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Review

Cell Surface NADH Oxidases (ECTO-NOX Proteins) with Roles in Cancer, Cellular Time-keeping, Growth, Aging and Neurodegenerative Diseases

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ECTO-NOX (because of their cell surface location) proteins comprise a family of NAD(P)H oxidases of plants and animals that exhibit both oxidative and protein disulfide isomerase-like activities. The two biochemical activities, hydroquinone [NAD(P)H] oxidation and protein disulfide–thiol interchange alternate, a property unprecedented in the biochemical literature. A tumor-associated ECTO-NOX (tNOX) is cancer-specific and drug-responsive. The constitutive ECTO-NOX (CNOX) is ubiquitous and refractory to drugs. The physiological substrate for the oxidative activity appears to be hydroquinones of the plasma membrane such as reduced coenzyme Q_{10}. ECTO-NOX proteins are growth-related and drive cell enlargement. Also indicated are roles in aging and in neurodegenerative diseases. The regular pattern of oscillations appears to be related to α-helix-β-structure transitions and serves biochemical core oscillator of the cellular biological clock. Period length is independent of temperature (temperature compensated) and synchrony is achieved through entrainment.

Keywords: ECTO-NOX; Coenzyme Q_{10}; Protein disulfide–thiol interchange; Cancer; Biological clock; Aging; Neurodegenerative diseases

INTRODUCTION

Our laboratory has described a family of NAD(P)H oxidase (NOX) proteins that exhibit both an oxidative and a protein disulfide isomerase-like activity.[1,2] These proteins are characterized by the property, unprecedented in the biochemical literature, of having two distinct biochemical activities, hydroquinone (NAD(P)H) oxidation and protein disulfide–thiol interchange, that alternate.[1–5] Present in both plants and animals, they have no flavin, heme nor none-heme iron prosthetic groups and do not require ancillary proteins for activation.[1]

Now referred to as ECTO-NOX proteins because of their cell surface location[6] and to distinguish them from the *phox*-NOX proteins of host defense,[7] they achieve protease (including proteinase K) resistance, impart protease resistance to protease-susceptible proteins, contain a copper site and form amyloid,[8] all of which are characteristics of prions. While activities have been most often measured as oxidation of NADH, the physiological substrate for the activity appears to be hydroquinones of the plasma membrane such as reduced coenzyme Q_{10}.[9]

At least two forms of ECTO-NOX activities have been distinguished on the basis of response to hormones, growth factors, capsaicin (8-methyl-*N*-vanillyl-6-noneamide),[10,11] and certain other quinone-site inhibitors or potential quinone-site inhibitors with anticancer activity.[1,12] The constitutive ECTO-NOX, designated CNOX, is hormone responsive and refractory to the quinone-site inhibitors.[1] A tumor-associated NOX (tNOX) is

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unregulated, refractory to hormones and growth factors and responds to inhibitors.[1] CNOX proteins are widely distributed and exhibit activity oscillations with period lengths of 24 min.[13] tNOX proteins are cancer specific and exhibit oscillations with period lengths of about 22 min, ca. 2 min shorter than those of CNOX.[2,14] The sequence data for tNOX are available from GenBank under Accession No. AF207881.

The period length of the activity oscillations of the NOX protein is independent of temperature (temperature compensated)[3–5,14] and the phase is entrainable.[5,14] These two characteristics, temperature compensation and entrainment (coupling the intrinsic clock to environmental cues), are two defining hallmarks of the biological clock.[15,16] ECTO-NOX synchrony through entrainment is achieved through autosynchrony in solution,[5] by coupling to red (plants)[17] and blue (plants and animals)[18] light photoreceptors and in direct response to melatonin (unpublished). Based on analyses of COS cells transfected with cDNAs generating tNOX proteins having periods longer or shorter than 24 min.[19] ECTO-NOX proteins provide a biochemical basis for the biological clock (the circadian period is 60 × the ECTO-NOX period).[20]

Measurement of ECTO-NOX Activities

The functional diagram summarizing the two activities of the ECTO-NOX proteins emphasizes distinct oxidative and protein disulfide–thiol interchange events that alternate (Fig. 1). The oxidative portion of the ECTO-NOX protein has been most often measured from the decrease in A_{340} from the oxidation of NADH (or NADPH). With turbid membrane preparations, use of a dual beam instrument with the photo multiplier tube proximal to the sample, is essential to reduce light scattering error in continuous measurements. Unfortunately, the Aminco SLM 2000 which is a double beam, dual wavelength grating instrument designed for these types of measurements is no longer commercially available. A suitable alternative may be the Spectronics UV550 which is a double beam diode array instrument with an accessory that allows placement of the sample proximal to the photomultiplier tube.

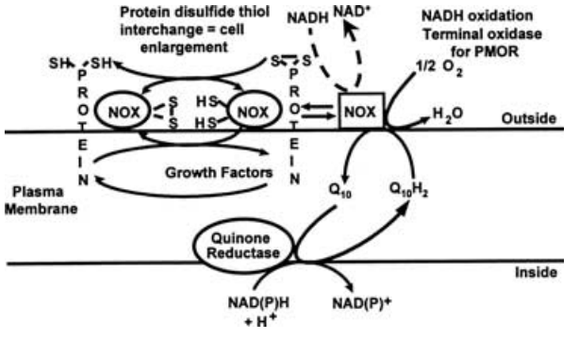


FIGURE 1 Relationships of the inside NAD(P)H quinone reductase and the membrane pool of coenzyme Q (Q_{10}) (Vitamin K_1 in plants) to shuttle electrons and protons across the plasma membrane. In this manner, the NOX protein functions as a terminal oxidase of plasma membrane electron transport donating electrons from cytosolic NADH either to molecular oxygen in a two electron transfer or to reduce protein disulfides. The left hand portion of the diagram summarizes reactions catalyzed by the protein disulfide–thiol interchange activities which occur in the absence of external reductants and have a role in growth. Growth factors appear to serve as switches to couple this part of the mechanism to cell enlargement.

For tissue, where the NADH solution is withdrawn and absorbance estimated at 1 min intervals, any spectrophotometer may be used because the samples are not turbid. The only complicating feature is that a blank of tissue without NADH must be subtracted.[17,18] The blank rate, while substantial due to leakage of A_{340} absorbing materials, is usually linear and an empirically determined constant A_{340} value is subtracted from each point to generate the decrease in A_{340} due to oxidation of NADH.

The continuous traces, even though illustrating the pattern of oscillatory activity, are difficult to interpret and to quantitate. For the latter, rates either consecutively determined for 5 min or for 1 min at intervals of 1.5 min are used.[2–5]

For the protein disulfide–thiol interchange activity, the restoration of activity to reduced, denatured and oxidized (scrambled) yeast RNase through reduction, refolding under non-denaturing conditions and reoxidation to form a correct secondary structure stabilized by internal disulfide bonds[21] is the most reliable assay. Hydrolysis of cCMP catalyzed by RNase is the end point estimated from the increase in A_{490}. An alternative assay which requires subtraction of a substrate blank uses dithiodipyridine where cleavage produces pyridinethionine which absorbs strongly at A_{340}.[22]

The statistical evaluation of an oscillatory activity that is neither sinusoidal nor monotonic (Fig. 2) is equally challenging. The period length over successive cycles usually can be determined by Fourier analysis. The reproducibility of the complexities within the major period is amenable to time series (decomposition) analysis in which successive periods are superimposed, the statistical agreement with the average activity pattern is calculated and an average "predicted" pattern is generated.[23] These statistical methods have been borrowed from economic forecasting where time series analysis is a widely used approach to analysis and prediction of seasonal trends.

