SUMMARY OF RESEARCH

A GRAVITY-RESPONSIVE TIME-KEEPING PROTEIN OF THE PLANT AND ANIMAL CELL SURFACE

NAG 2-1344

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1. Statement of the hypothesis, objective and value of the research.

The hypothesis under investigation wass that a ubiquinol (NADH) oxidase protein of the cell surface with protein disulfide-thiol interchange activity (= NOX protein) is a plant and animal time-keeping ultradian (period of less than 24 h) driver of both cell enlargement and the biological clock that responds to gravity. Despite considerable work in a large number of laboratories spanning several decades, this is, to my knowledge, our work is the first demonstration of a time-keeping biochemical reaction that is both gravity-responsive and growth-related and that has been shown to determine circadian periodicity. As such, the NOX protein may represent both the long-sought biological gravity receptor and the core oscillator of the cellular biological clock.

Completed studies have resulted in 12 publications and two issued NASA-owned patents of the clock activity. The gravity response and autoentrainment were characterized in cultured mammalian cells and in two plant systems together with entrainment by light and small molecules (melatonin). The molecular basis of the oscillatory behavior was investigated using spectroscopic methods (Fourier transform infrared and circular dichroism) and high resolution electron microscopy. We have also applied these findings to an understanding of the response to hypergravity. Statistical methods for analysis of time series phenomena were developed (Foster et al., 2003). Site-directed mutagenesis was used to generate NOX proteins with period length longer or shorter than 24 min which when introduced into COS cells generated circadian responses with period lengths corresponding longer or shorter than 24 h (60 X the NOX period length) (Morré et al., 2002). A role for bound copper in maintaining the 24 min period length was discovered together and a site-directed mutagenesis analysis of active site cysteines and functional domains was completed. A CNOX monoclonal antibody to soybean protein was obtained. The antibody was used in ongoing efforts to expression clone CNOX from plants as a first step toward site-directed mutagenesis and vector-forced mutational analysis of the role of CNOX in elongation growth, circadian function and gravity response in plants.

The importance of the work is underscored by recent report that, after three months in space, astronauts lose sleep and sleep less soundly because of the lack of gravity and the absence of day- and night cues that disrupt their internal clocks (Monk et al., 2001). While the biological clock continues to function in space, the results of this first long-term study demonstrate rapid deterioration of the body's daily rhythms and the need for ways to trick the internal clock into maintaining a strong 24 h cycle if longer space missions are to succeed. Our findings provide a biochemical approach to the development of just such a strategy.

We are confident that the ground-based studies completed under NAG 2-1344 will lead eventually to flight experiments to test our model and hypothesis for how multicellular organisms respond to gravitational perturbances. New precise monitoring systems for application to both humans and to non-human species are expected to derive from the work as well as significant contributions to understanding mechanisms that underlie space flight-related developmental changes in plants and biomedical and behavioral changes in humans.

The most important and unique feature of the research proposed lies in the opportunity, for the first time, to isolate and characterize a gravity responsive protein that may, in fact, be both the gravity-receptor molecule and the biological clock. The protein appears to be distributed widely among plant and animal cells where it is located on the external cell surface. The protein is involved in the growth process and is stimulated by relatively low imposed gravitational forces (Garcia et al., 1999; Bacon and Morré, 2001). In gravitationally-responsive plant parts, the activity of the protein responds to unit gravity (Garcia et al., 1999). The structural and functional implications of such a protein are far-reaching. They not only provide a conceptual frame work within which future micro-gravity research may be based but provide, as well, opportunities for chemical or genetic modulation of the receptor protein to enhance adaptation of astronauts, food plants and living organisms in general, to space flight conditions.

2. Review of research completed.

a) Summary

In work carried out under a previous NASA-sponsored project, a gravity-responsive protein with NADH oxidase activity at the external mammalian cell surface discovered from studies with isolated plasma membrane-derived endosomes from rat and bovine kidney and from HeLa (human cervical carcinoma) cells grown in culture. The initial observations were based on a response to imposed centrifugal forces (Morré et al., 1998a). A comparable activity was found for plants using a plasma membrane-derived endosome model (Morré et al., 1998b). Subsequent studies under NAG 2-1344extended the observations to isolated plasma membranes from HeLa cells and soybean tissues (Garcia et al., 1999). With the latter, a growth-related response to unit gravity was obtained (Garcia et al., 1999) as well as a response to imposed centrifugal forces. The cDNA to a mammalian form of the protein was cloned (GenBank Accession Number AF207881) and the protein expressed in bacteria (Chueh et al., 2002 a, b). The purified protein from soybean as well as the recombinant form of the protein from HeLa cells expressed in *E. coli* responded as well to low g centrifugal forces. A second important characteristic of the protein also given by the recombinant protein was a periodic alternation of two functional activities associated with the protein. The two functions were quinol (NADH) oxidation with concomitant transfer of protons and electrons to molecular oxygen or protein disulfides and protein disulfide-thiol interchange. The two activities alternated with a temperature compensated and entrainable period length of 24 min. The pattern of oscillations coincided with a similar pattern of oscillation with a period length of 24 min that characterize cell enlargement (growth) in plants (Morré, 1998; Morré et al., 2001a, 2002) and animal cells (Pogue et al., 2000). As such the activity has been suggested to function both as an ultradian core oscillator of the biological clock and of the enlargement phase of cell growth common to both plant and animal cells.

b) Plasma membrane NADH oxidase of soybean hypocotyls is gravity-responsive (Appendix Figures 1-8, Table 1).

i) Plant NOX activity responds to unit gravity

NADH oxidase measured with intact tissue sections and with isolated plasma membrane vesicles from etiolated hypocotyls of soybean (*Glycine max*) responds to gravity (Garcia et al., 1999). The response was one of inhibition of activity with low centrifugal g forces (< 100 g) and short stimulation times lying flat of 20 min or less at 1 g and one of stimulation with higher centrifugal forces and stimulation times of 3 min or longer at 1 g (Garcia et al., 1999). Everting the tissue sections resulted in stimulation of the activity with a lag of less than 20 min and returning the sections to the normal upright position resulted in a return to initial rates also with a lag of less than 20 min. Both the stimulated and non-stimulated activities oscillated with a period of 24 min.

ii) Plant NOX activity responds to imposed centrifugal force

When intact hypocotyl sections were centrifuged for brief periods of time at low g forces, NOX activity also was stimulated in a time- and relative g-force-dependent manner (Garcia et al., 1999). A similar response was given by NOX activity of plasma membrane vesicles isolated from hypocotyl segments.

The NOX activity of isolated vesicles of plasma membranes from soybean hypocotyls exhibited a response to centrifugation that was both g force- and time-dependent. These experiments were carried out with two spectrophotometers operated in parallel. With both instruments, the reaction mixtures were constituted, poured into tubes and transported from centrifuge to spectrophotometer in exactly the same manner, the only difference being that one was centrifuged and the other not. The centrifugal forces utilized were insufficient to sediment the membranes to form a pellet. The activity in response to centrifugation persisted for a time with a T ½ of ca 10 min for the return to basal levels. However, the centrifugation induced responses persisted for a time sufficient to carry out physical measurements of changes in secondary structure.

c) The gravity-responsive NOX activity oscillates with a temperature compensated period of 24 min (Appendix Figures 9-15, Table 2).

The NOX activity of etiolated hypocotyls of soybean oscillated with a period length of about 24 min or 60 times per 24 h day (Morré and Morré, 1998). The oscillations were temperature compensated such that the period remained constant at 24 min between 17 and 37°C, a temperature range over which enzymatic activity varied 4-fold (Q_{10} of 2). The oscillations were observed with intact tissue sections, with isolated plasma membrane vesicles and with the detergent-solubilized and partially purified enzyme. The oscillations were observed both with the oxidation of NADH and in the restoration of activity to scrambled ribonuclease used as a measure of a protein disulfide-thiol interchange activity also associated with the NOX protein (Morré et al., 1995a).

To illustrate the reproducibility of the periodic nature of the activity oscillations, rates were measured for plant plasma membranes simultaneously with two spectrophotometers and with reaction mixtures prepared in parallel. The two data sets yielded similar patterns of oscillations. The corresponding Fourier transforms of the oscillations given in Appendix Figures 14C and 14D verified that the oscillations were periodic.

The rate of NADH oxidation $\cong 1.6 + 1.9 \sin\left(\left(\frac{15.12\ \pi\ t}{180}\right) + 120\ \pi/180\right)$ where t =time in min and the value of 1.6 is the mean rate of NADH oxidation in the presence of 1 μM 2,4-D, a synthetic auxin regulator of plant cell enlargement that stimulates both growth and the NOX activity (Appendix Figrues 14A and 14B). The sine function described by the equation (dotted sine function) approximated the experimentally determined values of Appendix Figures 14a and 14B.

To allow for calculation of standard deviations in amplitude, a second set of two determinations was carried out under the same conditions as those for the first set. The second set of determinations was delayed to begin in phase with the first set. The specific activities of the four data sets were then averaged and standard deviations were calculated (Appendix Fig. 15A). The maxima and minima of the oscillations were significantly different and the composite Fourier analysis (Appendix Fig. 15B) yielded a mean frequency of 0.042 ± 0.001 cycles/min or an average period of 23.8 ± 0.3 min.

Fourier analyses were applied to triplicate data sets collected in the absence of 2,4-D at each of the 3 temperatures investigated (Appendix Table 2). The periods determined were about 24 min at 17°, 27° and 37° C and the mean period comparing the three different temperatures was 24.1 ± 0.3 min.

d) NOX activity of the bovine milk fat globule membrane a derivative of the mammary epithelial plasma membrane exhibits a 24 min temperature compensated period.

