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Ene-Reductase-Catalyzed Oxidation Reactions

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Dedicated to Prof. M.T. Reetz at the occasion of his 80th birthday

Ene-reductases from the old yellow enzyme (OYE) family have been traditionally employed in the reduction of conjugated C=C double bonds. This study explores the underutilized oxidative potential of OYEs, demonstrating their capability to catalyze the enantioselective desaturation of carbonyl compounds. Utilizing a deprotonated tyrosine residue as a catalytic base, we developed a method to enable OYE-catalyzed desaturation at ambient temperature and alkaline pH without the need for high-temperature conditions. Through screening of various OYE enzymes, we identified several candidates from different genera with enhanced desaturase activity across different substrates. This work broadens the scope of biocatalytic applications for OYEs, introducing a novel approach to the synthesis of chiral α,β-unsaturated carbonyl compounds.

1. Introduction

Old yellow enzymes (OYEs, E.C. 1.6.99.1) are flavin mononucleotide (FMN)-containing NAD(P)H oxidases capable of stereoselectively reducing conjugated C=C double bonds.[1,2] OYEs are on their way to becoming important industrial biocatalysts for the synthesis of fine chemicals and are already being scaled-up in the pharmaceutical industry.[1,3]

In contrast to the likewise NAD(P)H-dependent alcohol dehydrogenases, OYEs are so far almost exclusively used in the reductive direction (i.e., the NAD(P)H-driven reduction of double bonds). Literature examples using OYEs in the oxidative (i.e., desaturation) direction are scarce. In 1995, Massey and coworkers reported the dismutation of cyclohexanone derivatives.[4] The same group also employed synthetic FMN analogues with increased redox potentials to turn an OYE into a desaturase.[5] Later, Winkler and coworkers[6] and more recently, several groups[7–9] reported aerobic desaturations catalyzed by OYEs. Since only minor desaturation activities were observed using the well-known mesophilic OYE from *Bacillus subtilis* (YqjM),[10] Winkler suspected the endothermic reaction to depend on elevated temperatures to overcome the high activation energy of the reaction.[6] In parallel, an extensive study on the dismutation observed by Massey resulting in a nicotinamide cofactor-independent process has been carried out by Faber and coworkers.[11,12]

To protonate the enolate originating from the Michael-type hydride addition to the β-carbon atom of the activated alkene, OYEs typically utilize a tyrosine as a general acid (Scheme 1B).[13] We, therefore, hypothesized that in the oxidative direction, a deprotonated tyrosine may be crucial as a base to facilitate the enolization and thereby the hydride transfer to the oxidized flavin cofactor (Scheme 1C). Considering the pK_a of the tyrosine hydroxyl group to be in the range of pH 10–11 this would explain why so far this activity has not found widespread attention. Typically, OYEs investigated in the reductive direction exhibit pH optima from pH 6 to 8. Based on these observations, we wanted to further explore the role of the tyrosine as a base to promote the desaturation reaction catalyzed by OYEs and establish them as oxidative enzymes for further applications.

2. Results and Discussion

To test our hypothesis, we chose the OYE from *Thermus scotoductus* (*Ts*OYE).[14,15] for its thermostability and previously shown disproportionation activity. Using *rac*-2-methyl cyclohexanone as starting material, we first investigated the temperature- and pH-dependence of the *Ts*OYE-catalyzed desaturation reaction (Figure 1).

In line with the observations by Winkler and coworkers,[6] the rate of the *Ts*OYE-catalyzed reaction approximately doubled between 30 °C and 60 °C (Figure 1A). Increasing the pH value

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of the reaction mixture from 5.5 to 8.5, however, resulted in a more than 20-fold increase of the desaturation rate (Figure 1B). The reaction proceeded as kinetic resolution favoring the oxidation of the (*R*) enantiomer. The enantioselectivity, however, was rather modest ($E \approx 10$), possibly due to racemization of the starting material at alkaline values, as previously shown by Scrutton and coworkers.[16] Mitigation strategies such as in situ removal of the product or reduction of the carbonyl group have been established[17] and will be evaluated in future studies. However, we also observed double desaturation eventually yielding *o*-cresol as a major by-product. *o*-Cresol, however, inhibits OYEs,[8,18] which in principle may be overcome by (e.g., peroxygenase-initiated) radical polymerization (Supporting Information: Section 2.5). Nevertheless, we decided to focus on cyclopentanone as a model starting material to avoid undesired aromatization and related inhibition issues.

