J Bioenerg Biomembr (2010) 42:355–360
DOI 10.1007/s10863-010-9305-8

Essential role of copper in the activity and regular periodicity of a recombinant, tumor-associated, cell surface, growth-related and time-keeping hydroquinone (NADH) oxidase with protein disulfide-thiol interchange activity (ENOX2)

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Received: 24 March 2010 / Accepted: 8 July 2010 / Published online: 5 October 2010
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Abstract ECTO-NOX proteins are growth-related cell surface proteins that catalyze both hydroquinone or NADH oxidation and protein disulfide interchange and exhibit time-keeping and prion-like properties. A bacterially expressed truncated recombinant 46 kDa ENOX2 with full ENOX2 activity bound ca 2 moles copper and 2 moles of zinc per mole of protein. Unfolding of the protein in trifluoroacetic acid in the presence of the copper chelator bathocuproine resulted in reversible loss of both enzymatic activities and of a characteristic pattern in the Amide I to Amide II ratios determined by FTIR with restoration by added copper. The H546-V-H together with His 562 form one copper binding site and H582 represents a second copper site as determined from site-directed mutagenesis. Bound copper emerges as having an essential role in ENOX2 both for enzymatic activity and for the structural changes that underly the periodic alternations in activity that define the time-keeping cycle of the protein.

Keywords Hydroquinone (NADH) oxidase · tNOX or ENOX2 · Copper · Biological clock · Growth

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Introduction

Both animals and plants exhibit one or more hormone-responsive external plasma membrane hydroquinone oxidases (ECTO-NOX proteins) (Kishi et al. 1999) that use NADH as an alternative substrate (NADH oxidase = NOX) and carry out protein disulfide-thiol interchange (Morré 1998). There are at least two forms of ECTO-NOX or ENOX activities that may be distinguished on the basis of response to capsaicin and certain anticancer drugs. tNOX or ENOX2 (GenBank Accession No. AF207881) is drug inhibited and tumor associated (Morré et al. 1995). The other is constitutive, refractory to drugs and designated CNOX or ENOX1 (GenBank Accession No. EF432052) (Bruno et al. 1992; Jiang et al. 2008). ENOX2 activity has been found on the surface of cancer cell lines in culture (Morré et al. 1995), in cell culture media conditioned by growth of cancer cells (Morré 1995) and in sera of cancer patients (Morré et al. 1997). ENOX1, on the other hand, is present at the surface of all eukaryotic cells and tissues including non-cancer, cancer, plant and animal (Morré 1998)

as well as in conditioned culture media (Wilkinson et al. 1996). ENOX1 responds to hormones and growth factors but not to anticancer drugs. Bruno et al. (1992) compared NOX activities from plasma membranes isolated from rat liver (ENOX1) and plasma membranes from rat hepatomas (ENOX1+ENOX2). They found that the ENOX activities of the hepatoma plasma membranes were much less hormone responsive than those of liver. A similarly altered ENOX activity of the plasma membranes subsequently was observed with a variety of transformed cells and tissues (Morré 1998; Morré et al. 1995; Wang et al. 2001).

The principal characteristic used to distinguish ENOX2 activity from other ENOX activities was its response to capsaicin, a quinone-site inhibitor, and several other anticancer drugs potentially regarded as interacting with quinone sites (Morré 1998). Here, functional motifs and characteristics are reported for recombinant ENOX2 from cDNA obtained by expression cloning of a HeLa library using a ENOX2-specific monoclonal antibody. The findings show that the activity of this cell surface growth-related and time-keeping protein of cancer cells is dependent on the presence of a metal, most likely copper.

Single amino acid substitutions in histidines critical to the formation of potential copper binding ligands as well as reversible metal removal with the copper chelator bathocuproine and restoration of activity by readdition of copper provide additional evidence for an essential role for copper in both the overall oxidative activity catalyzed by the protein and in the periodic alternation of activities and secondary structural changes that characterize both ENOX2 and ENOX proteins in general.

The finding support a model depicting ENOX2 as a dimeric protein containing 4 coppers/dimer capable of carrying out concerted four electron transfers from NADH or ubiquinol to molecular oxygen as required to form water as occurs during the oxidative portion of the ENOX2 catalytic cycle (Orczyk et al. 2005).

