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ORIGINAL PAPER

Characterization of a soluble oxidoreductase from the thermophilic bacterium *Carboxydothermus ferrireducens*

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Abstract An NAD(P)H-dependent oxidoreductase has been purified approximately 40-fold from the soluble protein fraction of the dissimilatory iron-reducing, anaerobic, thermophilic bacterium *Carboxydothermus ferrireducens*. The enzyme, a flavoprotein, has broad-substrate specificity—reducing Fe^{3+}, Cr^{6+}, and AQDS with rates of 0.31, 0.33, and 3.3 U mg^{-1} protein and calculated NADH oxidation turnover numbers of 0.25, 0.25, and 2.5 s^{-1}, respectively. Numerous quinones are reduced via a two-electron transfer from NAD(P)H to quinone, thus participating in managing oxidative stress by avoiding the formation of semiquinone radicals.

Keywords Anaerobic bacteria · Biochemical characterization · Thermophiles and thermophilic enzymes · Oxidoreductase · Metal reduction · Oxidative stress · Gram-type positive · Flavoprotein · Quinones

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Abbreviations

NQO1	NAD(P)H:quinone oxidoreductase 1
CFOR	*Carboxydothermus ferrireducens* NAD(P)H-dependent oxidoreductase
AQDS	9,10-Anthraquinone-2,6-disulfonate
MES	2-(*N*-morpholino)ethanesulfonic acid
MOPS	3-(*N*-morpholino)propanesulfonic acid
TAPS	*N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
QH_2	Reduced quinones

Introduction

Although membrane-bound oxidoreductases, such as NADH-quinone oxidoreductases (analogous to mitochondrial complex I), are commonly found in bacteria and involved in the respiratory chain, few examples of soluble NAD(P)H-dependent oxidoreductases are known in prokaryotes (Thorn et al. 1995). In contrast to the NAD(P)H:quinone oxidoreductase 1 (NQO1) and zeta-crystallin, two of the best-studied eukaryotic examples that play important roles in oxidative stress responses (Ernster 1987; Rao et al. 1992), catalytic activities of bacterial soluble oxidoreductases are less well understood.

Bacterial soluble oxidoreductases may be involved in the electron transport chain and\or oxidative stress response. Quinones are important within the bacterial respiration and act as intermediates in cellular electron transport chains (Søballe and Poole 1999). Bacterial soluble (quinone) oxidoreductases have been proposed to prevent the formation of potentially toxic semiquinone radicals and reactive oxygen species, and homologs have been detected in numerous genomes (Wang and Maier

2004; Gonzalez et al. 2005; Mihasan et al. 2007; Chakraborty et al. 2008).

Here, we report on the properties of the purified NAD(P)H-dependent oxidoreductase (CFOR) from the cytoplasmic fraction of *Carboxydothermus ferrireducens* (Slobodkin et al. 1997), an anaerobic, thermophilic, dissimilatory iron-reducing bacterium belonging to the Phylum Firmicutes. The enzyme has a broad-substrate specificity—reduces Fe^{3+}, Cr^{6+}, AQDS, and numerous other quinones and might play some role in redox reactions and/or in minimizing oxidative stress by preventing the occurrence of semiquinones.

Materials and methods

Materials

Unless otherwise noted, all chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA. $FADH_2$ was prepared as previously described (Louie et al. 2003). Recombinant human NAD(P)H:quinone oxidoreductase, purified from *Escherichia coli* (Beall et al. 1994), was the generous gift of Dr. David Siegel, University of Colorado Health Sciences Center, Denver, Colorado, USA. The purified human NAD(P)H:quinone oxidoreductase was at a final protein concentration of 3.3 mg ml^{-1} in 25 mM Tris–HCl (pH 7.4) containing 250 mM sucrose and 5 μM FAD. Lyophilized guinea pig zeta-crystallin protein was the generous gift of Dr. J. Samuel Zigler, National Eye Institute (NIH), Bethesda, Maryland, USA. *C. ferrireducens* (DSM 11255), from the authors' laboratory culture collection, was cultivated as previously described (Slobodkin et al. 1997).