The ECTO-NOX Family

Based on detailed analyses as described above, a number of different ECTO-NOX forms have been

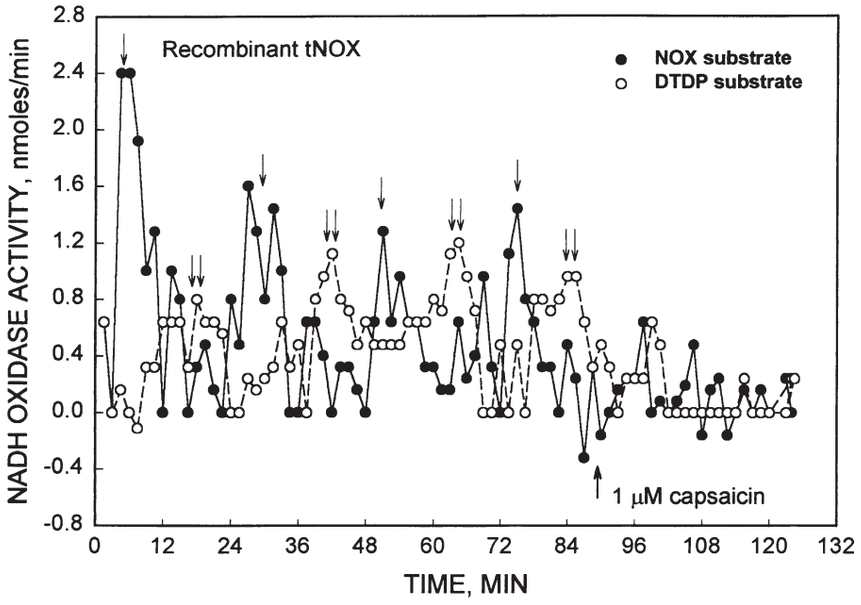


FIGURE 2 Simultaneous measurement of NADH oxidation (solid symbols and lines) and protein disulfide–thiol interchange (dotted line, open symbols), the latter using the dithiodipyridine (DTDP) substrate. As indicated by the arrows denoting rate maxima (single arrows for NADH oxidation, double arrows for protein disulfide–thiol interchange) the rate maxima of the two activities alternated. Capsaicin (1 μM) inhibited both enzymatic activities.

described.[1] They are differentiated on the basis of period length, response to inhibitors and activators and entrainment properties. The normal constitutive ECTO-NOX, CNOX, has a period length of 24 min,[3] is completely refractory to inhibitors, autoentrainable[5] and can be entrained (synchronized) by light and melatonin. Early studies suggested CNOX to be stimulated by a variety of hormones and growth factors[24] possibly by linkage of the ECTO-NOX protein to receptor molecules at the cell surface. The response to light also is receptor mediated (ECTO-NOX proteins have no chromophores) but the response to melatonin does not require association of the CNOX protein with a membrane and is presumed to be direct.

Cancer cells and tissues exhibit two ECTO-NOX forms.[14] Normal CNOX accounts for 40–60% of the NOX activity and a cancer-specific ECTO-NOX form, tNOX, accounts for the remainder. tNOX differs from CNOX in several important respects. Its activity period is shorter (22 vs. 24 min) and both the hydroquinone (NADH) oxidase and protein disulfide–thiol interchange activities are inhibited by several known quinone-site inhibitors all of which have anticancer activity.[1] Examples include adriamycin, the antitumor sulfonylureas, the antitumor vanilloids and capsaicionoids e.g. (capsaicin), the principal tea catechin, (−)-epigallocatechin-3- gallate, and several known differentiating agents including antitumor retinoids, sodium phenylacetate and calcitriol. These agents are largely without effect on the CNOX activity of normal cells. They inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting CNOX or growth of non-cancer cells. Drug inhibition of tNOX served as the defining tNOX characteristic used to guide its isolation and molecular characterization.[25]

The constitutive ECTO-NOX activity, that of CNOX, is universally present in all organisms thus far examined, plant, animal and bacteria.[26] The period length is approximately 24 h for all species and appears to be closely coupled both to biological time keeping as the biochemical basis for the biological clock[20] and as drivers of cell enlargement.[4,27,28]

Biochemistry

The alternation of enzymatic activities carried out by NOX proteins is unprecedented in the biochemical literature.[2] Based on studies with tNOX,[29,30] the specific activity of NADH oxidation by the pure protein is only 10–20 μmoles/min/mg protein with a turnover number (the number of substrate molecules converted to product per minute with the enzyme fully saturated with substrate) of between 200 and 500 (Table I). Rates of oxidation of natural hydroquinone or other substrates contained within the plasma membrane may be much higher.

Enzymatic assays with tNOX purified from the HeLa cell surface or purified recombinant tNOX are especially difficult. Greatest specific activities have been obtained with purified fractions from HeLa cells still complexed with GAPDH and other proteins prior to final purification

TABLE I Properties of ECTO-NOX proteins

Oxidative activity
Donor: Hydroquinone/NADH/NADPH/Dopamine
Acceptor: Molecular oxygen → H_2O/Protein disulfides
Protein disulfide–thiol interchange
Restore activity to scrambled RNase
Dithiodipyridine substrates
Two activities alternate to generate a period length of 24 min (22 min for tNOX, 26 min for arNOX)
Low turnover number: 200–500
Specific activity: 10–20 μmoles/min/mg protein
Two moles of zinc and potentially up to ca. 1 mole bound copper/mole of protein
No flavin and no cytochromes, heme or non-heme iron
Refractory to N-terminal sequencing
Protease resistant
Located at external cell surface
No GPI anchor or membrane-spanning domains
No ancillary proteins required for activity

and aggregation.[29,30] The purified tNOX tends to form insoluble aggregates of amyloid which, once formed, are devoid of enzymatic activity[30] and resistent to restoration of activity by disaggregation. The aggregates tend to dissociate at low temperature (freezing and thawing) but aggregates are reformed under normal conditions of NADH oxidase assay. The recombinant tNOX has been successfully assayed most often with dilute solutions (ca. 10 ng/ml).[2]

Electron donors for the oxidative activity include hydroquinones (reduced coenzyme Q for animals and reduced phylloquinone for plants) and NADH or NADPH. The reduced pyridine nucleotides, NADH and NADPH, are regarded as non-physiological substrates since reduced pyridine nucleotides in the concentrations required to sustain ECTO-NOX activities are encountered rarely, if at all, at the external cell surface. Reduced quinones [coenzyme QH_2 and phylloquinone (vitamin K_1H_2)] are abundant in plasma membranes of animals and plants, respectively, and can function as lipophilic trans-plasma membrane shuttles ferrying reducing equivalents from cytosolic NAD(P)H to molecular oxygen with the ECTO-NOX protein functioning as the terminal oxidase.[9,31] This trans plasma membrane electron transport chain is initiated at the cytosolic plasma membrane surface by quinone reduction catalyzed by NAD(P)H-quinone reductase.[32] These relationships among plasma membrane electron transport constituents and ECTO-NOX proteins are summarized in Fig. 1.

Molecular oxygen[33] and protein disulfide[34] both have been shown to function as electron acceptors for ECTO-NOX protein-catalyzed reactions with a stoichiometry of 1 NAD(P)H or hydroquinone oxidized per 1/2 O_2 or disulfide reduced.[33] Like NADH oxidation, oxygen consumption is periodic with a period length of 24 min.

All ECTO-NOX proteins thus far examined utilize either NADH or NADPH as electron donor. Most CNOX activities utilize NADH preferentially whereas tNOX activity is greatest with NADPH.[35] ECTO-NOX activity with NADPH as electron donor including recombinant tNOX is inhibited by diphenyliodonium (DPI). ECTO-NOX activity with NADH as electron donor is largely unaffected by DPI.[35] DPI is regarded widely as a specific inhibitor of flavin-containing oxidases. tNOX lacks both flavin and flavin-binding domains and ECTO-NOX activities in general are unaffected by added flavin (FMN or FAD). Yet, ECTO-NOX activities are inhibited by DPI with NADPH as substrate. DPI inhibition of ECTO-NOX activities, therefore, appears to involve some aspect of NADPH binding not encountered with NADH but unrelated to the presence of bound flavin.

The absence of iron-flavin prosthetic groups and the lack of a requirement for ancillary proteins distinguishes the ECTO-NOX proteins from the *phox*-NOX proteins of host defense.[7] The latter are located at the cytosolic plasma membrane surface[7] whereas the ECTO-NOX proteins are found exclusively at the external membrane surface.[6,36] An external plasma membrane location of the ECTO-NOX proteins has been demonstrated rigorously from direct assay with whole cells and tissues[4,37] (NADH is an impermeant substrate), assay of right-side-out (active) vs. inside-out (inactive) plasma membrane vesicles,[36] inhibition by antibodies,[38] use of impermeant inhibitors[39] and immuno-, cyto- and histochemistry.[38]

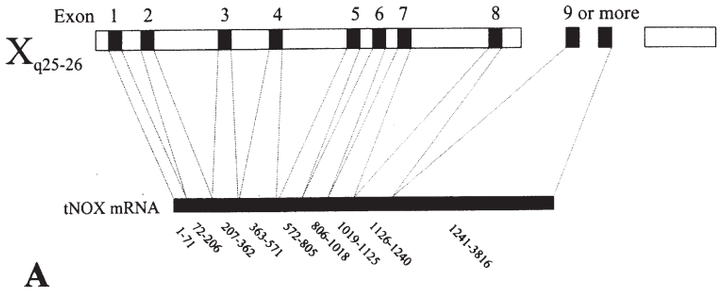
Since NOX proteins lack iron or iron sulfur clusters and still reduce oxygen, a metal in a redox site must be present. tNOX contains a copper site conserved with superoxide dismutase.[40] In early studies with tNOX purified from HeLa cells, the copper content was estimated to be about 1 mole bound copper/mole tNOX protein.[2] However, atomic absorption analyses with recombinant tNOX gives a ratio substantially <1. The basis for the discrepancy between these two measurements is unknown. tNOX does contain two putative zinc finger motifs. Zinc is present in recombinant tNOX preparations in the ratio of 2 moles of bound zinc/mole of protein. ECTO-NOX proteins are not general thiol oxidases nor do they normally function as perioxidases.

