide generation by flavin are also not excluded.

The table summarizes the data on superoxide generation by complex I in the NADH- and succinate-dependent reactions. Three mutated cytochromes *c* were suitable for quantitative measurements of superoxide generation by the respiratory chain. The rates of superoxide production with low concentrations of NADH (50 μM) as the substrate were significantly (about 1.5 times) higher than that observed upon succinate oxidation. This difference is

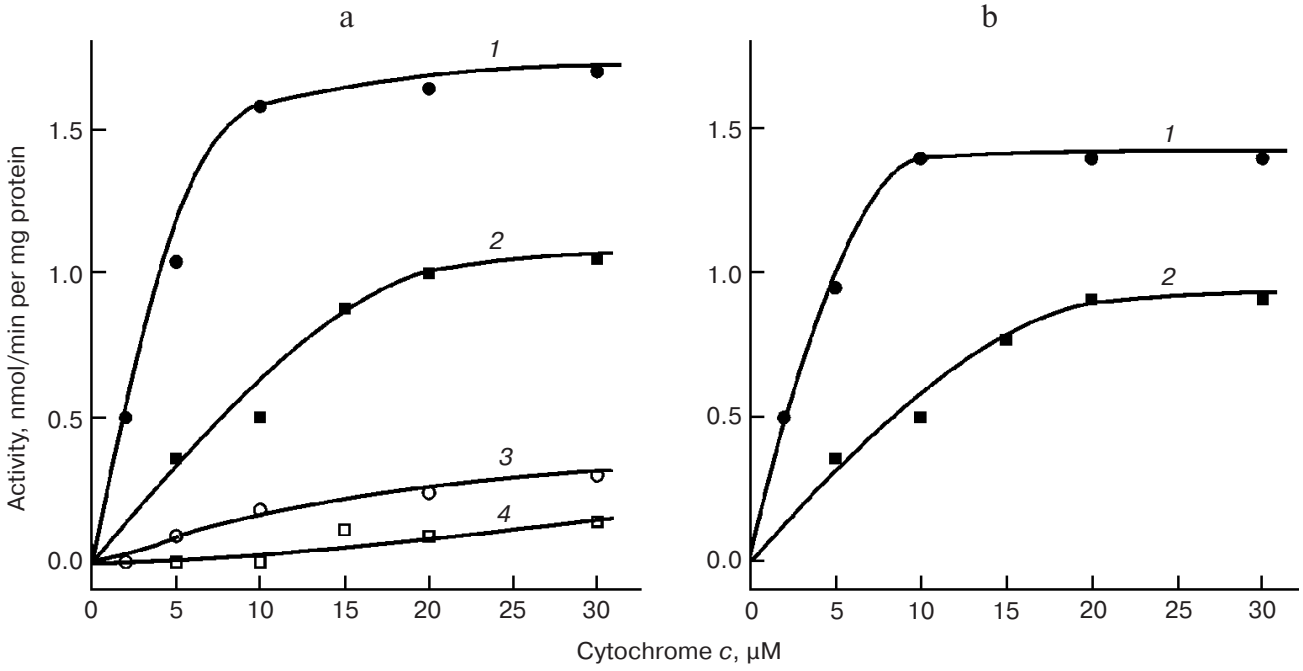

Fig. 4. Rates of reduction of K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K cytochrome *c* as a function of its concentration. a) The total succinate:cytochrome *c* reductase measured in the mixture containing 0.25 M sucrose, 50 mM Tris-HCl, pH 8.0, and 0.2 mM EDTA (with 5 mM succinate as a substrate) in the absence (*1* and *3*) or in the presence (*2* and *4*) of 100 mM potassium phosphate. Curves *3* and *4*, SOD-insensitive fractions. b) SOD-sensitive succinate:cytochrome *c* reductase (superoxide generation) in the absence (*1*) and in the presence (*2*) of 100 mM potassium phosphate.

most likely due to different degree of FMN reduction by NADH in the forward reaction (in the presence of rotenone) or by succinate in the reverse energy-dependent reaction. Significantly smaller effect of K-P_i on the NADH-dependent reaction as compared with than on the succinate-dependent reaction is in accord with the proposal on uncoupling effect of potassium phosphate.

The data on generation of hydrogen peroxide by complex I as measured by resorufin formation in the Amplex Red:H_2O_2 peroxidase reaction [31], which is the most sensitive assay for H_2O_2, are also presented in the table. The rates of hydrogen peroxide formation were about twice of those measured by cytochrome *c* reduction. It should be noted that the reduction of cytochrome *c* is a one-electron reaction, whereas two electrons are needed to reduce oxygen to hydrogen peroxide and the formation of one mol of H_2O_2 is equivalent to generation of two moles of superoxide radical (or two moles of the reduced cytochrome *c*). Oxidation of Amplex Red to resorufin at low hydrogen peroxide concentrations proceeds with the stoichiometry of 1 : 1.