An oscillating activity with a temperature compensated period of 24 min has been described for the NOX protein of the bovine milk fat globule membrane, a derivative of the mammary epithelial cell plasma membrane (Appendix Fig. 9, Morré et al., 2002). The period of 24 min remained unchanged at 17, 27 and 37° C whereas the amplitude doubled with each 10° C rise in temperature ($Q_{10} \cong 2$) as for soybean. The periodicity was observed with both intact milk fat globule membranes and with detergent-solubilized membranes.

e) The NOX activity of the HeLa cell surface exhibits a ca 24 min temperature compensated period and responds to gravity.

A mammalian counterpart to oscillating NOX activity with a temperature compensated period length of 24 min of plants was observed as well for HeLa cells (Wang et al., 2001). The period length of 24 min remained unchanged at 17, 27 and 37° C whereas the amplitude doubled with each 10° C rise in temperature ($Q_{10} \cong 2$). The periodicity was observed with the surface activity of whole cells and in a cell-free environment with right side-out plasma membrane fragments and with partially purified NOX protein. The oscillations were not the result of instrument variation or of chemical interactions among reactants in solution. Preparations with different periodicities entrained (synchronized) when mixed. The gravity-responsive cell surface NOX protein is the first reported example of an entrainable biochemical entity with a fully temperature-compensated period length potentially capable of functioning as an ultradian core oscillator of the circadian clock.

A response to imposed gravitational forces has been demonstrated for the NOX protein of plasma membranes of HeLa cells, a partially-purified fraction of NOX proteins from HeLa cells and for human recombinant NOX protein. When subjected to low centrifugal forces, i.e., < 500 g·min, the human NOX protein from all three sources responded with an enhanced rate of NADH oxidation. The enhanced activity persisted over 5-6 min and then slowly returned to basal levels with a T ½ of about 10 min.

f) The rate of plant and animal cell enlargement oscillates with a period length of about 24 min (Appendix Figures 16 and 17, Tables 3 and 4).

Since activity of the gravity-sensitive NADH oxidase was correlated with the rate of cell enlargement, experiments were designed to determine if the rate of cell enlargement also oscillated with a period length of 24 min. This expectation is borne out with elongation of hypocotyl sections of dark-grown soybeans (Morré, 1998; Morré et al., 2002), elongation of coleoptile sections of dark-grown maize and enlargement of individual epidermal cells of soybean (Morré et al., 2001a) as well as enlargement of CHO (Pogue et al., 2001) and HeLa (Wang et al., 2001) cells. Steady state cell enlargement in these cells and tissues is strikingly periodic and the pattern of oscillations parallels those of the cell surface NADH oxidation.

Oscillations in the rates of cell enlargement in CHO (Pogue et al., 2001) and HeLa cells (Wang et al., 2001) were monitored by light microscopy. The period lengths corresponded to those of the NADH oxidases present at the CHO and HeLa cell plasma membranes.

As both the gravity-responsive NOX activity and plant and animal cell enlargement exhibit oscillations with period lengths of 24 min, we postulate that gravity may be an important contributor to NOX synchrony and entrainment with important implications in cell growth, homeostasis and circadian control of cellular activities. A hypothesis that we are eager to test is that small molecules that mimic the effect of gravity on earth can be utilized in space to improve plant production and to facilitate traverse of the complete reproductive cycle and to enable space travelers to maintain normal biorhythms in a micro-gravity environment.

g) The NOX protein shed from cells and into the circulation.

The NOX protein is an ectoprotein anchored in the outer leaflet of the plasma membrane. As is characteristic of other examples of ectoproteins (sialyl and galactosyl transferase, dipeptidylamino peptidase IV, etc.), the gravity-responsive NOX protein is shed. It appears in soluble form in conditioned media of cultured cells and in sera. The serum presence of the NOX proteins seems to represent an origin due to shedding from cells and offers an opportunity to monitor NOX activities of human subjects based on serum analyses.

h) Mechanistic studies (Appendix Figures 19-29)..

The mechanisms whereby the NOX protein both responds to gravity and exhibits a fully temperature-compensated period length are, at the moment, speculative but appears to reside within the secondary or tertiary structure of the NOX protein. One scenario is that paired NOX subunits interact specifically to induce a random coil or α-helix to β-structure transformation.

The ensuing conformational change would be spontaneous and reversible. Upon reaching a certain conformation, the process would then reverse and, after 24 min, begin anew. Synchronous subunits might at some point dissociate from each other more readily than asynchronous subunits and then reassociate with subunits where folding and unfolding was less synchronous than with themselves. Association of two asynchronous subunits would then result in synchronization such that by association and reassociation, a membrane population or solution would become highly synchronized. The NOX protein is considered to be anchored to the membrane either by hydrophobic or electrostatic interactions that can be reversibly dissociated. The NOX protein appears to contain neither a GPI anchor or a transmembrane spanning domain. Both of these possibilities are ruled out by experiment and on a theoretical basis from the derived amino acid sequence and from the hydropathy plots of the derived amino acid sequence of the open reading frame of the cDNA (Morré et al., 2001b). As such, the NOX protein would be relatively mobile in and off the membrane. Subunits would be free to associate and dissociate as well as recruit unsynchronized new molecules coming from synthesis to replace shed molecules. A role of NOX as an ultradian time-keeping mechanism would also be served by the existence of a fully active but drug-responsive circulating form of the protein that retained periodicity (see above). The circulating form could move throughout the organism and associate with NOX subunits on the surfaces of cells to synchronize the entirety of NOX molecules within the cell and ultimately within the entire organism.

i) Cloning of the tumor-associated NOX protein (tNOX) from HeLa (human cervical carcinoma) cells and the constitutive NOX protein (CNOX) from soybean.

Expression screening of a HeLa cell library was used to isolate cDNA clones encoding a protein recognized by a monoclonal antibody to the human tNOX protein isolated from sera of cancer patients.

The amino acid sequence derived from the open reading frame of the cloned cDNA (Appendix Figure 30) contains conserved quinone binding site motif (drugs inhibitory to tNOX are considered to bind at the quinone site) and a conserved NADH-binding site. The protein contains 8 cysteines and a putative C-XXXX-C protein thiol-disulfide interchange motif distinct from that of protein disulfide isomerase and other known thiol oxido-reductases. A H(326)-V-H-P-F-G copper-binding motif in the deduced amino acid sequence of the NOX gene is highly conserved with the motif H-V-H-E-F-G in that of both human and chicken copper/zinc superoxide dismutase (Cu/Zn SOD). The third ligand for copper binding was located 15 amino acids downstream from the motif in chicken SOD. One histidine (histidine 355) located 14 amino acids downstream of the motif in tNOX, therefore, is the likely candidate for the third copper binding ligand in the NOX protein.

The antigen was a 34 kD peptide from sera of cancer patients (Chueh et al., 1997). This 34 kD peptide region had been associated previously with a sulfonylurea-binding protein from plasma membrane of HeLa cells and with the sulfonylurea-inhibited and/or capsaicin-inhibited NOX activity shed into culture media conditioned by growth of HeLa cells and of sera of cancer patients. Antisera generated to the 34 kD peptide inhibited and immunoprecipitated the sulfonylurea- or capsaicin-inhibited activity and cross-reacted with the 34 kD band from sera. Clones were selected on the basis of reactivity with 34 kD tNOX-enriched fractions prepared

from the HeLa cell surface. Secondary screening used inhibition of the drug-inhibited component of tNOX activity of pooled sera of cancer patients. The antisera were extensively characterized (Cho et al., 2002).

The periodic oscillations in enzymatic activities associated with the cellular NOX proteins has been observed with the recombinant tNOX generated from expression of the tNOX cDNA in bacteria (Chueh et al., 2002 a, b). The period length of the recombinant protein is the same as that of cellular tNOX and its identity as tNOX is confirmed by the total inhibition of the activity by 1 μM capsaicin.

A second unique feature of the cell surface tNOX activity is that the two activities associated with the cloned and expressed protein, the hydroquinone (NADH) oxidase activity and the protein disulfide-thiol interchange (dithiopyridine cleavage) not only oscillate but the two oscillatory activities alternate (Appendix Figure 20C). This represents an extraordinary observation and one unprecedented in the scientific literature.

j) Cloning of the constitutive NOX protein (CNOX) from plants.

In a parallel investigation, a monoclonal antibody was generated to the constitutive NOX activity of soybean plasma membranes (CNOX). This antibody was used to initiate expression cloning of the CNOX cDNA from a soybean library.

A total of 5 mice were immunized with right side-out plasma membrane vesicles as immunogen. One mouse yielded a monoclone to the constitutive NOX. The antibody had the required characteristics to proceed with library screening. The antibody completely blocked the 24 min pattern of oscillations of the column purified constitutive NOX activity from soybean (Appendix Figure 18), reacted on Western blots with the 48 kD CNOX peptide, immunoprecipated both the CNOX peptide and the activity in parallel and did not cross react with the *E. coli* NOX as required to have utility for expression cloning using the bacterial system.

k) Red light in plants and cysteine compounds in plants and animals set the period and cysteine compounds may have utility for growth of plants and other organisms in space.

The NADH oxidase activity of the plant cell surface clearly oscillates with a 24 min period. With plants of spinach or soybean, the oscillations of all plants within a single population are in phase (Morré et al., 1999a). Experiments were carried out to determine what stimulus in the environment was responsible for the synchrony. The findings demonstrate that light exposure of plants transferred from darkness initiates a maximum 12 min following the light exposure independent of the point in the cycle where the light was given (Morré et al., 1999a). Red light (650 nm, 10 min, 50 μM $m^{-2}s^{-1}$) was equivalent to white light. Blue light also phases the oscillations but apparently through a different chromophore than red light (Morré et al., Submitted b). Green or yellow light at a similar intensity as red or blue light were ineffective. Far-red illumination (730 nm, 5 min) appeared to be equivalent to darkness and was sufficient to condition the plants to respond to light by resetting the NOX cycle. Involvement of phytochromes, as the light detecting molecule, therefore, is indicated for the red light absorbing

chromophore. Since blue light sets the period in both plant cells and mouse skin, a blue light absorbing chromophore common to both plant and animal cells such as a cryptochrome is indicated for the blue light chromophore.