The desaturation of cyclopentanone proceeded smoothly at ambient temperature (30 °C) between pH 7 and 10 (Figure 2).

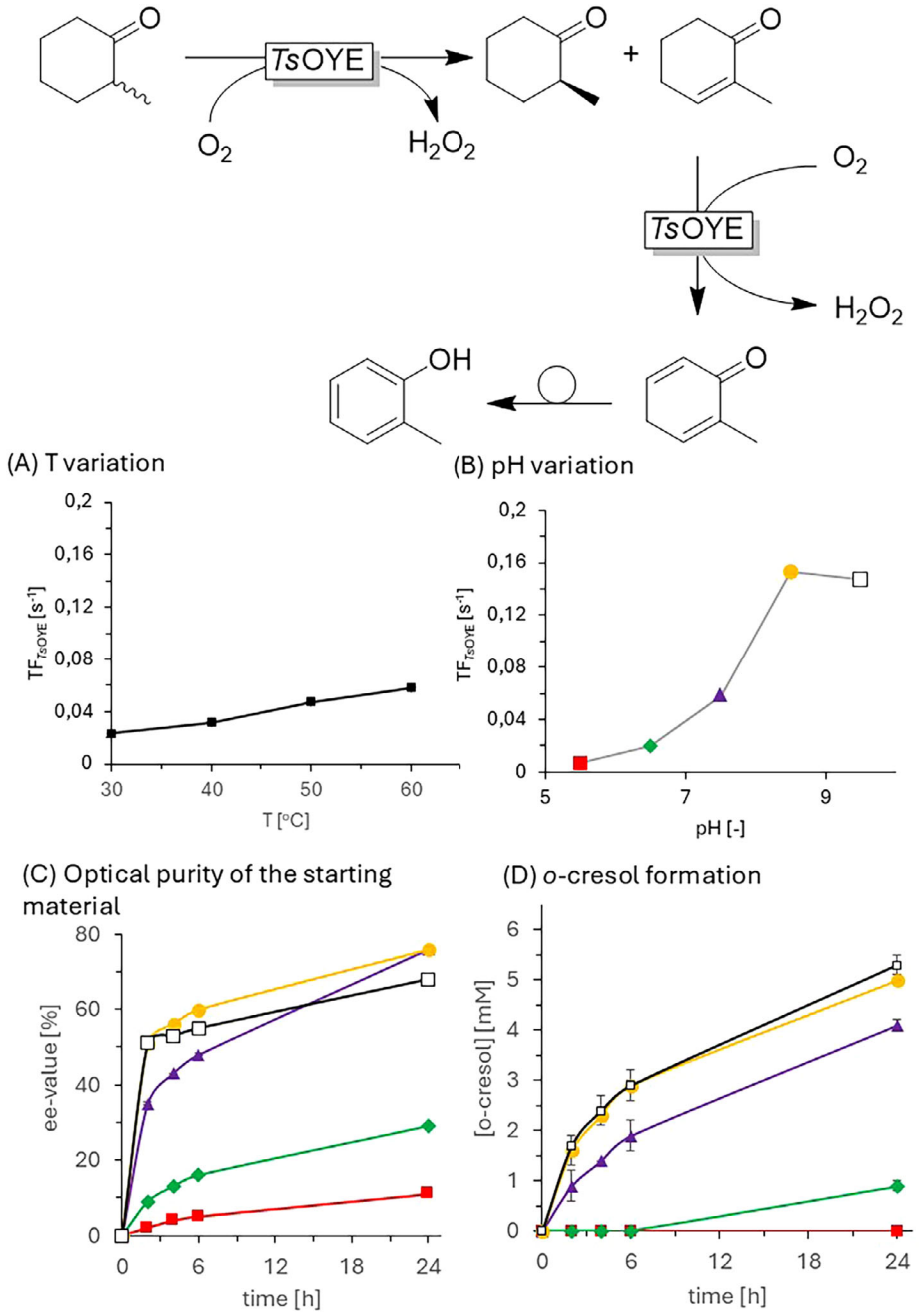
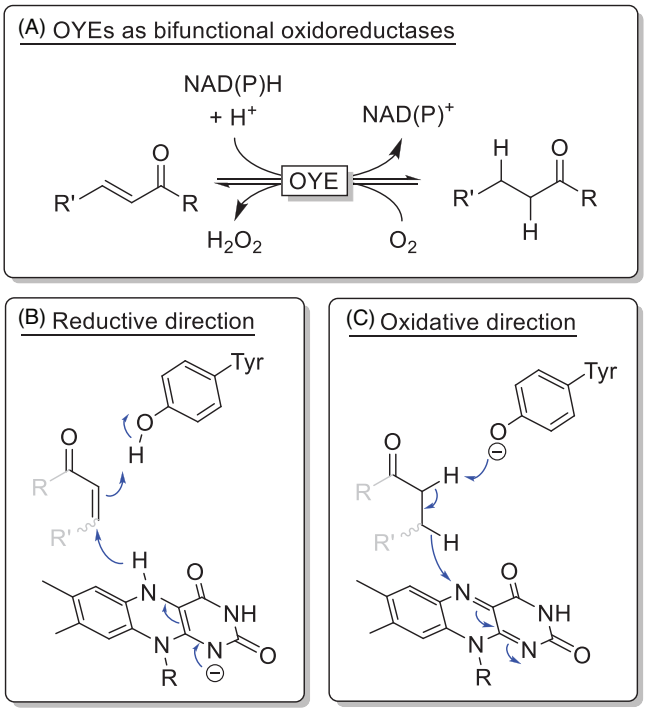


