BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

Reactive Oxygen Species Production by *Escherichia coli* Respiratory Complex I

Klaudia Frick, Marius Schulte, and Thorsten Friedrich*

Institut für Biochemie, Albert-Ludwigs-Universität, 79104 Freiburg, Germany

Supporting Information

ABSTRACT: Respiratory complex I couples the electron transfer exclusively from NADH to a quinone with the translocation of protons across the membrane. However, *Escherichia coli* adapts to imposed high cellular NADPH concentrations by selecting the mutations E183A^F and E183G^F that lead to a high catalytic efficiency of complex I with NADPH. Other mutations at position E183^F resulting in an efficient NADPH oxidation were not selected. Here we show that the naturally occurring variants exhibit a remarkably low level of production of reactive oxygen species, a byproduct of NAD(P)H oxidation, that besides high catalytic efficiency might be favored by natural selection.

The energy-converting NADH:ubiquinone oxidoreductase, respiratory complex I, couples the electron transfer from NADH to a quinone with the translocation of protons across the membrane.[1−4] Complex I is highly specific for NADH as the pyridine nucleotide substrate leaving NADPH as a very poor substrate.[5−8] NADH and NADPH are central players in redox metabolism. Despite their similar chemical structure, the nucleotides exert different metabolic functions. While NADH is engaged in catabolic processes, the major role of NADPH is to drive anabolic processes. For this reason, the NADH/NAD$^+$ couple is present in a more oxidized state in the cell than the NADPH/NADP$^+$ couple. NADH oxidation by complex I is coupled with the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide contributing to cellular oxygen stress.[9] It was proposed that ROS are produced at the nucleotide-binding site, namely the flavin cofactor, the primary electron acceptor of the complex. In mitochondria and bacteria living under normoxic conditions, ROS production comprises ∼1% of NADH oxidation (Figure 1).[10−12]

To decipher changes in metabolic networks involved in the adaptive evolution to increasing cellular NADPH concentrations, *Escherichia coli* was forced to enhance its NADPH production by the deletion of genes encoding the phosphoglucose isomerase of glycolysis and 6-phosphogluconate dehydrogenase of the Entner−Doudoroff pathway.[13] In this strain, the oxidation of 1 mol of glucose to acetyl-CoA is coupled to the production of 2 mol of NADH and NADPH, each.[13] Multiple clones adapted to the imposed NADPH stress, all by the E183A^F single mutation in complex I. (*E. coli* complex I consists of subunits NuoA−N, and the superscript marks the subunit containing the mutation.) From structural studies, it is evident that NuoF provides the NADH-binding site of complex I (Figure S1 of the Supporting Information).[14] E183^F forms two hydrogen bonds to the adenosyl-ribose in the active site of the native complex blocking the access of NADPH to the NADH-binding site.[5,13,14] The additional deletion of two genes encoding transhydrogenases that catalyze the reversible conversion of NADPH and NAD$^+$ to NADP$^+$ and NADH led to the production of 3 mol of NADPH and 1 mol of NADH by oxidation of 1 mol of glucose to acetyl-CoA.[13] *E. coli* adapted to this even higher NADPH stress by selection for the E183G^F mutation in addition to the E183A^F mutation already obtained with the other strain.[13] This excellent study led to the proposal that E183^F is a key residue in nucleotide discrimination by respiratory complex I.[13] However, the protein variants were not isolated and kinetically characterized. Variants E183D, -H, -N, and -Q^F generated by site-directed mutagenesis also showed an up to 200-fold increased catalytic efficiency with NADPH as the substrate.[5] Thus, the NADPH oxidoreductase activities of these variants were catalytically as competent as the ones that evolved under NADPH stress, but they were not positively selected.[5,13] To gain insights into the natural selection for the E183A^F and E183G^F variants at a molecular level, we here generated the corresponding mutations in *E. coli*, produced and isolated the variants, and measured the NAD(P)H-dependent activities of the preparations, including their side reaction to generate ROS.

