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Commentary

The Thermodynamics of Xanthine Oxidoreductase Catalysis

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ABSTRACT

Xanthine oxidoreductase is a complex enzyme found in a wide range of organisms. Recent interest in this enzyme stems from its ability to produce reactive oxygen species under a range of conditions. It is found as a homodimer, each unit containing a molybdopterin cofactor, two iron sulfur centers, and FAD. The enzyme can exist in two forms that differ primarily in their oxidizing substrate specificity. The dehydrogenase form preferentially utilizes NAD^+ as an electron acceptor but is able to donate electrons to molecular oxygen. Xanthine dehydrogenase from mammalian sources can be converted to an oxidase form that readily donates electrons to molecular oxygen, but does not reduce NAD^+. The catalytic mechanism of both forms of the enzyme can be described in terms of a rapid equilibrium model in which reducing equivalents are distributed rapidly between the different redox centers of the enzyme on the basis of their midpoint potentials. The present commentary gives a brief overview of the literature concerning the rapid equilibrium model and the differences between the two enzyme forms. NADH is also discussed in terms of an alternative to xanthine or hypoxanthine as an electron donor. Antiox. Redox Signal. 1, 371–379.

INTRODUCTION

XANTHINE OXIDOREDUCTASE (XOR) is a widely distributed enzyme found in organisms ranging from bacteria (Leimkuhler *et al.*, 1998) to humans (Kooij *et al.*, 1992), higher plants (Nguyen, 1986; Mendel and Schwarz, 1999), and invertebrates (Doyle *et al.*, 1996). The action of xanthine oxidoreductase was first observed in 1902 when it was shown that fresh milk could decolorize methylene blue in the presence of formaldehyde (Schardinger, 1902). The enzyme was then shown to convert hypoxanthine and xanthine to urate in the presence of oxygen or anaerobically in the presence of methylene blue (Morgan *et al.*, 1922). The high availability of xanthine oxidase from bovine milk has led to a wide body of research concerning its action. The enzyme from mammalian sources exists in two interconvertible forms, an oxidase form (XO) and a dehydrogenase form (XDH). It is a homodimer, with each subunit containing a molybdopterin cofactor, two iron sulfur centers, and one FAD. A wide range of reducing substrates can donate electrons to the molybdopterin cofactor (Hille, 1994), including purines, aldehydes, and pteridines. Some artificial dyes such as methylene blue are able to accept electrons from the iron sulfur centers (Salaris *et al.*, 1991). XO and XDH differ from each other primarily at the FAD site, where XO donates electrons solely to molecular oxygen and XDH preferentially donates electrons to NAD^+ (Saito and Nishino, 1989). The probable pathways of electron transfer are summarized in Figure 1.

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Recent interest in XO has been focussed on its ability to reduce molecular oxygen either by two electrons to hydrogen peroxide (H_2O_2), or by one electron to superoxide radicals (McCord and Fridovich, 1968; Hille and Massey, 1981; Porras *et al.*, 1981). This ability of XO to produce reactive oxygen species (ROS) has led a number of workers to implicate the enzyme in a range of pathological conditions, including postischaemia reperfusion injury (see Nishino *et al.*, 1997 and Zhang *et al.*, 1998 for reviews). As yet, the physiological role of XOR is unclear, although it is thought to play an important role in purine metabolism. The production of ROS by XO has also been suggested to have possible roles in a defense mechanism (Tubaro *et al.*, 1980) during infections, and in normal signal transduction (Suzuki *et al*, 1997).

