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Observation of a "blue" intermediate in the reaction between ferrylmyoglobin and thiocyanate. Deactivation of the hypervalent meat pigment as a step-wise process with slow intramolecular electron transfer

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Abstract

A "blue" reaction intermediate (absorption maximum at 835 nm) is observed when ferrylmyoglobin is reacted with excess thiocyanate in neutral aqueous solution. The intermediate is formed in a bimolecular process (first-order in thiocyanate and ferrylmyoglobin) with a rate constant ($0.119 \pm 0.006 M^{-1}s^{-1}$at 25°C) showing moderate temperature dependence (enthalpy of activation $\Delta H^{\#} = 45 \pm 6$ kJ mol^{-1}, entropy of activation $\Delta S^{\#} = -113 \pm 19$ J mol^{-1} K^{-1}), but decays in a first-order, highly temperature-dependent reaction ($1.53 \pm 0.05 \times 10^{-4}s^{-1}$ with $\Delta H^{\#} = 99 \pm 1$ kJ mol^{-1} and $\Delta S^{\#} = 14 \pm 3$ J mol^{-1} K^{-1}). Hypothiocyanite is not detected as an intermediate (as for peroxidase-catalyzed oxidation of thiocyanate), and the "blue" intermediate is proposed to be a protein radical with thiocyanate bound and which decays by intramolecular electron-transfer.

1. Introduction

Hypervalent forms of myoglobin and hemoglobin are receiving increasing attention as initiators of lipid and protein oxidation both in vivo under ischemic conditions (Arduni, Eddy, & Hochstein, 1990) and in meat during processing and storage (Kanner, German, & Kinsella, 1987; Skibsted, Mikkelsen, & Bertelsen, 1998). In the presence of hydrogen peroxide, myoglobins will crosslink certain proteins such as β-lactoglobulin and may transform other proteins such as bovine serum albumin into protein radicals with remarkably long half-lives of more than 10 min in aqueous solution at ambient conditions (Østdal, Daneshvar, & Skibsted, 1996; Østdal, Skibsted, & Andersen, 1997). The high abundance of myoglobin in mammalian tissue, together with the ubiquitous presence of peroxides, makes a detailed understanding of such pseudo-peroxidase activity important, both in relation to protection against oxdative stress in vivo, and in relation to quality deterioration in meat products. The hypervalent myoglobins, ferrylmyoglobin, MbFe(IV)=O, and the protein radical perferrylmyoglobin, ·MbFe(IV)=O, formed by reaction of myoglobin with hydrogen peroxide are deactivated by antioxidants such as flavonoids and ascorbate (Jørgensen & Skibsted, 1998; Kröger-Ohlsen & Skibsted, 1997). The deactivation mechanism is largely unknown, and we have embarked on kinetic investigations of reduction of MbFe(IV)=O by a variety of reductants in order to contribute to a better understanding. For thiocyanate, which notably is an important substrate for lactoperoxidase, we have made some intriguing spectroscopic observations, which were worthy of communicating, since we may have detected a reaction intermediate which is a protein radical with external reductant directly bound.

2. Materials and methods

Metmyoglobin (MbFe(III), horse heart, type III), was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium thiocyanate was from Fluka (Fluka Chemie, Buchs, Switzerland). 0.0200 M HCl was purchased from Bie & Berntsen Laboratory (Bie & Berntsen A/S,

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Rødovre, Denmark). All other chemicals (analytical grade) were from Merck (Darmstadt, Germany). The concentration of H_2O_2 (8 mM stock solution) was determined spectrophotometrically [$\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$ (Nelson & Kiesow, 1972)]. Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

2.1. Synthesis of ferrylmyoglobin

MbFe(III) dissolved in 5.0 mM phosphate buffer (pH 7.5) was purified by elution on a Sephadex G50 column (40×2.5 cm) (Pharmacia Biotech AB, Uppsala, Sweden). The purified MbFe(III) was diluted with the phosphate buffer to a concentration in the range of 190–280 μM as determined spectrophotometrically [$\epsilon_{525} = 7700\ M^{-1}\ cm^{-1}$ (Andersen & Skibsted, 1988)]. MbFe(III) was mixed with an equimolar amount of H_2O_2 and left at room temperature for 5 min before thermostatting at the temperature chosen for the subsequent reaction with SCN^- for 5 min.