Materials and methods

Recombinant ENOX2 protein was prepared from *E. coli* cells transfected with pET 43.1 a (+) reporter plasmid carrying the cDNA for the 46 kD truncated form of ENOX2 (nt 680 to 1855; aa 220 to 610) carrying Nus and His tags. Cells were grown in LB broth with ampicillin overnight, then lysed by French Press and insoluble material was removed by centrifugation. The pellet and supernatant were analyzed for the presence of tENOX2 protein by western blot analysis and silver staining.

The Nus- and His-tagged proteins were purified from the supernatant using a Ni-NTA Superflow column (QIAGEN) according to the manufacturer's protocol. Briefly, after equilibrating the column with 10 ml Buffer I (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0), the supernatant of the lysate was transferred to the equilibrated column and washed twice by 10 ml Buffer II (50 mM NaH_2PO_4, 300 mM NaCl and 200 mM imidazole, pH 8.0). The ENOX2 proteins were eluted with 50 mM NaH_2PO_4 containing 300 mM NaCl and 200 mM imidazole, pH 8.0.

The stcENOX2 (short truncated C-terminus ENOX2) protein is composed of 101 amino acids (starts with methionine and followed by ENOX2 K502 to F601). The calculated molecular weight is 11.47 kDa.

stcENOX2 was expressed in *E. coli* strain BL21 (DE3) transformed with plasmid—pET11-stcENOX2 nt 1526 to 1823). Bacterial cultures of LB medium containing ampicillin were grown to an OD_{600} of 0.8. Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.5 mM and the culture was grown for another 3 h. Cells were harvested by centrifugation and then lysed by French Press. Pellets (inclusion bodies) were collected after centrifugation and solubilized and refolded. Refolding of stcENOX2 was according to the procedure provided by Novagen (Madison, WI, USA). Briefly, the pellet was washed with 1% Triton X-100 and the recombinant stcENOX2 proteins were dissolved in 50 mM CAPS, pH 11, containing 0.3% *N*-laurylsarcosine (sodium salt). Proteins were then dialyzed in two changes of dialysis buffer (20 mM Tris-HCl, pH 8.5 containing 0.5 mM cysteamine and 0.05 mM cystamine) over a period of 14 h followed by dialysis in two changes of equilibration buffer (20 mM Tris-HCl, pH 7.5, 0.1 mM DTT). After dialysis, stcENOX2 protein was stored at −20°C.

Oligonucleotides to replace amino acid residues potentially involved in metal binding by site-directed mutagenesis were designed according to Braman et al. (1996). Histidines in the potential copper-binding domain were replaced by alanines (H246A, H546A, H562A, H582A) and a tyrosine was replaced by a phenylalanine (Y556F). Oligonucleotides for mutagenesis were as follows: H546A:5'-GGATTATCTCCACATTCCTTGCGGTTCACCCATTTGGAGC-3' (forward), 5'-GCTCCAAATGGGTGAACCGCAAGGAATGTGGAGATAATCC-3' (reverse); H562A: 5'-GAATACATCTGTTCCTACTTGGCCCGTCTTGATAATAAGATCTGC-3'(forward), 5'- GCAGATCTTATTATCAAGACGGGCCAAGTAGGAACAGATGTATTC-3' (reverse); H246A: 5'CCACCCCCAGTGGTCGCCTATTCAGATCATGAATGC-3' (forward), 5'-GCATTCATGATCTGAATAGGCGACCACTGGGGGTGG (reverse); H582A: 5'- CTCATGGGTAGACTCCAGGCTACCTTCAAGCAGGAAATG-3' (forward), 5'-CATTTCCTGCTTGAAGGTAGCCTGGAGTCTACCCATGAG-3' (reverse); Y556F: 5'- GCATTGAATACATCTGTTCCTTCTTG

CACCGTCTTG-3’ (forward), 5’- CAAGACGGTGCAA GAAGGAA CAGATGTATTCAATGC-3’ (reverse).

The mutagenesis was carried out using a QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All mutants were sent for DNA sequencing to confirm the correct replacements.

NADH oxidation was measured spectrophotometrically from the disappearance of NADH measured at 340 nm (Morré et al. 1995) or from the cleavage of dithiodipyridine (DTDP) (Morré et al. 1999). Proportionality of enzymatic activity to protein concentration with the purified tENOX2 or with the bacterial extracts was verified for both assays.

Proteins were estimated by the bicinchoninic acid (BCA) procedure (Smith et al. 1985) with bovine serum albumin as standard.