The only substances found so far that alter the pattern of oscillations are melatonin and valerian. These compounds do not change the period length but "set" the phase of the oscillatory patterns much like light. But unlike light these substances act directly on the NOX protein itself. A single addition of 1 μM melatoninresults in an activity maximum with activity maxima every 24 min thereafter. The response to valerian is similar to that for melatonin and has been demonstrated for soybean, HeLa and CHO cells and mouse skin.

FT-IR spectrometer used to measure protein changes in NASA centrifuge.

When subjected to low centrifugal forces (< 500 g min), a human recombinant ECTO-NOX protein (tNOX) responded with an enhanced rate of NADH oxidation. The enhanced activity persisted for 5 to 6 min after centrifugation and then slowly returned to basal levels with a $T_{1/2}$ of about 10 min. Normally ECTO-NOX proteins exhibit complex oscillatory behavior within each 24 min period consisting of five maxima unequally separated at intervals of 4 to 6 min. Each of the 5 maxima correspond to changes in the proportion of α-helix and β-structure determined by Fourier transform infrared (FT-IR) and circular dichroism spectroscopy. After centrifugation for 4 min at 100 g, the activity of the fourth upward inflection in each period was disproportionately increased and the third inflection appeared earlier. These two inflections are two of three inflections that coincide with maxima in protein disulfide-thiol interchange and correlate with growth.

Taken together, the findings suggest that the gravity response is confined to specific domains within the structure of the protein involved in the oscillatory behavior. To test this hypothesis, an Avatar 360 FT-IR spectrometer was mounted to the arm of the 20 G centrifuge (29 ft radius with Avatar's center mounted at 23 ft) at the NASA Ames Research Center, Moffett Field, CA. Greatest instrument stability was achieved with the mirror aligned tangential to the centrifuge rotation. The Avatar, fitted with a calcium chloride sample cell containing human recombinant tNOX protein was rotated at forces of up to 2.75 G_l (the instrument withstood 5 min at 3.5 G). An alteration in the FT-IR signal (Amide I/Amide II) was observed in proportion to G force that was retained by the protein following centrifugation (Appendix Figure 32) for a time before returning to the original baseline value. The findings confirm that ECTO-NOX proteins respond to normally sustainable G forces on earth and experienced in flight.

NOX proteins have many characteristics in common with prions. A distinguishing property of prions is the ability to undergo a permanent conformational change (to learn) and to transmit this conformational change to other proteins like itself (to teach). The response to gravity may be similar except that the memory component is transient and the protein in solution, at least, returns after a time to its original baseline value.

As controls, the recombinant tNOX protein was replaced by albumin or ribonuclease. Neither control protein exhibited the five-peak pattern of change in Amide I/Amide II nor

exhibited the G-force dependent alternations in peak amplitude observed with the recombinant NOX protein.

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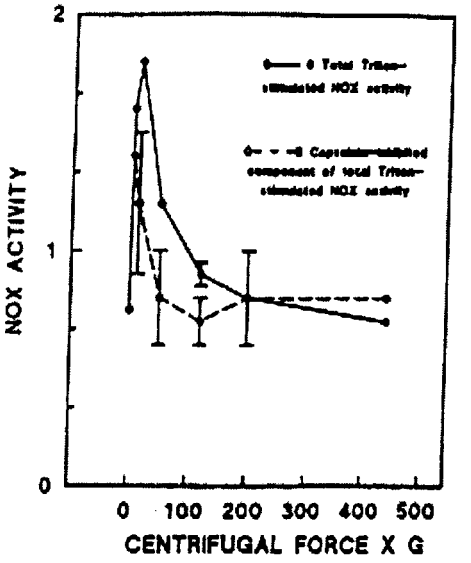


Fig. 1. Response to centrifugation of the Triton X-100-stimulated NADH oxidase (NOX) activity of a HeLa cell endosome fraction and inhibition by 1 μM capsaicin. The preparations were centrifuged in a swing-out rotor for 5 min during the resolution of the two phases. Units of specific activity are nmol/min per mg protein. From Morré et al. (1998a).

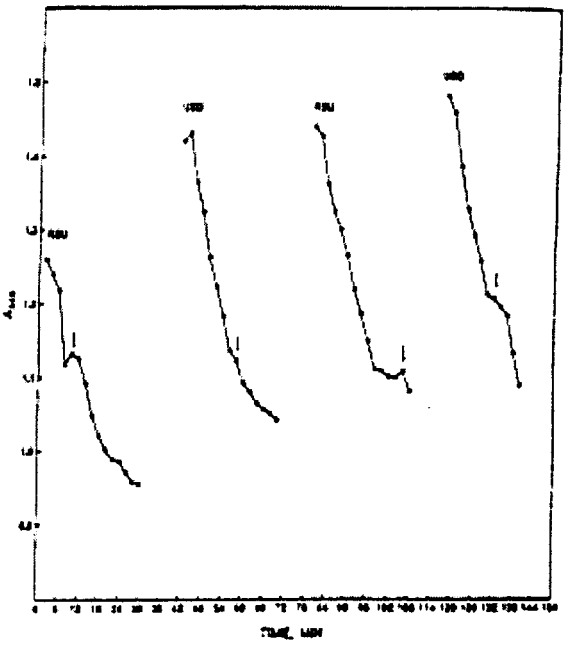


Fig. 2. Decrease in A_{340} as a measure of NADH oxidation by 20 1 cm sections of etiolated hypocotyl of soybean at 24°C in 4 ml of a solution containing 50 mM Tris-MES, pH 7, 1 mM KCN and 150 μM NADH. At intervals of 2 min the incubating solution was mixed and a 2.5 ml portion was transferred to a second cuvette and the absorbance at 340 nm was determined. The solution was then returned to the incubation vessel. The sections were oriented right side-up (RSU) for the first 30 min of incubation and then the sections were everted and incubated upside down (USD). After an additional 30 min, the sections were again oriented RSU, incubated for 30 min and, finally, incubated for 30 min USD. Fresh NADH was added after each inversion. The arrows mark the 24 min period of cyclic oscillations in the activity. Average rates of NADH oxidation averaged for 5 to 10 repetitions of the basic experiment are summarized in Table 1.

Table 1. Summation of NADH oxidation by averages of 1 cm soybean hypocotyl sections.
*NADH oxidation, nmoles/min/20 sections ± std. dev.

Cycles	n	RSU	USD	Δ	RSU	USD	Δ
One cycle	10	9.2 ± 1.8	13.2 ± 2.4	4.0 ± 1.2			
Two cycles	5	8.2 ± 0.000	12.0 ± 4.0	4.0 ± 2.2	9.0 ± 1.4	13.2 ± 3.0	3.2 ± 1.2

RSU = Right side up
USD = Upside down

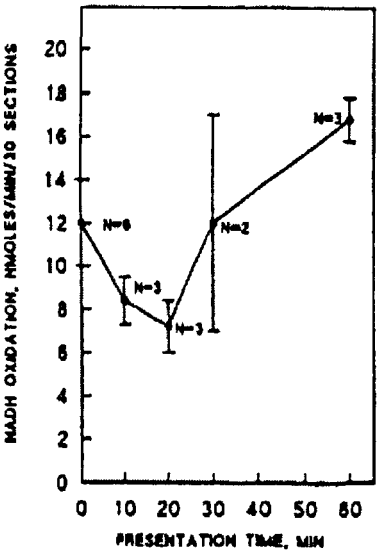
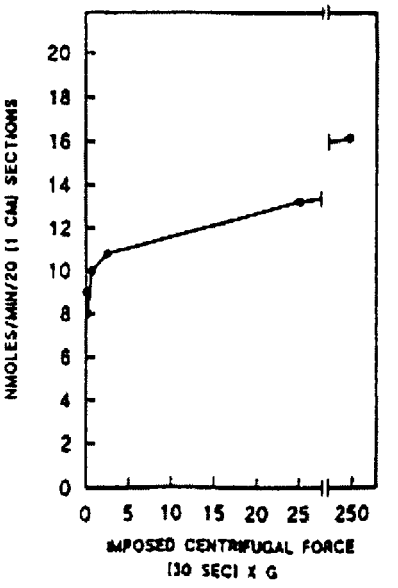


Fig. 3. NADH oxidation as a function of presentation time in min (lying flat). Values are average of 3 determinations ± standard deviations.

Fig. 4. NADH oxidation of 1 cm hypocotyl sections of soybeans as a function of centrifugal force (G) applied for 30 seconds. Rates are initial rates over 10 to 20 min following placement right side-up in the spectrophotometer cuvette. The sections were centrifuged lying flat orthogonal to the axis of rotation.



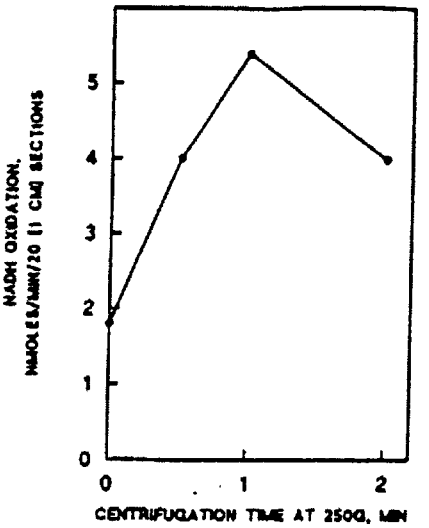


Fig. 5. NADH oxidation of 1 cm hypocotyl sections of soybean as a function of centrifugation time in min at 250 g. The sections were oriented in the centrifuge as described for Figure 4.

