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Effects of the Environment on Microperoxidase-11 and on Its Catalytic Activity in Oxidation of Organic Sulfides to Sulfoxides

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Microperoxidase-11 (MP-11, also known as heme undecapeptide of cytochrome *c*) was immobilized by encapsulation into sol-gel silica glass and by physisorption, chemisorption, and covalent attachment to silica gel. We then compared these species with one another and with dissolved microperoxidase-11 as catalysts for the sulfoxidation of methyl phenyl sulfide by hydrogen peroxide. MP-11 is prone to oligomerization in solution, both via axial ligation and via intermolecular interactions. When the ligation oligomerization is suppressed upon immobilization, heme becomes more accessible, and the sulfoxide yield increases 4−6 times, from 15% up to 95%. When the ligation oligomerization of dissolved MP-11 is suppressed by protonation and acetylation of amino groups and by addition of methanol, sodium dodecyl sulfate (SDS), or trifluoroethanol, the sulfoxide yield increases 3−5 times (up to 76%). The oligomerization via intermolecular interactions is important for preserving enantioselectivity in immobilized and dissolved MP-11. For MP-11 in amine-rich and especially alcohol-rich environments, the enantioselectivity is vanishingly low, presumably because amino and hydroxyl groups cause a conformation change in the catalyst. In other environments, the MP-11 species are aggregated via intermolecular interactions in micellar (SDS) solution and on the surface of the silica gel, or via axial ligation in aqueous buffer at pH 6.0. Under these conditions, the enantioselectivity is enhanced; the enantiomeric excess (ee) becomes as high as 46%. An understanding of the effects of the aggregation state and consequent properties on the catalytic activity of MP-11 allowed us to control the yield and enantioselectivity of sulfoxidation reaction.

Introduction

Microperoxidases are heme peptides obtained by proteolytic digestion of cytochrome *c*.[1−3] Those designated MP-8, MP-9, and MP-11 (Figure 1) contain the protein segments 14−21, 14−22, and 11−21, respectively. The heme is attached to the peptide via thioether linkages involving the side chains of Cys 14 and Cys 17. The imidazole group in the side chain of His 18 is one of the axial ligands; the other one can be water or an exogenous ligand such as cyanide, halide, or an amine.

Microperoxidases display peroxidase activity,[1−5] that is, they catalytically reduce hydrogen peroxide to water while oxidizing a substrate to its radical cation.[6−12] They also display oxygenase activity, akin to cytochrome P-450, in catalyzing sulfide oxidation, amine N-demethylation,

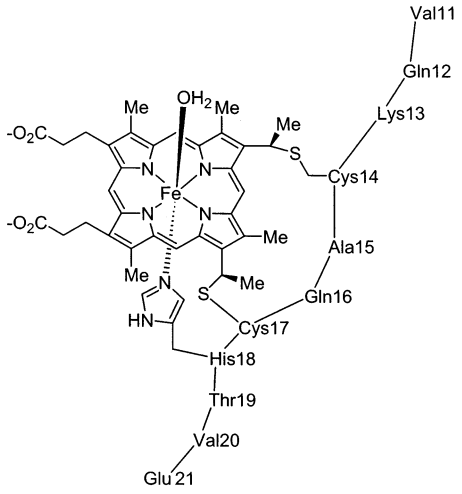

FIGURE 1. Structure of monomeric microperoxidase-11.

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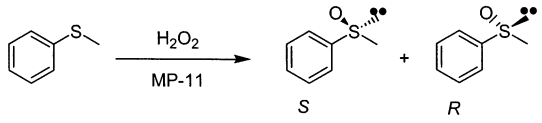
olefin oxidation, and aniline *p*-hydroxylation.[2,13−16] The ferryl form of the heme, created by dioxygen, H_2O_2, or other oxygen-atom donors, can catalytically transfer an oxygen atom to the substrate.[7,9,17−20]

Proteins and other biomolecules can be immobilized in various matrices by chemisorption, physisorption, covalent bonding, encapsulation, cross-linking, or combinations of these methods.[21] Much attention has recently been devoted to the development of the sol-gel methods for encapsulation of biomolecules.[22−25] Achievements in these studies opened many possibilities for basic and applied research in materials science, bioanalytical chemistry, biocatalysis, biotechnology, and environmental technology.[24,25] In our laboratory, proteins cytochrome *c*,[26,27] carbonic anhydrase,[28] horseradish peroxidase,[29] and lipase[30] were encapsulated into sol-gel glass. Research in our and other[21,22] laboratories has shown, however, that enzymes and even small molecules may behave differently when free in solutions and when immobilized. More basic research is needed before supported catalysts, biosensors, and other composite materials can be turned into practical chemical devices.