For unfolding and refolding, recombinant ENOX2 protein 46 kD (100 μg in a total volume of 200 μl) was combined with 0.2 μl trifluoroacetic acid and 0.4 ml 20 mM metal solution or water with or without 25 μM bathocuproine. After a 2 h incubation at room temperature, the material was dialyzed for 2 days against 15 mM Tris-MES, pH 7 and lyophilized.

ENOX2 protein from sera (the antigen sources for the monoclonal antibody) and recombinant ENOX2 were analyzed for copper, nickel, zinc and iron content by atomic absorption spectroscopy (AAnalyst #300, Perkin-Elmer Instruments, Shelton, CT). Standards were diluted from 1 g/L metal stock (Aldrich, Milwaukee, WI). To measure copper binding, ENOX2 and $CuCl_2$ were preincubated for 24 h at 4°C at a 10-fold molar ratio of excess $CuCl_2$. Samples were then dialyzed for 48 h against four changes of 20 mM Tris-HCl, pH 7.8. Protein samples analyzed contained between 0.5 μg/ml and 2 μg/ml bound copper, zinc or added nickel but no iron and were within the linear range of the assay.

The copper amounts of purified Nus tag protein and mutant proteins were determined by a colorimetric assay. To 100 μl protein (ca. 10 μM) samples were added to 25 μl 0.3 g/ml trichloroacetic acid, 25 μl 0.35 mg/ml L-dehydroascorbic acid and 160 μl 0.067 mg/ml acid disodium bicinchoninic containing 0.04 mg/ml NaOH and 0.17 mg/ml HEPES sodium salt. The mixture was incubated at room temperature overnight. Distilled water (2 ml) then was added and the absorbance values were recorded at 354 nm. The copper contents were estimated from a standard copper series from 5 μM to 30 μM assayed in parallel (Brenner and Harris 1995).

Infrared spectra were taken from recombinant tENOX2 solvated in deuterium oxide to minimize water absorption in the amide I/amide II region of the spectrum (1,700 to 1,499 cm^{-1}) with a Perkin-Elmer Fourier Transform Infrared Spectrophotometer (Model 1750). The data were analyzed using appropriate software and a workstation. Spectra were collected at 2 min to 3 min intervals and co-added over two to four successive cycles of oscillation. CaF_2 windows had a Teflon 9 mm spacer to provide a sealed, demountable cell. Data were collected over the IR region 4,000 cm^{-1} to 6,000 cm^{-1}. Regions of interest were copied, converted to absorbance and processed computationally for analysis. Real time full spectra were recorded both as a function of protein concentration and at different temperatures.

Results

Copper presence was necessary for ENOX2 activity (Fig. 1). In the folded native state both the recombinant and the cell surface-associated ENOX2 were refractory to inhibition by the copper chelator bathocuproine. The relative resistance of the ENOX2 activity to inhibition by the copper chelator bathocuproine suggests that the bound copper is located within a protected portion of the protein which is inaccessible to the chelator. It was first necessary to unfold the protein for the bathocuproine to bind and remove the ENOX2-bound copper. Nus-tagged ENOX2 when unfolded in the presence of trifluoroacetic acid retained activity after dialysis and at physiological pH. However, if the ENOX2 was unfolded by trifluoroacetic acid treatment in the presence of the copper chelator

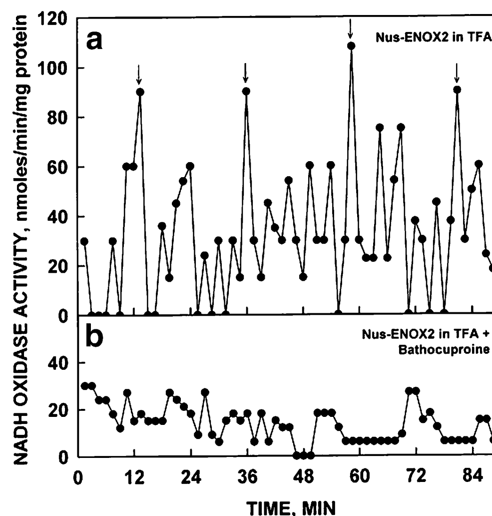

Fig. 1 Nus-ENOX2 activity unfolded in the presence of 0.1% trifluoroacetic acid (TFA) **a** or in the presence of 25 μM bathocuproine to remove bound copper (**b**). For each set of measurements, the TFA, bathocuproine and released copper were removed by dialysis prior to assay. Upon removal of the TFA by dialysis, activity in the absence of bathocuproine was retained (**a**). Upon removal of TFA from the bathocuproine-treated sample no activity was observed (**b**) but activity was restored by addition of 100 μM $Cu^{II}Cl_2$

bathocuproine, activity was lost. Activity was subsequently restored by refolding in the presence of copper at physiological pH (not shown). Refolding in the absence of copper did not restore activity.

