

Ultradian oscillators of the circadian clock in *Saccharomyces cerevisiae*

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ABSTRACT

The yeast, *Saccharomyces cerevisiae*, has an ENOX1 activity with a period length of 24 min similar to that of other eukaryotes. In contrast to other eukaryotes, however, *Saccharomyces cerevisiae* has a second ENOX1-like activity with a period length of 25 min. The latter is distinguishable from the traditional ENOX1 on the basis of the longer period length along with resistance to an ENOX1 inhibitor, simalikalactone D, and failure to be phased by melatonin. In addition, two periods are apparent in measurements of oxygen consumption indicating that the consumption of oxygen to water occurs independently by homodimers of both of the two forms of ENOX. Based on the measurements of glyceraldehyde-3-phosphate dehydrogenase, *S. cerevisiae* exhibits circadian activity maxima at 24 and 25 h together with a 40 h period possibly representing the 40 min metabolic rhythm of yeast not observed in our measurement of oxygen consumption and normally observed only with continuous cultures. The findings are indicative of at least three independent time-keeping systems being operative in a single cell.

Keywords: Biological Clock; ECTO-NOX (ENOX) Proteins; Ultradian Oscillator; Yeast (*Saccharomyces cerevisiae*)

1. INTRODUCTION

ECTO-NOX designates a family of cell surface proteins that exhibit a time-keeping function, oxidize reduced coenzyme Q or NAD(P)H and carry out protein disulfide-thiol interchange [1]. They are referred to as ECTO-NOX proteins because of their localization on the outer surface of the plasma membrane [2] and to distinguish

them from the phox-Nox proteins of host defense [3]. This external location of ECTO-NOX proteins has been demonstrated both in plants and animals.

At least three members of the ECTO-NOX (ENOX) family have been described: 1) the human constitutive NADH oxidase (ENOX1 or cNOX) (GenBank Accession No. EF432052), expressed in normal cells and located on chromosome 13q14.11; 2) the human tumor-associated NADH oxidase (ENOX2 or tNOX) (GenBank Accession No. AF207881), present on the surface of invasive cancer cells and located on chromosome Xq25-q26.2 and 3) the age-related NADH oxidase (arNOX or ENOX3), which is present only in individuals after the age of 30, in late passage cultured cells and in senescent plant parts [4]. Human ENOX1 shares 64% identity and 80% similarity with human ENOX2. Members of all three ENOX families contain an adenine nucleotide-binding site, a disulfide-thiol interchange site and two copper-binding motifs [5-7].

ECTO-NOX proteins differ from all other NAD(P)H oxidases in that they are able to reduce pyridine nucleotides in the absence of bound flavin [5]. Moreover, their activity is not inhibited by cyanide [8]. Also, ECTO-NOX proteins have properties of prions, including stability, protease resistance and ability to form amyloid [9].

Although enzymatic activity has been most often measured by monitoring NAD(P)H oxidation at 340 nm, the physiological substrates appear to be quinols (*i.e.*, ubiquinol or phytoquinols) [10,11]. The NADH binding site is on the outside of the cell where there is usually little or no NAD(P)H. Quinones, such as Coenzyme Q, act as trans-plasma membrane shuttles to transfer reducing equivalents from cytosolic NAD(P)H to external acceptors, including molecular oxygen and protein disulfide. ENOX1 and ENOX2 lack iron or iron sulfur clusters. However, the copper binding motifs revealed from sequence analysis and site-directed mutagenesis are necessary for electron transfer to molecular oxygen [6,7]. Based on size exclusion chromatography, the soluble forms of ENOX2 are predominantly dimers. Therefore,

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the ENOX proteins may be viewed as homodimeric proteins containing four coppers per dimer, thus carrying out four electron transfers to molecular oxygen as required to form water [6].

ECTO-NOX proteins exhibit a second catalytic activity that of protein disulfide-thiol interchange despite the absence of the CXXC motif common to most members of the protein disulfide isomerase family [5,6,12]. The two activities, hydroquinone oxidation and protein disulfide-thiol interchange, alternate with a period length that is characteristic for each family member. ENOX1 exhibits oscillations with a period length of 24 min, that of ENOX2 with a period length of about 22 min and that of arNOX with a period length of about 26 min [1].

Several lines of evidence demonstrate that ENOX1 and ENOX2 proteins are involved in at least two important cell functions. They drive the enlargement phase of cell growth and they are components of the biological clock. The regulation of the circadian oscillatory system is mainly ascribed to ENOX1. The two enzymatic activities catalyzed by ENOX1 alternate within a 24 min period: the first activity rests after 12 min and the second one begins in a cycle that confers a time-keeping function to the protein. This oscillation pattern is entrainable and temperature independent. Cells transfected with mutated ENOX cDNAs, which have oscillation period lengths of 22, 36 or 42 min, exhibited circadian period lengths of 22, 36 or 42 h for the cellular circadian biochemical marker, glyceraldehyde-3-phosphate dehydrogenase [13].

ENOX proteins are ubiquitous at the surface of all cells thus far studied [1]. They are protease and heat resistant [14] and respond to hormones [15]. Evidence suggests that ENOX1 serves as an ultradian oscillator of the biological clock in mammals [1] and a human protein with activity characteristics of ENOX1 has been cloned [6]. Whereas circadian rhythms in yeasts have been studied less widely than in mammals, there is no reason to suspect that *Saccharomyces cerevisiae* should be exempt from circadian regulation. Especially well studied in *S. cerevisiae* is the 40 min cycle of respiratory activity in continuous culture [Discussion].