A derivative from *E. coli* strain BW25113 was used, in which the *nuo* operon was deleted by genomic replacement methods.[5]

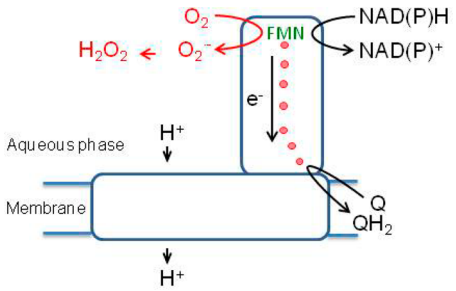

Figure 1. Scheme of respiratory complex I with the peripheral and membrane arm. The binding sites for the substrates are indicated; the iron−sulfur clusters are shown as red dots, and the quinone is abbreviated as Q. The membrane arm is involved in proton translocation. The side reaction of nucleotide oxidation at the flavin site leads to the production of ROS such as superoxide and hydrogen peroxide (red).

Received: February 17, 2015
Revised: April 15, 2015
Published: April 21, 2015

© 2015 American Chemical Society

DOI: 10.1021/acs.biochem.5b00160
Biochemistry 2015, 54, 2799−2801

Table 1. Kinetic Parameters of the NAD(P)H:Decyl-ubiquinone Oxidoreductase Activity of *E. coli* Complex I and Its Variants

	K_M (μM)		V_{max} (μmol min^{-1} mg^{-1})		k_{cat} (s^{-1})		k_{cat}/K_M (s^{-1} μM^{-1})	
variant	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH
parental	13 ± 1	1870 ± 30	2.9 ± 0.2	0.37 ± 0.1	26 ± 2	3.0 ± 1	2.0 ± 0.3	0.0018 ± 0.001
E183A^F	12 ± 1	8 ± 1	3.9 ± 0.1	3.9 ± 0.2	35 ± 1	35 ± 2	2.9 ± 0.2	4.35 ± 0.005
E183G^F	11 ± 1	33 ± 2	2.1 ± 0.1	2.4 ± 0.1	19 ± 1	21 ± 1	1.7 ± 0.2	0.65 ± 0.004
E183D^F[a]	5.8 ± 1	390 ± 30	4.2 ± 0.2	3.8 ± 0.2	37 ± 2	34 ± 2	6.4 ± 0.4	0.09 ± 0.006
E183H^F[a]	5.7 ± 1	25 ± 2	4.1 ± 0.2	1.1 ± 0.1	37 ± 2	10 ± 1	6.5 ± 0.3	0.40 ± 0.003
E183N^F[a]	14 ± 1	480 ± 50	1.2 ± 0.2	1.2 ± 0.3	11 ± 2	11 ± 3	0.8 ± 0.1	0.022 ± 0.006
E183Q^F[a]	12 ± 2	45 ± 4	3.1 ± 0.2	1.6 ± 0.1	28 ± 2	14 ± 1	2.3 ± 0.5	0.32 ± 0.003

[a]Data taken from ref 5.

Plasmid pCA24N*nuoF* was used to introduce the *nuoF* E183G and *nuoF* E183A mutations (Table S1 of the Supporting Information), and the mutations were introduced into complex I expression plasmid pBAD*nuo* using λ-Red-mediated recombineering (see the Supporting Information).[5] The complex and its variants were isolated as described previously.[5] Typical preparations are listed in Tables S2 and S3 (Supporting Information). The purity of the preparations was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Figure S2 of the Supporting Information). The NAD(P)H:decyl-ubiquinone oxidoreductase activity was measured as a decrease in the NAD(P)H concentration (see the Supporting Information). The rate of superoxide and H_2O_2 production was measured with the horseradish peroxidase-dependent oxidation of Amplex Red (see the Supporting Information).[10−12]

The E183A^F and E183G^F mutations did not significantly alter the K_M of complex I with NADH or the V_{max} of the NADH:decyl-ubiquinone oxidoreductase reaction. Thus, the catalytic efficiency of this reaction was 85% of the parental protein with the E183G^F variant and 145% with the E183A^F variant (Table 1). Impressively, the K_M toward NADPH was 60- and 230-fold increased, respectively, and the V_{max} with NADPH was enhanced 7−10-fold. The catalytic efficiency of the NADPH:decyl-ubiquinone oxidoreductase activity of the E183G^F variant was 360-fold enhanced and that of the E183A^F variant >2400-fold (Table 1). Hence, the naturally occurring mutations generate an energy-converting NADPH:ubiquinone oxidoreductase as proposed previously.[13] The E183A^F variant shows by far the highest K_M with NADPH in comparison to those of the variants that were produced by site-directed mutagenesis[5] and the E183G^F variant, the adaption to the highest imposed intracellular NADPH concentrations, the second best. The E183A^F variant is the only variant with a higher catalytic efficiency with NADPH than with NADH. Thus, a catalytically highly competent complex I evolved at elevated intracellular NADPH concentrations, and a further increase in NADPH concentration led to the additional evolution of a complex I with a lower affinity for NADPH but a still high V_{max} of the reaction (Table 1).