Enzyme assays

Unless otherwise noted, all enzyme assays were performed in duplicate under anoxic conditions (N_2-sparged Hungate tubes) at 50°C for 15 min. Assay mixtures for kinetic analysis (1.0 ml) contained: 20 mM Tris–HCl, and 40 mM $MgCl_2$ (pH 6.5); 0.01–10 mM NADH or NADPH; 1.0–10 μM FAD; 0.2 mM ferrozine; 0.01–500 μM Fe^{3+} citrate; and 0.5–50 μg of protein. For the determination of the pH-dependence of the enzymatic activity, a combination of buffers (20 mM MES, 20 mM Tris–HCl, 20 mM MOPS, 20 mM TAPS) was used and appropriately adjusted using 1.0 M HCl and 1.0 M NaOH. The pH^{25C} measurements were done at 25°C with a pH meter calibrated at 25°C and a pH electrode acclimated at 25°C using standards corrected for temperature. The reaction was initiated by the addition of protein. Activity was monitored on a Beckman DU-64 spectrophotometer by following the reduction of Fe^{3+} to Fe^{2+} using ferrozine (Dailey and Lascelles 1977), and NADH oxidation. Blanks, lacking either enzyme or substrate, were run routinely. The activity of the blank (reaction mixture minus the enzyme) was subtracted from all observed and reported activity assay measurements. One milliunit (mU) is the amount of enzyme catalyzing the release of 1 nmol of product per min.

Product inhibition studies were performed as previously described (Onyenwoke and Wiegel 2007). Assay mixtures for inhibition studies employing EDTA, NTA, or metals ($CuSO_4 \cdot 5H_2O$, $ZnSO_4 \cdot 7H_2O$, $NiCl_2 \cdot 6H_2O$, Na_2CrO_4, MnO_2, Na_2SeO_3, or Co-EDTA) and contained 100 nM ferric citrate, 5 μM FAD, 50 μM NADH, and 2.5 μg protein.

Decrease in NADH absorbance at A_{340} was used to follow the reduction of quinones and various other metal ions (all 50 μM) by purified CFOR in the absence of iron and ferrozine. To further corroborate the loss of NADH absorbance assays, the reduction of the following substrates were determined as described previously: 9,10-anthraquinone-2,6-disulfonate (AQDS) (Lovley et al. 1996), manganese (Brewer and Spencer 1971), cobalt (Caccavo et al. 1996), chromium (Urone 1955), and arsenic (Johnson 1971).

A quinone (50 μM 1,2-naphthoquinone) reduction assay utilizing cytochrome *c* as a trap for semiquinone generation was used to discriminate between a possible one or two electron transfer mechanism used by the CFOR. Increase in reduced cytochrome *c* was followed at A_{550} (Rao et al. 1992; Gonzalez et al. 2005). Purified CFOR was tested along with two controls: guinea pig zeta-crystallin protein (Rao et al. 1992) and human NAD(P)H:quinone oxidoreductase (Ernster 1987), which employ one- and two-electron mechanism, respectively.

Purification of a soluble oxidoreductase with Fe^{3+}-reducing activity from *C. ferrireducens*

Harvested cells were washed and re-suspended in 20 mM Tris–HCl buffer [10% (v/v) glycerol, pH 8.5] to a final concentration of 1.0 g cell mass ml^{-1}. All purification steps were performed aerobically at room temperature (~25°C) unless otherwise noted. Cell lysate was prepared from 20 g wet weight cell mass in 20 ml volume by two passages through a French pressure cell at 5,500 kPa. Cell debris and membranes were removed by stepwise centrifugation (10,000*g*; 30 min followed by 105,000*g*; 1 h; 4°C). The supernatant was used for subsequent purifications. All buffers contained 10 μM FAD. All columns and containers were wrapped in aluminum foil to prevent degradation of FAD by light. The 20 ml supernatant was applied to a Q-Sepharose Fast Flow column (1.0 × 30 cm; Amersham Biosciences, Piscataway, NJ) previously equilibrated with