THE RAPID EQUILIBRIUM MODEL

The rapid equilibrium model for the action of xanthine oxidase arose from the observation that internal electron transfers between the molybdopterin cofactor, the iron sulfur centers, and the flavin group in bovine milk XO are much more rapid than the turnover rate (Edmondson *et al.*, 1973). The kinetics of the reduction of the enzyme by xanthine and equilibrium titration of the enzyme with dithionite were studied in detail using optical and electron paramagnetic resonance (EPR) spectroscopy (Olson *et al.*, 1974). The observed changes in spectral properties were accounted for by oxidation–reduction equilibria, which give rise to 36 different possible states of the enzyme at different levels of reduction. Thus, with intramolecular electron transfer not being rate limiting, the appearance of reduced acceptor groups is simply a function of their relative reduction potentials. The observed time course for the reduction of both the flavin and iron sulfur centers during reduction of the enzyme by xanthine were accurately calculated by adopting a model in which three moles of substrate each donate a pair of electrons in a consecutive process. Electrons are known to be donated to the enzyme by xanthine via the molybdopterin cofactor (Komai *et al.*, 1969; Bray, 1975), and to be donated from the enzyme to molecular oxygen via the FAD group (Komai *et al.*, 1969). Thus, with the rough order of electron affinity constants being FAD ≥ Fe/S > Mo, the iron sulfur centers were proposed to act as an electron sink, functioning to maintain molybdenum in its oxidized state for reaction with xanthine and flavin in its reduced state for reaction with molecular oxygen.

In support of the rapid equilibrium hypothesis, a number of investigators have measured the midpoint potentials for the redox centers in xanthine oxidase using a variety of techniques. Using EPR spectroscopy to monitor the redox states of the enzyme, the following midpoint potentials were estimated by potentiometric titration at pH 8.2 (Cammack *et al.*, 1976): Mo^{VI}/Mo^{V}, −355 mV; Mo^{V}/Mo^{IV}, −355 mV; $Fe/S\ I_{ox/red}$, −343 mV; $Fe/S\ II_{ox/red}$, −303 mV; $FAD/FADH^{\bullet}$, −351 mV; $FADH^{\bullet}/FADH_2$, −236 mV Measurements made using microcoulometry (Spence *et al.*, 1982) yielded the following values at pH 7.7: Mo^{VI}/Mo^{V}, −375 mV; Mo^{V}/Mo^{IV}, −405 mV; $FAD/FADH_2$, −280 mV; $Fe/S\ I_{ox/red}$, −320 mV; $Fe/S\ II_{ox/red}$, −230 mV. It was also found that each of these potentials was shifted to a more negative value (25–75 mV) when the enzyme was titrated at pH 8.9. Thus, both sets of measured potentials support the approximate order of midpoint potentials of FAD ≥ Fe/S > Mo in the rapid equilibrium model. There are some discrepancies between measurements, but these might be explained by the fact that the EPR measurements

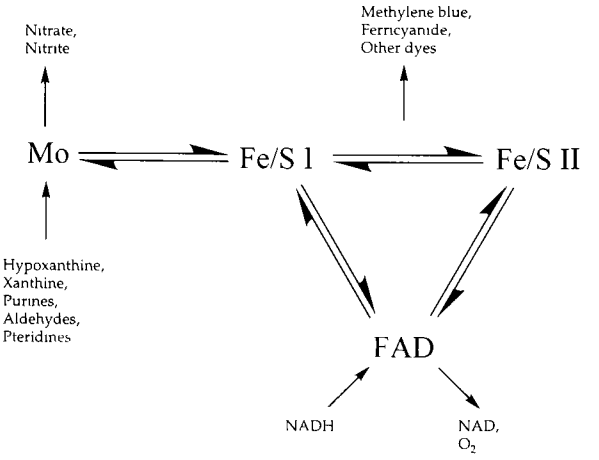

FIG. 1. A scheme showing the probable pathways of inter- and intramolecular electron transfer of xanthine oxidoreductase (see text).

were made at cryogenic temperatures, whereas the microcoulometry carried out by Spence *et al.* was carried out at 25°C. Thus, the overall midpoint potential for the two-electron reduction of the molybdenum center is above that for the xanthine/urate couple at the pH values studied (E_m xanthine/urate = −410 mV at pH 7.65 and −441 mV at pH 8.07; Clark, 1960), favoring the reduction of the enzyme by xanthine.