In this report, we have identified two different constitutive oscillatory ENOX proteins of *S. cerevisiae* one with a period length of 24 min and a second with a period length of about 25 min. Both proteins catalyze the oxidation of both externally supplied NAD(P)H to NAD(P)⁺ and hydroquinone and carry out protein disulfide interchange. These two forms of ENOX activity together with an already known ultradian rhythm with a period length of 40 min in *S. cerevisiae* generate three independent sets of potential ultradian time keeping oscillations.

2. METHODS

2.1. *Saccharomyces* Growth and Preparation

The yeast strains were maintained on YEPD agar plates and stored at 4°C. Cells were grown at room temperature with shaking in rich media (YEPD) for 1 - 2 days to attain ca 2×10^8 cells/ml. ENOX proteins of yeast are exposed at the external surface of the yeast cells such that activities may be measured with unbroken cells. Additionally, the yeast cells were heated for 1 h at 70°C to inactivate heat-sensitive enzymes prior to analysis. This was to prevent yeast contamination in the open laboratory. ENOX proteins resist heating at 70°C for one h. Results were similar with unheated yeast cells.

2.2. NAD(P)H Oxidase Assay

Heat-inactivated yeast cells (ca. 10^7 cells in 50 μ l) were combined with 2.5 ml 50 mM Tris-Mes buffer (pH 7.0), 2 mM KCN and 150 μ M NADH. To refold and renature the ENOX proteins of the heat-inactivated yeast, reduced glutathione (GSH) was added to a final concentration of 100 μ M, and incubated for 10 min to reduce disulfides to allow for refolding. Next H₂O₂ was added to a final concentration of 0.03% followed by incubation for 10 min to reform disulfides to stabilize the protein in a correctly folded conformation.

NADH oxidation was measured in a Hitachi Model U3210 spectrophotometer at 340 nm, continuously for one min at 1.5 min intervals for two h using an extinction coefficient of 6.21 mM⁻¹·cm⁻¹. Inhibitors were added at 60 min and data were collected for a further 60 min. A second machine of the same model with the vehicle alone added at 60 min served as the control.

2.3. Hydroquinone Oxidation

Reduced coenzyme Q₁₀ was prepared fresh by addition of an equal volume of 0.25% NaBH₄ under nitrogen to a 7.5 mM stock solution of coenzyme Q₁₀ in ethanol. After several min of incubation, 0.1 volume of 0.1 N HCl was added to degrade the excess NaBH₄. The reaction mixture for measuring the oxidation of the reduced coenzyme Q₁₀ (Q₁₀H₂) contained heat-inactivated yeast, ca. 10^7 cells in 2.5 ml of 50 mM Tris-Mes buffer, pH 7.0 and 0.08% Triton X-100 to maintain the Q₁₀H₂ in suspension. The reaction was started with the addition of 40 μ l of 5 mM Q₁₀H₂. The disappearance of reduced CoQ was measured at both 290 and 410 nm using an extinction coefficient of 0.805 mM⁻¹·cm⁻¹.

2.4. Dithiodipyridine (DTDP) Cleavage

Heat-inactivated yeast (ca. 10^7 cells in 50 μ l) were added to 2.5 ml of 50 mM Tris-Mes, pH 7.0. The reaction was pre-incubated with 0.5 μ mol of 2,2'-dithiodipyridine

(DTDP) in 5 μ l DMSO to oxidize residual thiols. After 10 min of incubation, a further 3.5 μ mol of DTDP were added in 35 μ l of DMSO to start the reaction. The increase in absorbance due to the cleavage of DTDP was monitored at 340 nm using a millimolar extinction coefficient of 6.21.

2.5. Glyceraldehyde-3-Phosphate Dehydrogenase Assay

Yeast were grown in 200 ml minimal SD minus uracil or SR minus uracil media (Open Biosystems, Huntsville, AL) on a shaker at 200 rpm at room temperature for 2 - 3 days at 25°C. Samples of 50 μ l were collected every 2 h and snap frozen at -80°C. Time windows were used to collect the samples, with the flow between collections returned to the flask. Although this was a batch culture, the small sample sizes did not reduce the total volume of the culture significantly, and each sample had approximately the same total protein content.

To prepare the samples for the assay, the yeast were thawed and 50 μ l of Y-PER (Yeast Protein Extraction Reagent, Pierce) were added and the solution was vortexed for 20 min to lyse the yeast and release the cell contents. Glyceraldehyde-3-phosphate dehydrogenase activity was assayed in a reaction solution containing 100 μ l lysed yeast, 0.1 M Tris-HCl, 0.5 mM EGTA, pH 8.0, 1 mM Na₂HAsO₄, 2 mM NAD⁺ and 3 mM glyceraldehyde-3-phosphate. NAD⁺ reduction was determined from the increases in absorbance at 340 nm at 37°C measured over 5 min using a millimolar extinction coefficient of 6.21.

2.6. Statistical Analyses

Results were analyzed using fast Fourier transform and decomposition fits [16]. To determine the period length of activity oscillations, the fast Fourier transforms were with a user-defined transform in Sigma Plot 8.0. Decomposition fits on both the enzyme activity and the growth determinations used MINITAB and were based on the period length established by Fourier analysis.