Recombinant 46 kDa Nus-ENOX2 when refolded in the presence of excess copper followed by exhaustive dialysis to remove unbound copper yielded 2.1±0.4 mole of bound copper/mole of recombinant protein (Table 1). In the presence of stoichiometric amounts of copper during refolding, 2.3±0.2 mole copper was bound/mole of protein. Similar results were obtained with a short truncated C-terminus ENOX2 that contained the two putative copper binding sites determined from site-directed mutagenesis (see below). With bathocuproine present during refolding, in the absence of added copper, 0.8±0.2 mol of copper remained per mol of protein (Table 1), an amount insufficient to support enzymatic activity (Fig. 1).

Excess copper caused the ENOX2 protein to form aggregates. Aggregates were typically amyloid fibrils as shown by electron microscopy (del Castillo-Olivares et al. 1998) and were readily collected by centrifugation at 10,000×*g*. This response to copper may be different from copper bound in the catalytic sites.

In addition to 2 moles of copper per mole of protein, recombinant ENOX2 contained 2 moles of zinc per mole of protein but no iron or nickel (Table 2). The presence of iron or nickel above the limit of detection of 0.02 nmoles or 0.00015 nmoles/mole protein, respectively, were not observed. With copper-loaded recombinant ENOX2, 2 moles nickel/mole of protein were bound with 20 mM nickel added. In the absence of added nickel the amount present was less than 150 µmoles/mole of ENOX2. When assayed in the presence of 20 mM copper, nickel or zinc without refolding or prior metal removal, the specific activities observed were 63, 48 and 53 nmole/min/mg protein and not significantly different. With copper and zinc, the period length observed was 22 min. With nickel addition, the period length increased from 22 min to 24 min.

A further indication of the importance of copper to ENOX2 function was provided by experiments where changes in Amide I to Amide II ratios were monitored by FTIR (Fig. 2). With the wild type, the ratio varied between 1.70 and 1.72 generating a regular recurring pattern of maxima (Fig. 2a). After bathocuproine treatment, a more random pattern of change with a much reduced amplitude was observed (Fig. 2b). Also with the H562A and the H564A replacements, no regular pattern of oscillations in Amide I-Amide II ratio could be discerned (not shown).

Four amino acid substitutions in full length ENOX2 H246A, H546A, H562A, H582A and H546A and H582A in 46 kDa truncated ENOX2 resulted in loss of NADH oxidase activity (Table 3, Fig. 3). With the H546A, H562A and H582A substitutions, ≤ 1 mol copper/mol protein was bound compared to 1.7±0.3 for wild type and 1.8±0.2 for the Y556F substitution which did not affect ENOX2 activity (Table 4).

Table 2 Zinc, iron and nickel contents of recombinant ENOX2

Metal	Moles metal/mole protein
Zn (*n*=6)	2.2±0.7
Fe (*n*=6)	0.02±0.02
Ni (*n*=3)	<0.00015

Table 1 Bound copper comparing Nus-tagged recombinant ENOX2 and short truncated C-terminus ENOX2 (stcENOX2)

	µmoles		Ratio Cu:Protein
	Protein	Copper	
Nus-tagged ENOX2			
TFA only	2.0±0.2	4.6±0.5	2.3±0.2
TFA + Bathocuproine	4.2±2.0	3.5±2.0	0.8±0.2
TFA + $CuCl_2$	5.0±3.0	10.3±3.0	2.1±0.4
stcENOX2			
TFA only	23±8	39±9	1.7±2
TFA + Bathocuproine	23±2	18±1	0.8±0
TFA + $CuCl_2$	14±3	35±2	2.5±0.7
stcENOX2 H546A+H582A			
TFA only	39.5±0.4	9.1±0.4	0.23±0.01
TFA + Bathocuproine	48.0±1.3	10.2±0.4	0.25±0.03
TFA + $CuCl_2$	37.7±1.7	9.1±0.3	0.24±0.02