buffer A (20 mM Tris buffer with 10% (v/v) glycerol, pH 8.5). Proteins were eluted using a linear gradient of 0–0.6 M NaCl in 100 ml buffer A. Proteins with Fe^{3+} reduction activity eluted between 0.3 and 0.6 M NaCl. Corresponding fractions were pooled, dialyzed (Spectra/Por 12-14 kDa cut-off dialysis tubing; Spectrum, Rancho Dominguez, CA) overnight against 3 × 400 ml buffer B (20 mM sodium phosphate, 10% (v/v) glycerol, pH 7.0) and loaded onto a HiTrap Blue HP column (1.6 × 2.5 cm; Amersham Biosciences, Piscataway, NJ) previously equilibrated with buffer B. After washing with five column volumes of buffer B, proteins were eluted with 30 ml buffer B + 1.0 mM NADH. The eluate was dialyzed overnight against 2 × 400 ml buffer B, concentrated to 5 ml using a 400 ml Amicon 8400 ultra-filtration stirred cell (Millipore, Bedford, MA) with a 10 kDa cut-off ultra-filtration Amicon PM membrane, and loaded onto a Sephacryl S-300 HR column (1.6 × 50 cm; Amersham Biosciences, Piscataway, NJ). Proteins were eluted with equilibration buffer B + 0.15 M NaCl; yielding the gel-electrophoretically pure enzyme.

Determination of molecular weight

The native molecular weight (M_r) of the enzyme was determined using gel filtration (Sephacryl S-300 HR); and apoferritin (443 kDa), alcohol dehydrogenase (150 kDa), and bovine serum albumin (66 kDa) as molecular weight standards. The subunit molecular weight was determined using SDS-PAGE [12%] (Laemmli 1970) employing a Bio-Rad broad range molecular marker kit (Hercules, CA). Proteins were stained using the Pierce (Rockford, IL) GelCode stain reagent.

Determination of flavin and metal content

The flavin content of the purified CFOR was extracted with perchloric acid and analyzed by high-pressure liquid chromatography (HPLC) using a C-18 reversed-phase column (25 × 4.6 cm) with a 20 mM ammonium acetate mobile phase (pH 6.0) containing 21% acetonitrile (v/v) at a flow rate of 0.6 ml/min (Hausinger et al. 1986). Absorbance at 280 nm was detected using a Dynamax UV detector (Rainin Instruments Company Inc., Woburn, MA). Peak areas were calculated using a DataJet SP4600 Integrator (Spectraphysics, San Jose, CA). Various concentrations of FAD (retention time = 4.88 min) and FMN (retention time = 5.71 min) were prepared as standards to identify, and to determine the amount of, any possible bound flavin. Metal content was determined using both the iron quantitation method of Fish (1988) and inductively coupled plasma mass spectrometry analysis (Chemical Analysis Laboratory, UGA, Athens, GA). Total protein was determined using the Bio-Rad protein assay kit (Richmond, CA) with bovine serum albumin as standard. The cofactor content of the CFOR was calculated based on a molecular weight of 45 kDa.

Determination of *N*-terminal amino acid sequence

For *N*-terminal amino acid sequencing, the purified CFOR was transferred from SDS-PAGE (12%) gels to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) and stained (1% Amido black). Selected bands were excised. The amino acid sequence was determined using automated Edman degradation (J. Pohl, Microchemical and Proteomics Facility, Emory University, Atlanta, GA).

Kinetic data analysis

Data were expressed as the various double-reciprocal plots and subsequent re-plots (Segel 1975). Nonlinear regression analysis was performed with the kinetic software package Aca-Stat 5.1.25.

Results and discussion

Purification and physical and biochemical characteristics of the *C. ferrireducens* oxidoreductase (CFOR)

An NAD(P)H-dependent oxidoreductase (CFOR) localized in the soluble protein fraction of *C. ferrireducens* was purified and characterized. SDS-PAGE analysis showed a single subunit of ~45 kDa, whereas the native molecular weight was ~200 kDa—suggesting a homotetrameric enzyme. Employing chromatography (Table 1), the CFOR was purified to gel-electrophoretic purity. Activity was stimulated by the addition of FAD. Either NADH or NADPH could serve as an electron donor; however activity was ~25% less when NADPH served as the electron donor instead of NADH. HPLC analysis of perchloric acid-extracted CFOR indicated each subunit contained 0.9 ± 0.09 FAD. Therefore, purification was performed in the presence of 10 μM FAD. ICP-MS analysis of purified, and distilled water-dialyzed CFOR, yielded 0.96 ± 0.04 Fe per subunit of enzyme. The ferrozine assays indicated 0.98 ± 0.05 Fe per subunit (Fish 1988).