3. RESULTS

3.1. NAD(P)H Oxidase Assays

Standard NAD(P)H oxidase activity assays in which the disappearance of NADH was measured over one min at 1.5 min intervals in heat-inactivated yeast revealed two sets of oscillations based on the recurrence of two maxima separated by 6 min of similar but not identical period lengths. One set denoted by ① and ② in **Figure 1(a)** exhibited a period length of 24 min. The period length of the second set denoted by A and B in **Figure 1(a)** was longer and nearer to 25 min. This double period in yeast

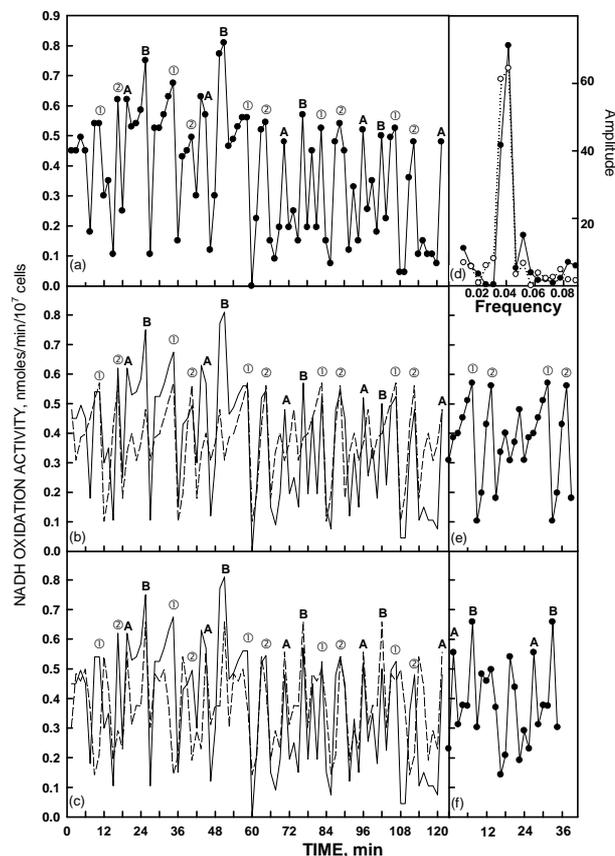


Figure 1. Oscillatory pattern of NADH oxidation (ENOX activity) of heat killed yeast (a) and statistical analysis indicating two activities of non-identical period lengths (b)-(f). ① and ② indicate the first and second maxima of the ENOX activity producing a 24 min period and A and B indicate a second activity producing a 25 min period. ① and ② are separated from each other by 6 min as are (a) and (b); (b) Decomposition (time series) fit using a 24 min period length from Fourier analysis; (c) Decomposition fit using a 25 min period from Fourier analysis. In (b) and (c) the dashed lines are the decomposition fits and the solid lines are the values of (a); (d) Fourier analysis comparing the maxima labeled ① and ② (solid symbols and lines) yielding a period length of 24 min and those labeled A and B in Part A (open symbols and dashed lines) yielding a period length of 25 min; (e) Decomposition fit for B; (f) Decomposition fit for C. Three measures of the accuracy of the statistically fitted values are provided in **Table 1**.

increases the complexity of the data over that of mammalian cells and plants which normally exhibit only a single set of oscillations with a period length of 24 min.

To resolve the two oscillatory patterns, fast Fourier (**Figure 1(d)**) and time series analyses (decomposition fits) (**Figures 1(e)** and **(f)**) were employed as used conventionally to resolve patterns of oscillations that are neither sinusoidal nor monotonic [16]. Fourier analyses (**Figure 1(d)**) partially resolved the two oscillatory patterns with the pattern labeled A and B yielding a period with a slightly lower frequency (longer period) than the

pattern labeled ① and ②. Decomposition fits for a 24 min period length based on the Fourier analysis clearly superimposed the maxima labeled ① and ② but not those labeled A and B (**Figure 1(b)**) whereas decomposition fits for a 25 min period length based on the Fourier analysis coincided with the maxima labeled A and B but not those labeled ① and ② (**Figure 1(c)**). The recurring patterns predicted by the time series analysis are reproduced in **Figure 1(e)** for the 24 min oscillations (maxima ① and ②) and in **Figure 1(f)** for the 25 min oscillations (maxima A and B). Statistical measures of accuracy are summarized and explained in **Table 1**. With the exception of **Figure 2**, **Figures 3-8** were analyzed in a manner identical to that for **Figure 1** with comparable measurements of accuracy.

3.2. Circadian Day

To link the two ENOX activities and the extra maxima corresponding to a 40 min ultradian period to the circadian clock, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was used. GAPDH activity peaks once within each 24 h period making it an ideal marker of circadian day length [17]. Morr  *et al.* [13] reported that the ENOX1 period in min equals the circadian day length in h for a series of site directed C → A replacements in ENOX2 where ultradian period lengths of 22, 36 and 45 min resulted in circadian period lengths of 22, 36 and 45 h. Yeast cultures grown in glucose sampled every 2 h for 88 h and assayed for GAPDH activity revealed maxima of GAPDH activity at 24, 25 and 40 h (**Figure 2**). The two hour sampling interval was sufficient to resolve the different maxima observed as each maximum was represented by several points defining

both the ascending and the descending limbs of each maximum. The observed two maxima of 24 and 25 corresponded to the 24 and 25 min ultradian rhythms based on NADH oxidation. The 24 min period is denoted by ① and ②, the 25 min period is denoted by A and B. The 40 min period, denoted by 40 in the figure, may correspond to the 40 min metabolic rhythm of yeast thus far observed only with continuous cultures.