Spectra of MP-11 and its reactivity have been studied, but the structure and intermolecular interactions have not been correlated with effectiveness of MP-11 as a catalyst for oxidation of organic substrates. In this work, we compare catalytic properties of free (dissolved) and variously immobilized microperoxidase-11. We chose a simple reaction, oxidation of methyl phenyl sulfide (thioanisole) to sulfoxide, shown in eq 1.[31−33] Our goal was not to optimize this particular reaction, but to use it as an indicator of the state of the catalyst. Because the sulfoxide yield depends on the efficiency of the oxygen-atom transfer, and the degree of asymmetric induction depends on the chirality of the catalyst, we can infer the state of the MP-11 from its catalytic activity in various media.



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Experimental Procedures

Chemicals. Reagents were obtained from commercial sources and used without further purification. The silica gel had an average particle size of 40 μm in the range 32−63 μm and a surface area of 550 m^2/g. Distilled water was demineralized to an electrical resistivity greater than 17 MΩ cm.

Instruments. Ultraviolet−visible (UV−vis) spectra were recorded with a Perkin-Elmer Lambda 18 spectrophotometer. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz; the integration error was 5−10%. X-band electron paramagnetic resonance (EPR) spectra were recorded at 120 K. Fourier transform infrared (FTIR) spectra of Nujol mulls and diffuse-reflectance infrared Fourier transform (DRIFT) spectra were recorded with an IR spectrometer equipped with a TGS detector in the main compartment and a MCT detector in the auxiliary module (AEM), which housed a Harrick diffuse-reflectance accessory. The DRIFTS samples were held in the Harrick microsampling cup. Diffuse-reflectance UV−vis spectra were recorded with a spectrophotometer fitted with a 60 mm integrating sphere accessory. Each sample was spooned into a powder holder and analyzed in a % reflectance mode. Circular dichroism (CD) spectra were recorded using 0.1 mm quartz cuvette. Gas chromatography−mass spectrometry (GC-MS) experiments were done with a triple-quadrupole mass spectrometer attached to a gas chromatograph. The system was configured in the electron ionization mode. The first quadrupole was used as a mass analyzer to scan the *m*/*z* values from 35 to 650 at a rate of 1.2 scan/s. The second and third quadrupoles were kept in the RF-only mode. Unit mass resolution was achieved using perfluorokerosene as the calibration and tuning reference. The GC starting temperature was kept at 110 °C for 1 min and then raised to 170 °C at a rate of 8 °C/min. A Chiraldex G-PN GC column (30 m × 0.32 mm) was used to separate the sulfoxide enantiomers. Lyophilization (freeze-drying) was done at −70 °C.

Encapsulation of Microperoxidase-11 in Sol-Gel Silica Glass. A modification of a published method[34] was used. Aqueous MP-11 (5.80 mM, 1.00 mL), aqueous sodium fluoride (1.00 M, 0.200 mL), and aqueous poly(vinyl alcohol) (4% w/w, 0.400 mL) were combined with 0.128 mL of water. While the mixture was vigorously stirred with a vortex shaker, tetramethyl orthosilicate (1.827 g, 12 mmol) was added. After ca. 10 s, when the mixture turned into a clear homogeneous solution and warmed up, it was placed into an ice bath. The gelation occurred ca. 10 s later, and the mixture was kept in the ice bath for 5 min. The sealed reaction vessel was kept at rt for 24 h and then opened. The gel was air-dried at 40 °C for 96 h. The resulting glass was ground in a mortar and shaken with 10.0 mL of water at rt for 2 h. The solid was filtered off, washed with water, acetone, and hexane, and dried for 12 h at 40 °C. The final product was a powder of a light reddish-brown color. Elemental analysis found 3.81 C, 1.51 H, and 0.10 N; the first and the last result correspond to 3.39 μmol of MP-11 per gram of solid.

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Preparation of Aminopropyl-Functionalized Silica Gel. Silica gel (10.0 g) was activated[35,36] by refluxing with 100.0 mL of concentrated hydrochloric acid, washed with deionized water, and dried for 6 h at 190 °C. Elemental analysis found 0.00 C, 0.77 H, and 0.00 N. A suspension of activated silica gel in 90.0 mL of a 10% (v/v) solution of 3-aminopropyltriethoxysilane in dry toluene was refluxed for 2 h under a nitrogen atmosphere. Aminopropyl-functionalized silica gel was filtered off, washed with toluene and acetone, and carefully dried for 8 h at 115 °C to afford 9.27 g of an off-white solid. Elemental analysis found 5.24 C, 1.39 H, and 1.57 N; the first and the last result correspond to 1.12 mmol of NH_2 groups per gram of solid. Both IR and DRIFT spectra of aminopropyl-functionalized silica gel exhibited bands at 3368 and 3298 cm^{-1}, characteristic of an NH_2 group.

Covalent Attachment of Microperoxidase-11 to Aminopropyl-Functionalized Silica Gel. To a solution of MP-11 (41.6 mg, 21.6 μmol) in 84.0 mL of a 0.050 M HEPES buffer at pH 7.5 were added EDC (161.0 mg, 0.84 mmol) and aminopropyl-functionalized silica gel (305.0 mg, 0.342 mmol of NH_2 groups). The reaction mixture was stirred at rt for 3.5 h. The modified silica gel was filtered off, washed with the HEPES buffer and acetone, and air-dried for 30 min to afford 261.8 mg of a dark reddish-brown solid. Elemental analysis found 6.86 C, 1.59 H, and 2.05 N; the first and the last result correspond to 17.1 μmol of MP-11 per gram of solid.