The CFOR exhibited maximal activity around pH 6.5 and 50°C (Figs. 1a, b). Enzymatic activity was inhibited 25, 60, and 75% by 50, 100, and 1,000 μM EDTA, respectively, and 25 and 50% by 10 and 100 μM NTA, respectively. When added to ferrozine assays, 100 μM nickel inhibited enzymatic iron reduction by 60%, whereas the addition of 100 and 1,000 μM manganese increased iron reduction by 60 and 100%, respectively. The addition

Table 1 Purification of the CFOR

Purification step	Amount of protein (mg)	Total activity[a] (mU)	Sp act (mU mg^{-1})	Purification factor (fold)	Yield (%)
Soluble fraction	680	10,200	15	1	100
Q-Sepharose	99	7,920	80	5	78
Blue Sepharose	13.5	7,020	520	35	69
Sephacryl S-300 HR	4.5	2,520	560	37	25

[a] Assays contained 50 μM NADH, 5 μM FAD, and 100 nM Fe^{3+} citrate

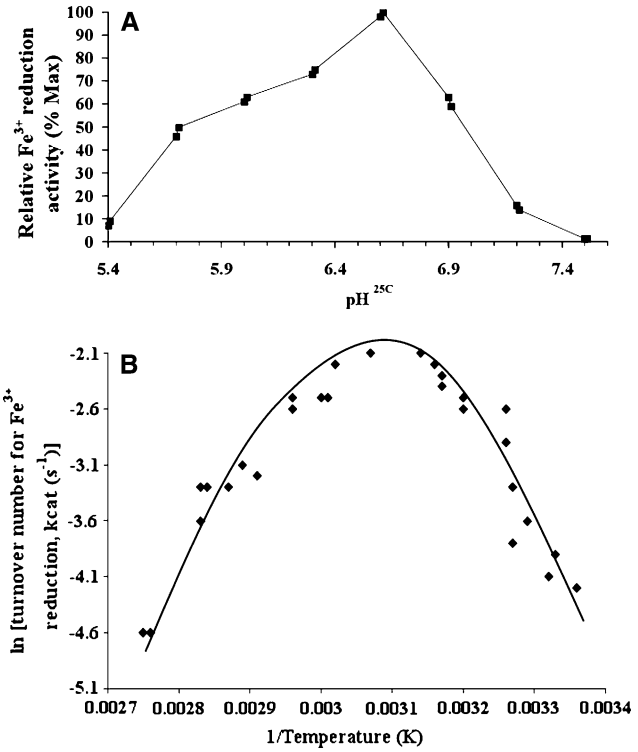

Fig. 1 The effect of pH and temperature on the CFOR. **a** The effect of pH on the NAD(P)H-dependent Fe^{3+} reduction activity of the CFOR. **b** Arrhenius plot of temperature dependence of the CFOR. Assay mixtures contained: 2.5 μg of protein, 5 μM FAD, 0.05 mM NADH, and 100 nM Fe^{3+} citrate

of 1 mM chromium, selenium, cobalt, copper, or zinc had no effect on the enzymatic activity.