3.3. DTDP Cleavage

As measured by cleavage of the substrate 2,2'-dithiodipyridine (DTDP), three maxima separated by 4.5 min were generated by mammalian ENOX1 [6]. Yeast also exhibited this activity (**Figure 3**), with two sets of 3 maxima labeled ③, ④ and ⑤ and, C, D and E, each separated by 4.5 min, an activity that alternates with the two peaks of the oxidative activity for the two sets of oscillations [1]. DTDP cleavage was analyzed statistically as for **Figure 1** with a similar outcome and comparable accuracy values (**Table 1**).

3.4. Hydroquinone Oxidation

Although externally-supplied NADH was used to assay ENOX activity, hydroquinones resident in the plasma membrane are most likely the natural substrate [10]. When reduced coenzyme Q₁₀ was assayed, two sets of oxidative maxima were seen (**Figure 4**) similar to those observed with NADH (**Figure 1**). Within each set, two maxima each separated by 6 min were observed. Statistical analyses of both **Figures 4(a)** and **(b)** resolved the two sets of oscillations into series having distinct period lengths and accuracy values similar to those of **Figure 1** (**Table 1**).

Table 1. Accuracy measures as determined by decomposition (time series) analyses.*

	24 min			25 min		
	MAPE	MAD	MSD	MAPE	MAD	MSD
Figure 1	66.442	0.139	0.029	74.314	0.143	0.035
Figure 3	55.092	0.003	<0.001	58.190	0.003	<0.001
Figure 4(a)	142.843	0.002	<0.001	187.67	0.002	<0.001
Figure 4(b)	113.758	0.002	<0.001	144.993	0.002	<0.001
Figure 5	51.632	0.003	<0.001	61.761	0.003	<0.001
Figure 6	23.825	0.003	<0.001	39.491	0.003	<0.001
Figure 7(a)	144.450	0.003	<0.001	219.036	0.003	<0.001
Figure 8	29.506	30 min 0.058	0.007	23.364	32 min 0.050	0.005

*Decomposition analyses decompose a time series into trend, cyclic, and error components. The measured values of **Table 1** evaluate the accuracy of the fitted values and include MAPE (mean of the average percent error), MAD (mean average deviation), and MSD (mean standard deviation). The fitted values by the additive model used agreed closely with the experimental data. Overall the accuracy measures provided show a strong positive relationship between the actual and predicted values. By the statistical criteria, the model performs exceptionally well with the p-values approaching zero for a reasonable level of precision [16].

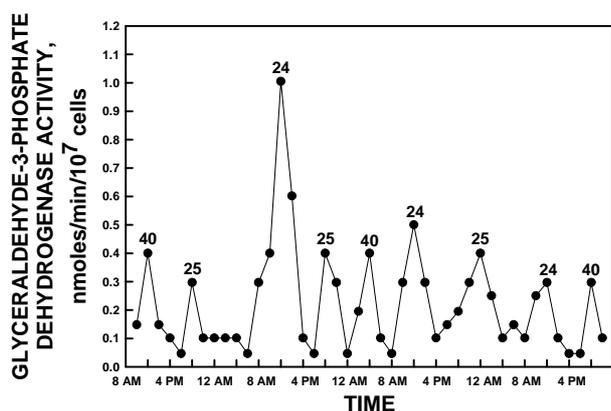


Figure 2. GAPDH activity of yeast grown in glucose. GAPDH activity peaked at intervals of 24 h, 25 h and 40 h. The 40 h maxima appear to correspond to the 40 min period normally observed only with continuous cultures [20,21].

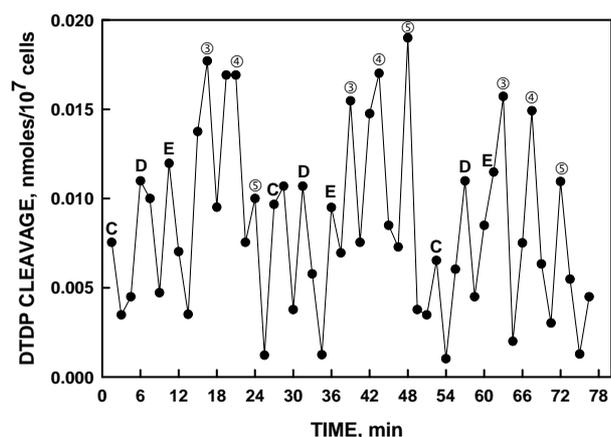


Figure 3. Heat-inactivated yeast exhibited disulfide-thiol interchange activity determined from the cleavage of a synthetic dithiodipyridine DTDP substrate which alternate with the oxidative activity (Morré *et al.*, 1999). ③, ④ and ⑤ are from the 24 min period and D, E and F are from the 25 min period. Data were analyzed as for **Figure 1** and the measures of the accuracy of the statistically fitted values are provided in **Table 1**.

