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SHORT COMMUNICATION

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The flavo-enzyme xanthine oxidase is under circadian control in the marine alga *Gonyaulax*

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Abstract The activity of xanthine oxidoreductases (xanthine oxidase, XO, EC 1.2.3.2 and xanthine dehydrogenase, XDH, EC 1.1.1.204) in partially purified extracts of *Gonyaulax polyedra* was measured over 24 h both in a light:dark cycle and in constant light. This is the first demonstration of xanthine oxidoreductase in a unicellular alga. The activity of the O_2-dependent form (XO) was found to be 15 times higher in light than in darkness. The same time-of-day specific differences persisted in constant light, demonstrating a control of XO by the circadian clock. In contrast, the activity of the NAD-dependent form (XDH) is not under circadian control. Because pharmacological inhibition of XO also blocks the effect of blue light on the *Gonyaulax* circadian clock, the possible relationship between XO and light reception in this unicellular alga will be discussed.

Introduction

Circadian clocks are found in all phyla, from cyanobacteria to humans. They allow organisms to compartmentalize different biological processes into the appropriate phases of the 24-h day (Pittendrigh 1993; Sweeney 1987). Circadian clocks comprise a rhythm generator, as well as inputs and outputs (clock-controlled processes). Via the inputs, they are synchronized (entrained) with the environment by specific signals (*zeitgeber*). Light is a strong zeitgeber for all circadian systems (Roenneberg and Foster 1997), and we are beginning to understand the molecular mechanisms of light entrainment (Iwasaki et al. 2000; Lee et al. 1996; Schmitz et al. 2000; Yanovsky et al. 2000; Zylka et al. 1998). There are common features for the circadian systems investigated so far. Components of circadian light input pathways are often themselves under clock control (for a review, see Roenneberg and Merrow 2001). The circadian photoreceptors are often different from those used for spatial orientation (for a review, see Roenneberg and Foster 1997). And, often more than one photic input converges onto the circadian clock (for a review, see Roenneberg and Merrow 2000).

In higher plants, at least four light receptors contribute to circadian entrainment and photoperiodism (Roenneberg and Merrow 2000; Yanovsky et al. 2000), and at least two do so in the marine unicellular alga *Gonyaulax polyedra* (Roenneberg and Deng 1997). In the latter case, it has been shown that the two inputs control different parts of the circadian system, involving at least two oscillators (Morse et al. 1994; Roenneberg and Morse 1993). One of the light inputs is both red *and* blue sensitive, the other responds mainly to short wavelength light.

In the course of characterizing the different photoreceptors in *Gonyaulax*, we found that allopurinol, an inhibitor of the flavo-enzyme xanthine oxidase (XO), can specifically block the blue-sensitive light input of the *Gonyaulax* circadian system (Deng and Roenneberg 1997). This suggests that a xanthine oxidoreductase or one of its substrates and products is involved in this light transduction process. Xanthine oxidoreductases are part of a large family of enzymes found in all phyla, from bacteria to humans, which contain flavins and/or pterins as cofactors (Parks and Granger 1986; Woolfolk and Downard 1977). Among the various plant and animal species, xanthine oxidoreductase is described as a homodimer of approximately 300 kD (Nishino 1994; Parks and Granger 1986; Sarnesto et al. 1996). This enzyme exists in two forms, as an O_2-dependent oxidase (XO) and an NAD-dependent dehydrogenase (XDH). The conversion between the two forms has been well documented in different organisms (Hille and Nishino 1995; Nishino 1994; Waud and Rajagopalan 1976). XDH is

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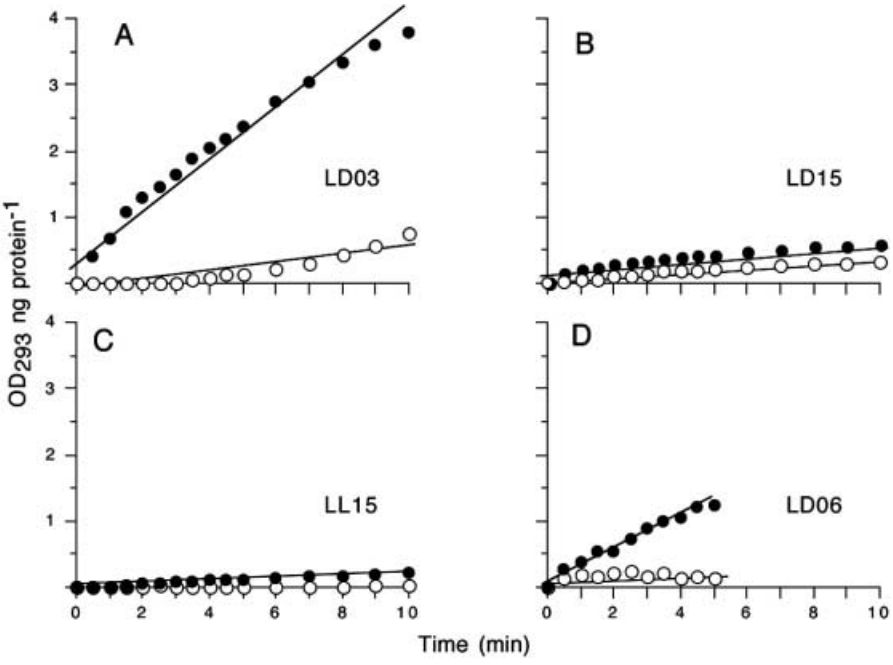