Kinetic studies

Kinetic studies were performed using a three substrate, steady-state analysis (Table 2, as described by Segel (1975) and Onyenwoke and Wiegel (2007)]. Best-fit plots for (1) NADH saturation in the presence of various concentrations of Fe^{3+} citrate, and (2) Fe^{3+} citrate saturation in the presence of a series of [NADH] suggest a random sequential mechanism (Cleland 1977) for the binding of Fe^{3+} citrate and NADH. Plots of (1) FAD with a series of [Fe^{3+} citrate] and (2) AQDS and Cr^{6+} saturation in the

Table 2 Kinetic parameters of the CFOR

	K_m^{app} (μM)	V_{max}^{app} (mU mg^{-1} protein)	k_{cat}^{a} (s^{-1})
Fe^{3+} reduction			
Fe^{3+}	0.014	480	0.36
FAD	1.2	230	0.18
NADH	62	310	0.25
Cr^{6+} reduction			
Cr^{6+}	0.6	1,000	0.75
FAD	2.0	330	0.25
NADH	76	330	0.25
AQDS reduction			
AQDS	0.025	2,500	1.9
FAD	5.8	2,500	1.9
NADH	26	3,300	2.5

Data are expressed as the various double-reciprocal plots and subsequent re-plots (Segel 1975)

[a] k_{cat}s (turnover numbers) were calculated by determining the amount of CFOR, based on the native molecular weight of the enzyme (200 kDa) and assuming one catalytic site per CFOR tetramer, present in the reaction mixture

The k_{cat}s were then determined from the V_{max}^{app} values

presence of various concentrations of NADH were also indicative of a sequential reaction mechanism, although the plot for Cr^{6+} saturation indicated an ordered sequential mechanism (Cleland 1977) (detailed plots not shown).

Product inhibition studies indicated that NAD^+ was a mixed type inhibitor (Segel 1975) versus NADH with an apparent $K_I' = K_I$ when Fe^{3+} citrate was at a sub-saturating level (100 nM) and Fe^{2+} an uncompetitive inhibitor versus Fe^{3+} when NADH was present at a subsaturating level (50 μM). Taken together, these two plots indicate the reaction mechanism is ordered sequential (Segel 1975). However, the kinetics of $FADH_2$ versus FAD suggested activation instead of inhibition by $FADH_2$.

Amino acid sequence analysis of the enzyme

The *N*-terminal sequence of the CFOR, Met-Asn-Lys-Tyr-Val-His-Ala-Val-Pro-Asn-Phe, was used to search databases [SwissProt/TrEMBL (http://us.expasy.org/sprot/)

and GenBank (http://www.ncbi.nlm.nih.gov/)] for similar proteins. The *N*-terminal sequence showed 100 and 72% identity to the annotated glutamate formiminotransferase of *Carboxydothermus hydrogenoformans* (305 amino acids, predicted molecular weight ~34 kDa) (Wu et al. 2005) and phosphotransferase EII fragment of *Mycoplasma capricolum* (118 amino acids, predicted molecular weight ~13 kDa) (Bork et al. 1995), respectively. Recombinant expression of the protein was attempted using a pQE-30 vector (Qiagen, Valencia, CA) for the expression of a 6×His-tagged protein in *E. coli* strain M15[pREP4] competent cells (Qiagen, Valencia, CA). The recombinant protein was purified using Ni-affinity chromatography (Amersham Biosciences, Piscataway, NJ). The purified recombinant protein was colorless, whereas the native purified protein is a flavoprotein (yellow in color) containing FAD, and enzymatically inactive. Attempts to reconstitute the recombinant enzyme by incubating with the FAD cofactor were unsuccessful in restoring activity.