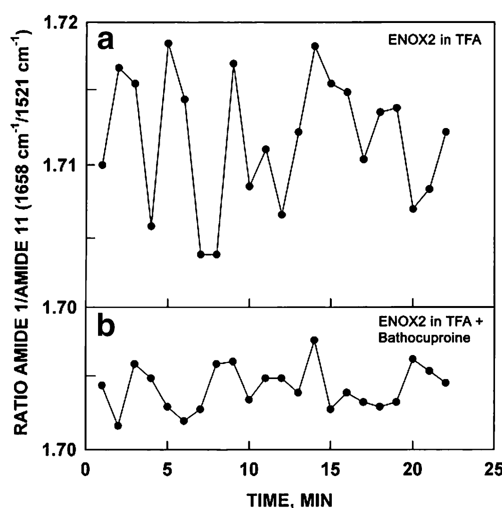

Fig. 2 As in Fig. 1 except ratios of Amide I to Amide II of recombinant Nus-ENOX2

Table 3 Specific activities of mutated recombinant Nus-tagged ENOX2 to identify copper binding sites

Protein	N	nmol/min/mg
WT	8	50±10
H246A	3	6±6[a]
H546A	3	8±8[a]
Y556F	3	60±8
Y560A	6	50±13
H562A	3	8±8[a]
H582A	6	10±10[a]
H546A+H582A	3	5±4[a]

[a] Significantly different from wild type (WT) $p<0.001$

Table 4 Bound copper comparing Nus-tagged tENOX2 and mutant ENOX2 proteins. (n=4)

	Protein (μM)	Copper (μM)	Ratio
WT	8.7	15±3	1.7±0.3
H246A	22.4	24±6	1.1±0.3
H546A	11.4	10±1	0.9±0.1
Y556F	8.0	14±2	1.8±0.2
Y560A	14.0	31±5	2.2±0.3
H562A	2.5	2.4	0.9[a]
H582A	22.6	23±2	1.0±0.1
H546A+H582A	13.8	4±1	0.3±0.1

[a] Determined independently by inductively coupled plasma atomic emission spectroscopy

Discussion

We have reported previously that both ENOX2 isolated from patient sera or recombinant ENOX2 contained bound copper but no zinc or iron from analyses by atomic absorption spectroscopy (Chueh et al. 2002). Additionally, the presence of Cu^{II} in ENOX2 has been confirmed by extended X-ray fine structure analysis (Morré et al. 2007). Pre-edge at the Cu K-edge and EXAFS K^2 weighted ($X^{(k)}$) spectra for a 0.1 M aqueous solution of $CuCl_2$ at 25°C and 170 bar in the presence or absence of ENOX2 with or without NADH revealed the sole species observed was the hexaaqua Cu^{II}. Measurements were recorded every 1.5 min over 90 min.

The spectral changes observed by FTIR were assumed to represent oscillating α-helix-β-sheet transitions. In these experiments absorbance was determined at 1,658 cm^{-1} as a measure of Amide I absorption and compared to Amide II absorption at 1,521 cm^{-1}. Generally, the changes observed in Amide I and in Amide II appeared to be related inversely (Kim et al. 2005). Because of the apparent inverse relationship and also for the Amide II absorbance to serve as a reference, the ratios of absorbance at 1,658 cm^{-1} snf 1,521 cm^{-1} were calculated to determine the oscillatory pattern. The maxima occurred at intervals of about 4.2 min but were not evenly spaced and created a 5-peak pattern where two of the maxima were separated by 5 min and 3 of the maxima were separated by 4 to 4.5 min. This asymmetric 5-peak pattern is characteristic of ENOX-related activity oscillations (Morré and Morré 2003). The observed spectral changes are reproducible and offer a means of functional assessment not afforded by other methods of assay. Since the protein appears to undergo such oscillatory changes in secondary structure within each 22 min cycle, crystallization of the protein may be difficult due to the constant motion. From this standpoint the availability of the H546A+H582A double mutant may facilitate crystallization of the recombinant protein for structural studies.

The H546-V-H-P-F-G motif is conserved in the copper-binding site of both human and chicken superoxide dismutases (Shininá et al. 1996) although ENOX proteins lack superoxide dismutase activity. The H546V together with the His562 was suggested to serve as a binding site potentially important to the reduction of oxygen (Chueh et al. 2002). The ability of recombinant protein to bind copper in excess of 1 mol copper/mol of protein (1.3 to 1.5 mol copper even in the absence of unfolding and refolding in the presence of excess copper followed by dialysis), suggests the presence of a second site important for oxygen binding. For example, the H582A replacement resulted in loss of enzymatic activity as copper binding capacity was reduced from about 2 mol copper/mol protein to about 1 mol copper/mol protein. The double mutant H546A+H582A reduced the copper binding capacity even further to 0.3 mol copper/mol protein.

