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Purified NADH-cytochrome b_5 reductase is a novel superoxide anion source inhibited by apocynin: sensitivity to nitric oxide and peroxynitrite

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ABSTRACT

Cytochrome b_5 reductase (Cb_5R) is a pleiotropic flavoprotein that catalyzes multiple one-electron reduction reactions with various redox partners in cells. In earlier work from our laboratory, we have shown its implication in the generation of reactive oxygen species (ROS), primarily a superoxide anion overshoot peak, which plays a major role as a triggering event for the acceleration of apoptosis in cerebellar granule neurons in culture. However, the results obtained in that work did not allow us to exclude the possibility that this superoxide anion production could be derived from Cb_5R acting in concert with other cellular components. In this work, we have purified Cb_5R from pig liver and we have experimentally shown that this enzyme catalyzed NADH-dependent production of superoxide anion, assayed with cytochrome *c* and nitroblue tetrazolium as detection reagents for this particular ROS. The basic kinetic parameters for this novel NADH-dependent activity of Cb_5R at 37 °C are V_{max} = 3.0 ± 0.5 μmol/min/mg of purified Cb_5R and K_M(NADH) = 2.8 ± 0.3 μM NADH. In addition, we report that apocynin, a widely used inhibitor of nonmitochondrial ROS production in mammalian cell cultures and tissues, is a potent inhibitor of purified Cb_5R activity at the concentrations used in the experiments done with cell cultures. In the presence of apocynin the K_M(NADH) value of Cb_5R increases, and docking simulations indicate that apocynin can bind to a site near to or partially overlapping the NADH binding site of Cb_5R. Other ROS, such as nitric oxide and peroxynitrite, have inhibitory effects on purified Cb_5R, providing the basis for a feedback cellular protection mechanism through modulation of excessive extramitochondrial superoxide anion production by Cb_5R. Both kinetic assays and docking simulations suggest that nitric oxide-induced nitrosylation (including covalent adduction of nitroso functional groups) of Cb_5R cysteines and peroxynitrite-induced tyrosine nitration and cysteine oxidation modified the conformation of the NADH binding domain leading to a decreased affinity of Cb_5R for NADH.

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Cytochrome b_5 reductase (Cb_5R)[1] is a flavoprotein implicated in multiple one-electron reduction reactions with various redox partners in cells and, owing to this, Cb_5R can be seen as a multifunctional and pleiotropic enzyme. The NADH-dependent Cb_5R activity is involved in multiple metabolic pathways, such as cholesterol biosynthesis [1], desaturation [2] and elongation of fatty acids [3], P450-dependent reactions [4], and methemoglobin reduction [5]. The physiological relevance of Cb_5R is well highlighted by the type II form of recessive congenital methemoglobinemia, in which a systemic deficiency of both soluble and membrane-bound isoforms of NADH-dependent Cb_5R leads to cyanosis associated with severe mental retardation and neurologic impairment [6,7].

In addition to the endoplasmic reticulum, Cb_5R has also been shown to be present in other subcellular locations [8]. Associated with the outer membrane of mitochondria, Cb_5R is the NADH-consuming component uncoupled with oxidative phosphorylation, i.e., insensitive to the respiratory chain inhibitors antimycin A, amytal, and rotenone [9]. Cb_5R bound to the plasma membrane not only is the enzyme responsible for most of the NADH-dependent

Abbreviations: Cb_5R, cytochrome b_5 reductase; Cyt *c*, cytochrome *c*; DAF, 4,5-diaminofluorescein; DMF, *N,N*-dimethylformamide; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; IgG, immunoglobulin G; NAD^+/NADH, nicotine adenine dinucleotide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EZ-Link biotin–HPDP, *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide; HRP, horseradish peroxidase; IP, immunoprecipitation; NOR-4, (±)-(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexen-1-yl-nicotinamide; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PDB, Protein Data Bank; PEG, polyethylene glycol; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene difluoride; TX-100, Triton X-100; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase

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methemoglobin reductase activity in erythrocytes but also has a prominent role in recycling endogenous antioxidants, such as vitamin E and ascorbate, using NADH as the electron donor [10,11]. In addition to erythrocytes, this protein can be found at the plasma membrane of hepatocytes [12], endothelia [13], and neurons [14]. Cb_5R is also a component of the so-called redox chain of the plasma membrane present in plant and mammalian cells [15]. More recently, it has been shown that Cb_5R clusters within cholesterol-rich and caveolin-rich lipid raft-associated submicrodomains of the plasma membrane [13,14,16]. This association is mediated by the interaction of Cb_5R with caveolins, the caveolin binding site to Cb_5R being close to the NADH binding site of this flavoprotein [17]. It is to be noted that cytochrome b_5 is a key element in cholesterol synthesis from lanosterol [18], and caveolins are known to play a relevant role in cholesterol traffic within the cells [19].

In previous work we have shown that Cb_5R can account for most of the NADH-dependent superoxide anion production of the neuronal plasma membrane, being largely responsible for the superoxide anion overshot that is observed in an early stage of the apoptosis of cerebellar granule neurons induced by extracellular potassium deprivation [14,17], a well-established model for neuronal apoptosis [20]. This ROS burst is a critical event triggering the advance of the apoptosis into the irreversible steps mediated by caspase activation [21,22]. In addition, NADPH oxidases, in particular NOX2, have been also shown to be sources of superoxide anion ($O_2^{\bullet -}$) in the plasma membrane of cells under other apoptotic stimuli [23] and in other types of neurons [24]. However, it is to be noted that one of the inhibitors more often used to discriminate between these alternate $O_2^{\bullet -}$ sources, i.e., diphenyleneiodonium, has a well-known spectrum as a pan-inhibitor of flavoproteins, including Cb_5R activity [25]. Moreover, one of the most frequently used inhibitors of NOX activity is apocynin, a member of the vanillin compounds family with inhibitory effects on the plasma membrane NADPH oxidase activity of various cell types [26]. On these grounds, the putative activity of Cb_5R as an enzymatic source of superoxide anion and the possibility that apocynin can also be an inhibitor of this enzyme deserve to be experimentally measured and assessed, as to the best of our knowledge these points have not been reported elsewhere.

On the other hand, neuronal nitric oxide synthase (nNOS) is also associated with caveolin-rich lipid raft submicrodomains of the neuronal plasma membrane [27], where Cb_5R also clusters [14,16,17]. However, the putative regulation of Cb_5R activity by nitric oxide ($NO^{\bullet}$) is an issue that has been overlooked until now, despite the fact that the recent discovery of a regulatory role of Cb_5R in controlling the release of $NO^{\bullet}$ from some forms of hemoglobin in endothelia [28] also suggests that this activity could have a cross talk regulation with different sources of $NO^{\bullet}$. Moreover, because of colocalization of nNOS and Cb_5R within caveolin-rich submicrodomains the production of superoxide anion linked to a truncated electron flux across plasma membrane-bound Cb_5R can lead to the focalized production of peroxynitrite, a very harmful cytotoxic agent [29]. Let us note that the rate constant for the reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ to produce peroxynitrite is $6.7 \times 10^9\ M^{-1}\ s^{-1}$ [30], i.e., the production of peroxynitrite is a diffusion-controlled reaction [29]. As $NO^{\bullet}$ reacts with $O_2^{\bullet -}$ threefold faster than with superoxide dismutase (SOD) ($k = 2.3 \times 10^9\ M^{-1}\ s^{-1}$), $NO^{\bullet}$ is the only known biomolecule capable of competing with SOD for available $O_2^{\bullet -}$ [31]. Therefore, micromolar concentrations of peroxynitrite are likely to be reached only in cell compartments in which $NO^{\bullet}$ and $O_2^{\bullet -}$ are produced simultaneously.