The mechanisms of electron transfer within xanthine oxidase have also been studied by pulse radiolysis (Hille and Anderson, 1991). *N*-Methylnicotinamide radical and 5-deazaflavin radical were generated radiolytically and used to reduce the enzyme rapidly at pH 6.0 and pH 8.5. At both pH values, all rate constants for internal electron transfer between specific centers were found to be rapid relative to enzyme turnover. Electron equilibration between the molybdopterin and one of the Fe/S centers was found to be particularly rapid, and a model was developed in which the Fe/S centers play a role in mediating the transfer of electrons between the molybdopterin and the flavin centers. This model is supported by a study of the magnetic interactions between the redox centers of xanthine oxidase (Barber *et al.*, 1982) in which the distances between the various centers were estimated from the relaxation behavior of the electron paramagnetic resonance signals of the enzyme. The following distances were obtained: Mo-Fe/S I, 11 Å; Fe/S I-Fe/S II, 15 Å; FAD-Fe/S I, 16 Å; FAD-Fe/S II, 16 Å. No interaction was detected between Mo and FAD.

Further pH jump experiments (Hille, 1991) lend particularly elegant support to the rapid equilibrium model. It has been shown that the reduction of each of the redox centers in xanthine oxidase is associated with a protonation step, and therefore their midpoint potentials show pH-dependent behavior (Barber and Siegel, 1982; Porras and Palmer, 1982). The spectral changes resulting from a rapid increase or decrease in pH were monitored and shown to be consistent with the known standard midpoint potentials and pK_a values of the redox centers of the enzyme. Thermodynamic control of the kinetic properties of XO was shown with the bovine milk enzyme by manipulation of the redox properties with the use of chemically modified flavins (Hille and Massey, 1991). Steady-state analysis revealed a sigmoidal dependence of V_{max} on the flavin midpoint potential. It was shown that the inactive forms of the enzyme containing low potential flavins were able to react with both xanthine and molecular oxygen. The observed pattern of activity was accurately predicted in terms of a model in which the catalytic rate is attenuated by the amount of partially reduced enzyme generated during turnover possessing an unfavorable distribution of reducing equivalents among the redox centers of the enzyme. Such a model is based entirely upon the rapid equilibrium hypothesis.

XDH AND XO INTERCONVERSIONS

XOR can be isolated from mammalian sources in the dehydrogenase form if purification is carried out nonproteolytically and in the presence of thiol reagents. It was noted in 1968 (Della Corte and Stirpe, 1968a) that xanthine dehydrogenase activity in rat liver extracts was slowly converted to xanthine oxidase activity on storage at −20°C. Further investigation (Della Corte and Stirpe, 1968b) showed that an XDH- to-XO conversion could also be achieved by incubation with proteolytic enzymes. Solvents were also noted to cause an XDH-to-XO conversion (Della Corte and Stirpe, 1969). Thus, as both proteolysis and solvent extraction had been extensively used during the purification of XO from bovine milk, it was suggested that the mammalian enzyme exists predominantly as XDH *in vivo*, which is then converted to XO during purification.

XDH-to-XO conversion was also carried out by oxidation of rat liver extracts with thiol reagents (Della Corte and Stirpe, 1972), and unlike with proteolyzed XO, the conversion was found to be reversible by reduction with dithioerythritol. Conversion of XDH from rat liver to XO with 4,4′-dithiodipyridine (Saito, 1987) was accompanied by the formation of approximately 8 moles of 4-thiopyridone and four disulfide bonds per mole of FAD, although it was suggested that at least three of the sulfhydryl groups are nonessential for the conversion of XDH to XO.

If purified nonproteolytically, XOR can be isolated in its dehydrogenase form from both bovine and human milk (Hunt and Massey, 1992; Sanders *et al.*, 1997). XDH from these sources can also undergo the above interconversions. Such interconversions are not observed for XDH from chick or pigeon liver (Della Corte and Stirpe, 1972), which do not have an oxidase form. Limited proteolysis of rat liver XDH showed that conversion to XO is associated with cleavage of the peptide chain at two positions (Amaya *et al.*, 1990), giving rise to three fragments with molecular weights of 20, 40, and 85 kDa from the amino terminus to the carboxyl terminus, respectively. The NAD binding site of the enzyme was shown to be associated with the 40-kDa fragment. Chicken liver XDH can be proteolyzed to give three domains of approximately the same size as for the rat liver enzyme (Sato *et al.*, 1995), although with no associated conversion to XO. For both enzymes, dissociation of the proteolytic fragments only occurs under denaturing conditions.