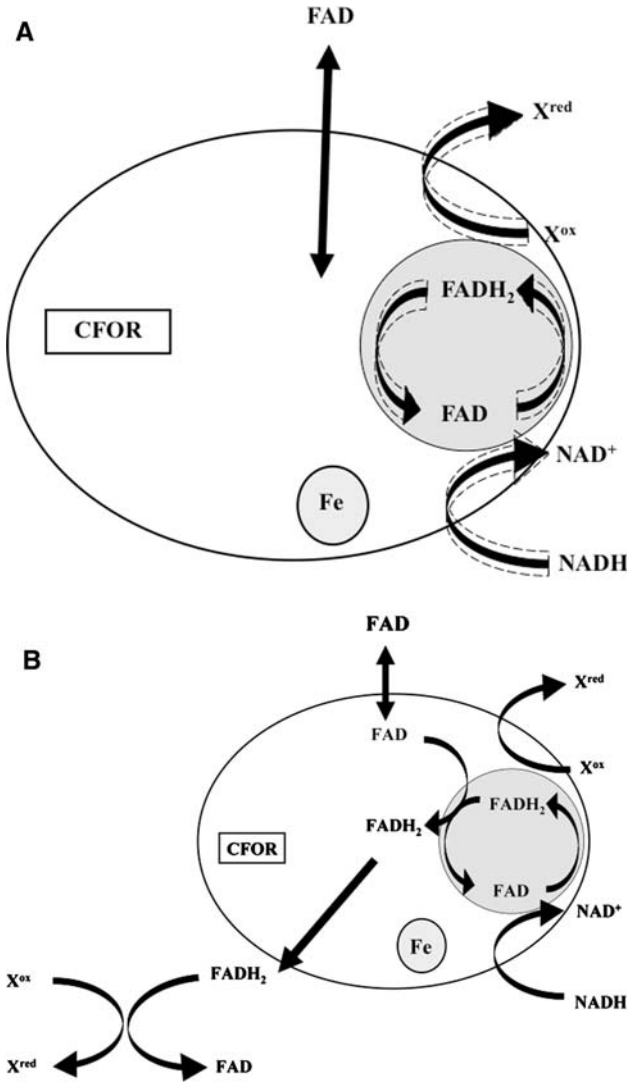

Fig. 2 Proposed mechanisms of substrate reduction by the CFOR. "*X*" refers to the substrate, e.g., Fe^{3+}, Cr^{6+}, or AQDS, reduced (X^{red}) or oxidized (X^{ox}). **a** Substrate is reduced via the oxidation of NADH with the enzyme-bound FAD (*large*, *gray circle*) serving as a cofactor, i.e., electron mediator. Bound (with proposed structural role) Fe (*smaller*, *gray circle*). Exogenous FAD (*line with arrows on both ends*) increases enzymatic activity (indicated by *thicker*, *dotted-line arrows*) and possibly occupies a secondary site on the CFOR. **b** Alternatively, exogenous FAD increases activity because the CFOR additionally functions as a flavin reductase. The exogenous FAD is reduced by the CFOR. The reduced FAD in turn reduces the substrate "*X*"

Enzymatic activities and possible physiological functions

From biochemical and kinetic data, including the broad substrate specificity, two possible mechanistic models are proposed (Fig. 2a, b).

1. The CFOR can function as an FAD reductase (Fontecave et al. 1994; Fig. 2b), as is supported by the $FADH_2$-dependent activation of Fe^{3+} reduction. $FADH_2$ could then non-enzymatically reduce compounds such as Fe^{3+}, Cr^{6+}, AQDS, or other quinones. However, the specific FAD reduction activity, 14 mU mg^{-1} protein with no additional electron acceptor added to the assay, is significantly below other observed activities (Table 3). Also, high affinity

Table 3 Enzymatic activities associated with the CFOR

NAD(P)H-dependent enzymatic activity (substrate is listed)	Avg sp act[a] (mU mg^{-1})	Relative activity[b]
AQDS reduction	2,100 ± 50	3.8
1,2-Naphthquinone reduction	870 ± 80	1.6
Cr^{6+} reduction	650 ± 30	1.2
Fe^{3+} reduction	560 ± 55	1.0
2-Hydroxy-1,4-naphthquinone reduction	300 ± 32	0.54
Duroquinone reduction	250 ± 70	0.45
As^{5+} reduction	210 ± 65	0.38
Fumarate reduction	210 ± 25	0.38
Cu^{2+} reduction	180 ± 20	0.32
1,4-Naphthquinone reduction	180 ± 14	0.32
Co^{3+} reduction	150 ± 16	0.27
Mn^{4+} reduction	130 ± 55	0.23
Benzoquinone reduction	130 ± 30	0.23
Menadione reduction	110 ± 28	0.20
Se^{6+} reduction	None detected	0

[a] Assays contained 50 μM NADH and 5 μM FAD and are the representative values of duplicate samples. Decrease in NADH absorbance at A_{340} was used to follow the reduction of the quinones and various metal ions. One milliunit (mU) is the amount of enzyme catalyzing the release of 1 nmol of product per min