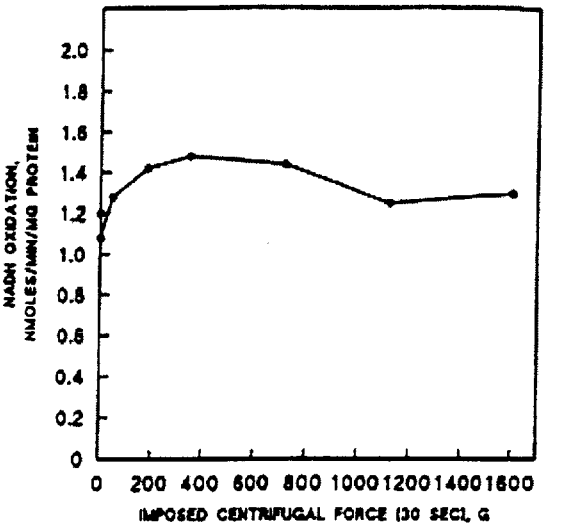


Fig. 6. NADH oxidation by right side-out plasma membrane vesicles as a function of centrifugal force (G) applied for 30 sec. The forces and times were insufficient to pellet the membranes. Assays were over 10 min following centrifugation. An uncentrifuged preparation was assayed in parallel and manipulated exactly as the centrifuged preparation except not centrifuged.

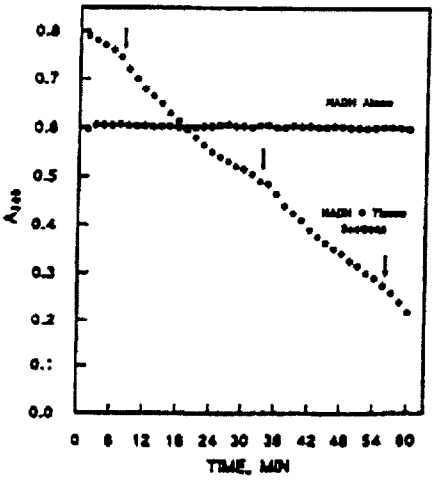


Fig. 7. Decrease in A_{340} as a measure of NADH oxidation in the absence (open symbols) or presence (closed symbols) of 40 1-cm sections of etiolated hypocotyls of soybean at 17°C. Forty sections were placed in 4 ml of solution containing 30 mM Tris-Mes, pH 7, 0.6 mM KCN and 90 µM NADH in a spectrophotometer cuvette. At intervals of 1.5 min, the incubating solution was mixed, a 2.9 ml portion was transferred to a second cuvette and the absorbance at 340 nm was determined. The solution was then returned to the incubation vessel. A sinusoidal fluctuation was observed with an approximate period of 24 min. Arrows indicate activity minima. The average rate of NADH oxidation over the 60 min observation period was 6 nmoles/min/40 1-cm sections.

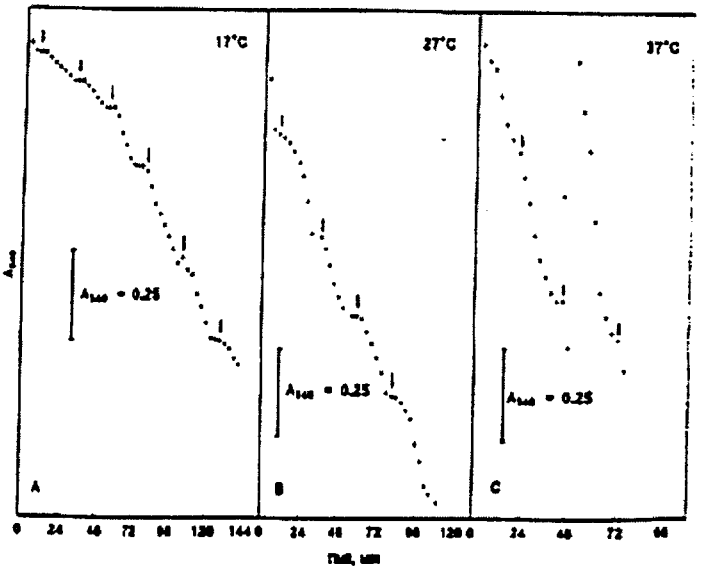


Fig. 8. NADH oxidation rates determined at intervals of 1 min by 40 1-cm sections of etiolated hypocotyls of soybean as the decrease in A_{340} measured comparing 17, 27 and 37°C. Conditions were as for Figure 1 except that additional NADH was added every 15 min at 37°C, every 30 min at 27°C and every 60 min at 17°C to maintain the NADH level above the previously determined K_m of about 100 µM. The absorbance changes due to NADH addition were excluded from the composite traces. The second trace at 37°C is a continuation of the first but the data are presented as two traces to accommodate to the same scale as for 17°C and 27°C. Average rates of NADH oxidation measured over the times indicated were 4 nmoles/min/40 1-cm sections at 17°C, 7.2 nmoles/min/40 1-cm sections at 27° and 15.6 nmoles/min/40 1-cm sections at 37°C. Yet the period remained constant at about 24 min. Arrows indicate activity minima. A. 17°C. B. 27°C. C. 37°C.

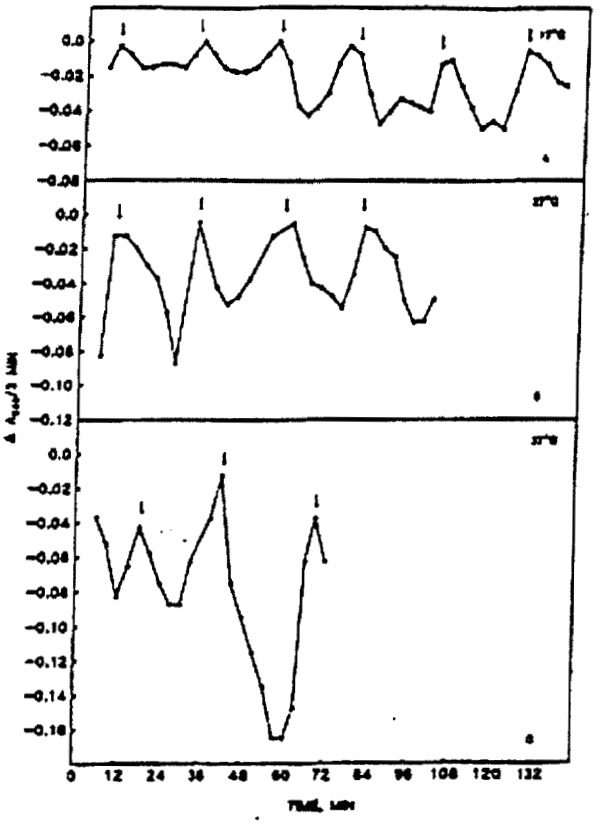


Fig. 9. As in Figure 8, except that rates of NADH oxidation were calculated from the change in A_{340} using the technique of numerical averaging. Rates at any point were calculated according to the formula:
$n = [A_{340\ n-11} - A_{340\ n+11}]/2$.

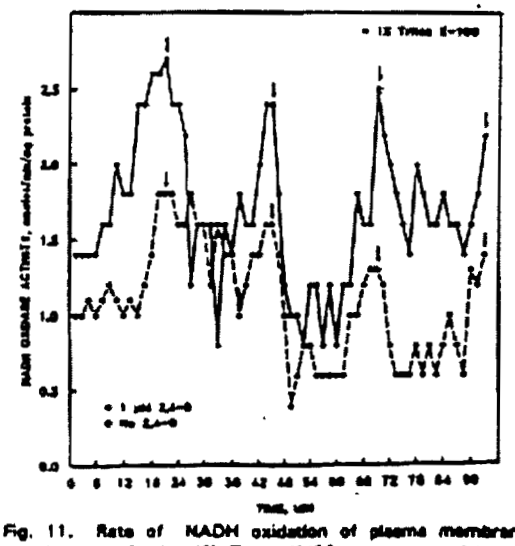


Fig. 11. Rate of NADH oxidation of plasma membrane vesicles solubilized in 1% Triton X-100 and measured in the absence (open symbols) or presence (solid symbols) of 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The two assays were from the same preparation of plasma membranes and were analyzed simultaneously using side-by-side Hitachi Model U3210 spectrophotometers. The activity oscillated with maxima (arrows) at ca. 24 min.

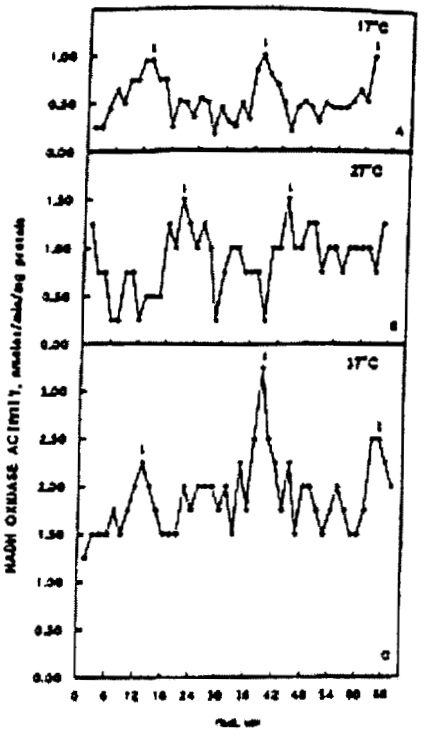


Fig. 10. Rates of NADH oxidation of right side-out plasma membrane vesicles isolated from 1 cm soybean hypocotyl sections by partition in a two-phase polymer system. The reaction mixture in a final volume of 3.5 ml, contained 50 mM Tris-Mes, pH 7, 1 mM KCN, 150 μM NADH and 40 to 50 μg plasma membrane protein. NADH oxidation was determined from the decrease in absorbance at 340 nm using a Hitachi U3210 spectrophotometer equipped with a water jacketed sample cell for temperature control with constant stirring. Arrows indicate activity maxima. A. 17°C. B. 27°C. C. 37°C.