The observed proteolytic conversion of XDH to XO along with the ability of XO to produce both H_2O_2 and superoxide, led to a proposed role of the enzyme in postischaemia reperfusion injury (McCord, 1985). According to this hypothesis, influx of Ca^{2+} into the cell during ischemia activates calcium-dependent proteases and XDH is proteolytically converted to XO. Levels of hypoxanthine also build up during ischemia, and so upon recirculation a burst of xanthine oxidase activity gives rise to ROS, which cause tissue damage. A number of potential problems with this hypothesis have been reviewed (Nishino *et al.*, 1997; Zhang *et al.*, 1998). Proteolytic conversion of XDH to XO has been shown to be rather slow (Frederiks and Bosch, 1996). XDH does have some inherent oxidase-type activity that gives rise to superoxide (Nishino *et al.*, 1989; Harris and Massey, 1997). However, this activity might be expected to be inhibited by the levels of NAD^+ found *in vivo*. Another potential problem is the low levels of XOR activity measured in all human tissues other than liver and intestine (Kooij *et al.*, 1992). XOR of low reactivity toward xanthine has been isolated from human heart (Abadeh *et al.*, 1993) and milk (Sanders *et al.*, 1997). In the case of human milk xanthine oxidase, the enzyme was shown to be lacking the molybdopterin cofactor (Godber *et al.*, 1997).

THE ORIGINS OF DEHYDROGENASE AND OXIDASE SPECIFICITY

A comparison of some of the properties of XDH and XO from rat liver (Saito and Nishino, 1989) revealed differences in the environment of the flavin. XDH showed considerable formation of a neutral semiquinone species during reductive titrations and during steady-state turnover with xanthine and molecular oxygen as substrates, whereas XO did not. Flavin semiquinone has been shown to react more slowly with molecular oxygen than fully reduced flavin (Porras *et al.*, 1981), which may therefore be a factor in the comparatively low oxidase activity of XDH. Differences in the flavin environment between the two enzyme forms were further highlighted by a study using chemically modified flavins as active site probes (Massey *et al.*, 1989; Saito *et al.*, 1989). It was shown that deflavo-XO from bovine milk or rat liver binds flavins with ionizable groups (-OH or -SH) in the 6- or 8-positions in their anionic forms, whereas deflavo-XDH from rat liver, chicken liver, or bovine milk binds the same flavins in their neutral forms. The pK_a of the ionizable group was found to be perturbed by greater than or equal to 4 pH units on binding to XDH, which was attributed to the presence of a strong negative charge in the flavin binding site of XDH that is absent in XO (Massey *et al.*, 1989; Hunt and Massey, 1992).

The midpoint potentials for the redox centers of XDH have been measured. A potentiometric titration of XDH from turkey liver (Barber *et al.*, 1977) yielded the following values at pH 8.2 and 25°C: Mo^{VI}/Mo^{V}, −350 mV; Mo^{V}/Mo^{IV}, −362 mV; FAD/FADH•, −359 mV; FADH•/$FADH_2$, −366 mV; Fe/S $I_{ox/red}$, −295 mV; Fe/S $II_{ox/red}$, −292 mV. The midpoint potentials of the molybdopterin are very close to those measured for bovine milk XO under the same conditions by the same investigators (see above). The midpoint potentials for the Fe/S centers are slightly higher than in XO,

but the major difference is in the value of the $FADH^{\bullet}/FADH_2$ potential, which is 130 mV lower than the corresponding one for bovine milk XO. The midpoint potentials of the Fe/S centers and FAD of XDH from bovine milk have also been measured (Hunt *et al.*, 1993), thus allowing a more direct comparison between the two enzyme forms. The potentials of the Fe/S centers were found to be the same as those measured for XO from bovine milk (−310 mV and −235 mV for Fe/S I and Fe/S II, respectively, at pH 7.5 and 25°C). The FAD/$FADH^{\bullet}$ potential was found to be −270 mV, close to the corresponding value for XO, but the $FADH^{\bullet}/FADH_2$ potential was found to be considerably lower, at −410 mV. The large separation between the two flavin half-potentials results in a 90% stabilization of the neutral semiquinone. With these measured potentials for the flavin and Fe/S centers, a potential of −420 mV for a reducible disulfide, and −320 mV for the molybdopterin center, the electron distributions from a reductive titration of the enzyme could be accurately predicted using the rapid equilibrium model.