3.5. Response to Melatonin

Melatonin shifts the period of ENOX1 in mammalian cells [18]. When melatonin (final concentration 10 μ M) was added to the NADH oxidase assay of mammalian ENOX1, a new maximum of NADH oxidase activity appeared exactly 24 min after melatonin addition. In yeast, the 24 min ENOX1 period responded to melatonin in this manner, denoted by ① and ② before melatonin addition and M after melatonin addition (**Figure 5(a)**). The initial 24 min period denoted ① and ② was suppressed immediately following melatonin addition (**Figure 5(b)**). The third maximum labeled ① just prior to melatonin addition was present but no third maximum ② was observed. The 24 min periodic oscillations then re-

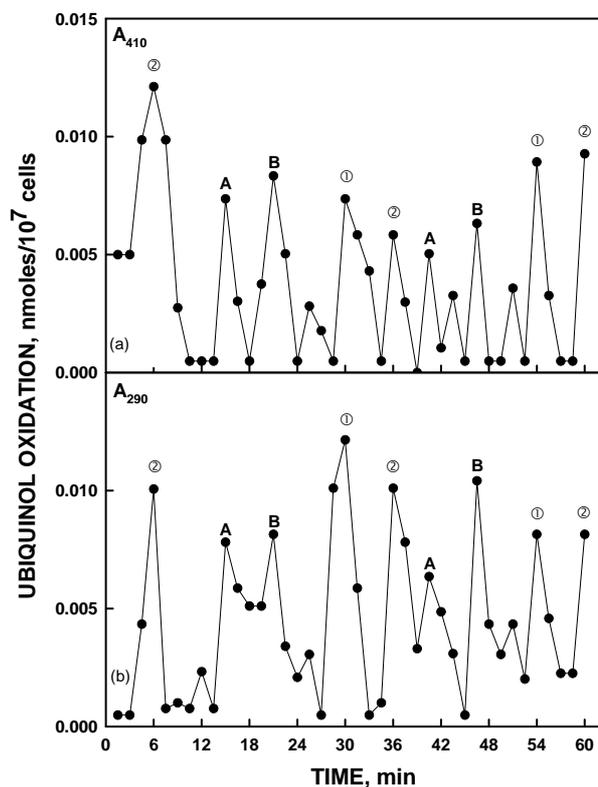


Figure 4. Heat-inactivated yeast oxidized reduced coenzyme Q measured either by an increase in A_{410} (a) or by a decrease in A_{290} (b). As with NADH oxidation of **Figure 1**, the activity oscillated with two sets of prominent maxima separated by 6 min. There were two maxima within each set of oscillations separated by 6 min. Those labeled ① and ② generated a period of 24 min in length. Those labeled A and B generated a period of 25 min in length. Accuracy measures of the decomposition fits are provided in **Table 1**.

sumed exactly 24 min following addition of melatonin 1.5 min out of phase with the original 24 min oscillations. In contrast, melatonin addition did not affect the oscillations with the 25 min period length (A and B) (**Figure 5(c)**).

3.6. Simalikalactone D

An extract from the leaves and bark of *Quassia amara L.* containing the ENOX 1 inhibitor, simalikalactone D, that inhibits the activity of ENOX1 in mammalian and plant preparations [19], when added to the NADH oxidase assay inhibited the activity represented by the 24 min ENOX period (① and ②), **Figure 6(b)** but was without effect on the ENOX activity represented by the 25 min period length (A and B, **Figure 6(c)**).

3.7. Copper Dependence

The mammalian form of ENOX1 binds 2 moles of copper/mole of protein and copper is essential for its activity

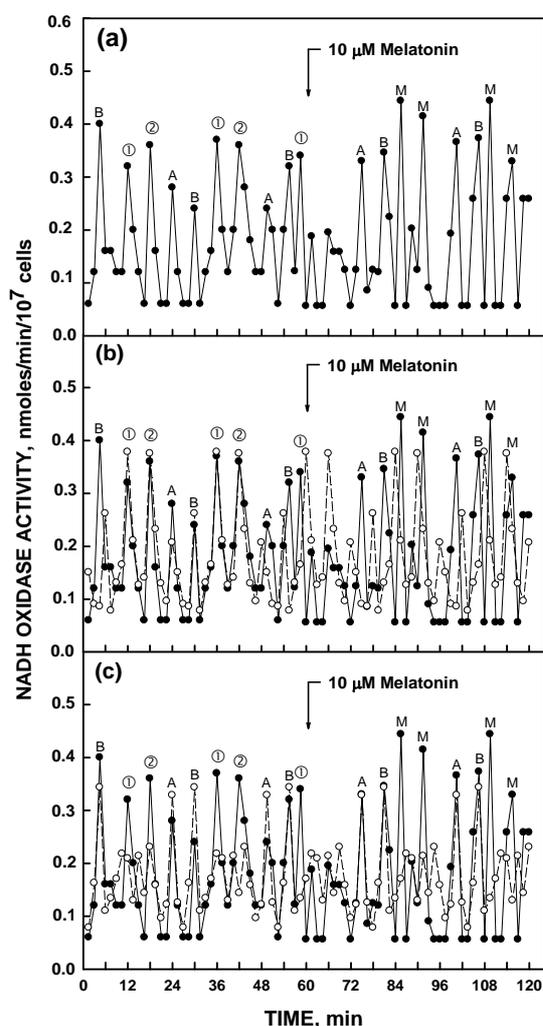


Figure 5. Heat-inactivated yeast ENOX1 activity was phased by addition of 10 μM melatonin. The activity exhibiting a 24 min period labeled ① and ② was shifted as shown by the 24 min decomposition fits (open circles, dashed lines) (b). In contrast the activity exhibiting a 25 min period length (A and B) was not phased by melatonin addition as shown by the 25 min decomposition fits (open circles, dashed lines). (c). The two maxima observed post melatonin addition (labeled M) corresponded to neither. Accuracy measures are given in **Table 1**.