Fig. 1A–D Measurements of XO activity in partially purified *Gonyaulax* extracts (see Materials and methods section). *Filled circles* in panels **A** to **C** show reactions with xanthine as substrate, *open circles* without substrate. **A** Cells extracted 3 h into the light phase of a light:dark cycle (LD03); **B** cells extracted 3 h into the dark phase of a light:dark cycle (LD15); **C** cells extracted after 15 h in constant light (LL15), corresponding to the "subjective night"; **D** XO activity (measured at LD06) of extracts incubated with the XO inhibitor allopurinol (*open circles*) and without (*filled circles)*

converted to XO either reversibly by oxidation of free sulfhydryl groups (Hunt and Massey 1992) or irreversibly through proteolysis by a calcium-dependent protease (calpain) (Nishino 1994; Olanow 1993; Sarnesto et al. 1996; Waud and Rajagopalan 1976).

Among other biochemical steps, the enzymes of this family catalyze the conversion of hypoxanthine via xanthine to uric acid, which is involved both in the purine and the pterin metabolism (Forrest et al. 1956; Ziegler 1987). Xanthine oxidoreductases consist of two identical subunits each containing three domains, which bind a flavin, a pterin, and two non-identical Fe_2S_2 centers, respectively (Nishino 1994). Their kinetics and regulation have been extensively studied in prokaryotes (R Wagner et al. 1984; Woolfolk and Downard 1977), fungi (Lyon and Garret 1978), higher plants (Corpas et al. 1997), and animals (Nishino 1994).

The effects of allopurinol on light transduction in *Gonyaulax* raise two questions: (1) does *Gonyaulax* show enzymatic activity of either of the xanthine oxidoreductases and (2) is this activity under circadian control as are many components of circadian light input pathways? Here, we show for the first time that both the XO and the XDH form exist in *Gonyaulax*, and that the activity of only one of them, XO, is under the control of the circadian clock.

Materials and methods

Culture conditions have been described elsewhere (Roenneberg and Deng 1997). For experiments under constant light (LL), cells were kept in 150 µE m^{-2} s^{-1} for the XO measurements and in 30 µE m^{-2} s^{-1} for measuring XDH activity. This lower fluence was chosen due to the results of pilot experiments which showed no circadian variations in constant 150 µE m^{-2} s^{-1}. Since bright light can suppress some circadian outputs, we performed the final XDH experiment at a lower fluence.

In light:dark cycles (zeitgeber conditions), the onset of light is defined as ZT_0 (ZT, zeitgeber time). In constant light, when the clock runs free (LL), the endogenous period is divided into 24 circadian hours, regardless of its period length in real hours (e.g., 19 or 27 h). The first onset of light under these constant conditions is defined as CT0 (CT, circadian time), and the first half of each cycle is referred to as subjective day, the second as subjective night. However, because the data produced here in constant light differ only by 12 h from those in LD, we presented the hours in constant light as LL, and those in zeitgeber conditions as LD.