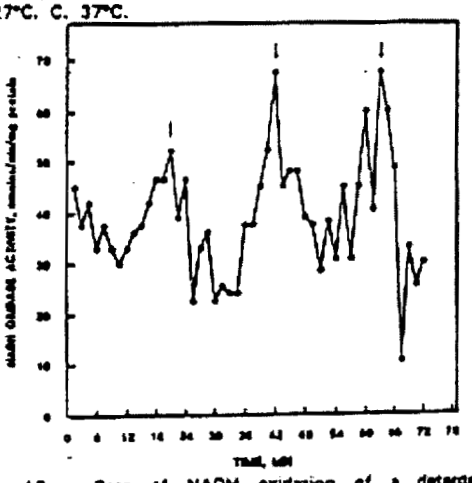


Fig. 12. Rate of NADH oxidation of a detergent-solubilized and affinity-purified fraction enriched in the auxin hormone-stimulated NADH oxidase activity. Assay was as described for Figure 10. The activity of the solubilized and partially purified enzyme exhibited the same ca. 24 min periodicity as was observed with the intact hypocotyl sections (Figs. 8 and 9) and with the isolated plasma membranes vesicles (Fig. 10). The experiment was done twice. The average period length with the solubilized and partially-purified protein was 23.8 ± 0.4 min.

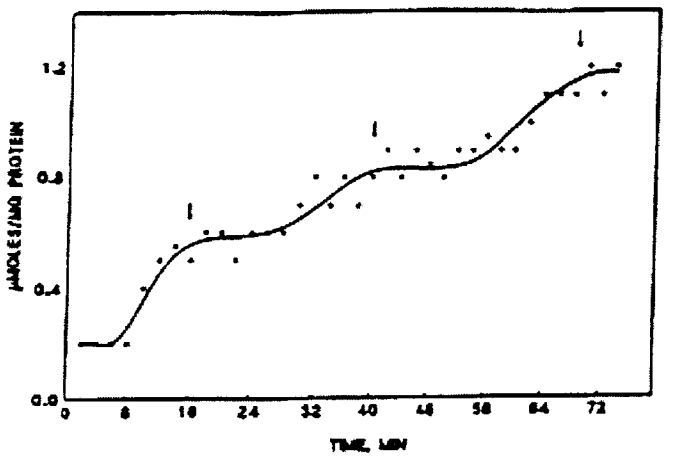


Fig. 13. Time course of protein disulfide-thiol-interchange activity estimated from the activation of scrambled RNase in the absence of cCMP substrate. Membranes (400 μg of protein) were incubated together with scrambled (inactive) RNase for the times indicated in the presence or absence of a balanced redox buffer of freshly prepared 1 μM GSH plus 1 μM GSSG and in the presence of 1 μM 2,4-D. After the times of preincubation indicated, cCMP substrate was added, the RNase activity was assayed over 2 min following the preincubation, and the rate was determined. The rate of activation was approximately linear but fluctuated with a ca. 24 min period. Arrows indicate minima in the rate of activation of RNase activity.

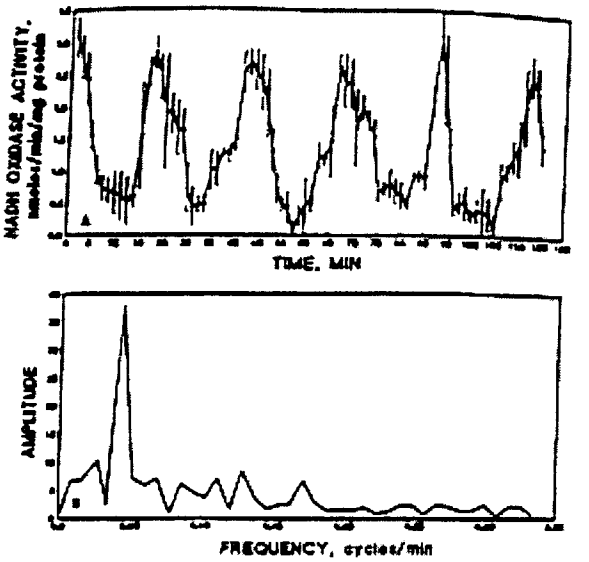


Fig. 15. As in Fig. 14 except means of four sets of da representing those of Fig. 14 plus a second set determinations with the same plasma membran preparation and begun with the periodicity in phase wi the first set of determinations. A. Results are given a means ± standard deviations among the fo determinations to shown the reproducibility of the patte of the periodicity. B. The Four transforms of the averaged data. T maximum amplitude coincided with frequency of 0.042 ± 0.001 cycles/min or period length of 23.8 ± 0.3 min. From Mor and Morré (in press).

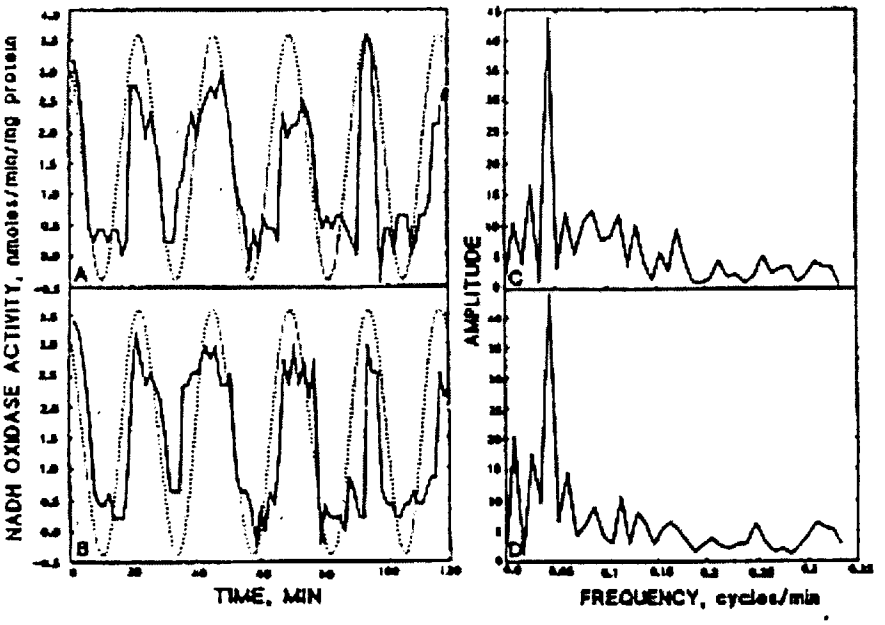


Fig. 14. Two parallel determinations of the rate of NADH oxidation by soybean plasma membranes with time over 120 min in the presence of 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (A and B) as described in Fig. 10 and the corresponding Fourier transforms (C and D). The two determinations were carried out simultaneously using two side-by-side Hitachi U3210 spectrophotometers. Rates of NADH oxidation were measured over 1 min at intervals of 1.5 min. The maximum amplitude from the Fourier analysis coincided with a frequency of about 0.042 cycles/min and corresponded to a period length of 23.8 min. A sine function was fitted to the data (dotted curve) according to the equation given in the text.

Table 2. Fourier analyses of NADH oxidase periodici comparing 17°, 27° and 37° C (n=3). The period w determined from the frequencies observed at maxim amplitude.

Temperature	Frequency (cycles min⁻¹)	Period (min)
17° C	0.041 ± 0.002	24.4 ± 1.3
27° C	0.0415 ± 0.003	24.1 ± 1.9
37° C	0.042 ± 0.004	23.8 ± 2.4
Mean	0.0415 ± 0.0006	24.1 ± 0.3

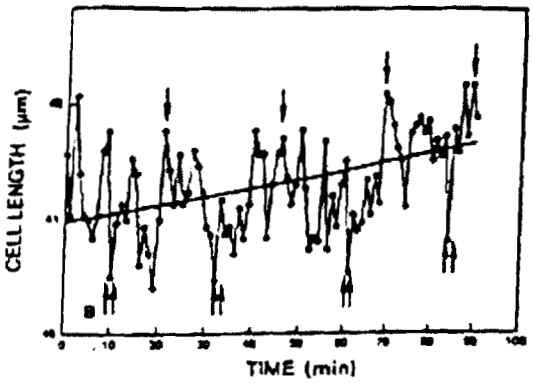


Fig 16. Periodic enlargement of an epidermal cell of soybean as a function of time over 90 min determined by video-enhanced microscopy. Length measurements were taken at intervals of 1 min. Single arrows indicate maximum cell lengths based on decomposition fits. Double arrows indicate minima. These maxima and minima recurred every 24 min. From Morré et al. (2001a).

Table 3. Fourier analyses of NADH oxidase periodicities of soybean plasma membranes comparing 17°, 27° and 37° C (n=3). The period was determined from the frequencies observed at maximum amplitude (Morré and Morré, 1998).

Temperature	Frequency (cycles min^{-1})	Period (min)
17° C	0.041 ± 0.002	24.4 ± 1.3
27° C	0.0415 ± 0.003	24.1 ± 1.9
37° C	0.042 ± 0.004	23.8 ± 2.4
Mean	0.0415 ± 0.0005	24.1 ± 0.3

Table 4. Period length of cell elongation of soybean cells determined from differential interference contrast images recorded at 1 min intervals as in Figure 8.