[6]. Alteration of the two copper sites by site-directed mutagenesis or copper removal by bathocuproine inactivated both human ENOX1 [6] and ENOX2 [7] such that little or no oscillatory activity remained. To determine if the yeast ENOX forms also required copper, to 500 μl heat-inactivated yeast were added 1 μl of trifluoroacetic acid to unfold the protein in the presence or absence of 15 μl of 10 mM bathocuproine, a copper chelator, to remove the copper. Yeast ENOX activity was diminished when bathocuproine was present (**Figure 7**). TFA alone did not reduce activity and activity could be restored to

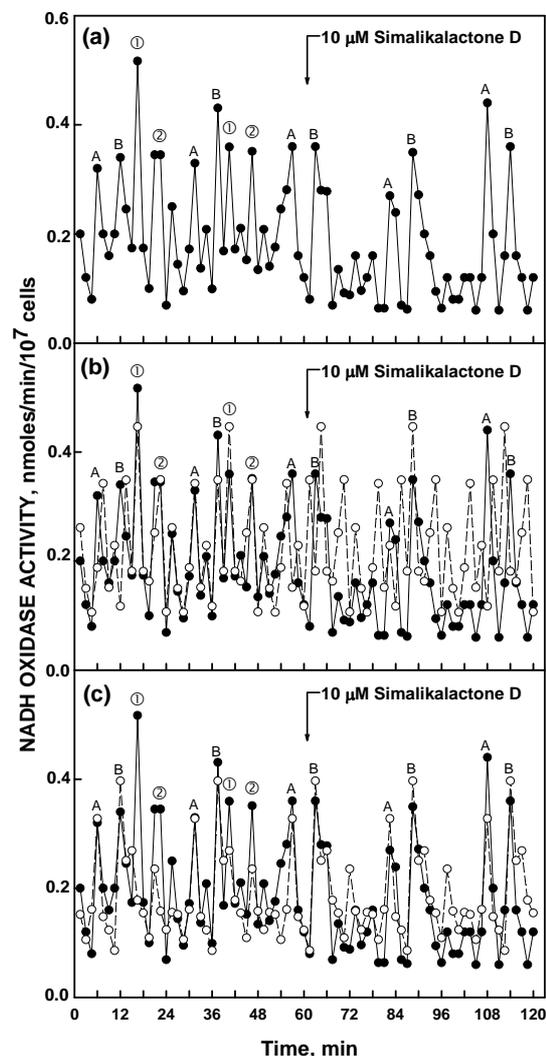


Figure 6. Inhibition of ENOX activity of heat-inactivated yeast inhibited by simalikalactone D. (a) The ENOX activity (① and ②) with a period length of 24 min was inhibited by simalikalactone D as shown by the 24 min decomposition fits (open circles, dashed lines); (b) The second activity (A and B with a period length of 25 min period) was not; (c) Accuracy measures are given in **Table 1**.

TFA and bathocuproine treated preparations by addition of copper. In the presence of TFA, both ENOX1 activities were present (**Figure 7(a)**). In the presence of bathocuproine (**Figure 7(b)**), both ENOX1 activities were reduced or eliminated. All that remained were two maxima separated by 40 min which may correspond to the fundamental respiratory oscillation of 40 min normally seen only with continuous cultures [20,21].

3.8. Temperature Independence of Period Length

The period lengths of the two ENOX forms in yeast were

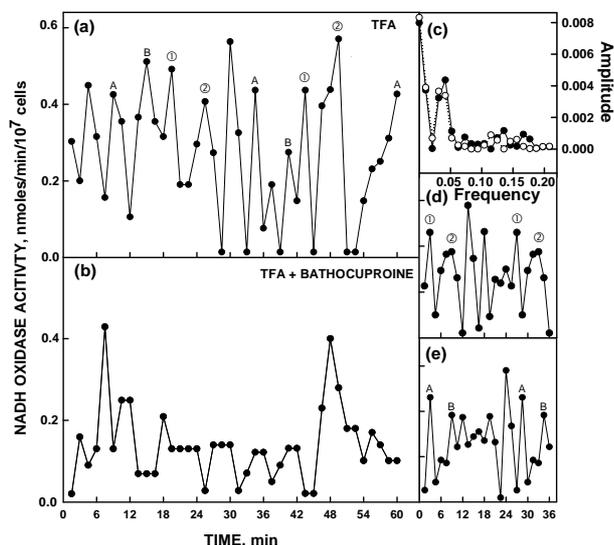


Figure 7. Heat-inactivated yeast ENOX activity with TFA ± bathocuproine. (a) In the presence of TFA, the activity with both the period length of 24 min (① and ②) as determined from the corresponding Fourier analysis and decomposition fits and the activity with the 25 min (A and B) periodic activities were unaffected; (b) When assayed with TFA and bathocuproine, the 24 min and 25 min periods were greatly reduced or absent. Two maxima separated by 40 min remained, however. (c) Fourier analysis comparing the maxima labeled (① and ②) in part A (solid symbols and lines) and those labeled A and B in part A (open symbols and dashed lines) yielded a period length of 24 min. (d) Decomposition fit for the maxima labeled (① and ②); (e) Decomposition fit for the data labeled A and B. Accuracy measures are given in **Table 1**.

temperature independent (**Table 2**), a hallmark of the biological clock and a characteristic of mammalian and plant ENOX1. The period length of the oscillations did not change over the range of 17°C to 37°C. In contrast, as is typical of both biochemical and chemical reactions, the amplitude doubled with a 10°C increase in temperature.

3.9. Protease Resistance

A hallmark of the ENOX family of proteins is that of protease resistance. When the yeast ENOX forms were assayed after digestion with proteinase K or trypsin, the activity was unaffected even when unfolded in the presence of trifluoroacetic acid prior to addition of protease (**Table 3**).