Figure 1. Characterization of the *Ts*OYE-catalyzed desaturation of *rac*-2-methylcyclohexanone. Top: Overall reaction including the kinetic resolution of the starting material and further oxidation of 2-methylcyclohexanone as well as isomerization to *o*-cresol. General conditions if not stated otherwise: [*Ts*OYE] = 10 μM, [2-methylcyclohexanone] = 10 mM, [FMN] = 1 mM, 50 mM MOPS-NaOH containing 5 mM $CaCl_2$, pH 7.5. (A) Variation of the reaction temperature (pH 7.5); (B) variation of pH (at 60 °C), pH 5.5: ■$_{red}$ pH 6.5: ϒ$_{green}$, pH 7.5:▲$_{purple}$, pH 8.5: ●$_{orange}$, pH 9.5: □$_{transparent}$; (C) optical purity of the starting material at different pH values; and (D) *o*-cresol formation at different pH values. For further experimental data, see Supporting Information: Section 2.1.



Scheme 1. Dual reductive and oxidative activity of OYEs and proposed rationale for an alkaline pH optimum for the desaturation reaction in the oxidative direction.

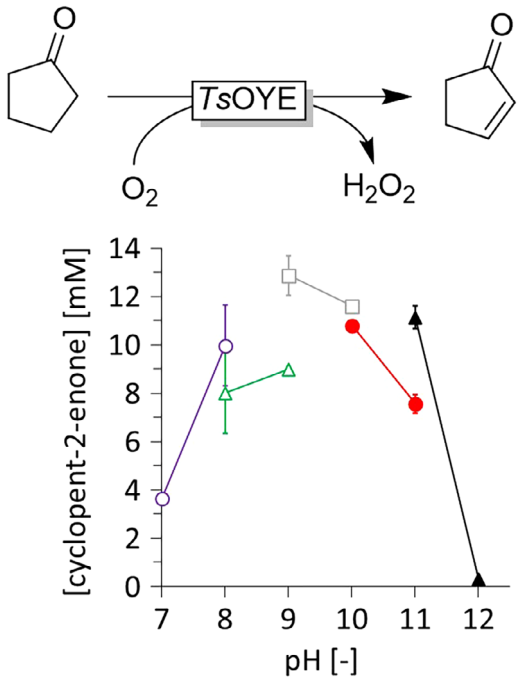


Figure 2. Influence of pH on the product formation of the *Ts*OYE-catalyzed aerobic desaturation of cyclopentanone. Conditions: [cyclopentanone] = 25 mM, [*Ts*OYE]= 10 μM, 30 °C, 22 h, 50 mM buffer: ($\circ_{pink}$) MOPS-NaOH (3-(*N*-morpholino)propanesulfonic acid), ($\triangle_{green}$) Tris-HCl (tris(hydroxymethyl)aminomethane) (12 mM), ($\square_{grey}$) CHES-NaOH (2-(*N*-cyclohexylamino)ethanesulfonic acid), ($\bullet_{red}$) CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid), and ($\blacktriangle_{black}$) PIP (piperazine), 2.5% v/v DMSO.