Temperature	Period (min)
17° C	24.4 ± 1.3
27° C	24.1 ± 1.9
37° C	23.8 ± 2.4
Mean	24.1 ± 0.3

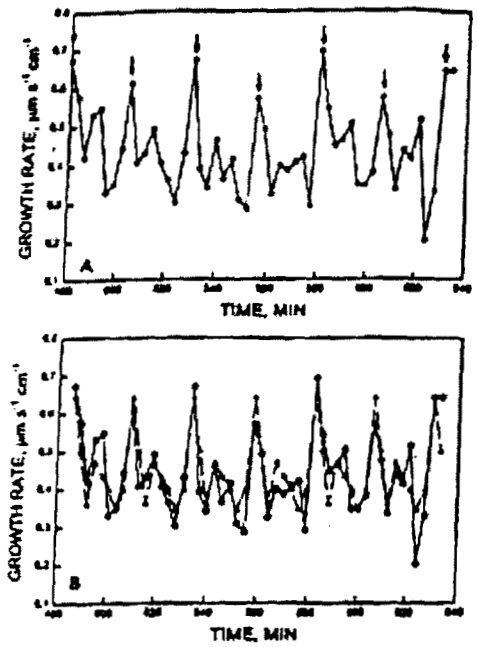


Fig. 17. Elongation growth of 0.5 cm sections of dark-grown seedlings of soybean measured at 2 min intervals using a multichannel auxinometer as described by Lüthen and Böttger (1992). Each result is for four 0.5 cm sections. Elongation rate fluctuated with a period length of 24 min (A). The sections were equilibrated for 180 min after which 1 µM 2,4-D was added. Steady state elongation rates between 430 and 630 min are shown. (B) Time series (decomposition) analysis to verify the reproducibility of the pattern of oscillations also confirmed by Fourier analysis (From Morré et al., 2001b).

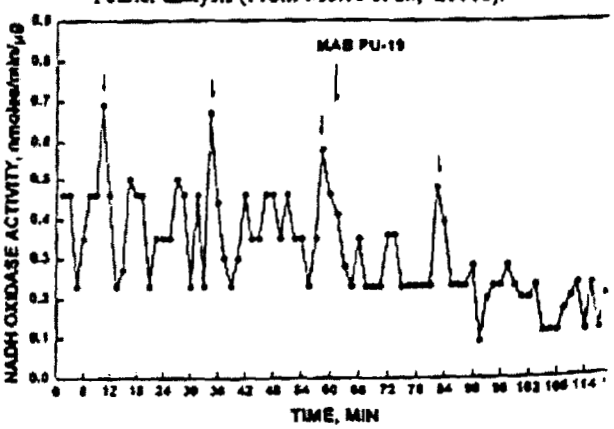


Fig. 18. Antibody inhibition of the activity of the 2,4-D-unresponsive NOX protein purified by concanavalin A column chromatography from solubilized plasma membranes of etiolated hypocotyls of soybean. The periodic component (24 min period length) is reduced by soybean CNOX-specific MAB PU-19 in the first cycle after MAB addition and completely eliminated in subsequent cycles.

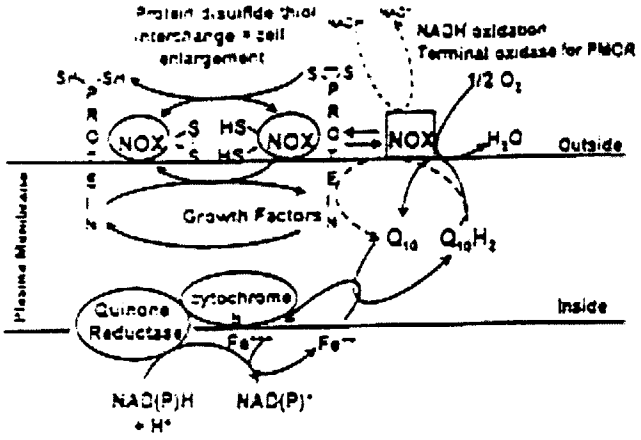


Fig. 19. A functional diagram summarizing the physiological and multifunctional properties of the drug- and hormone-responsive plasma membrane NADH oxidase. The right hand side of the diagram illustrates the spatial relationships of the inside NAD(P)H:quinone reductase, the membrane pool of coenzyme Q (Q_{10}) (Vitamin K_1 in plants) and the external NADH oxidase (NOX) protein across the plasma membrane. In this manner, the NOX protein could function 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 the two reactions catalyzed by the protein disulfide-thiol interchange and thiol-disulfide oxido-reductase activities. The interchange activity which occurs in the absence of external reductants is postulated to play a role in growth. Growth factors appear to serve as switches to emphasize this part of the mechanism (Morré, 1998a).

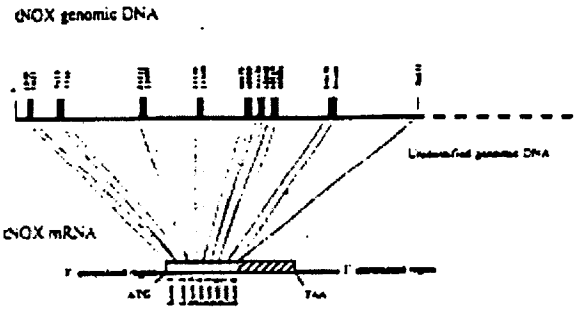


Fig. 21. Exon-intron organization of the gene coding for human tNOX. The solid line represents the genomic DNA and cDNA regions cloned while the dashed line represents uncharacterized cDNA sequences. Closed boxes in the genomic DNA map represent the 8 identified protein-coding exons. Open boxes in the cDNA map represent the eight identified protein-coding exons and the hatched box in the cDNA map represents the protein-coding region.

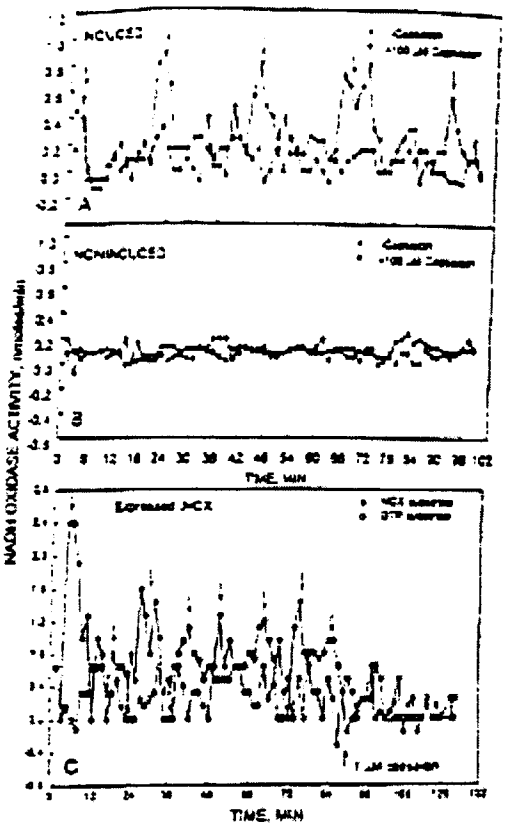


Fig. 20. The activity of the cloned and expressed protein from HeLa also is periodic. The periodic variation in the rate of oxidation of NADH as a function of time over 100 min showing 5 maxima (3 min, 24 min, 47 min, 69 min and 93 min). Within a time of 93 min, four maxima were observed with an average period length of about 23 min per period. The activity was completely blocked by the tNOX inhibitor capsaicin at a concentration of 100 μM. The paired sets of data were collected simultaneously using two Hitachi U3074 spectrophotometers. Oxidation of NADH at 340 nm was measured. A. The enzyme source was a crude preparation from bacteria expressing tNOX cDNA from a HeLa (human) library induced to express the protein by addition of IPTG. B. As in A but in the absence of inducer to serve as a control for machine and reagent variation. C. As in A except the activities were measured simultaneously as a function of time using two different spectrophotometers each with identical amounts of the bacterially expressed tNOX protein. The solid curve shows oxidation of NADH measured as in A. The dotted curve shows the cleavage of a dithiodipyridine (DTP) substrate which is a measure of the thiol-disulfide interchange activity. Both activities show a 23 min periodicity when rates were determined for 90 min except that the activity maxima were 1/2 period out of phase with each other. Both activities were inhibited by 1 μM capsaicin added at 96 min (Morré, 1998a).

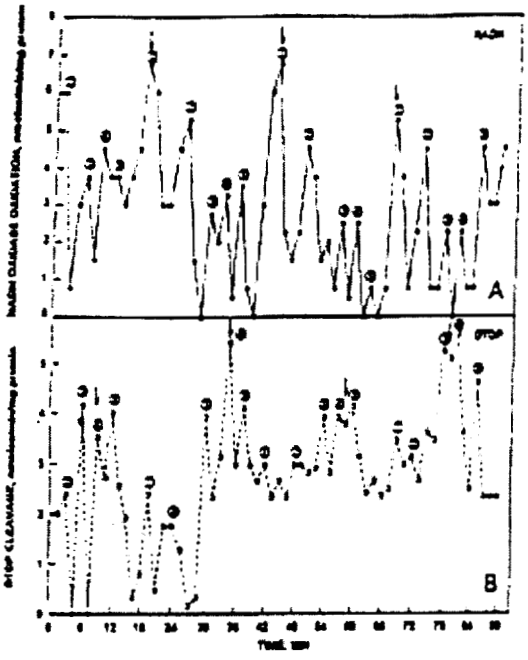


Fig. 23. As in Figure 2. A. NADH oxidation determined by the decrease in A_{340} (upper curve). Maxima (arrows) were at 18, 42 and 66 min with secondary maxima at 24, 48 and 72 min. Three minor peaks completed each 24 min period. B. Disulfide-thiol interchange activity measured simultaneously in parallel as an increase in A_{340} from the cleavage of dithiodipyridine (DTDI). Major peaks are at 6, 9 and 12 min and at 24 min intervals thereafter with minor peaks at 18 and 24 min and at 24 min intervals thereafter. Clearly, the two activities, NADH oxidation (A) and DTD cleavage (B), alternate.