The above measurements indicate an overall midpoint potential for the two-electron reduction of the flavin of bovine milk XDH of −340 mV. This potential is thus well poised for the reduction of NAD (E_m = −335 mV at pH 7.5) during turnover. The equivalent midpoint potential of bovine milk XO at −255 mV (Porras and Palmer, 1982), is too high for efficient oxidation of the flavin. However, the redox properties of the enzyme-bound flavin are modified by the binding of pyridine nucleotides to chicken liver XDH (Schopfer *et al.*, 1988; Nishino and Nishino, 1989). Binding of aminopyridine adenine dinucleotide (AAD), an analog of NAD that does not undergo electron transfer with XDH, causes large perturbations of the visible spectrum of the enzyme and a greater separation of the flavin half-potentials, giving rise to a greater stabilization of the neutral semiquinone. In contrast, the binding of NAD causes only minor changes in the enzyme absorbance spectrum. Reductive titration indicated that the FAD/$FADH^{\bullet}$ potential is raised by NAD binding, but the $FADH^{\bullet}/FADH_2$ potential is raised to a greater extent, thus destabilizing the neutral semiquinone. The addition of AAD or NAD to XO had no observed effect.

To distinguish between the thermodynamic and substrate binding determinants of XOR specificity, the redox potentials of XDH and XO from bovine milk were manipulated by the use of chemically substituted flavins (Harris *et al.*, 1999). Both XDH and XO containing 1-deaza-FAD were shown to have a flavin midpoint potential similar to that of native XDH, while XDH and XO containing 8-CN-FAD were shown to have flavin midpoint potentials higher than that of native XO. Both the low-potential XO (containing 1-deaza-FAD) and the high-potential XDH (containing 8-CN-FAD) showed essentially no xanthine/NAD activity. This activity was detected for 1-deaza-XDH. High-potential XDH (containing 8-CN-FAD) showed a 2.5-fold increase in xanthine/oxygen activity as compared to native XDH, and high potential XO (containing 8-CN-FAD) showed normal xanthine/oxygen activity. The binding constants for NAD to both oxidized XDH and oxidized XO were also measured, by ultrafiltration and isothermal calorimetry titration. NAD was found to bind to XDH with a K_d of approximately 220 μM, with no detectable binding of NAD to XO by either method. Thus, it is apparent that both a low flavin midpoint potential and a suitable binding site for NAD are necessary for xanthine/NAD activity. Xanthine/oxygen activity only requires a reduced flavin. The K_d for the binding of NAD to reduced XDH has been determined kinetically to be 25 μM (Harris and Massey, 1997). From these data and a midpoint potential of −340 mV for the unbound flavin, a midpoint potential of −370 mV can be calculated for the NAD-bound flavin based on a thermodynamic box (Harris *et al.*, 1999). This apparent 30-mV fall in flavin midpoint potential would aid the reduction of NAD, although a decrease in the flavin midpoint potential is in direct contrast to observations made for the rat liver enzyme (Nishino and Nishino, 1989).

NADH AS AN ELECTRON DONOR

The finding that the midpoint potentials of the flavin of XDH and the NAD/NADH redox

couple are very similar suggests that oxidized XDH should be reducible by NADH. Indeed, it was found (Schopfer *et al.*, 1988) that the reaction of oxidized chicken liver XDH with NADH brings the enzyme to the same redox state as the reaction of reduced XDH with excess NAD, *i.e.*, to the 2-electron reduced level. Thus, XDH should be able to utilize NADH as a reducing substrate during turnover. NADH oxidase activity has been detected for rat liver XDH (Waud and Rajagopalan, 1976) and bovine milk XO (Nakamura, 1991), and was shown to be associated with substantial production of superoxide anion for bovine milk XO and XDH (Nakamura, 1991; Harris and Massey, 1997).