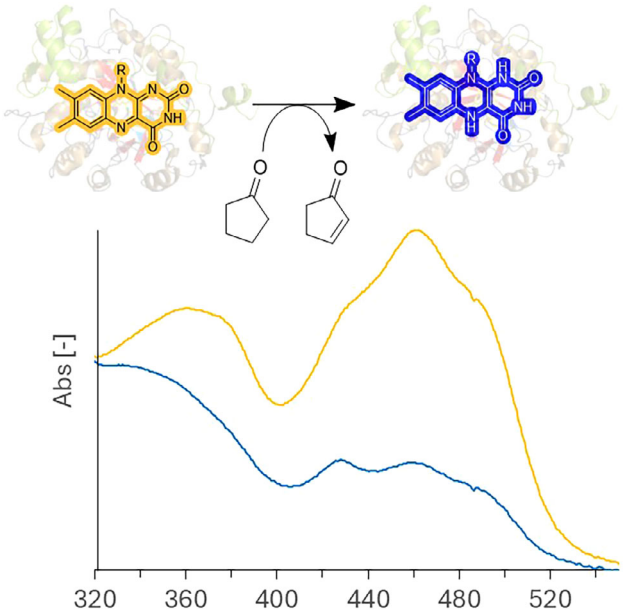


Figure 3. UV spectra of oxidized *Ts*OYE before (yellow) and after incubation (blue) with cyclopentanone under anaerobic conditions. Conditions: 50 mM CHES-NaOH pH 9, [*Ts*OYE] = 40 μM, [cyclopentanone] = 25 mM, 30 °C, the blue spectrum was recorded ca. 1 min after addition of cyclopentanone.

To confirm the catalytic mechanism suggested in Scheme 1, we performed a spectroscopic experiment exposing *Ts*OYE to cyclopentanone under anaerobic conditions (Figure 3). Indeed, the characteristic UV absorption maxima of oxidized FMN in *Ts*OYE flavins around 360 and 460 nm disappeared immediately after addition of the starting material. It is worth mentioning that the decolorization (i.e., reduction of enzyme-bound FMN) was observed in alkaline media only (Supporting Information: Section 2.2).

We qualitatively detected H_2O_2 in this reaction (Supporting Information: Section 2.3), thereby supporting our assumption of substrate to flavin hydride transfer (yielding in a reduction of FMN and concomitant decolorization) and aerobic re-oxidation. In this context, a negative influence of H_2O_2 (or other intermediate reactive oxygen species) may also negatively impair enzyme stability and/or product purity.[19]

The catalytic role of Tyr177 was further confirmed by evaluating *Ts*OYE-Y177F, in which the tyrosine was exchanged for a phenylalanine. Using this mutant, only trace amounts of the desaturation product were observed (Supporting Information: Section 2.4).

To estimate the kinetic parameters of the *Ts*OYE-catalyzed desaturation reaction, we performed initial rate measurements with varying cyclopentanone substrate concentration (Figure 4). We determined a K_M value of 11.2 ± 1.1 mM and a k_{cat} of 0.60 ± 0.01 s^{-1}.

Compared to the published values for the reduction of cyclohexanone[14] of ca. 4 mM and 110 s^{-1}, respectively, this corresponds to a similar affinity but a significantly reduced catalytic activity.