[b] Represents the ratio of activity to the Fe^{3+} reductase activity

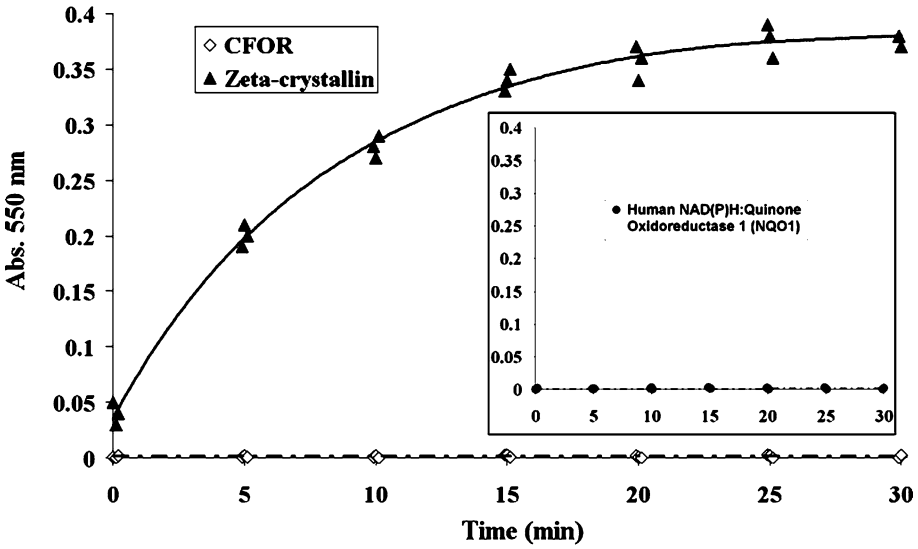

Fig. 3 Determining the mechanism of quinone reduction for the CFOR. The reduction of cytochrome *c* was monitored spectrophotometrically by the increase in absorbance at 550 nm during the reduction of 50 μM 1,2-naphthoquinone. The appearance of reduced cytochrome *c* indicates at least partial one-electron transfer, whereas the lack of this species in a reaction signifies a two-electron mode of quinone reduction. *Filled triangle* and *open diamond* indicate cytochrome *c* reduction by guinea pig zeta crystallin and the CFOR, respectively. *Inset*, the reduction of cytochrome *c* by NAD(P)H:quinone oxidoreductase (*closed circle*). Axes labeling is the same in the *inset* figure

for other substrates, and low observed K_ms for Fe^{3+}, Cr^{6+}, and AQDS, suggest a more direct role for the CFOR in their reduction.

2. The data suggest the CFOR is a quinone oxidoreductase as the highest observed activities were with quinones (Table 3). Apart from their obvious role in respiration, reduced quinones (QH_2) scavenge lipid peroxyl radicals and thereby prevent lipid peroxidation (Forsmark-Andrée et al. 1995; Søballe and Poole 1999). The soluble human NAD(P)H:quinone oxidoreductase (NQO1; EC 1.6.99.2) maintains the pool of QH_2 and other reduced quinones by catalyzing a two-electron transfer to protect cells from reactive oxygen species and semiquinones (Beyer et al. 1996). However, it should be noted that the protection provided by this mechanism will depend heavily on the reactivity of the hydroquinone species generated (Ross and Siegel 2004). In contrast, zeta-crystallin acts through an one-electron transfer process to produce a semiquinone radical that may be further reduced to a hydroquinone or oxidized back to a quinone (Thorn et al. 1995; Siegel et al. 2004). Using these enzymes as controls, the CFOR exhibited a two-electron mechanism (Fig. 3). Both NQO1 (including azo dyes, superoxide, chromium, and iron) (Thorn et al. 1995; Ernster 1987; Onyenwoke and Wiegel 2007; Siegel et al. 2004; Petrilli and de Flora 1988) and the CFOR have broad substrate spectra (Table 3). Thus, the CFOR may function analogously to NQO1 and other bacterial quinone oxidoreductases protecting against cytotoxic and carcinogenic effects (Wang and Maier 2004; Gonzalez et al. 2005; Mihasan et al. 2007; Sedláček et al. 2009).

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