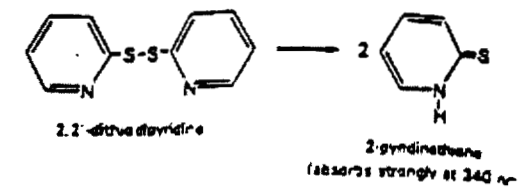


Fig. 22. Dithiodipyridine substrate generating 2 moles of 340 nm-absorbing 2-pyridinethione and cleaved as a measure of the disulfide-thiol interchange activity of the NOX protein.

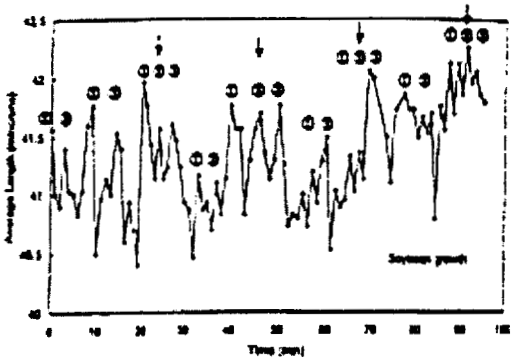
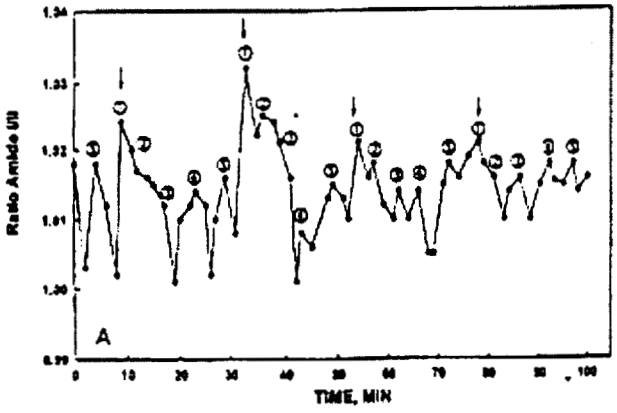


Fig. 24. Increase in length (enlargement) of a single cell of an epidermal strip removed from the surface of a dark grown seedling of soybean as determined by image enhanced light microscopy. Cell enlargement proceeds in bursts every 12 min as for animal cells in culture (Fig. 9) separated by rest periods where the cells actually shrink. As with NOX activity, each 24 min period 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 (e.g., Fig. 15B). The two maxima contained within the resting period correlate with the two maxima of NADH oxidation (e.g., Fig. 15A).



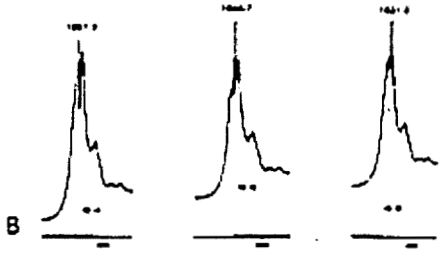


Fig. 25. Fourier transform infra red analyses of recombinant tNOX. Sixty-one 1 min scans taken 1.5 min apart over 100 min are illustrated. A. The ratio of the amide I (1645) to amide II (1545) absorbances varied with maxima at 24 min intervals as indicated by the arrows. B. Within the amide I region (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.

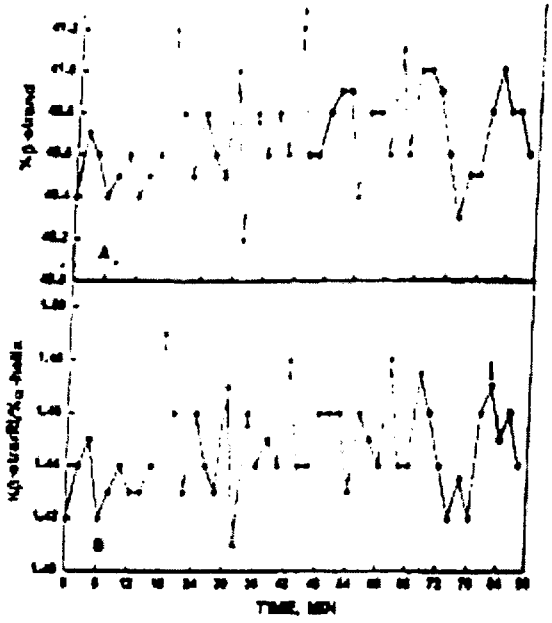


Fig. 26. Percent β-strand (A) and ratio of percent β-strand/α-helix (B) of soluble recombinant tNOX prior to acquisition of proteinase K-resistance. Single arrows denote maxima in β-strand structure spaced at intervals of 22 min. The double peak at 62 and 68 min (to right of 3rd arrow) is a recurrent feature possibly associated with time keeping. See also, Fig. 2 (24 and 30 min).

Fig. 27. Image-enhanced electron microscopy of recombinant truncated tNOX (ttNOX) negatively stained with 1% uranyl acetate. Image enhancement was achieved by superposition of 4 images aligned to a common morphological feature the central channel filled with electron dense uranyl acetate in A and the stained-filled discontinuity in the circular profile of B. The image in A is interpreted as a top view of the functional ttNOX dimer lying flat. The image in B is interpreted as a view from the top of a ttNOX monomer standing upright. Scale bar = 0.01 μm.

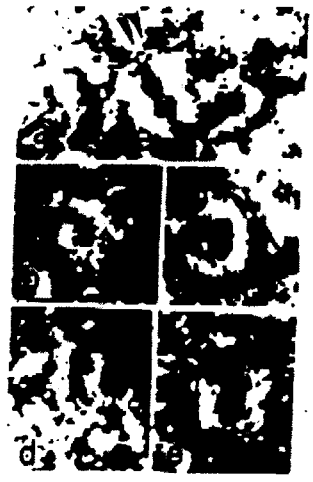


Fig. 28. Negatively stained images of recombinant ttNOX in closed (right) and open (left) configurations. A. The two dominant views are seen in the top panel consisting of an apparent hollow cylinder seen in lying flat (double arrowheads) or from the top (single arrowheads). b and d. closed configuration. c and e. Open configuration.

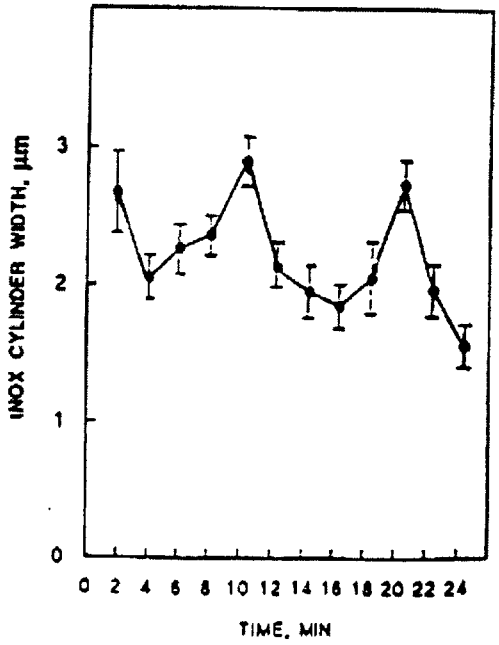


Fig. 29. Diameters of single recombinant ttNOX particles measured from preparations stained and photographed at two min intervals. Diameters varied from <2 (b. and d. of Fig. 28) to 2.8 (c. and e. of Fig. 28) nm