3.10. Period Length in Deuterium Oxide

Organisms grown in heavy water (deuterium oxide or D₂O) exhibit a circadian day lengthened in period length by about 25% [23,24]. When mammalian ENOX was assayed in heavy water, it exhibited a period length of about 30 min [25]. Heat-inactivated yeast assayed in heavy water yielded period lengths of approximately 30

Table 2. ENOX1 period length of *Saccharomyces cerevisiae* is independent of temperature (n = 4).

Temperature	Period length ¹		Amplitude A ²	
	ENOX 1/2	ENOX A/B	ENOX 1/2	ENOX A/B
17°C	24 min	25 min	0.22 ± 0.05	0.24 ± 0.06
27°C	24 min	25 min	0.48 ± 0.09	0.43 ± 0.03
37°C	24 min	25 min	1.0 ± 0.10	0.80 ± 0.12

¹Period length monitored over time intervals as long as 6 h varied with a cumulative error of less than one min; ²Nanomoles/min/10⁶ cells.

Table 3. Protease resistance of yeast ENOX forms of heat-inactivated *Saccharomyces cerevisiae* (n = 4).

Treatment	Specific Activity, nmoles/min/50 µl		Inhibition%	
	ENOX 1/2	ENOX A/B	ENOX1/2	ENOX A/B
None	0.3 ± 0.13	0.32 ± 0.23		
Proteinase K	0.26 ± 0.07	0.295 ± 0.09	9	8
Trypsin	0.3 ± 0.12	0.32 ± 0.15	0	0
Trypsin + TFA*	0.28 ± 0.13	0.30 ± 0.12	7	6

*Trifluoroacetic acid.

min for the maxima labeled ① and ② and of approximately 32 min (31.25 min) for the maxima labeled A and B (**Figure 8**).

4. DISCUSSION

Mammalian ENOX1 exhibits several defining characteristics to indicate that yeast contain not one but two ENOX forms similar to the mammalian ENOX1. The ENOX activities of yeast are at the external surface of the plasma membrane so that activity may be measured with intact cells using external NADH, a membrane impermeant substrate. The activities are protease and heat (80°C) resistant in keeping with data from mammalian sources [26-28]. In mammalian and plant preparations of ENOX1, 5 maxima are seen, in which the first two dominant maxima are separated by 6 min from each other, and the remaining 3 subordinate maxima are separated from each other and from the first two maxima by 4.5 min [1]. Because of the presence of two ENOX1-like activities, one with a 24 min period length and one with a 25 min period length, only the first two dominant maxima of each of the two ENOX cycles are clearly resolved in the experiments with wild type yeast. However, the existence of the subordinate intervening maxima is indicated in the decomposition fits averaged from the primary data (**Figures 1(e) and (f); Figures 7(d) and (e)**).

Analysis of the statistical validity of asymmetric, non-sinusoidal oscillatory patterns such as those exhibited by ENOX proteins is limited primarily to Fast Fourier and decomposition (seasonal forecasting) types of analyses [16]. When applied to the data presented herein, the data

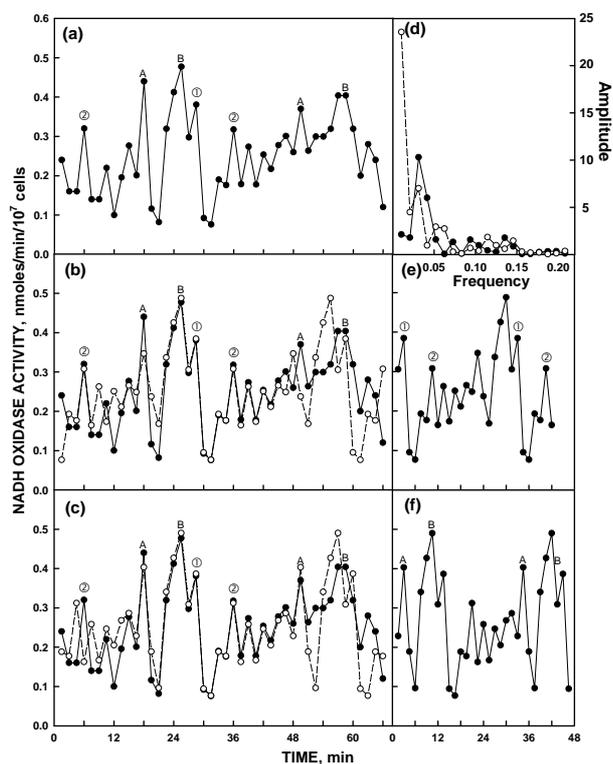


Figure 8. ENOX activity of heat-inactivated yeast assayed in D_2O . (a) Heat-inactivated yeast exhibited two increased period lengths as is characteristic of ENOX1 proteins when assayed in the presence of heavy water. ① and ② exhibited a period of ca. 30 min as compared to 24 min in H_2O and A and B exhibited a period of ca. 32 min as compared to 25 min in H_2O ; (b) Decomposition fits (open circles, dashed lines) using a 30 min period length from Fourier analysis; (c) Decomposition fits (open circles, dashed lines) using a 32 min period length; (d) Fourier analysis comparing the maxima labeled (① and ②) (solid symbols and lines) and the maxima labeled A and B (open symbols, dashed lines); (e) Decomposition fit for B; (f) Decomposition fit for C. Accuracy measures are given in **Table 1**.

were resolved into two recurrent patterns, one with a period length of 24 min and one with a period length of 25 min. Three statistical measures of reproducibility MAPE (mean absolute percentage error), MAD (mean average deviation) and MSD (mean standard deviation) comparing successive periods from the same data sets revealed a mean standard deviation of 5% or less for each of the several data sets amenable to this type of analysis.