2.2. pH measurements

pH was measured relative to concentration standards (0.0100 and 0.00100 M HCl, ionic strength 0.16 adjusted with NaCl), employing the definition $pH = -\log[H^+]$. pH was measured with a Hamilton 640.238-100 combination glass electrode (Hamilton Bonaduz AG, Bonaduz, Switzerland) connected to a Metrohm 713 pH-meter (Metrohm, Herisau, Switzerland).

2.3. UV–Vis measurements

A spectrum of the reaction intermediate was obtained after correction for contribution from other components in the solution (phosphate buffer, thiocyanate and NaCl) and correction for other heme species. This latter correction was obtained using the spectrum of a mixture of MbFe(III) and MbFe(IV)=O constructed from their pure spectra (i.e. spectra of freshly prepared solutions) and calculated concentrations (Miller et al., 1993) at the time of maximum intermediate concentration from the recorded spectrum of the reaction mixture (70.0 μM total myoglobin, 10 mM SCN^-, 25.0°C, pH = 7.40, I = 0.16, 20 mM phosphate buffer). Control experiments for the formation of $OSCN^-$ were carried out using two cuvettes with 0.1 and 1.0 cm path lengths, respectively, containing the same reaction mixture (40.0 μM MbFe(IV)=O, 10 mM SCN^-, 25.0°C, pH = 7.40, I = 0.16, 20 mM phosphate buffer) allowing simultaneous detection of the "blue" intermediate (835 nm, 1 cm path length) and $OSCN^-$ [235 nm, 0.1 cm path length; (Pruitt & Tenovuo, 1982)]. A HP 8453 UV–Vis diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA) was used for all measurements.

2.4. Kinetic experiments

Solutions of SCN^- were freshly prepared each day to yield the final conditions pH = 7.40 ± 0.05, I = 0.16 ± 0.01, 20 mM phosphate buffer in 1:1 mixture with ferrylmyoglobin. Cuvettes with 1.5 ml SCN^- solution of different concentrations were thermostatted in the cell holder of an HP 8453 UV-Vis diode array spectrophotometer (Hewlett–Packard, Palo Alto, CA) before addition of 1.5 ml prethermostatted MbFe(IV)=O solution, and the recording of spectra of stirred solutions (450–1100 nm) were started after mixing. The conversion of MbFe(IV)=O to MbFe(III) was followed at 580 nm, while the decay and formation of the intermediate was followed at 835 nm.The reaction temperature was measured in the cuvettes after completion of the reaction, and pH was subsequently measured in thermostatted samples of the reaction mixtures.

2.5. Data analysis

Data were analysed using the PC Pro-K Global Analysis software package obtained from Applied Photophysics Ltd. (Applied Photophysics, Leatherhead, UK) and the Origin 5.0 (patch 2) obtained from Microcal (Microcal Software, Inc., Northampton, MA).

3. Results

Reduction of ferrylmyoglobin by thiocyanate followed first-order kinetics as evaluated using the PC Pro-K Global Analysis software package for analysing data in the interval 500–650 nm, the spectral region where the conversion of MbFe(IV)=O to MbFe(III) is normally followed (Kröger-Ohlsen & Skibsted, 1997). The observed pseudo first-order rate constant depended linearly on the concentration of thiocyanate in agreement with a second order reaction and a value of $8.7\times 10^{-2} M^{-1}s^{-1}$ was obtained at 25°C:

$$\frac{d[MbFe(IV)=0]}{dt} = -k_1[MbFe(IV)=O][SCN^-] \qquad (1)$$

However, a reaction intermediate with maximum absorbance at 835 nm was formed during the thiocyanate-mediated conversion of ferrylmyoglobin to metmyoglobin. On a longer timescale (Fig. 1), this "blue" intermediate, the spectrum of which is shown in Fig. 2 at the time for its maximal concentration, decayed.