The H246 site found in full length recombinant ENOX2 (Fig. 3) does not appear in either the truncated ENOX2 proteins with full activity analyzed or in the 34 kDa functional ENOX2 corresponding to the ENOX2 protein

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  1 MQRDFRWLWV YEIGYAADNS RTLNVDSTAM TLPMSDPTAW ATAMNNLGMA
 51 PLGIAGQPIL PDFDPALGMM TGIPPITPMM PGLGIVPPPI PPDMPVVKEI
101 IHCKSCTLFP PNPNLPPPAT RERPPGCKTV FVGGLPENGT EQIIVEVFEQ
151 CGEIIAIRKS KKNFCHIRFA EEYMVDKALY LSGYRIRLGS STDKKDTGRL
                                                  246
201 HVDFAQARDD LYEWECKQRM LAREERHRRR MEEERLRPPS PPPVVHYSDH
251 ECSIVAEKLK DDSKFSEAVQ TLLTWIERGE VNRRSANNFY SMIQSANSHV
301 RRLVNEKAAH EKDMEEAKEK FKQALSGILI QFEQIVAVYH SASKQKAWDH
351 FTKAQRKNIS VWCKQAEEIR NIHNDELMGI RREEEMEMSD DEIEEMTETK
401 ETEESALVSQ AEALKEENDS LRWQLDAYRN EVELLKQEQG KVHREDDPNK
451 EQQLKLLQQA LQGMQQHLLK VQEEYKKKEA ELEKLKDDKL QVEKMLENLK
                                                  546
501 EKESCASRLC ASNQDSEYPL EKTMNSSPIK SEREALLVGI ISTFLHVHPF
             562                    582
551 GASIEYICSY LHRLDNKICT SDVECLMGRL QHTFKQEMTG VGASLEKRWK
601 FCGFEGLKLT
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Fig. 3 Full length amino acid sequence of the ENOX2 protein. Histidine to alanines substitutions by site-directed mutagenesis resulting in loss of NADH oxidase activity are *underlined*

expressed by the cancer-specific exon 4 minus splice variant (Tang et al. 2007).

Despite the fact that ENOX proteins lack flavin and/or electron carriers other than copper (Chueh et al. 2002), they do reduce molecular oxygen to water (Chueh et al. 2002). Thus, during the oxidative portion of the ENOX activity cycle, the transfer of electrons and protons to molecular oxygen is catalyzed with a stoichiometry of 2 mol of $NADH+H^+$ oxidized to 1 mol of O_2 reduced. Oxygen uptake by plasma membranes prepared from HeLa cells is inhibited by the antitumor sulfonylurea LY181984 with approximately the same dose response (Fig. 1 of Morré et al. 1998) as are other aspects of ENOX2 activity (compare to Fig. 6 of Tang et al. 2007). Thus, ENOX2 and ENOX proteins in general must bind oxygen. While some role for zinc in oxygen binding not involving a change in oxidation state cannot be completely ruled out, addition of only copper was sufficient to restore full activity to the ENOX2 proteins depleted of metals by unfolding in the presence of bathocuproene. The absence of iron or iron-binding motifs in the ENOX2 proteins adds to the argument for copper involvement in their oxidative activities (MacBeth et al. 2000). Zinc is normally coordinated by two cys and two his ligands or by four cys ligands (Lee et al. 1989). Two such cys domains, C103XXC and C506XXXC occur in ENOX2. However, the consensus sequence for zinc finger domains $YXCX_{2,4}CX_3FX_3X_2HX_{3-5}H$ is missing from ENOX2.

The possibility of a second copper site is supported as well in that the ENOX2 protein bound approximately 2 mol of copper/mol protein. As the prevailing soluble form of ENOX2 proteins appears to be a dimer based on size exclusion chromatography (del Castillo-Olivares et al. 1998), the presence of two coppers per monomer permits a model depicting ENOX2 as a dimeric protein containing four coppers/dimer capable of carrying out concerted four electron transfers from NADH or reduced coenzyme Q directly to molecular oxygen as required to form water.