Determination of XO activity

Cells from two 800 ml cultures were harvested on filter paper (Whatman no.1 or Scheichen & Schuell 5955, cat. no. 300412), scraped off, and resuspended in 2 ml Eppendorf tubes in extraction buffer (100 mM pyrophosphate, 1 mM EDTA, 0.001% β-mercaptoethanol and 2 mM cystein, at pH 8.0). Extractions and precipitations were carried out at 4°C and enzyme assays at 25°C. Cells were broken by vortexing (5×20 s) with glass beads (0.45–0.5 mm; Braun Melsungen, Germany, cat. no. 854170/1) previously washed in extraction buffer. Cell debris was removed by centrifugation at 1,000 rpm for 1 min, and the supernatant was further centrifuged at 6,000 rpm for 10 min. To the final supernatant, ammonium sulfate (AS) was added stepwise to 40% and 65% with intermittent centrifugations. Each precipitation step was allowed to incubate for 15 min with gentle shaking, then centrifuged at 14,000 rpm for 30 min. The precipitate of the 40–65% AS-cut, which contained the XO activity, was resuspended in extraction buffer and then both desalted and concentrated by ultrafiltration (Filtron, Microsep 100 kD). For the assays of XO activity (Heinz and Reckel 1983; Pateman et al. 1964), the collected material was suspended in reaction buffer (100 mM pyrophosphate, 1 mM EDTA, pH 8.0; with 100 µM xanthine as substrate and 39 units of catalase as well as 0.03% H_2O_2 for the supply of O_2). XO activity was measured photometrically for 10 min via the formation of uric acid (OD_{293}, Du-64, Beckmann). One unit of XO is defined as the amount of enzyme required to increase the optical density by 0.041/min at 25°C. Protein concentrations were determined quantitatively (Bradford 1976).

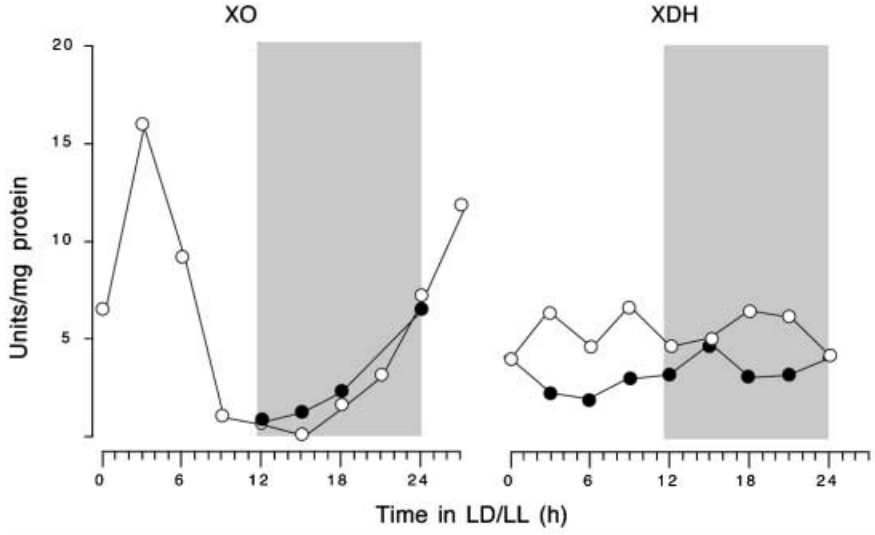

Fig. 2A, B Time-of-day and light dependent activity of the xanthine oxidoreductases in *Gonyaulax polyedra*. **A** XO activity in entrained light:dark conditions (LD 12:12, filled circles) and in constant light conditions (LL, open circles). **B** same as **A** but for the xanthine dehydrogenase from of the enzyme. Depending on condition (LD or LL), *shaded areas* correspond to darkness or to subjective night

Determination of XDH activity

Harvested cells were resuspended in 1.5 ml Eppendorf tubes in extraction buffer (120 mM dipotassium phosphate, 8.5 mM potassium phosphate, 5 mM EDTA and 3 mM dithiothreitol, pH 8.3) and broken down as described above. Cell debris was removed by quick-spin centrifugation at 14,000 rpm for 10 s. The supernatant was further centrifuged at the same speed for 10 min. The final supernatant (crude extract) was used for the activity determination of XDH (Lyon and Garret 1978). Assays of XDH activity were started by adding 30 µM xanthine and 100 µM NAD^+. XDH activity was determined by measuring the formation of uric acid as described above. One unit of XDH is defined as the amount of enzyme required for increasing the optical density by 0.003/min at 25°C.

OD-measurements for both enzyme assays were performed with and without substrate (see examples in Fig. 1A–C), and activities were corrected accordingly.

Results

XDH and XO are two forms of xanthine oxidoreductases catalyzing reactions in the purine and pyrimidine metabolism in various organisms. XDH activity was found in crude cell extracts of *G. polyedra*, while XO activity could only be measured after partial purification (AS 40–65%, see Fig. 1). Both forms were completely inhibited by 1 mM allopurinol (see example for XO in Fig. 1D; Deng 1997). When cells were kept in a light:dark cycle (LD), XO-activity was found to be 15 times higher in the light phase (with a maximum at LD03) than in darkness (compare Figs. 1A, B 2). These time-of-day specific differences are not a consequence of the light regime because they persist when cells are kept in LL (Figs. 1C, 2A) and are, thus, controlled endogenously by the circadian clock. In contrast to the O_2-dependent XO, the NAD-dependent XDH shows only marginal activity fluctuations in either LD or LL (Fig. 2B), indicating that XDH activity is neither controlled by light nor by the circadian clock.