The ECTO-NOX protein disulfide–thiol interchange activity is similar to that catalyzed by a protein disulfide isomerase (PDI). tNOX, however, lacks the characteristic- C-X-X-C- PDI motif.[41] The interchange results in no net oxidation or reduction of protein thiols and does not require exogenous donors or acceptors. NAD(P)H, for example, is not required. With the dithiodipyridine substrates, reductive cleavage of the dithio bond is equivalent to the reductive cleavage of a protein disulfide

and occurs at the expense of protein thiols. Thiol reducing agents such as reduced glutathione react spontaneously with the dithiodipyridines and must be avoided in the assays.

While the two C-X-X-X-X-C motifs characteristic of flavoproteins are missing from tNOX, the redox active disulfide of thioredoxin reductase from the malaria parasite *Plasmodium falciparum* contains a motif C88-X-X-X-X-C93[42] similar to one found in tNOX. Together with a downstream His509, the motif was shown to be a putative proton donor/acceptor. Either the C88A or the C93A replacement resulted in complete loss of enzymatic activity.[42] A C535-X-X-X-X-C540 motif in the same protein was shown to be involved in substrate coordination and/or electron transfer.[43] A C535A replacement did result in diminution of enzymatic activity but the C540A replacement did not.[43] Thus, either or both of the two comparable motifs present in tNOX, C505-X-X-X-X-C510 or C569-X-X-X-X-C575, alone or together with downstream histidines provide potential active sites for protein disulfide–thiol interchange. With tNOX, the C505A and C569A replacements lost activity as with the C535A replacement above for the *P. falciparum* protein but the C510A and C575A did not as with the above C540A replacement for the *P. falciparum* protein.

The various tNOX functional motifs of the 34 kDa processed form of tNOX are illustrated in Fig. 3. The correctness of the various assignment has, for the most part, been confirmed by site-directed mutagenesis.[19] While amino acid replacements that block oxidation of reduced pyridine nucleotide by tNOX also eliminated protein disulfide–thiol interchange and vice versa,[19] the two activities appear to occur independently. One can be measured in the absence of the other. Also Alzheimer's Aβ peptide carries out oscillatory oxidation of NADH (see below) but lacks



A

- **Processed molecular weight of 33.5 kDa (ttNOX)**
- **Functional motifs:** **Quinone binding site (EEMTE)**
  Potential PDI motif (CXXXXC)
  Copper binding site (HVH) and H 343
  Adenine binding site (TGVGASL)

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  1  M L A R E E R H R R R M E E E R L R P P S P P P V V H Y S D
 31  H E C S I V A E K L K D D S K F S E A V Q T L L T W I E R G
 61  E V N R R S A N N F Y S M I Q S A N S H V R R L V N E K A A
 91  H E K D M E E A K E K F K Q A L S G I L I Q F E Q I V A V Y
121  H S A S K Q K A W D H F T K A Q R K N I S V W C K Q A E E I
151  R N I H N D E L M G I R R E E E M E M S D D E I E E M T E T
                                          Quinone binding site
181  K E T E E S A L V S Q A E A L K E E N D S L R W Q L D A Y R
211  N E V E L L K Q E Q G K V H R E D D P N K E Q Q L K L L Q Q
241  A L Q G M Q Q H L L K V Q E E Y K K K E A E L E K L K D D K
271  L Q V E K M L E N L K E K E S C A S R L C A S N Q D S E Y P
                                  Potential PDI motif
301  L E K T M N S S P I K S E R E A L L V G I I S T F L H V H P
                                                Copper binding site
331  F G A S I E Y I C S Y L H R L D N K I C T S D V E C L M G R
361  L Q H T F K Q E M T G V G A S L E K R W K F C G F E G L K L
391  T          Adenine (NADH) binding site
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B

FIGURE 3 Sequence information. (A) tNOX gene on human chromosome X. (B) Deduced amino acid sequence of the bacterially expressed 46 kDa functional C-terminus of tNOX. The complete sequence data for the full length 70.1 kDa protein are available from GenBank under Accession No. AF207881.

a cysteine residue and does not carry out protein disulfide–thiol interchange.

Periodicity

A defining feature of the ECTO-NOX proteins is that the two enzymatic activities they catalyze, hydroquinone (NADH) oxidation and disulfide–thiol interchange, alternate within a 24-min period (Fig. 2). The length of the period is temperature independent and the phase is entrainable, both features of the biological clock.

The NOX oscillations are not simple sine functions. Rather they represent complex 2 + 3 oscillatory patterns (Fig. 4).[2,4,44] In the oxidizing portion of the cycle, hydroquinone or NADH oxidation is normally represented by two maxima of variable height ratios (see below) followed by three minor oscillations. When disulfide–thiol interchange is measured, the two maxima of NADH oxidation coincide exactly with minor oscillations whereas the three minor oscillations of NADH oxidation now coincide with major oscillations in disulfide–thiol interchange (Fig. 4 A–C). Each of the components of the 2 + 3 pattern of oscillations repeats with a precise and temperature-compensated (independent of temperature) period length of approximately 24 min for CNOX, 22 min for tNOX or 26 min for the age-related ECTO-NOX (arNOX).

While the 2 + 3 pattern is an immutable feature of ECTO-NOX function, variations in the relative peak heights of the two maxima of NADH oxidation vary. NADH oxidation by CNOX exhibits either a single dominant maximum within each 24 min period of two, often nearly equal, maxima separated by about 9 min within each 24 min period. Both pattern extremes have been observed with CHO cells and plasma membranes of soybean. In those preparations exhibiting the two maxima pattern, the relative activities of the two maxima vary with

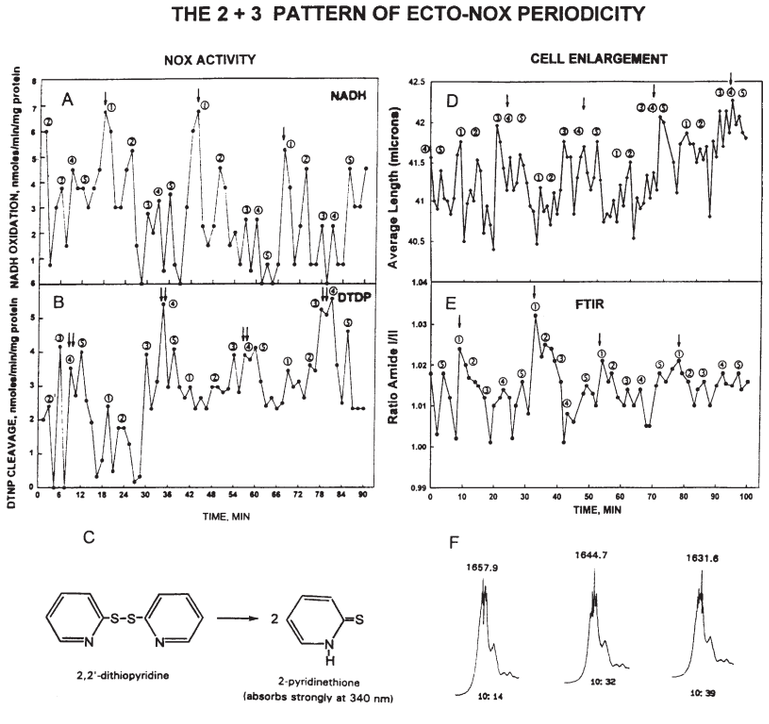

FIGURE 4 Details of the 3 + 2 pattern of NOX activity oscillations. A, B as in Fig. 2. (A) NADH oxidation determined by the decrease in A_{340} (upper curve). Maxima (arrows) were at 18, 42 and 66 min with secondary maxima at 24, 48 and 72 min. Three minor peaks completed each 24 min period. (B) Disulfide–thiol interchange activity measured simultaneously in parallel as an increase in A_{340} from the cleavage of dithiodipyridine (DTDP). Major peaks are at 6, 9 and 12 min and at 24 min intervals thereafter (double arrows) with minor peaks at 18 and 24 min and at 24 min intervals thereafter. Clearly, the two activities, NADH oxidation (A) and DTDP cleavage (B), alternate. (C) Dithiodipyridine DTDP substrate generating 2 moles of 340 nm-absorbing 2-pyridinethionine and cleaved as a measure of the disulfide–thiol interchange activity of the NOX protein. (D) Increase in length (enlargement of a single cell as determined by image enhanced light microscopy). Cell enlargement proceeds in bursts every 12 min separated by rest periods where the cells actually shrink. As with NOX activity, each 24 min period (single arrows) is comprised, on average, of 5 resolvable maxima separated by minima. Three maxima are contained within the elongation phase and correspond to the protein disulfide–thiol interchange determined in parallel (B). The two maxima contained within the resting period correlate with the two maxima of NADH oxidation (A). (E) Fourier transform infrared analyses of recombinant tNOX. Sixty-one 1 min scans taken 1.5 min apart over 100 min are illustrated. The ratio of the amide I (1645)–amide II (1545) absorbances varied with maxima at 22 min intervals as indicated by the arrows. (F) Within the amide I region (below), peak absorbance varied between 1658 and 1630 indicative of alternating α-helix-β-sheet transitions. Concanavalin A, cytochrome c or albumin when analyzed in parallel showed no such pattern.