A detailed study of the NADH oxidase activity of both XDH and XO from human milk has been carried out (Sanders *et al.*, 1997). Both forms of the enzyme oxidize NADH to NAD and reduce molecular oxygen to superoxide and H_2O_2, and both enzyme forms were inhibited by NAD. The NADH oxidase activity of XDH was greater than that of XO and followed Michaelis Menten kinetics, with a K_m of 1.23 μM for NADH and a V_{max} of 0.33 μmol $min^{-1}mg^{-1}$. The NADH oxidase activity of XO does not follow Michaelis Menten kinetics and requires higher concentrations of NADH to turn over. Thus, despite the more favorable redox potential for oxidizing NADH, XO is a much less effective NADH oxidase, presumably due to the lack of a binding site for pyridine nucleotides. Studies of the oxygen-dependence of NADH activity (Harris and Massey, 1997; Sanders *et al.*, 1997) reveals a very high K_m for molecular oxygen, such that the response of this activity to oxygen concentration would be essentially linear under physiological conditions. The NADH oxidase activity of the enzyme is expected to be associated solely with the flavin center of the enzyme, as was demonstrated by the use of specific inhibitors that react with known centers of the enzyme (Sanders *et al.*, 1997). Given the lack of reactivity of XOR from human heart and milk toward xanthine, the NADH activity of XOR was suggested as an alternative mechanism for the production of ROS, giving rise to post-ischemia reperfusion injury. This enzyme activity might be expected to be effectively inhibited by the levels of NAD found *in vivo*, although the ratio of NAD to NADH falls dramatically in hypoxic tissues (Nishino and Tamura, 1991). The proposed mechanism also has no requirement for conversion of XDH to XO because XDH is the more effective source of ROS.

Nitrate and nitrite are also able to act as electron acceptors from NADH-reduced XOR. Reduction of nitrate by XO was demonstrated by the accumulation of nitrite under rigorously anaerobic conditions (Fridovich and Handler, 1962). XO has also been shown to oxidize NADH in the presence of nitrate under anerobic conditions, and nitrate was shown to be a competitive inhibitor of turnover of the enzyme with xanthine and molecular oxygen (Hackenthal and Hackenthal, 1966). By comparison with nitrate reductases (Hille, 1996), it seems likely that nitrate and nitrite react with the molybdopterin center of the enzyme. More recently, the reduction of therapeutic organic nitrate, nitroglycerin, inorganic nitrate, and nitrite by XOR has been investigated (Millar *et al.*, 1998). Turnover requires both the presence of NADH as a reductant and anerobic conditions, and each substrate gives rise to the production of NO. The interaction of each of these substrates with the molybdopterin center of the enzyme was demonstrated with the use of site-specific inhibitors, and by inhibition of NO production by xanthine. Such enzymic activities might be expected to inactivate XOR in the light of the finding that NO irreversibly inhibits both XO and XDH (Ichimori *et al.*, 1999). However, inhibition is pronounced only in the presence of an electron donor showing that only the reduced form of the enzyme is inhibited, and with a second-order rate constant of 14.8 $M^{-1}s^{-1}$ for the reaction between reduced XO and NO, such inactivation would be extremely slow under physiological conditions. The inactivated enzyme was shown to be of the "desulfo" form, *i.e.*, enzyme lacking a catalytically essential sulfur atom that is coordinated to the molybdenum atom of the active enzyme (Massey and Edmondson, 1970; Turner *et al.*, 1989).

PERSPECTIVES

XDH has been isolated from a number of organisms. These enzymes are highly similar in terms of both structure and function. XDH

from mammalian sources can be converted to an oxidase form (XO), which has many properties in common with XDH, but differs substantially in properties associated with the environment of the flavin, where the enzyme interacts with molecular oxygen and pyridine nucleotides. XDH exhibits xanthine/NAD activity, and to a lesser extent xanthine/oxygen activity. XO exhibits xanthine/oxygen activity, but no xanthine/NAD activity. In addition, both forms of the enzyme exhibit NADH oxidase activity, which is inhibitable by NAD. The reactions of this rather complex enzyme can be explained in terms of a rapid equilibrium model in which reducing equivalents equilibrate rapidly among the redox centers of the enzyme according to their midpoint potentials. All forms of oxidase activity give rise to both superoxide anion and H_2O_2. In addition, both XDH and XO can produce nitric oxide (NO) under suitable conditions. The production of both ROS and NO by XOR are of potential physiological and therapeutic importance.

ABBREVIATIONS

AAD, aminopyridine adenine dinucleotide; EPR, electron paramagnetic resonance; Fe/S, iron sulfur center; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase

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