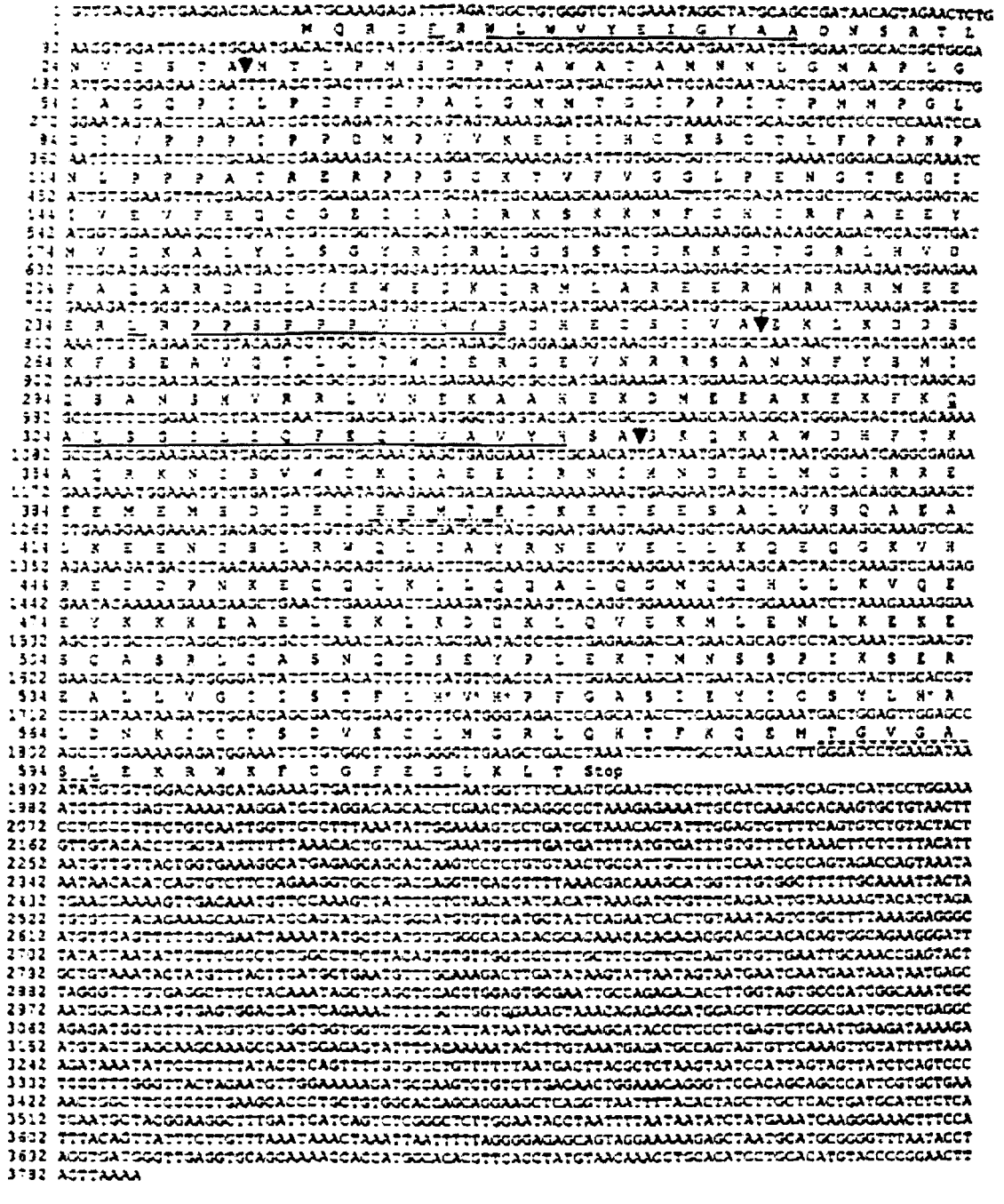
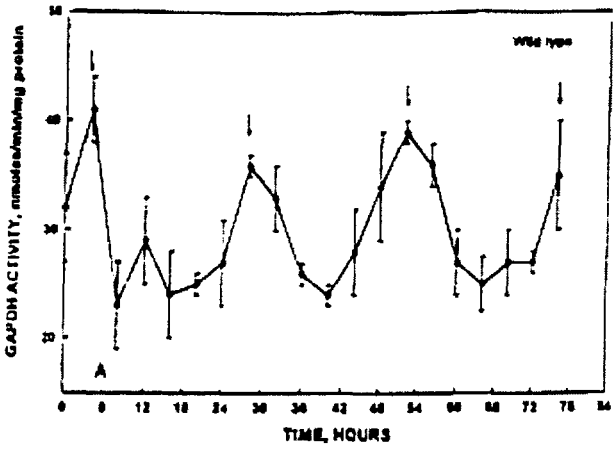
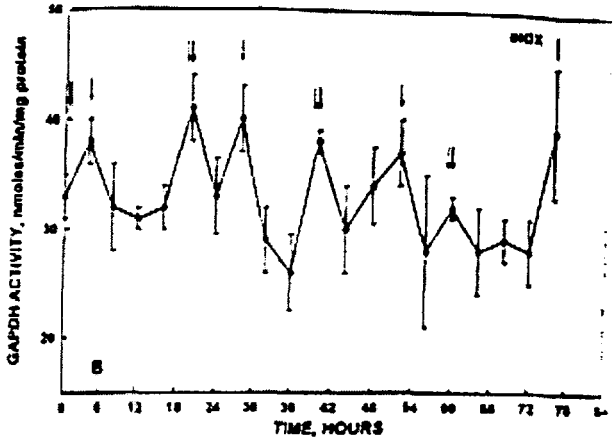


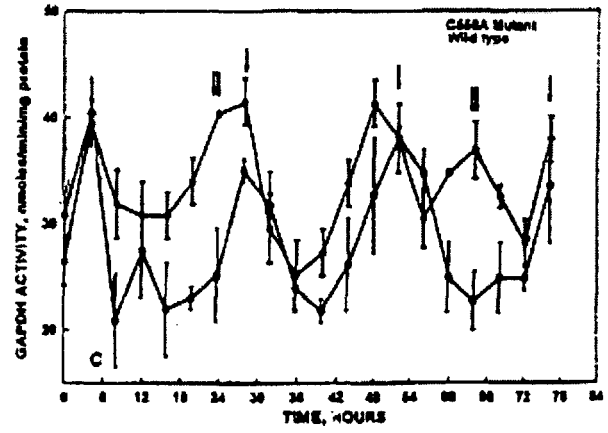
Fig. 30. Nucleotide and deduced amino acid sequences of the tNOX cDNA. The first potential translation initiation site is indicated at nucleotides 23-25 (ATG) with termination at 1855-1857 (TAA). Putative signal peptides are underlined and the signal peptide cleavage sites are indicated by arrows. The putative quinone-binding sequence, E394EMTE, is denoted by a long dash-dot dot line. The H546VH and down stream H562 copper sites are shown by asterisks. The adenine nucleotide (NADH) binding sequence, T589GVASL, is underlined by dashes. The sequence data is available from GenBank under Accession No. AF207881.



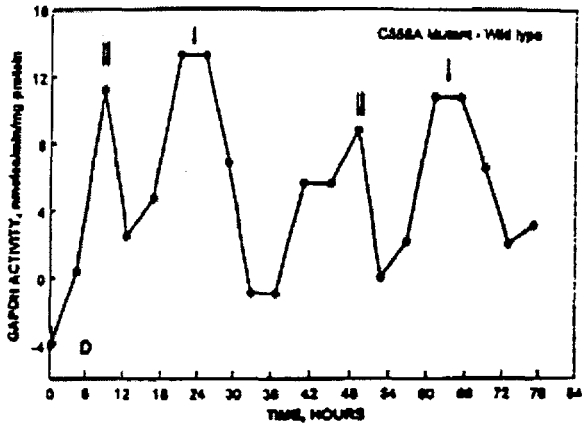
Glyceraldehyde-3-phosphate dehydrogenase activity of COS cells oscillates with a circadian period of 24 h (arrows)



COS cells transfected with tNOX cDNA exhibit two circadian clocks – one of 24 h (single arrows) corresponding to the CNOX period X 60 and one of 22 h (double arrows) corresponding to the tNOX period X 60.



COS cells transfected with the C558 replacement giving a tNOX activity with a 42-min period compared to wild type



When the GAPDH activities of wild type COS cells Are subtracted from those carrying the C558A mutant with a 42 min period, the difference shows a circadian period length of 42 h (NOX period X 60)

Fig. 31. Preliminary studies with tNOX cysteine to alanine replacements show that NOX period length determines the length of the circadian period. Glyceraldehyde-3-phosphate dehydrogenase, a cellular house keeping protein whose activity oscillates with a 24 h circadian period, was used to monitor cellular clock function. A. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in wild type COS cells exhibits a precise circadian period length of 24 h. A minor period length of 22 h corresponds to that for endogenous tNOX (COS cells are SV-40 transformed) (double arrows). B. GAPDH activities of COS cells transfected with ttNOX cDNA overexpressing the 34 kD tNOX protein with a 22 min period length. The transfected cells express both 24 h (single arrows) and 22 h (double arrows) circadian periods. C. GAPDH activities of COS cells transfected with mutant ttNOX cDNA overexpressing the C558A mutant tNOX protein with a period length of 42 min. The transfected cells express both 24 (single arrows) and 42 h (double arrows) circadian periods. D. GAPDH specific activities of wild type subtracted from those expressing the C558A mutant tNOX with a 42 min period length. The difference shows a circadian period length of 42 h.

These experiments have been repeated subsequently with one of the tNOX cysteine to alanine replacements exhibiting a 36 min period length (C575A). These transfectants now exhibit GAPDH activity with a period length of 36 h in addition to the wild type period length of 24 h.

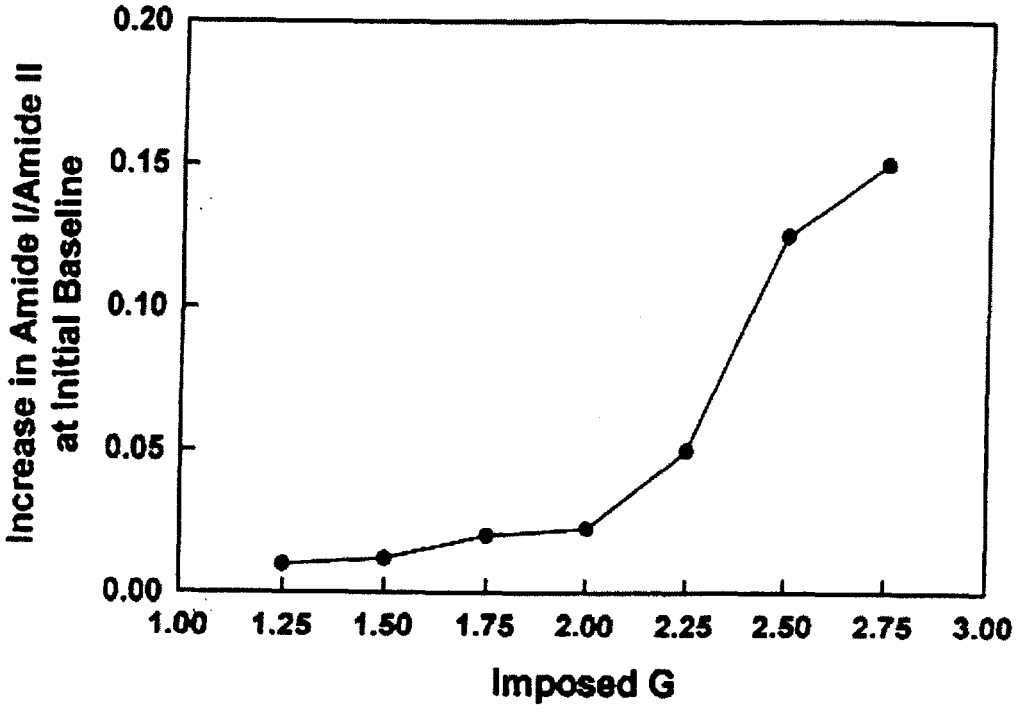


Figure 32. Retention of a conformational change by recombinant tNOX in response to increasing imposed centrifugal force (Imposed G) over 5 minutes as measured using a centrifuge-mounted Avatar. The values reported are the increase in Amide 1/Amide II ratio determined at 1650 cm^{1}, respectively, after return to unit gravity following a 5 min centrifugation to generate the imposed G forces indicated.