The formation and decay of this reaction intermediate was followed at 835 nm and the spectrophotometric time traces recorded at 835 nm were analysed using the absorbance at 613 nm as a spectral reference correcting for protein denaturation, which led to a slight increase in absorbance. Formation and decay

of the intermediate by consecutive first order reactions would result in the expression for absorbance as function of time:

$$A_{835,t} = \alpha \exp(-k_1 t) + \beta \exp(-k_2 t) + A_{835,\infty} \quad (2)$$

where α and β are the constants

$$\alpha = \frac{(\epsilon_I - \epsilon_A)k_1 + (\epsilon_A - \epsilon_P)k_2}{k_2 - k_1}[A]_0 \quad (3)$$

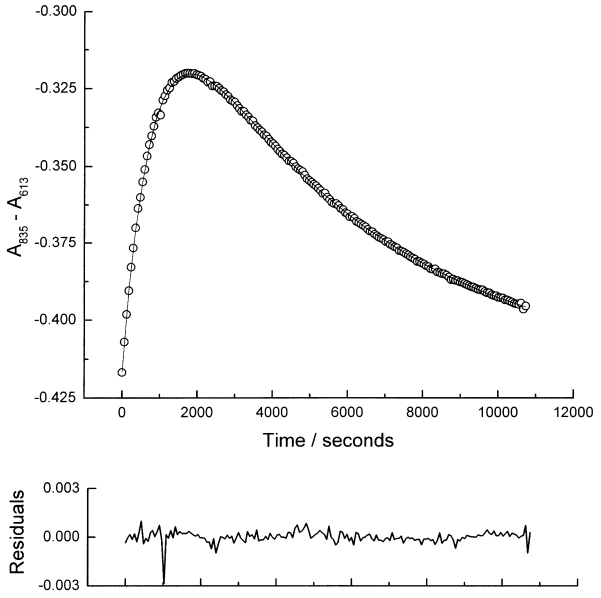

Fig. 1. Circles: Relative absorbance at 835 nm during reaction between 75.0 μM MbFe(IV)=O and 13 mM thiocyanate at pH 7.35 (0.020 M phosphate buffer) and 26.8°C at ionic strength 0.16 (NaCl) corrected for slightly increasing degree of denaturation by subtraction of absorbance at 613 nm. Full line: non-linear fit of the expression $A(t) = \alpha \exp(-k_1 t) + \beta \exp(-k_2 t) + A_\infty$, from which a pseudo-first-order rate constant for the formation of the intermediate $k'_1 = 1.20 \times 10^{-3} \pm 7 \times 10^{-6} s^{-1}$ and a first-order rate constant for the decay of the intermediate $k_2 = 1.94 \times 10^{-4} \pm 2 \times 10^{-6} s^{-1}$ are obtained. Lower panel shows residuals of the fit.

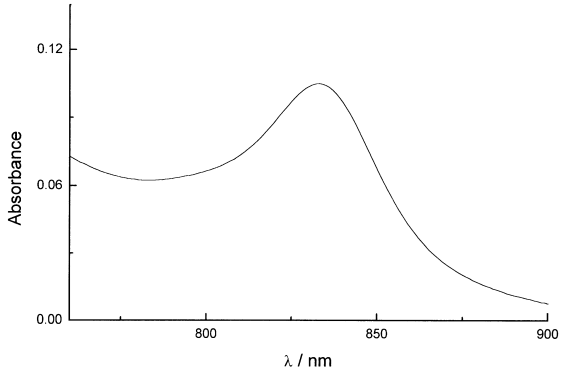

Fig. 2. Spectrum of the "blue" intermediate at its maximal concentration [70.0 μM total myoglobin, 10 mM SCN^-, 25.0°C, $pH = 7.40$, $I = 0.16$ (NaCl), 20 mM phosphate buffer].

$$\beta = \frac{(\epsilon_P - \epsilon_I)k_1}{k_2 - k_1}[A]_0 \quad (4)$$

and ϵ_A, ϵ_I, and ϵ_P are the absorptivities of the reactant, the intermediate, and the product, respectively, and $[A]_0$ is the initial concentration of the reactant (Skibsted, 1979). k_1 and k_2 are the (pseudo) first-order rate constants for formation and decay of the intermediate. Dual solutions exist, however, to this expression, and correct assignment of the two rate constants to the two reaction steps relies on further information such as dependence of one of the rate constants on reactant concentrations.