DISCUSSION

We have succeeded in engineering of three mutated forms of cytochrome *c*, which are acceptable for direct measurement of superoxide generation rates in samples containing highly active natural cytochrome *c* reductases. The key criterion for the specificity of any red-ox reagent in oxidation or reduction by superoxide is the sensitivity of the reaction to SOD. The data presented in the table show the differences in this criterion for acetylated and three genetically engineered cytochromes *c*, although the measured SOD-insensitive subtracted rates were the same, as expected. The reaction rates were well reproducible for either chemically modified or for genetically mutated forms. For the analytical purpose the mutated forms, which react specifically (up to 90% SOD-sensitivity) are evidently superior over acetylated cytochrome *c*, which shows significantly lower specificity (20-65% SOD-sensitivity under different assay conditions). The mutated cytochrome *c* that bears eight substitutions (K8E/K27E/E62K/E69K/K72E/K86E/K87E/E90K) can be used as new specific probe for superoxide. Also this protein is better suited for superoxide assay in low ionic strength media than acetylated cytochrome *c*. Interestingly, the substitution of even five lysyl residues (at positions 8, 27, 72, 86, 87) does not prevent the natural reactivity of cytochrome *c* with the respiratory chain components (see SOD-insensitive reaction shown in Fig. 3).

The data on significant contribution of electrostatic interactions involved in the formation of reactive complexes of cytochrome *c* with its natural red-ox partners [35, 39, 40] and the information on the role of lysine residues positively charged cluster interactions with the negatively charged clusters in partner structures [32-35] were used for the engineered constructions reported in this paper. The aim of mutagenesis was to eliminate electrostatic interactions between cytochrome *c* and complex III having retained its total positive charge evidently needed for rapid reaction with superoxide anion. This approach happened to be sufficient for our particular goal. It should be noted, however, that all types of interaction, electrostatic, hydrophobic, hydrogen bonds, are involved in the specific protein–protein interactions and the reactive complex formation usually proceed as a multistep process [39, 41]. Recently, atomic structure of the reduced cytochrome *c* (isoform 1)–reduced dimeric complex III of *Saccharomyces cerevisiae* has been solved at 1.9 Å [42]. The structure of this "supercomplex" shows that cytochrome c_1 of complex III interacts with yeast cytochrome *c* isoforms 1 and 2 via hydrophobic residues located in the heme-containing cleft, and this interaction is stabilized by complementarily located negatively charged E201, D231, D232, and E99 of cytochrome c_1 and positively charged K17(11), K92(86), K93(87), K95(89) of cytochrome *c* (numbering of horse heart cytochrome *c* is given in parentheses). The yeast cytochrome *c* residues A87(81), V34(28), T18(12), and R19(13), which are involved in hydrophobic interactions within the complex, are not conserved and correspond to I81, T28, Q12, and K13 in horse heart cytochrome *c*; this is also true for the residues K17(11), K95(89) (responsible for electrostatic interactions) which correspond to I11 and E89, respectively, for the mammalian cytochrome. Clearly, the involvement of particular amino acid residues in the specific interactions within the mammalian complex III–cytochrome *c* supercomplex should await its atomic resolution.

An apparent quantitative discrepancy between the rates of superoxide and hydrogen peroxide generation (see the table) deserves brief discussion. If the formation of hydrogen peroxide is a secondary process resulting from the superoxide dismutase reaction only, the same rates (in terms of normalized electron equivalents) measured as cytochrome *c* reduction or resorufin formation are expected. However, the data presented in the table show that this was not the case. The simplest proposal that superoxide formed partially escapes the cytochrome *c* reduction in the spontaneous dismutation reaction, i.e. cytochrome *c* does not quench superoxide completely, contradicts the data shown in Fig. 4: the increase of cytochrome *c* concentration did not increase its SOD-sensitive reduction rate. Two conceivable explanations for apparent quantitative discrepancy can be offered. If superoxide generation ($FMNH^-$–oxygen interaction) occurs in a cavity that is not accessible for the solvent and free diffusion is limited, the spontaneous dismutation of superoxide within the cavity would result in simultaneous appearance in the surrounding medium of hydrogen peroxide and superoxide that would react with cytochrome *c*. The other possibility

is that the primary $FMNH^-$–oxygen adduct is decomposed via two pathways: one leading to hydrogen peroxide formation and the other leading to superoxide generation [43]. A possibility of the presence of two oxygen reactive sites in complex I can also not be excluded. Recently, the Cambridge group reported that the ratio between superoxide and hydrogen peroxide production rates for isolated bovine heart and *E. coli* complexes I are significantly different [44]. On the other hand, our data for the membrane-bound complex I in SMP (table) differs from those reported for isolated complex I derived from the same specie (bovine heart) [44]. This may suggest that the ratio between the rates of H_2O_2 and O_2^- generation is a parameter highly sensitive to subtle alterations of complex I structure upon its solubilization.

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