the second maximum normally being subordinate to the first. It is as though the second maximum may vary in activity from just above background to nearly equal to the first maximum. Neither the underlying cause nor the consequence of these differences have been elucidated.

Since blanks without NADH or without an enzyme source did not exhibit oscillatory absorbance changes,[3,45] the oscillations observed with the complete system are inherent in the cell surface NADH oxidase protein itself and are not a function of machine variation or more complex environments. The oscillatory behavior is similar for partially purified NOX proteins released from the HeLa cell surface as for the NOX activity of isolated plasma membrane vesicles and of whole cells. As discussed below, the 2 + 3 pattern of oscillations is seen as well for cell enlargement (Fig. 4D).

Fine Structure of 2 + 3 Pattern. Molecular Basis for the 2 + 3 Pattern

The 22 min periodic behavior exhibited by pure recombinant tNOX protein is accompanied by recurring patterns of Fourier transform infrared (FTIR) (Fig. 4 E–F) and circular dichroism (CD) (Fig. 5) spectral changes suggestive of α-helix-β-structure transformations. The percentage of β-structure varies between 40.2 and 41.2% of the proteins secondary structure in generating the oscillations as determined from CD or a change of 1% in a protein of 391 amino acids. An amino acid replacement at H322 within the putative copper site eliminated the periodic oscillations suggesting that bound copper may be involved in maintaining the oscillations. Replacement at H343 altered but did not eliminate the oscillatory behavior of the 46 kDa functional C-terminus of tNOX (Fig. 3). Based on the studies with other oscillatory amyloid-forming proteins (Table II), the regions involved in the oscillations may be no more than 40–50 amino acids in length.

Temperature Compensation and Entrainment

The period lengths of the activity oscillations of ECTO-NOX proteins are independent of temperature (temperature compensated),[3–5,45] i.e. the period length is the same, for example, at 14, 24 and 34°C. Amplitude, on the other hand, doubles for each 10°C rise in temperature yielding a Q_{10} of 2 as is characteristic of most chemical reactions. A period length independent of temperature is a characteristic known only for one other biological phenomenon, the biological clock.

How temperature compensation is achieved in maintaining a constant period length and precise temperature-independent time keeping are unknown. The working model is that of a spring where the traverse path shortens as temperature is lowered but reversal is initiated at the same level of force-extension at any particular temperature.

NOX synchrony and entrainment (coupling the intrinsic clock to environmental clues) is achieved through auto-synchrony in solution[5], by coupling to red (plants)[17] or blue (plants and animals)[18] light photoreceptors and in direct response to melatonin (unpublished). If two NOX preparations with different periods are mixed, the two

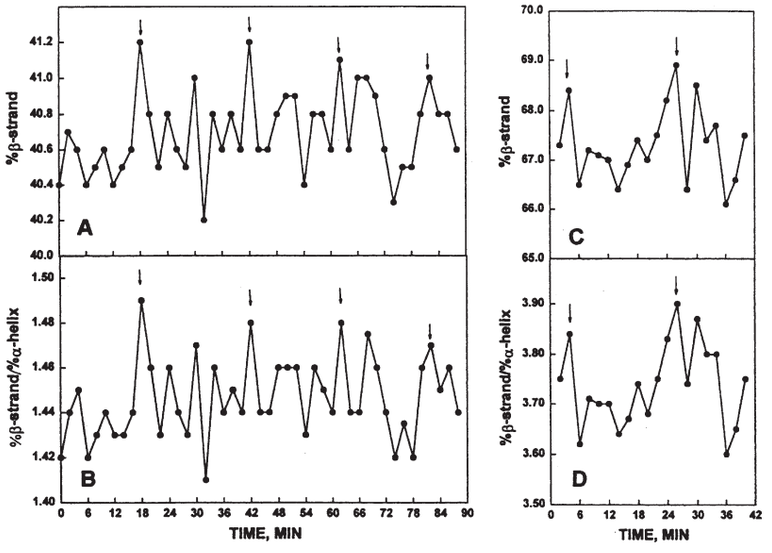

FIGURE 5 Percent β-strand (A) and ratio of percent β-strand/α-helix (B) of soluble recombinant tNOX prior to acquisition of proteinase K-resistance. Single arrows denote maxima in β-strand structure spaced at intervals of 22 min. The double peak at 62 and 68 min (to right of 3rd arrow) is a recurrent feature possibly associated with time keeping. See also, C and D at 24 and 30 min. C. Percent β-strand (C) and ratio of percent β-strand/α-helix (D) of recombinant tNOX after refolding and acquisition of proteinase K-resistance. Single arrows denote maxima in β-strand structure with a period length of ca. 22 min.

TABLE II Amyloid-forming proteins that exhibit periodic (copper-dependent) oscillations in NADH oxidation

CNOX (CLOX)*	24 min	ECTO-NOX proteins
tNOX†	22 min	
arNOX‡	26 min	
Aβ 1–42	24 min	Alzheimer's
Mouse prion	24 min	Spongiform encephalopathies
α-Synuclein	54 min	Parkinson's

*Constitutive NOX (normal cells and tissues). †Tumor NOX (cancer specific). ‡Age-related NOX (aged >60 y individuals).

preparations will entrain and, after several hours, reach a common 22 (tNOX), 24 (CNOX) or 26 (arNOX) min period length. CNOX, tNOX or arNOX preparations do not cross entrain, however. Light entrainment is receptor-mediated while melatonin affects CNOX (both plant and animal) directly as do valerian preparations but neither melatonin nor valerian entrain with tNOX or arNOX. The mechanism of autoentrainment, while extremely important to achievement of NOX synchrony is unknown except that cysteine residues of interacting NOX proteins appear to be involved.

ECTO-NOX Protein Function

A rather large body of published evidence, taken together, suggest that ECTO-NOX proteins are essential to three very important areas of cell function: (1) to drive the enlargement phase of cell growth, (2) as a terminal oxidase for plasma membrane electron transport and (3) as the ultradian (period length <24 h) core oscillator of the biological clock. These features of NOX function are implicit in Fig. 1 and summarized in the three sections that follow.

Enlargement Phase of Cell Growth

When a cell divides, the resultant small cells must enlarge to reach some minimal size in order to divide again.[46] With plant stems and roots, cell enlargements of 20- to 200-fold are frequently associated with the overall growth process.

In early studies summarized by Morré[1] growth and ECTO-NOX activity were highly correlated. For both excised plant parts and animal cells in culture, treatment and conditions that simulated the NADH oxidase stimulated growth whereas treatments and conditions that inhibited the NADH oxidase inhibited cell growth. With cell enlargement in plants, the reverse also was true. Conditions and treatments that stimulated cell enlargement also stimulated NADH oxidase and conditions and treatments that inhibited cell enlargement inhibited NADH oxidase. Included in the latter category were touch,[47] gravity,[48,49] decreased turgor[50] and response to auxin regulators of cell enlargement both natural, indole-3-acetic acid (IAA) and synthetic, 2,4-dichlorophenoxyacetic acid (2,4-D).[51] With plant stems, the auxins stimulate the NADH oxidase directly and promote cell enlargement. With roots, where auxins traditionally inhibit cell enlargement, the NADH oxidase also was inhibited by auxin.[49] Both stimulations and inhibitions were log dose-dependent.