Although the catalytic efficiencies of the other variants investigated here such as the E183H^F variant were on the same order of magnitude as that of the E183G^F variant, these mutations were not observed during the evolutionary adaption to cellular NADPH stress.[13] We wondered whether this may be connected with the detrimental production of ROS by complex I (Figure 1 and Table 2).

In the absence of nucleotides, no ROS production was observed in the Amplex Red assay. With NADH, the *E. coli* complex I transferred approximately 1% of the electrons from NADH to O_2. This rate was 18-fold enhanced using NADPH as a substrate. The NADH-dependent ROS production was enhanced in all variants but to a different extent. While it was approximately doubled in most variants, it was 12-fold increased with the E183D^F variant. Compared to that of the parental protein, the NADPH-dependent ROS production by the E183D^F variant is nearly doubled while it is only approximately one-third with all other variants (Table 2). The E183A^F and E183G^F variants show remarkably low levels of ROS production with NADPH as a substrate that were <10% of that of the parental complex. It is noteworthy that the level of ROS production by these two variants with NADPH is even slightly lower as with NADH as a substrate.

Table 2. H_2O_2 Production per Oxidized NAD(P)H by Complex I and Its Variants

	H_2O_2 production per oxidized nucleotide (%)	
variant	with NADH	with NADPH
parental	0.8 ± 0.08	14.1 ± 0.4
E183A^F	1.6 ± 0.1	1.2 ± 0.1
E183G^F	1.6 ± 0.1	1.3 ± 0.1
E183D^F	9.5 ± 0.9	24.4 ± 1.7
E183H^F[a]	2.1 ± 0.2	3.6 ± 0.2
E183N^F	2.2 ± 0.2	4.1 ± 0.2
E183Q^F	2.1 ± 0.2	4.9 ± 0.3

[a]Data taken from ref 5.

The E183H and -Q^F variants exhibit a higher k_{cat} with NADH as the parental protein, and their level of NADH-dependent ROS production is only slightly enhanced. However, these residues are not found at position 183 in any NuoF homologue in the databases, most likely because the mutations would lead to a loss of the enzyme's specificity for NADH over NADPH.

The kinetic data indicate that the production of ROS is enhanced in variants with a low affinity for the substrate NADPH and a high turnover number. For example, the E183D^F variant with a V_{max} of 3.8 units/mg and a K_M of 390 μM produces 5 times more ROS than the E183H^F variant with a V_{max} of 1.1 units/mg and a K_M of 25 μM. With the assumption that the mutations induce only local changes in the protein structure, we propose that it is the binding of the pyridine nucleotide to subunit NuoF that determines the accessibility of oxygen to the NADH-binding side.[5] Accordingly, the mutations give raise to a conformational flexibility at the NADH-binding site, allowing several binding modes of the nucleotide that result in an easy or blocked access of oxygen to the flavin. This implies that there are several possible mutations around the NADH-binding side that lead to enhanced ROS production.