As seen in Fig. 1, the expression fits well to spectral data. One of the rate constants was found to depend linearly on the thiocyanate concentration under the present conditions of large excess of thiocyanate (Fig. 3), whereas no concentration dependence was found for the other constant (Fig. 4). The constant depending on thiocyanate concentration had, for 25°C the value 0.119 M^{-1} s^{-1} (as interpolated from the Arrhenius plot of Fig. 5) in fair agreement with the value 8.7×10^{-2} M^{-1} s^{-1} obtained for the decay of MbFe(IV)=O in the spectral region 500–650 nm. This allows the assignment of k'_1 (Fig. 3) for the formation of the intermediate to the faster reaction and k_2 for the decay of the intermediate to the slower reaction, independent of thiocyanate concentration (Fig. 4). In agreement with this assignment, increasing thiocyanate concentration was found to result in earlier appearance of the absorption maximum. Second-order rate constants, k_1, for formation of the "blue" intermediate are thus given by the slopes of the lines in Fig. 4. Arrhenius plots for

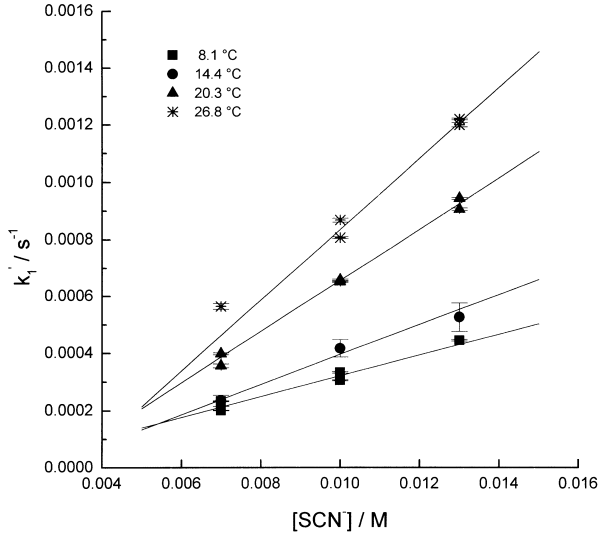

Fig. 3. Linear dependence of the pseudo-first order rate constants (k'_1) assigned to formation of the intermediate in the reduction of MbFe(IV)=O by SCN^- for conditions of excess of SCN^-. For each temperature the second-order rate constant k_1 is given by the slope of the straight line.

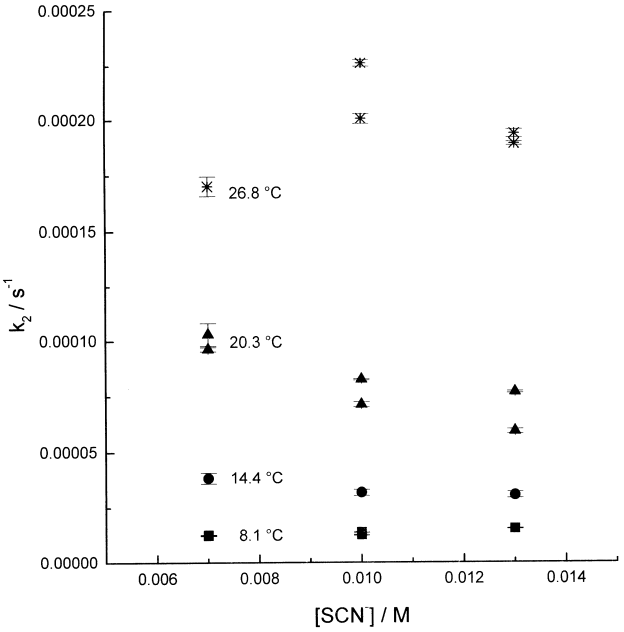

Fig. 4. The first-order rate constants observed for decay of the intermediate in the reduction of MbFe(IV)=O by SCN^- at varying concentrations of excess of SCN^-. No systematic dependence is seen for k_2 with varying concentration.

formation and decay of the "blue" intermediate are shown in Fig. 5 and activation parameters for the two reactions are listed in Table 1.