Based on the large body of correlative data available, studies were carried out to determine if the oscillations in ECTO-NOX enzymatic activities were reflected in rates of cell enlargement. Using area measurements from image-enhanced light microscopy (plants and animals)[4,27] and length measurements of stem sections measured using a sensitive transducing system,[28] enlargement rates were measured at intervals of 1–3 min.

These studies reveal clearly that rates of cell enlargement also fluctuate with a 24 min period in plants and normal mammalian cells in culture. With cancer cells that contain both CNOX (24 min period) and tNOX (22 min period), cell enlargement exhibits both 24 and 22 min oscillations in the rates of cell enlargement.[45]

Maximum rates of cell enlargement correlate with the portion of the NOX cycle involved in protein disulfide–thiol interchange (Fig. 4). During the electron transport phase of the cycle involving hydroquinone oxidation, cell enlargement rests. The period length of cell enlargement, like that of the enzymatic activities of ECTO-NOX proteins is independent of temperature (temperature compensated).[4,27,28]

The functioning of ECTO-NOX proteins in driving cell enlargement can be understood in the context of a model proposed much earlier[52] (Fig. 6) and based on several decades of indirect evidence. Irrespective of any model, the disulfide–thiol interchange portion of the NOX cycle drives cell enlargement in a mechanism involving the breakup and formation of disulfide bonds in membrane proteins. The mechanism applies equally to plant and animal cells and is not cell wall- or exocalyx-based. Overexpression of tNOX cDNA in COS cells led to a more rapid rate of cell enlargement and several-fold increase in cell volume compared to non-transfected COS cells [Unpublished].

Terminal Oxidase of Plasma Membrane Electron Transport

CNOX and other ECTO-NOX proteins present at the cell surface and capable of oxidizing reduced quinones[9] has offered an opportunity to formulate, for the first time, a complete electron transport chain from the cytosol to oxygen at the cell surface with the ECTO-NOX (CNOX) protein acting as the terminal oxidase. The plasma membrane electron transport would serve to oxidize the NADH accumulated from

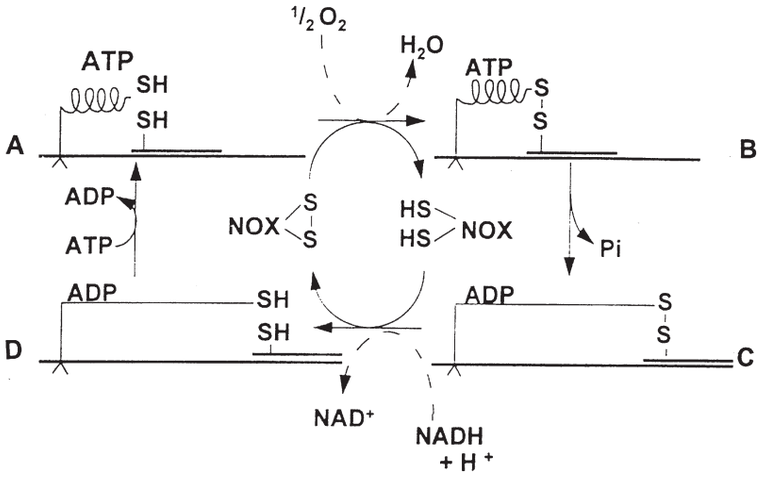

FIGURE 6 Physical membrane displacement model to explain NOX functions in growth.[52] (A) A hypothetical membrane protein with a dynamic coiled region with ATP bound. (B) One end of the protein is permanently anchored (via the cytoskeleton?) and cannot slip. (C) The ATP is hydrolyzed and the coiled region extends, displacing the bound protein within the membrane. (D) The disulfide is reduced, releasing the bound protein, after which the coiled region binds another ATP which is exchanged for ADP. The coil re-forms, and the system is ready to displace another membrane protein. Repeated cycles would result in membrane displacement or, with proper orientation, in the formation of membrane vesicles or blebs.

the glycolytic production of ATP which is necessary to maintain NAD^+/NADH homeostasis essential for survival at points in the cytosol distant from mitochondria.

NADH oxidation by ECTO-NOX proteins may be coupled to an amiloride-insensitive proton transport[53] that leads to alkalinization of the cytosol[54] and may contribute to an increased membrane potential. The released energy from the NADH oxidation might be coupled to growth, for example, or be utilized to drive proton transport.[55] Cell enlargement is an energy-requiring process and conservation of energy of plasma membrane electron transport to drive the energy-requiring steps of cell enlargement would represent a totally new paradigm in biochemistry if correct.

Biochemical Basis for the Biological Clock

ECTO-NOX proteins of plant and animal cells exhibit stable and recurring patterns of oscillations with potentially clock-related, entrainable and temperature compensated period lengths (24 min for the constitutive CNOX). To test the hypothesis that ECTO-NOX proteins act as ultradian biochemical oscillators that underlie the cellular biological clock, COS cells were transfected with cysteine to alanine replacement cDNAs encoding ECTO-NOX proteins having period lengths of 22, 36 or 42 min.[19] We used activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a common clock-regulated housekeeping protein, as a measure of the cellular biological clock.[20] The transfected COS cells exhibited circadian period lengths of 22, 36 or 42 h for GAPDH activity in addition to their endogenous 24 h circadian period.[20] Since expression of single oscillating NOX proteins determined the period length of a well-established circadian biochemical marker, we conclude that ECTO-NOX proteins provide a biochemical basis for the operation of the biological clock.[20]

As such, the ECTO-NOX proteins provide a biochemical oscillator as an alternative to current clock models that suggest the biological clock to result from the operation of transcriptional feed back loops with time delays in protein translation and transport back to the nucleus and where activities fluctuate as a function of the light–dark cycle.[56,57] Other experiments show that clock related transcription factors, NPAS2 and Clock, are intracellular redox sensors.[58] Their cytosolic DNA binding activities are influenced by the redox status of NAD(H) and/or NADP(H) which, in turn, would be expected to be influenced by ECTO-NOX-driven plasma membrane electron transport.

Whereas the circadian day is 60 times the ECTO-NOX period, how the cell is able to record 60 ECTO-NOX periods to generate the circadian day and have it conform to the normal light–dark cycle may very well reside in some cumulative response in the ECTO-NOX activity cycle which remains to be elucidated.

Role of ECTO-NOX Proteins in Disease

The most thoroughly studied involvement of an ECTO-NOX protein in disease has been that of tNOX and cancer.[1,38] More recently, tNOX has been shown

to exhibit properties of a prion and various amyloid generating proteins of neurodegenerative diseases (Alzheimer's β-amyloid, Parkinson's α-synuclein) and including mouse prion have properties in common with NOX proteins. We also have described an aging-related ECTO-NOX potentially involved in the generation of reactive oxygen species at the cell surface.

tNOX and Cancer

The cancer-associated, drug-responsive and constitutively-activated ECTO-NOX form has been cloned and expressed.[2] Designated tNOX, it is present at the cell surface of invasive human cancers.[1,38] tNOX is shed into the circulation and, together with the cell surface form, provides a potential drug, vaccine and diagnostic target for cancer.[38] The normal constitutive NOX (CNOX) forms are hormone- and growth factor-regulated and responsive to agents that control growth and development.[24] When a cell divides, it must reach some certain minimal size to divide again.[46] When one or more NOX proteins are inhibited, cell enlargement is slowed or blocked.[54,59–61] The resultant small cells fail to divide and ultimately undergo programmed cell death (apoptosis).[54,59–61]

The drug responsive tNOX appears to arise as a splice variant from a single tNOX gene different from that encoding CNOX and is delivered to the cell surface as a processed 34 kDa ectoprotein [Unpublished]. The cDNA encoding tNOX cloned from HeLa cells, has been expressed in bacteria and mammalian cells.[2, Unpublished] Recombinant tNOX proteins exhibit the same periodic alternation of activities and drug response as the ECTO-NOX proteins of membranes, cells and tissues.[2]

The ECTO-NOX form associated with human cancers, tNOX, differs from the constitutive form of normal cells (CNOX) in several important respects.[1] Its activity period is shorter (22 vs. 24 min) and both the hydroquinone (NADH) oxidase and protein disulfide–thiol interchange activities are inhibited by a series of known quinone-site inhibitors many of which have anticancer activity.[1] Examples include adriamycin,[62] the antitumor sulfonylureas,[60] the antitumor vanilloids (capsaicin),[59] the principal tea catechin, (−)-epigallocatechin-3-gallate,[61] and several known differentiating agents including antitumor retinoids, sodium phenylacetate and calcitriol.[1] These agents are largely without effect on the CNOX activity of normal cells. They inhibit both tNOX and growth of cancer cells at potentially therapeutic dosage levels without inhibiting CNOX or growth of non-cancer cells. Drug inhibition of tNOX served as the defining tNOX characteristic used to guide its isolation and molecular characterization.[25]