DOI: 10.1021/acs.biochem.5b00160
Biochemistry 2015, 54, 2799−2801

The function of E183^F as a molecular device to discriminate between NADH and NADPH in complex I (Figure S1 of the Supporting Information) was proven by studies involving adaptive evolution, structural biology, and site-directed mutagenesis.[5,13,14] During adaptive evolution to NADPH stress, only the E183A^F and E183G^F mutants emerged.[13] The kinetic data presented here show that these mutations lead to the highest catalytic efficiencies of complex I with NADPH of all variants investigated. The catalytic efficiency of the E183A^F variant is extraordinary compared to those of all other variants. However, the efficiency of the E183G^F variant is on the same order of magnitude as that of the E183H^F and E183Q^F variants, although the latter mutations were not observed at cellular NADPH stress.[13] The significantly low level of production of deleterious ROS by the E183A^F and E183G^F variants with NADPH as a substrate might explain why only these mutations are selected under evolutionary pressure. The 3−4-fold lower level of ROS production by the E183A^F and E183G^F variants compared to that by the E183H, -N, and -Q^F variants may be regarded as a minor difference. However, over time, enhanced ROS production might lead to the accumulation of ROS, inhibiting cell growth and contributing to cell death.[15,16] The E183A^F and E183G^F mutations ensure an efficient and productive oxidation of NADPH coupled with the generation of a proton gradient.[5] At the same time, the superoxide production is kept at a very low level to protect the cell from noxious ROS. Thus, mutations are selected according to the catalytic efficiency and in addition according to the cellular safety of the altered reaction, namely the energy-converting NADPH:ubiquinone oxidoreductase activity.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2, Tables S1−S3, and Methods. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00160.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: friedrich@bio.chemie.uni-freiburg.de.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (DFG).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Sascha Jurkovic for his help in preparing the figures.

■ REFERENCES

(1) Friedrich, T. (2014) On the mechanism of respiratory complex I. *J. Bioenerg. Biomembr. 46*, 255−268.

(2) Hirst, J. (2013) Mitochondrial complex I. *Annu. Rev. Biochem. 82*, 551−575.

(3) Sazanov, L. A. (2012) in *A structural perspective on respiratory complex I* (Sazanov, L. A., Ed.) Springer, Dordrecht, The Netherlands.

(4) Brandt, U. (2006) Energy converting NADH:quinone oxidoreductase (complex I). *Annu. Rev. Biochem. 75*, 69−92.

(5) Morina, K., Schulte, M., Hubrich, F., Dörner, K., Steimle, S., Stolpe, S., and Friedrich, T. (2011) Engineering the Respiratory Complex I to Energy-converting NADPH:ubiquinone oxidoreductase. *J. Biol. Chem. 286*, 34267−34634.

(6) Vinogradov, A. D. (1998) Catalytic properties of the mitochondrial NADH-ubiquinone oxidoreductase (complex I) and the pseudo-reversible active/inactive enzyme transition. *Biochim. Biophys. Acta 1364*, 169−185.

(7) Hatefi, Y., and Hanstein, W. G. (1973) Interactions of reduced and oxidized triphosphopyridine nucleotides with the electron-transport system of bovine heart mitochondria. *Biochemistry 12*, 3515−3522.

(8) Djavadi-Ohaniance, L., and Hatefi, Y. (1975) Oxidation of NADPH by submitochondrial particles from beef heart in complete absence of transhydrogenase activity from NADPH to NAD. *J. Biol. Chem. 250*, 9397−9403.

(9) Balaban, R. S., Nemoto, S., and Finkel, T. (2005) Mitochondria, oxidants, and ageing. *Cell 120*, 483.

(10) Kussmaul, L., and Hirst, J. (2006) The mechanism of superoxide production by NADH:ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *Proc. Natl. Acad. Sci. U.S.A. 103*, 7607−7612.

(11) Pryde, K. R., and Hirst, J. (2011) Superoxide is produced by the reduced flavin in mitochondrial complex I: A single, unified mechanism that applies during both forward and reverse electron transfer. *J. Biol. Chem. 286*, 18056−18065.

(12) Galkin, A., and Brandt, U. (2005) Superoxide radical formation by pure complex I (NADH:ubiquinone oxidoreductase) from *Yarrowia lipolytica*. *J. Biol. Chem. 280*, 30129.

(13) Auriol, C., Bestel-Corre, G., Claude, J.-B., Soucaille, P., and Meynial-Salles, I. (2011) Stress-induced evolution of *Escherichia coli* points to original concepts in respiratory cofactor selectivity. *Proc. Natl. Acad. Sci. U.S.A. 108*, 1278−1283.

(14) Berrisford, J. M., and Sazanov, L. A. (2009) Structural basis for the mechanism of respiratory complex I. *J. Biol. Chem. 284*, 29773−29783.

(15) Murphy, M. P. (2009) How mitochondria produce reactive oxygen species. *Biochem. J. 417*, 1−13.

(16) Imlay, J. A. (2015) Diagnosing oxidative stress in bacteria: Not as easy as you might think. *Curr. Opin. Microbiol. 24*, 124−131.

DOI: 10.1021/acs.biochem.5b00160
Biochemistry 2015, 54, 2799−2801