The constitutive form of the ENOX protein, CNOX or ENOX1, has been cloned and characterized (GenBank Accession No. EF432052). The sequence was similar to that of ENOX2 (64% identity and 80% similarity). ENOX1 required bound copper for activity and bound 2 mol copper/mol protein (Jiang et al. 2008) as well. An H579VHPFG sequence of ENOX1 corresponding to H546VHPFG sequence of ENOX2 was present and required for activity. His562 is absent from ENOX1. A second site, H260YSEH, present in ENOX1 when mutated also resulted in loss of enzymatic activity. The H260YSEH site is represented in ENOX2 by the sequence H246YSDH. His582 is missing from the structure of ENOX1. Thus, both ENOX1 and ENOX2 have been shown experimentally to bind at least 2 mol copper/mol protein with the HVHPFG site being common to both. ENOX1 lacks His562 and His582 whereas 46 kDa ENOX2 which exhibits full enzymatic activity lacks the H260YS(E,D)H site common to both full length proteins due to the truncation.

Acknowledgements We thank Ania Kempa-Steczko, Bernard Shen, Thomas DeLuca, Emily Bacon, Lian-Ying Wu, Kevin Kowalski, Thomas Evans, Juliana Lawler, and Elyse Freiberger for contributing to the findings and Peggy Runck for assistance with preparation of the manuscript.

References

Braman J, Papworth C, Greener A (1996) Meth Mol Biol 57:31–44
Brenner AJ, Harris ED (1995) Anal Biochem 226:80–84
Bruno M, Brightman AO, Lawrence J, Werderitsh D, Morré DM, Morré DJ (1992) Biochem J 284:625–628
Chueh P-J, Kim C, Cho N, Morré DM, Morré DJ (2002) Biochemistry 41:3732–3741
del Castillo-Olivares A, Yantiri F, Cheuh P-J, Wang S, Sweeting M, Sedlak D, Morré DM, Burgess J, Morré DJ (1998) Arch Biochem Biophys 358:125–140
Jiang Z, Gorenstein NM, Morré DM, Morré DJ (2008) Biochemistry 47:14028–14038
Kim C, Layman S, Morré DM, Morré DJ (2005) Dose Response 3:391–413
Kishi T, Morré DM, Morré DJ (1999) Biochim Biophys Acta 1412:66–77
Lee MS, Gippert GP, Soman KV, Case DA, Wright PE (1989) Science 245:635–637
MacBeth CE, Golombek AP, Young VG Jr, Yang C, Kuczera K, Hendrich MP, Borovik AS (2000) Science 289:938–941
Morré DJ (1995) Biochim Biophys Acta 1240:201–208
Morré DJ (1998) In: Asard E, Bérczi A, Caubergs RJ (eds) Plasma membrane redox systems and their role in biological stress and disease. Kluwer Academic, Dordrecht, pp 121–156
Morré DJ, Morré DM (2003) Free Radical Res 37:795–808
Morré DJ, Chueh P-J, Morré DM (1995) Proc Natl Acad Sci USA 92:1831–1835
Morré DJ, Caldwell S, Mayorga A, Wu L-Y, Morré DM (1997) Arch Biochem Biophys 342:224–230
Morré DJ, Chueh P-J, Lawler J, Morré DM (1998) J Bioenerg Biomemb 30:477–487
Morré DJ, Gomez-Rey ML, Schramke C, Em O, Lawler J, Hobeck J, Morré DM (1999) Mol Cell Biochem 207:7–13
Morré DJ, Heald S, Coleman J, Orczyk J, Zhang Z, Morré DM (2007) J Inorg Biochem 101:715–726
Orczyk J, Morré DM, Morré DJ (2005) Mol Cell Biochem 273:161–167
Shininá ME, Carlini P, Polticelli F, Zappacosta F, Bossa F, Calabrese FL (1996) Eur J Biochem 237:433–439
Smith PK, Krohn RI, Hermanson GT, Mailia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Anal Biochem 150:70–76
Tang X, Tian Z, Chueh P-J, Chen S, Morré DM, Morré DJ (2007) Biochemistry 46:12337–12346
Wang S, Pogue R, Morré DM, Morré DJ (2001) Biochim Biophys Acta 1539:192–204
Wilkinson FE, Kim C, Cho N, Chueh P-J, Leslie S, Moya-Camarena S, Wu L-Y, Morré DM, Morré DJ (1996) Arch Biochem Biophys 336:275–282