The tNOX gene consists of at least nine exons that combine to yield 1830 bp open reading frame and 70.1 kDa protein comprised of 610 amino acids. It has been expressed in *Escherichia coli*, COS and MCF-10A cells. The expressed protein exhibits the same characteristics of alternation of the two activities and drug response as the cell surface form. Identified functional motifs include a quinone binding site, an adenine nucleotide binding site, a CXXXXC cysteine pair as a potential protein disulfide–thiol interchange site and a copper binding site conserved with superoxide dismutase. Correctness of functional domains is well supported by site-directed mutagenesis studies.[2,19]

The tumor form of the NOX proteins, designated tNOX, is blocked by quinone site inhibitors with anticancer activity (antitumor sulfonylureas, antitumor vanilloids, e.g. capsaicin, antitumor quassinoids, antitumor catechins, e.g. (−)-epigallocatechin-3-gallate which is the active principle of green tea, adriamycin, cis platinum).[1,59–62] As already mentioned, the predominant NOX of non-cancer cells (the constitutive NOX or CNOX) is drug resistant. With antimetabolites (e.g. methotrexate) and antimitotic (e.g. tamoxifen) agents as clear exceptions, several known anticancer agents may act at least in part through tNOX inhibition. Evidence for a cell surface site to explain the anticancer activity of adriamycin has been in the literature since the early 1980s.[62] Thus, tNOX emerges as an important potential drug target.

NOX proteins are released from cells into the circulation. Sera of cancer patients contain both tNOX and CNOX proteins.[38,63,64] Sera of healthy volunteers or of patients with diseases other than cancer contain only the CNOX form. tNOX has been found in sera of patients with all major forms of cancer including leukemia and lymphomas[38,63,64] and serves as the basis for a cancer diagnostic protocol under development. A 22 min NOX period generates a 22 h circadian day observed in activity patterns of some cancer patients.[65]

ECTO-NOX Proteins have Characteristics of Prions

The ability to form insoluble aggregates and the presence of bound copper are ECTO-NOX protein characteristics. Other unusual characteristics exhibited by tNOX and shown with other ECTO-NOX proteins include resistance to proteases, resistance to cyanogen bromide fragmentation, and an ability to form amyloid filaments closely resembling those of transmissible spongiform encephalopathies.[30] Additionally tNOX imparts protease resistance to a normally protease-susceptible protein[8] as is

characteristic of PrP^{sc} (PrP^{res}), the presumed infective and proteinase K-resistant form of the scrapie prion.

Aging

An aging-related ECTO-NOX protein (arNOX) found in human sera and buffy coat fractions of individuals >60 y generates superoxide as measured by reduction of ferricytochrome c and is capable of oxidizing circulating lipoproteins and other extracellular targets with a potential role in atherogenesis.[66] Activity is inhibited by both superoxide dismutase (SOD) and coenzyme Q_{10}. Coenzyme Q_0, Q_2 and Q_6 do not inhibit the activity. The activity, measured by reduction of cytochrome c, is reduced or absent from sera and/or buffy coat fractions of younger individuals (20–40 y) but appears to be widely distributed among other aged systems including late-passage cultured cells and senescing plant organs.

CNOX and other ECTO-NOX proteins have been postulated to link the accumulation of lesions in mitochondrial DNA to cell surface accumulations of reactive oxygen species as one consequence of their role as a terminal oxidase in a plasma membrane electron transport chain.[1,67] Cells with functionally deficient mitochondria become characterized by an anaerobic metabolism. NADH accumulated from the glycolytic production of ATP and an elevated plasma membrane electron transport activity become necessary to maintain the NAD^+/NADH homeostasis essential for survival.[68] Previous findings demonstrate that the hyperactivity of the plasma membrane electron transport system and ultraviolet irradiation results in an NADH oxidase activity capable of cell surface generation of reactive oxygen species.[67] This would serve to propagate the aging cascade both to adjacent cells and to oxidize circulating lipoproteins.

ar-NOX proteins generate superoxide directly. NADH or NADPH are not required or involved in the superoxide generation. They exhibit an oscillating pattern but with a period length of ca. 26 min. Like other ECTO-NOX proteins, both the activity and the susceptibility to inhibition by superoxide dismutase and by coenzyme Q were retained following proteinase K digestion. The superoxide generated is not only active in the reduction of ferricytochrome c but also in the reduction of tetrazolium salts such as XTT (Na 3′-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid) leading to colored formazan formation. Other NOX proteins lack this activity.

With buffy coats, the source of electrons would be plasma membrane electron transport. With sera, the ultimate source of electrons for the reduction of ferricytochrome c appears to be protein thiols. Protein thiols occur in sera at levels sufficient to sustain the activity of the enzyme for several months.

Neurodegenerative Disorders

That tNOX and prions share a set of similar properties was summarized in the preceding section. Subsequently, recombinant mouse prion protein was examined for ECTO-NOX activities. The recombinant mouse prion exhibited a copper dependent pattern of oscillating and alternating NADH oxidase and disulfide–thiol interchange with a 24 min period indistinguishable from that of tNOX. tNOX aggregates in the form of enzymatically inactive amyloid rods and open cylinders (rings) were observed by high resolution electron microscopy. These structures were virtually indistinguishable from corresponding aggregates associated with neurodegenerative amyloid-forming proteins.

As a result of structural similarities, observations were extended to Alzheimer's Aβ 1–43 peptide and α-synuclein of Parkinson's disease. The Alzheimer's Aβ peptide exhibits copper-dependent oscillations in NADH oxidase activity similar to that of tNOX and the mouse prion also with a 24 min period length. The Aβ peptide both lacks a cysteine and is unable to carry out disulfide–thiol interchange. α-Synuclein also has a copper-dependent and oscillating NADH oxidase activity but the period length is 54 min. The α-synuclein has not been evaluated for disulfide–thiol interchange activity. These various relationships are summarized in Table II.

SUMMARY

What emerges is the potential for a moderately large family of amyloid-forming proteins all capable of binding copper and exhibiting oscillatory oxidation of NADH of low specific activity. The oscillations are copper dependent as is the oxidative capacity. Complete amino acid sequences are known for each of the proteins listed in Table II. There is virtually no sequence similarity. Even though all bind copper, the copper binding strategies differ. For tNOX, the putative copper site is a HVH and a 3rd H downstream conserved with superoxide dismutase. The mouse prion utilizes the octarepeats. For α-synuclein and Alzheimer's Aβ, different combinations of histidines or tyrosines are utilized. It is unlikely that CNOX will exhibit substantial sequence similarity to tNOX based on data base searches and antisera specificity. Yet all examples in Table II bind adenine nucleotides (NADH) although the amino acid sequences within the putative binding regions differ. Probably the best source of a new set of tools to understand ECTO-NOX periodicity and oxidative

function are now provided by the Alzheimer's Aβ peptides of 43 ca. amino acids in length. The copper site is known and a putative NADH site is found near the C-terminus as in tNOX in the vicinity of M-35. We are hopeful that a detailed analysis of various modifications within the Aβ peptide will begin to shed light on how ECTO-NOX proteins keep time and carry out their oxidative functions.

An important emerging function beyond the oxidative response contributing to neurodegenerative pathology is the ability of ECTO-NOX proteins, prions and other amyloid-forming proteins to "remember".[69] ECTO-NOX protein entrainment both in solution and as receptor-mediated events involves memory (learning) encoded within a protein molecule and then capable of being imparted to other protein molecules (teaching) to achieve a synchronous population. In a similar manner, prions correctly folded to be proteinase K resistant, impart this property to other prion molecules initially protease susceptible to amplify the protein species associated with neurodegeneration. Thus ECTO-NOX proteins and prions and possibly all amyloid forming proteins share the property of protein memory and transmissible alterations passed from one protein to another through an entire population of molecules (learning and teaching). Even plant ECTO-NOX proteins may possess such properties. We have evidence that the auxin hormone responsive dNOX form when treated with the herbicide 2,4-D becomes hyperactive. In the hyperactive state, dNOX recruits non-hormone-responsive CNOX molecules into the hyperactive state until nearly all NOX proteins are functioning synchronously and hyperactively as the molecular basis for how 2,4-D herbicide kills plants. The basic strategy, once again, is that of the prion model involving modification of a certain subset of NOX proteins which then remember and recruit other family members into their modified aberrant state. In the normal situation these phenomena may be beneficial and important, for example to growth control and developmental processes. When carried to extremes, as with herbicide treatment, scrapie or mad cow disease, the result is a pathological state leading to the demise of the affected plant, animal or human.