Both of the two patterns of ENOX activity, one with a period length of approximately 24 min and one with a period length of approximately 25 min share features in common with mammalian ENOX1. In addition to NADH oxidation, both activities cleave the dithiodipyridine (DTDP) substrate indicative of protein disulfide-thiol exchange and oxidize hydroquinone indicating that both proteins share the dual functionality of other ECTO-

NOX family members. These alternating activities are unprecedented in the biochemical literature and, thus far, are unique to the ENOX family of proteins [1,15].

More compelling evidence came from measurements of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity as an indicator of circadian day length [17]. GAPDH activity cycles with an activity maximum every 24 h in mammalian cells and is controlled by components of the circadian clock. Previous work has shown that the ENOX1 period in minutes equals the circadian day length in h [13]. When GAD(P)H activity of a yeast culture was measured over time, maxima indicating circadian day lengths of 24 h, 25 h and 40 h were revealed corresponding to the 24 min, 25 min and 40 min period lengths of yeast ultradian oscillators. This indicates that yeast contain at least three separate clock rhythms, each regulating GAPDH activity independently of the other. In fact, *Saccharomyces cerevisiae* has three genes encoding GAPDH proteins, TDH1, TDH2 and TDH3 [29]. TDH2 and TDH3 appear to have redundant activities but TDH1 is active in stationary phase and in response to stress [30]. Which of these several GAPDH proteins are clock regulated has not been determined.

Despite important similarities, the two yeast ENOX activities are not identical. Upon addition of melatonin, mammalian ENOX1 exhibits a new maximum exactly 24 min after melatonin addition as is characteristic of ENOX1 of other organisms [18]. In yeast, the activity with the 24 min period length but not that with the 25 min period was phased by melatonin. Following melatonin addition neither the maxima with a 24 min period (**Figure 5(b)**) nor the maxima with a 25 min period (**Figure 5(c)**) aligned with the new maxima labeled M that occurred exactly 24 min post melatonin addition. The maxima labeled M based on previous experience with melatonin phasing of mammalian ENOX1, represent the previous maxima of the 24 min period now missing from the post melatonin data as evidenced from the decomposition fits. Yeast are not known to synthesize melatonin. However, the dose response to melatonin was similar to that of other plant and vertebrate ENOX1 proteins such that a response of the ENOX1 of yeast to melatonin was to the melatonin of the preparation and not to a contaminant.

The 24 h rhythm in GAPDH activity corresponded to the 24 min ENOX1 activity as observed in other organisms, and accounted for one of the two ENOX activities demonstrated here for heat-inactivated yeast. It was blocked by simalikalactone D and phased by melatonin whereas the activity with the 25 min period length was not. Thus we suggest that the 25 h rhythm corresponds to a second ENOX activity, which in addition to a slightly longer (25 min) period length, responded differently from ENOX1 to ENOX modulators.

In contrast to melatonin and simalikalactone D which

affected only the ENOX1 with the 24 min period, the periods of both ENOX forms of *S. cerevisiae* were lengthened by assay in D₂O. The latter underscores the vital role of water associated with the ENOX1 protein-bound copper hexahydrate in the oscillatory process [31].

Among the first defined clock-related oscillation in yeast, known as the respiratory oscillation, was a 40 min cycle of dissolved oxygen levels in continuous culture [20-22,32-35]. After establishing that the oscillations were driven by some aspect of ethanol metabolism [33], acetaldehyde and sulfide were found to be responsible for synchronizing the cell population in continuous culture [22]. Also, the period length of this oscillation was temperature independent, indicating a clock function [22]. In addition, a mutant in which the *GTS1* gene, which has homology to period genes in other organisms [36] was deleted, produced a shortened period, about 20 min, with a destabilization in the amplitude of the oscillation every 11 h [34].

Maxima separated by 40 min and not assignable to either the ENOX form with the 24 min period or the ENOX form with the 25 min period were normally not observed. Only in **Figure 7** following bathocuproine inhibition of both ENOX activities was the presence of maxima corresponding to the 40 min metabolic rhythm [20-22] observed.

It is interesting that in yeast, ENOX1 inhibitors do not inhibit growth, as they do in mammalian and plant cells [19]. This attribute is probably due to the difference in the mechanism of enlargement. Plant and mammalian cells enlarge by physical membrane displacement [37-39] while yeast enlarge by tip growth, or budding [40,41]. In mammalian cells, the cell enlargement function of ENOX1 is central to its activity and essential for cell viability. In yeast, the enlargement function is no longer a primary function, so the other activities of ENOX1 may be studied separated from cell growth. As ENOX1 activity was blocked without affecting growth, deletion mutants to identify the genes encoding the two ENOX forms of *S. cerevisiae* provide a route to ENOX identification independent of protein purification and peptide sequencing [Dick *et al.* in preparation]. In continuing studies, mutant yeast strains exhibiting only the ENOX activity with the 24 min period and lacking the ENOX activity with the 25 min period have been investigated along with mutant yeast strains with the 25 min period and lacking the 24 min period. The ENOX1 form with the 24 min period has been identified as YML117W and the ENOX form with the 25 min period has been identified as YDR005C. These findings will be the subject of subsequent publications.

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