Next, we investigated the scope of *Ts*OYE-catalyzed desaturation reactions (Figure 5). Quite expectedly, the scope of carbonyl compounds that could be converted into the corresponding

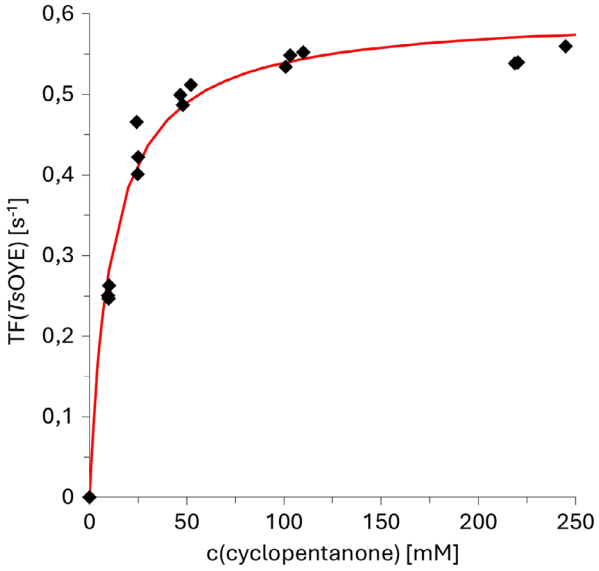


Figure 4. Reaction rate depending on the cyclopentanone substrate concentration. Conditions: 50 mM CHES-NaOH pH 9, [*Ts*OYE] = 10 µM, 30 °C, 2.5% v/v DMSO. After 1 h of incubation, reaction mixtures were analyzed by GC. The red curve is a fit of the Michaelis–Menten equation according to the parameters determined using Igor Pro 7 (www.wavemetrics.com, Oregon, USA).

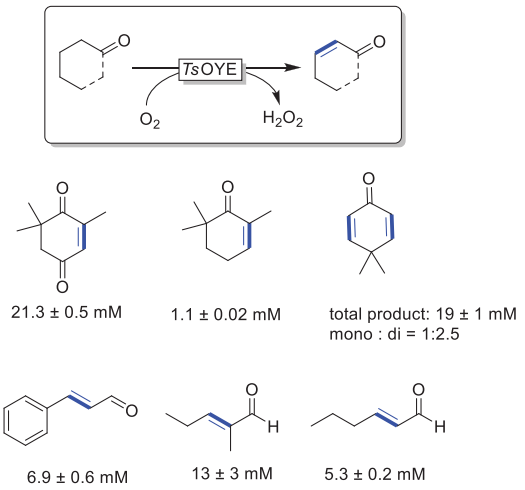


Figure 5. Preliminary substrate scope of the *Ts*OYE-catalyzed desaturation reaction; GC yields are shown. Conditions: [substrate] = 25 mM, [*Ts*OYE] = 10 µM, 30 °C, 24 h, 50 mM CHES-NaOH pH 9, 2.5% v/v DMSO (see Supporting Information: Sections 2.6, 3.1–7).

α,β-unsaturated carbonyl products was identical with *Ts*OYE's substrate scope in the reductive direction.[20] Obviously, this represents only a first evaluation of possible starting materials and future studies will focus more on expanding the substrate and enzyme-scope as well as gaining further understanding of the factors influencing the reaction rate.

Finally, we wondered whether this desaturation activity may be unique to thermophilic OYEs (as previously suggested) or whether the simple pH shift may be applicable to a broader range of OYEs. Therefore, we evaluated the ene reductase library from the company Seqens, a collection covering a broad biodiversity of OYEs that has been recently assessed in the reduction