References

[1] Morré, D.J. (1998) "NADH oxidase: a multifunctional ectoprotein of the eukaryotic cell surface", In: Asard, H., Bérczi, A. and Caubergs, R., eds, Plasma Membrane Redox Systems and Their Role in Biological Stress and Disease (Kluwer Academic Publishers, Dordrecht, The Netherlands), pp 121–156.

[2] Chueh, P.-J., Kim, C., Cho, N., Morré, D.M. and Morré, D.J. (2002) "Molecular cloning and characterization of a tumor-associated, growth-related and time-keeping hydroquinone (NADH) oxidase (NOX) of the HeLa cell surface", *Biochemistry* **41**, 3732–3741.

[3] Morré, D.J. and Morré, D.M. (1998) "NADH oxidase activity of soybean plasma membranes oscillates with a temperature compensated period of 24 min", *Plant J.* **16**, 279–284.

[4] Pogue, R., Morré, D.M. and Morré, D.J. (2000) "CHO cell enlargement oscillates with a temperature-compensated period of 24 minutes", *Biochim. Biophys. Acta* **1498**, 44–51.

[5] Morré, D.J., Lawler, J., Wang, S., Keenan, T.W. and Morré, D.M. (2002) "Entrainment in solution of an oscillating NADH oxidase activity from the bovine milk fat globule membrane with a temperature-compensated period length suggestive of an ultradian time-keeping (clock) function", *Biochim. Biophys. Acta* **1559**, 10–20.

[6] Morré, D.J. (1995) "NADH oxidase activity of HeLa plasma membranes inhibited by the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea (LY181984) at an external site", *Biochim. Biophys. Acta* **1240**, 201–208.

[7] Lambeth, J.D., Cheng, G., Arnold, R.S. and Edens, W.A. (2000) "Novel homologs of gp91 phox", *TIBS* **25**, 459–461.

[8] Kelker, M., Kim, C., Chueh, P.-J., Guimont, R., Morré, D.M. and Morré, D.J. (2001) "Cancer isoform of a tumor-associated cell surface NADH oxidase (tNOX) has properties of a prion", *Biochemistry* **40**, 7351–7354.

[9] Kishi, T., Morré, D.M. and Morré, D.J. (1999) "The plasma membrane NADH oxidase of HeLa cells has hydroquinone oxidase activity", *Biochim. Biophys. Acta* **1412**, 66–77.

[10] Morré, D.J., Chueh, P.-J. and Morré, D.M. (1995) "Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture", *Proc. Natl Acad. Sci. USA* **92**, 1831–1835.

[11] Morré, D.J., Sun, E., Geilen, C., Wu, L.-Y., de Cabo, R., Krasagakis, K., Orfanos, C.E. and Morré, D.M. (1996) "Capsaicin inhibits plasma membrane NADH oxidase and growth of human and mouse melanoma lines", *Eur. J. Cancer* **32A**, 1995–2003.

[12] Morré, D.J., Wu, L.-Y. and Morré, D.M. (1995) "The antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N*′-(4-chlorophenyl)urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membrane", *Biochim. Biophys. Acta* **1240**, 11–17.

[13] Morré, D.J., Sedlak, D., Tang, X., Chueh, P.-J., Geng, T. and Morré, D.M. (2001) "Surface NADH oxidase of HeLa cells lacks intrinsic membrane binding motifs", *Arch. Biochem. Biophys.* **392**, 251–256.

[14] Wang, S., Pogue, R., Morré, D.M. and Morré, D.J. (2001) "NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms", *Biochim. Biophys. Acta* **1539**, 192–204.

[15] Edmunds, L.N. Jr., (1988) Cellular and Molecular Basis of Biological Clocks (Springer-Verlag, New York/Berlin/Heidelberg), p 497.

[16] Dunlap, J.C. (1996) "Genetics and molecular analysis of circadian rhythms", *Annu. Rev. Genet.* **30**, 579–601.

[17] Morré, D.J., Morré, D.M., Penel, C. and Greppin, H. (1999) "NADH oxidase periodicity of spinach leaves synchronized by light", *Int. J. Plant Sci.* **160**, 855–860.

[18] Morré, D.J., Penel, C., Greppin, H. and Morré, D.M. (2002) "The plasma membrane-associated NADH oxidase of spinach leaves responds to blue light", *Int. J. Plant Sci.* **163**, 543–547.

[19] Chueh, P.-J., Morré, D.M. and Morré, D.J. (2002) "A site-directed mutagenesis analysis of tNOX functional domains", *Biochim. Biophys. Acta* **1594**, 74–83.

[20] Morré, D.J., Chueh, P.-J., Pletcher, J., Tang, X., Wu, L.-Y. and Morré, D.M. (2002) "Biochemical basis for the biological clock", *Biochemistry* **41**, 11941–11945.

[21] Lyles, M.M. and Gilbert, H.F. (1991) "Catalysis of the oxidative unfolding of ribonuclease A by protein disulfide isomerase: dependence of the rate on the composition of the redox buffer", *Biochemistry* **30**, 613–619.

[22] Morré, D.J., Gomez-Ray, M.L., Schrmke, K.C., Em, O., Lawler, J., Hobeck, J. and Morré, D.M. (1999) "Use of dipyridyl-dithio substrates to measure directly the protein disulfide–thiol interchange activity of the auxin stimulated NADH: protein disulfide reductase (NADH oxidase) of soybean plasma membranes", *Mol. Cell. Biochem.* **207**, 7–13.

[23] Foster, K., Anwar, N., Pogue, R., Morré, D.M. and Morré, D.J. (2003) "Decomposition analyses applied to a complex

ultradian biorhythm—the oscillating NADH oxidase activity of plasma membranes having a potential time-keeping (clock) function", *Nonlinearity Biol. Tox. Med* **1**.

[24] Bruno, M., Brightman, A.O., Lawrence, J., Werderitsh, D., Morré, D.M. and Morré, D.J. (1992) "Stimulation of NADH oxidase activity from rat liver plasma membranes by growth factors and hormones is decreased or absent with hepatoma plasma membrane", *Biochem. J.* **284**, 625–628.

[25] Chueh, P.-J., Morré, D.J., Wilkinson, F.E., Gibson, J. and Morré, D.M. (1997) "A 33.5 kDa heat-and protease-resistant NADH oxidase inhibited by capsaicin from sera of cancer patients", *Arch. Biochem. Biophys.* **342**, 38–47.

[26] Sedlak, D., Morré, D.M. and Morré, D.J. (2001) "A drug-unresponsive and protease-resistant CNOX protein from human sera", *Arch. Biochem. Biophys.* **386**, 106–116.

[27] Morré, D.J., Pogue, R. and Morré, D.M. (2001) "Soybean cell enlargement oscillates with a temperature-compensated period of ca. 24 min", *In Vitro Cell. Dev. Biol. Plant* **37**, 19–23.

[28] Morré, D.J., Ternes, P. and Morré, D.M. (2002) "Cell enlargement of plant tissue explants oscillates with a temperature-compensated period length of ca. 24 min", *In Vitro Cell. Dev. Biol. Plant* **38**, 18–28.

[29] Yantiri, F. and Morré, D.J. (2001) "Isolation and characterization of a tumor-associated NADH oxidase (tNOX) from the HeLa cell surface", *Arch. Biochem. Biophys.* **391**, 149–159.

[30] del Castillo-Olivares, A., Chueh, P.-J., Wang, S., Sweeting, M., Yantiri, F., Sedlak, D., Morré, D.M., Burgess, J. and Morré, D.J. (1998) "A drug-responsive and protease-resistant 34 kDa NADH oxidase from the surface of HeLa cells", *Arch. Biochem. Biophys.* **358**, 125–140.

[31] Bridge, A., Barr, R. and Morré, D.J. (2000) "The plasma membrane NADH oxidase of soybean has vitamin K_1 hydroquionone oxidase activity", *Biochim. Biophys. Acta* **1463**, 448–458.

[32] Forsmark-Andree, P., Dallner, G. and Ernster, L. (1995) "Endogenous ubiquinol prevents protein modification accompanying lipid peroxidation in beef heart submitochondrial particles", *Free Radic. Biol. Med.* **19**, 749–757.

[33] Morré, D.J., Chueh, P.-J., Lawler, J. and Morré, D.M. (1998) "The sulfonylurea-inhibited NADH oxidase activity of HeLa cell plasma membranes has properties of a protein disulfide–thiol oxidoreductase with protein disulfide–thiol interchange activity", *J. Bioenerg. Biomembr.* **30**, 477–487.