The major aims of this work were: (i) to experimentally measure and demonstrate the capability of purified Cb_5R to act as a NADH-dependent source of $O_2^{\bullet -}$; (ii) to evaluate the possibility that its contribution to the overall production of this reactive oxygen species could have been underestimated by the use of nonspecific inhibitors of other $O_2^{\bullet -}$-generating systems in cells; and (iii) to study the modulation of Cb_5R activity by the reactive nitrogen species nitric oxide and peroxynitrite.

Material and methods

Purification of microsomal Cb_5R from pig liver

To prepare fresh liver microsomes the procedure described by Mihara and Sato was followed [32]. A fresh pig liver weighing around 1.5 kg from a recently killed animal was purchased from a slaughterhouse. The liver was chopped and washed in KCl 1.15% and homogenized with a tissue grinder in 10 mM Tris-acetate buffer, 1 mM EDTA, 0.25 M sucrose, 1 mM PMSF, and 1 mM DTT (pH 7.4). After homogenization the lysate was centrifuged at 960*g* for 10 min. The supernatant was separated from the pellet and centrifuged at 18,000*g* for 15 min. The supernatant was carefully separated from the mitochondrial pellet and filtered with cheesecloth. This second supernatant was centrifuged at 105,000*g* for 60 min using a Beckman type Ti-55.2 Ti rotor at 4 °C. The microsomal pellet was suspended in storage buffer composed of 100 mM Tris-acetate, 1 mM EDTA, 0.1 mM DTT, and 20% glycerol (pH 8.1), and samples were frozen until use at −80 °C.

Solubilization of microsomes and polyethylene glycol (PEG) fractionation

For solubilization of microsomes the procedure described in [33], with recommendations defined in [34], was followed. Microsomes were diluted with storage buffer to a 25 mg/ml concentrated solution. To this solution 100 mM KCl and cholate in a ratio 2.5 to 1 with respect to the protein were added. After this step, the suspension was stirred for 1 h at 4 °C. After this time, 50% PEG6000 solution was added slowly until a final concentration of 6% was reached and left under stirring for 45 min more. The solution was later centrifuged at 9150*g* for 30 min at 4 °C. To the supernatant 50% PEG was added slowly under stirring to give a final PEG concentration of 12%. The mixture was stirred for 45 min and centrifuged at 9150*g* for 30 min at 4 °C. The sticky 6–12% precipitate was suspended in 10 mM Tris-acetate, 1 mM EDTA, 20% glycerol (pH 8.1), and 10% TX-100 was added to give a 0.5% final concentration solution to solubilize the pellets. This solution was left under stirring at 4 °C overnight.

DE-52 cellulose column

Cb_5R was separated from other solubilized proteins by the use of a DE-52 chromatographic support (Whatman) as described in [35]. A 5 × 20-cm column was equilibrated with 10 mM Tris-acetate and 1 mM EDTA (pH 8.1), and then the sample was loaded onto the column. Thereafter, a gradient was developed using 200-ml fractions composed of 10, 25, 50, 75, 100, and 200 mM Tris-acetate and 1 mM EDTA, 0.5% TX-100, and 0.2% deoxycholate (pH 8.1). The highest NADH-dependent ferricyanide reductase activity was eluted in the fractions collected after the application of 200 mM Tris-acetate buffer solution and were dialyzed at 4 °C against 10 mM potassium phosphate buffer, 1 mM EDTA, and 0.1 mM DTT (pH 6.5).

Hydroxylapatite column

The dialyzed Cb_5R was loaded onto a first hydroxylapatite column (2 × 10 cm; Calbiochem), equilibrated with 10 mM potassium phosphate (pH 6.5) and 1 mM EDTA. A gradient was developed using 10, 30, and 70 mM potassium phosphate buffer, 1 mM

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