The oxidation of thiocyanate by lactoperoxidase results in formation of unstable $OSCN^-$, which is seen as a transient absorption at 235 nm (Pruitt & Tenovuo, 1982). No such absorption band was observed during oxidation of thiocyanate by ferrylmyoglobin.

4. Discussion

Muscle membranal lipid peroxidation has been found to be initiated by hydrogen peroxide-activated metmyoglobin through formation of the hypervalent myoglobins ·MbFe(IV)=O and MbFe(IV)=O (Harel & Kanner, 1985). Oxidation of substrates such as cytoplasmic reductants and membranal phospholipids by MbFe(IV)=O is acid-catalyzed, and it has been suggested, that a protein radical cation is the actual reactant

$$MbFe(IV)=O + H_3O^+ \rightleftarrows {}^{\cdot+}MbFe(III)-OH+H_2O \quad (5)$$

and that, in neutral solution, a similar intramolecular electron transfer

$$MbFe(IV) = O \rightleftarrows {}^{\cdot+}MbFe(III) - O^- \quad (6)$$

for some substrates (but not for others) is rate determining (Kröger-Ohlsen & Skibsted, 1997). A similar reaction mechanism has been proposed for certain microperoxidases (Low, Winkel, & Gray, 1996). For MbFe(IV)=O, the reactive protein cation radical

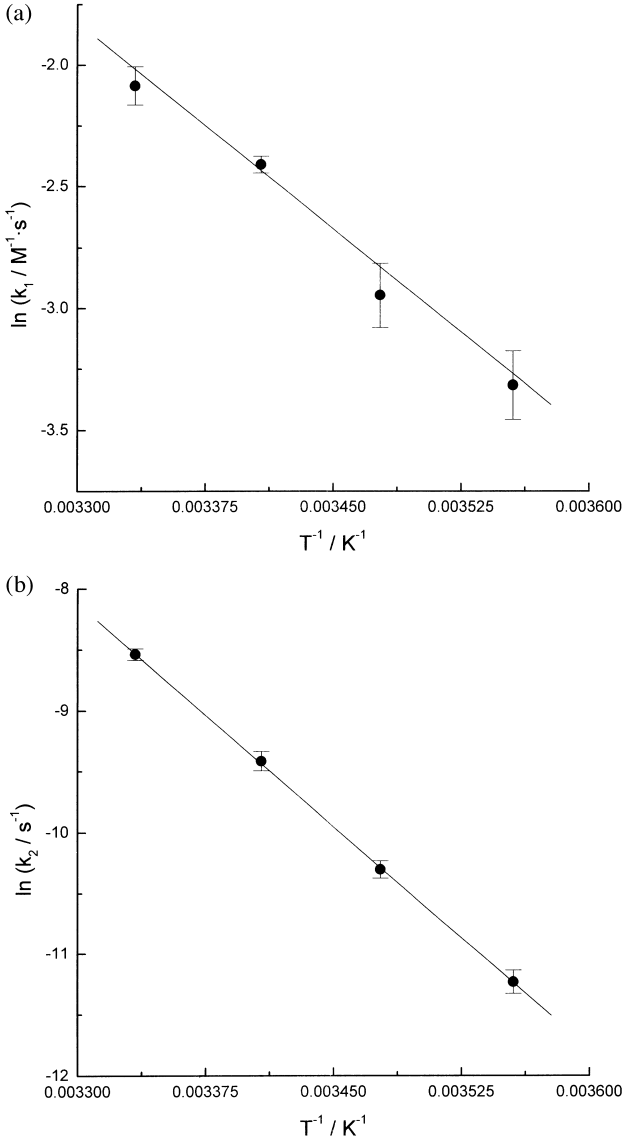

Fig. 5. Arrhenius plots for (a) formation and (b) decay of the intermediate in the reduction of MbFe(IV)=O by SCN^-.

${}^{\cdot+}$MbFe(III)$-O^-$ subsequently accepts an electron from an external reductant in a bimolecular reaction to yield a one-electron oxidized substrate as was shown for ascorbate by ESR-spectroscopy (Kröger-Ohlsen & Skibsted, 1997).