[34] Chueh, P.-J., Morré, D.M., Penel, C., DeHahn, T. and Morré, D.J. (1997) "The hormone-responsive NADH oxidase of the plant plasma membrane has properties of a NADH: protein disulfide reductase", *J. Biol. Chem.* **272**, 11221–11227.

[35] Morré, D.J. (2002) "Preferential inhibition of the plasma membrane NADH oxidase (NOX) activity by diphenyleneiodonium chloride with NADPH as donor", *Antioxid. Redox Signal.* **4**, 207–212.

[36] Morré, D.J. and Morré, D.M. (2000) "Applications of aqueous two-phase partition to isolation of membranes from plants: a periodic NADH oxidase activity as a marker for right side-out plasma membrane vesicles", *J. Chromatogr. B* **743**, 377–387.

[37] Hicks, C. and Morré, D.J. (1998) "Oxidation of NADH by intact segments of soybean hypocotyls and stimulation by 2,4-D", *Biochim. Biophys. Acta* **1375**, 1–5.

[38] Cho, N.M., Chueh, P.-J., Kim, C., Caldwell, S., Morré, D.M. and Morré, D.J. (2002) "Monoclonal antibody to a cancer-specific and drug-responsive hydroquinone (NADH) oxidase from the sera of cancer patients", *Cancer Immunol. Immunother.* **51**, 121–129.

[39] Kim, C., MacKellar, W.C., Cho, N., Byrn, S.R. and Morré, D.J. (1997) "Impermeant antitumor sulfonylurea conjugates that inhibit plasma membrane NADH oxidase and growth of HeLa cells in culture. Identification of binding proteins from sera of cancer patients", *Biochim. Biophys. Acta* **1324**, 171–181.

[40] Schininá, M.E., Carlinin, P., Polticelli, F., Zappacosta, F., Bossa, F. and Calabrese, L. (1996) "Amino acid sequence of chicken Cu, Zn-containing superoxide dismutase and identification of glutathionyl adducts at exposed cysteine residues", *Eur. J. Biochem.* **237**, 433–439.

[41] Ohnishi, K., Niimura, Y., Hidaka, M., Masaki, H., Suzuki, H., Uozumi, T. and Nishino, T. (1995) "Role of cysteine 337 and cysteine 340 in flavoprotein that functions as NADH oxidase from *Amphibacillus xylanus* studied by site-directed mutagenesis", *J. Biol. Chem.* **270**, 5812–5817.

[42] Gilberger, T.W., Bergmann, B., Walter, R.D. and Muller, S. (1998) "The role of the C-terminus for catalysis of the large thioredoxin reductase from *Plasmodium falciparum*", *FEBS Lett.* **425**, 407–410.

[43] Kliman, R.M. and Hey, J. (1993) "DNA sequence variation at the period locus within and among species of the *Drosophila melanogaster* complex", *Genetics* **133**, 375–387.

[44] Sun, P., Morré, D.J. and Morré, D.M. (2000) "Periodic NADH oxidase activity associated with an endoplasmic reticulum fraction from pig liver. Response to micromolar concentrations of retinal", *Biochim. Biophys. Acta* **1498**, 52–63.

[45] Wang, S., Pogue, R., Morré, D.M. and Morré, D.J. (2001) "NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms", *Biochim. Biophys. Acta* **1539**, 192–204.

[46] Baserga, R. (1985) *The Biology of Cell Reproduction* (Harvard University Press, Cambridge).

[47] Barr, R., Garcia, C. and Morré, D.J. (2000) "Touch-sensitive NADH oxidase activity of pea and cucumber tendrils and of soybean hypocotyl sections", *Int. J. Plant Sci.* **161**, 387–391.

[48] Garcia, C., Hicks, C. and Morré, D.J. (1999) "Plasma membrane NADH oxidase is gravi-responsive", *Plant Physiol. Biochem.* **37**, 551–558.

[49] Bacon, E. and Morré, D.J. (2001) "Plasma membrane NADH oxidase of maize roots responds to gravity and imposed centrifugal forces", *Plant Physiol. Biochem.* **39**, 487–494.

[50] Morré, D.J. and Brightman, A.O. (1997) "Parallel inhibition by external osmotica of auxin-induced elongation in stem sections and auxin-stimulated NADH oxidase activity of plasma membrane vesicles from soybean hypocotyls", *Plant Physiol. Biochem.* **35**, 311–319.

[51] Barr, R., Morré, D.J. and Crane, F.L. (2000) "Oxidation of NADH by hypocotyl segments of soybean is stimulated by 2,4-D", *Plant Physiol. Biochem.* **38**, 739–745.

[52] Morré, D.J. (1994) "The hormone- and growth factor-stimulated NADH oxidase", *J. Bioenerg. Biomembr.* **26**, 421–433.

[53] Sun, E., Lawrence, J., Morré, D.M., Sun, I., Crane, F.L., MacKellar, W.C. and Morré, D.J. (1995) "Proton release from HeLa cells and alkalization of cytoplasm induced by diferric transferrin or ferricyanide and its inhibition by the diarysulfonylurea antitumor drug *N*-(4-methylphenylsulfonyl)-*N*-(chlorophenyl)urea (LY181984)", *Biochem. Pharmacol.* **50**, 1461–1468.

[54] Morré, D.M. and Morré, D.J. (1995) "Mechanism of killing of HeLa cells by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N*-(chlorophenyl)urea (LY181984)", *Protoplasma* **184**, 188–195.

[55] de Grey, A.D.N.J. (1999) "The mitochondrial free radical theory of aging", *R.G. Landes*, (Austin, TX), pp 104–110.

[56] Konopka, R.J. and Benzer, S. (1971) "Clock mutants of *Drosophila melanogaster*", *Proc. Natl Acad. Sci.* **68**, 2112–2116.

[57] Young, M.W. (2000) "Life's 24-hour clock: molecular control of circadian rhythms in animal cells", *Trends Biochem. Sci.* **25**, 601–606.

[58] Rutter, J., Reick, M., Wu, L.C. and McKnight, S.L. (2001) "Regulation of clock and NPAS2 DNA binding by the redox state of NADH cofactors", *Science* **293**, 510–514.

[59] Morré, D.J., Chueh, P.-J. and Morré, D.M. (1995) "Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture", *Proc. Natl Acad. Sci. USA* **92**, 1831–1835.

[60] Morré, D.J., Wu, L.-Y. and Morré, D.M. (1995) "The antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N'*-(chlorophenyl)urea (LY181984) inhibits NADH oxidase activity of HeLa plasma membrane", *Biochim. Biophys. Acta* **1240**, 11–17.

[61] Morré, D.J., Bridge, A., Wu, L.-Y. and Morré, D.M. (2000) "Preferential inhibition by (−)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture", *Biochem. Pharmacol.* **60**, 937–946.

[62] Morré, D.J., Kim, C., Paulik, M., Morré, D.M. and Faulk, W.P. (1997) "Is the drug-responsive NADH oxidase of the cancer cell plasma membrane a molecular target for adriamycin?", *J. Biomembr. Bioenerg.* **29**, 269–280.

[63] Morré, D.J. and Reust, T. (1997) "A circulating form of NADH oxidase activity responsive to the antitumor sulfonylurea *N*-(4-methylphenylsulfonylurea)-*N'*-(chlorophenyl)urea (LY181984) specific to sera from cancer patients", *J. Biomembr. Bioenerg.* **29**, 281–289.

[64] Morré, D.J., Caldwell, S., Mayorga, A., Wu, L.-Y. and Morré, D.M. (1997) "NADH oxidase activity from sera altered by capsaicin is widely distributed among cancer patients", *Arch. Biochem. Biophys.* **342**, 224–230.

[65] Levi, F. (2000) "Marked 24-h rest/activity rhythms are associated with better quality of life, better response, and longer survival in patients with metastatic colorectal cancer and good performance status", *Clin. Cancer Res.* **6**, 3038–3045.

[66] Morré, D.M., Lenaz, G. and Morré, D.J. (2000) "Surface oxidase and oxidative stress propagation in aging", *J. Exp. Biol.* **203**, 1513–1521.

[67] Morré, D.J., Pogue, R. and Morré, D.M. (1999) "A multifunctional ubiquinol oxidase of the external cell surface and sera", *BioFactors* **9**, 179–187.

[68] Larm, J.A., Vaillant, F., Linnane, A.W. and Lawen, A. (1994) "Upregulation of the plasma membrane oxidoreductase as a prerequisite for the viability of human Namalwa rho^{o} cells", *J. Biol. Chem.* **269**, 30097–30100.

[69] Tompa, P. and Friedrich, P. (1998) "Prion proteins as memory molecules: an hypothesis", *Neuroscience* **86**, 1037–1043.