Discussion

Here we show for the first time the existence of the two enzymatic forms of xanthine oxidoreductases in a unicellular alga, exemplified by the marine dinoflagellate *Gonyaulax polyedra*. The pronounced circadian rhythm in XO activity (Fig. 2) cannot be explained by a time-of-day specific availability of substrates or products in cell extracts because the enzymatic measurements were performed in partial protein purifications collected and washed on ultrafiltration membranes. The circadian regulation of XO must, therefore, be due to protein concentration or modification, as is the case for several enzymes and other proteins in *Gonyaulax* (see review in Roenneberg et al. 1998). Although the conversion mechanisms between XO and XDH are not known in *Gonyaulax*, they are unlikely to provide a simple explanation for the circadian activity of XO; in this case, one would expect the concentrations of the two enzymes to oscillate in anti-phase.

The circadian rhythm of XO activity, with its peak in the subjective day, coincides with the circadian time courses of other clock-regulated metabolic pathways in *Gonyaulax* (Roenneberg et al. 1998). XO activity is highly O_2-dependent and photosynthesis is higher during the subjective day. XO (more than XDH) is a major source of superoxide and hydroxyl free radicals (McCord 1985) and the activity of superoxide dismutase, which functions as superoxide radical scavenger, is also higher during the subjective day (Colepicolo et al. 1992). Finally, nitrate reductase is another "day-active" enzyme in *Gonyaulax* (Ramalho et al. 1995; Roenneberg and Rehman 1996) containing the same cofactors as xanthine oxidoreductase.

Both XO and XDH strongly depend on the cell's redox state, which has long been known to be circadian in photosynthesizing organisms (E Wagner et al. 1984). We have recently found that extracellular pH in *Gonyaulax* cultures undergoes circadian oscillations in constant light

(B. Eisensamer and T. Roenneberg, unpublished results). These extracellular changes reflect a cellular proton flux in the millimolar range. In addition, induced (extracellular) pH changes shift the phase of the circadian pH and bioluminescent glow rhythm. This feedback indicates that metabolism makes a substantial contribution to the expression of circadian rhythmicity within a cell.

Our experiments with the XO-inhibitor allopurinol indicate that this oxidase is somehow involved in the blue-light reception of the *Gonyaulax* circadian system (Deng and Roenneberg 1997). Both XO activity and the responsiveness of the circadian blue-light input are rhythmic (Roenneberg and Deng 1997); although the XO peaks during the subjective day, the largest phase shifts are found during the subjective night. There are several possibilities to explain how light reception and XO activity could be connected.

First, the enzyme could be directly involved. Cryptochrome, which also contains flavin and pterin as cofactors, has been proposed as circadian light receptor in plants (Somers et al. 1998) and insects (Stanewsky et al. 1998). In general, redox-active enzymes often contain flavins and pterins, or molybdenum and cytochrome *b* as cofactors. Theoretically, they all are capable of absorbing blue light. Nitrate reductase, for example, was, also suggested to be involved in light reception in the fungus *Neurospora* (Ninnemann 1979). However, it is still disputed whether all oxidoreductases actually can serve as light receptors in vivo (Galland 1992; Lucas and Foster 1998).

Secondly, the relationship between XO and light perception could be based on the fact that this enzyme also catalyzes reactions within the metabolism of the blue-light-absorbing pterins themselves (Forrest et al. 1956; Ziegler 1987).

Finally, the involvement of XO in light reception could be indirect via its products and substrates. XO has been shown to catalyze peroxisomal reactions, e.g., glyoxylate to oxalate (Yanagawa et al. 1990), and substrates of peroxisomal metabolism also affect the *Gonyaulax* clock in a light-dependent fashion (M. Merrow and T. Roenneberg, unpublished results). The metabolism of peroxisomes is also related to nitrogen metabolism and photorespiration in plants. In *Gonyaulax*, nitrate and other substrates of the nitrogen assimilation pathway affect phase and period of the circadian clock in a light-dependent manner (Roenneberg and Rehman 1996). One indication that circadian light responses could be influenced by redox comes from a recent molecular finding (Rutter et al. 2001). The light-dependent expression of the clock gene, *per*, is involved in light resetting of the mammalian circadian clock (Shigeyoshi et al. 1997), and the DNA-binding activity of the transcription factors that control the expression of *per* (either CLOCK or NPAS2 together with their dimeric partner BMAL1) is regulated by redox.

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