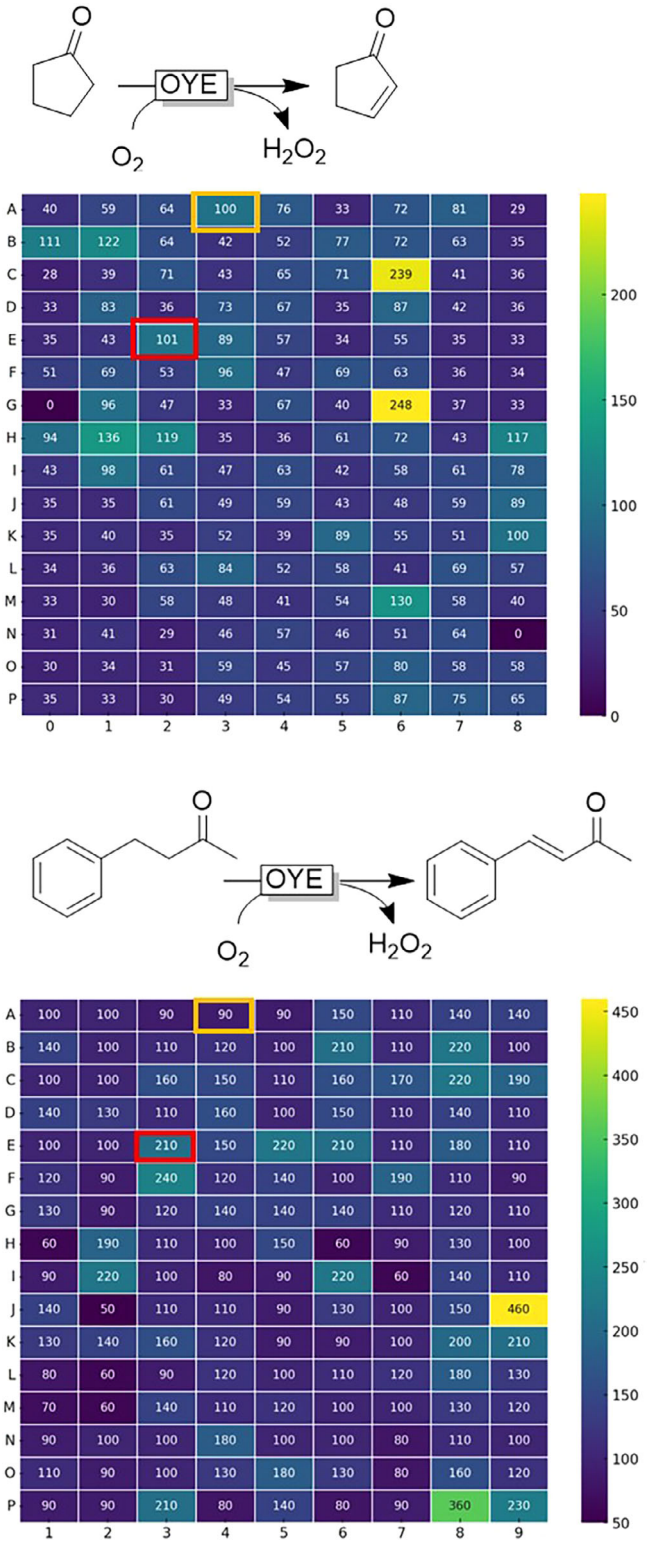


Figure 6. Results from screening the Sequens OYE library for desaturation activity. Conditions: [recombinant "wet" *Escherichia coli* cells] = 10–20 g/L; 50 mM CHES-NaOH pH 9, 30 °C, 5% v/v DMSO. Emphasized entries: Orange encircled: *Ts*OYE, red encircled: *Gk*OYE; C6: yeast OYE, G6 and N4: bacterial OYE, M4: plant OYE. Numbers shown represent the product concentration relative to the *Ts*OYE sample (A4). Absolute numbers are given in the Supporting Information: Section 2.7).

of cyclopropenyl esters and ketones, and cyclobutenones.[21,22] The screening of these enzymes in the desaturation of cyclopentanone and 4-phenyl-2-butanone (Figure 6 and Figure S9) showed several active enzymes. This screening shows the variability of OYEs to catalyze desaturation depending on the substrate and OYE family. For the desaturation of cyclopentanone, clear hits C6 (yeast OYE) and G6 (bacterial OYE) were identified, outperforming *Ts*OYE and *Gk*OYE. Whereas for 4-phenyl-2-butanone, M4 and N4 candidates (both arising from yeasts) gave promising conversions.

3. Conclusions

Overall, we have demonstrated that OYEs can be used as a desaturase in the oxidative direction. A simple pH shift enables this "new" reactivity and opens up new possibilities for biocatalysis using OYEs. These mechanistic insights enabled the discovery of new OYEs that catalyze the desaturation of carbonyl compounds and can be used in further applications.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supporting material of this article.

Keywords: Biocatalysis · Desaturation · Kinetic resolution · Old yellow enzyme · Oxidation

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