Thiocyanate is a substrate for lactoperoxidase in milk but, in contrast to this enzyme, MbFe(IV)=O as a pseudo-peroxidase was not found to yield hypothiocyanite, $OSCN^-$, as a reaction intermediate (Pruitt & Tenovuo, 1982). In contrast, the reaction clearly proceeded through an intermediate with unexpected spectroscopic properties. This "blue" intermediate was formed in a second-order reaction with activation parameters (Table 1) surprisingly similar to those for reduction of MbFe(IV)=O by β-lactoglobulin ($\Delta H^{\#}$ = 45kJmol^{-1}, $\Delta S^{\#} = -93$ J mol^{-1} K^{-1}; Østdal et al., 1996) and ascorbate ($\Delta H^{\#} = 45$ kJ mol^{-1}, $\Delta S^{\#} = -85$ J mol^{-1} K^{-1}; Kröger-Ohlsen & Skibsted, 1997). It seems safe to

Table 1
Rate constants and activation parameters at 25.0°C for formation and decay of the "blue" intermediate (with absorption maximum at 835 nm) formed during reduction of ferrylmyoglobin by thiocyanate at pH = 7.40 and ionic strength 0.16 (NaCl)

	Formation of intermediate (M^{-1} s^{-1})	Decay of intermediate (s^{-1})
Rate constant[a]	0.119 ± 0.006	$1.53\times10^{-4} \pm 5\times10^{-6}$
$\Delta H^{\#a}$ (kJ/mol)	45 ± 6	99 ± 1
$\Delta S^{\#a}$ (J/(mol K))	-113 ± 19	14 ± 3

[a] Estimated from Arrhenius plots of Fig. 5 according to $k = A\exp(-E_a/RT)$ using transition state theory and given with standard error of estimate.

conclude, that a common reaction is rate determining for these reactions also, since, for SCN^-, the rate of formation of the "blue" intermediate is very similar to the rate of transformation of iron(IV) to iron(III). We suggest that the forward reaction of the equilibrium of Eq. (6) is this common rate determining step for reduction of MbFe(IV)=O to MbFe(III) by ascorbate, by β-lactoglobulin and by SCN^-. The notable difference is, however, that, for SCN^-, the transition state for the subsequent bimolecular (and fast) reaction is not leading directly into a one-electron oxidized substrate (i.e. ·SCN) and MbFe(III), but into a relatively stable intermediate, which subsequently decomposes in a reaction, probably to yield ·SCN and MbFe(III). The reaction sequence is accordingly:

$$MbFe(IV) = O \overset{k_1}{\rightarrow} {}^{\cdot+}MbFe(III) - O^- \quad (7)$$

$${}^{\cdot+}MbFe(III)O^- + SCN^- + 2H^+ \overset{fast}{\longrightarrow} \quad (8)$$

$$(NCS^-, {}^{\cdot+}MbFe(III)OH_2) \overset{k_2}{\rightarrow} \quad (9)$$

$$\cdot SCN + MbFe(III)OH_2 \quad (10)$$

in which (NCS^-, ${}^{\cdot+}MbFe(III)OH_2$) is the "blue" intermediate. The absorption centered around 835 nm is low-energetic as would be expected for electron-transfer in a sulfur ion-stabilized cation radical. The mechanism described by the reactions of Eqs. (7)–(10) explains the following experimental observations: (i) the common activation parameters for reduction of MbFe(IV)=O by the widely differing reductants SCN^-, ascorbate and β-lactoglobulin with the foreward reaction of the equilibrium of Eq. (6) as rate determining; (ii) the second-order kinetics of formation of the "blue" intermediate with a very similar rate constant as for reduction of iron(IV) to iron(III) and understandable on the basis of a fast reverse reaction of the equilibrium of Eq. (6) competing with the fast reaction of Eq. (8); and (iii) the first-order kinetics of the disappearance of the "blue" intermediate indicative of intramolecular electron transfer with a large activation barrier ($\Delta H^{\#} = 99 kJ\ mol^{-1}$).

The unexpected observation of a transient in the reduction of MbFe(IV)=O by thiocyanate has thus provided important mechanistic information of key reactions in the coupling between pigment oxidation and other oxidative processes in muscles and meat. Although the nature of this intermediate is still speculative, we tentatively assign this intermediate as a thiocyanate derivative of a protein or a phorphyrin radical.

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