Adsorption of Microperoxidase-11 onto Silica Gel and Aminopropyl-Functionalized Silica Gel. Silica gel (6.000 g) or its aminopropyl-functionalized derivative (6.417 g) was added to a stirred solution of MP-11 (22.33 mg, 11.6 μmol for normal "loading"; 197.84 mg, 10.28 μmol for high "loading") in 15.0 mL of water or, for normal loading only, in 80% (v/v) methanol in water. The resulting suspension was lyophilized for 36 h to afford a uniformly colored reddish-brown solid. The normal and high loadings correspond to 1.93 and 17.1 μmol of MP-11 per gram of solid, respectively.

N-Acetylation of Microperoxidase-11.[37] Solution of MP-11 (50.0 mg, 26.0 μmol) in 130.0 mL of deionized water was combined with 130.0 mL of a saturated sodium acetate solution, and the resulting mixture was cooled to 0 °C. Acetic anhydride (16.25 mL, 172.2 mmol) was added in five portions at 10 min intervals. The reaction mixture was kept at 4 °C for 15 h and lyophilized for 70 h. Residue from the first lyophilization was dissolved in 100.0 mL of a 0.100 M aqueous sodium hydroxide and subjected to flash chromatography on Sephadex-10 with 0.100 M sodium hydroxide as an eluent. The eluate was lyophilized for 72 h. Residue from the second lyophilization was washed with four 50 mL portions of water in an Amicon 8050 ultrafiltration cell fitted with a YC05 membrane, which has a cutoff of 500 D. Final lyophilization for 100 h yielded 37.9 mg (73%) of N-acetylated microperoxidase-11. Concentration of its aqueous solutions was determined spectrophotometrically[37] using $\epsilon_{550} = 31.2\ mM^{-1}\ cm^{-1}$.

General Procedure for Oxidation of Methyl Phenyl Sulfide. All experiments were done in triplicate. Microperoxidase-11 in various forms (1.16 μmol) was dissolved or suspended in 3.50 mL of a solvent (see footnotes to Table 2 for details). To the stirred solution or suspension of the catalyst was added 36 μmol (4.27 μL) of thioanisole (methyl phenyl sulfide) neat or dissolved in dichloromethane. Hydrogen peroxide (240 μL of a 0.150 M aqueous solution, 36 μmol) was added in six portions, at 6 min intervals. The reaction was stopped 1.0 h after the first addition of hydrogen peroxide by extracting the reaction mixture with three 1.0 mL portions of dichloromethane; for reactions in alcoholic solutions, the extraction solvent was a 1:1 (v/v) mixture of dichloromethane and hexane. After the organic phase was dried over anhydrous magnesium sulfate, the solvent was removed by rotary evaporation. Residue was dissolved in CD_2Cl_2, and the compounds were identified by their GC retention times, mass spectra, and ^1H NMR chemical shifts in comparison with authentic samples. The yield of sulfoxide was measured by ^1H NMR. We checked the efficiency of the extraction step (i.e., mass balance) by spiking the organic phase with a known amount of internal standard (thioanisole) and comparing the ^1H peak integrals for thioanisole and corresponding sulfoxide before and after spiking. Thus, we estimated the recovery of more than 97% of organic material from the reaction mixture. The enantiomeric excess of (*S*)-sulfoxide was measured by chiral GC-MS, as described above, in the Instruments section. When the catalyst was scarce, reactions were run at 1/10 of the stated scale, and extraction was done directly with CD_2Cl_2. For recycling experiments with suspended catalyst, the catalyst was filtered off, washed with a small amount of solvent and resuspended in the same solvent. For recycling experiments with dissolved catalyst, the aqueous phase after methylene extraction was quickly frozen until the next cycle. Amount of unreacted hydrogen peroxide in the aqueous phase was estimated from the sulfoxide yield, and sufficient hydrogen peroxide was added in the subsequent cycle to achieve 1:1 initial ratio of thioanisole to hydrogen peroxide in the reaction mixture.

Preparative-Scale Oxidation of Methyl Phenyl Sulfide. These experiments were done in duplicate, on a scale 10 times larger than that stated above, for both reaction and workup. Sulfoxide was separated from unreacted sulfide by column chromatography (hexanes/ethyl acetate, gradient from 4:1 to 0:1). After the removal of the solvent and drying in vacuo, methyl phenyl sulfoxide was isolated as a yellowish oil. For reaction in 50 mM sodium phosphate buffer at pH 6.0, the sulfoxide yield was 7.2−9.1 mg (16 ± 2%). For reaction in 80:20 water/methanol, the sulfoxide yield was 18−21 mg (39 ± 3%).

Assignment of Absolute Configuration in the Predominant Enantiomer of Methyl Phenyl Sulfoxide. An equimolar mixture of racemic and (*S*)-methyl *p*-tolyl sulfoxide dissolved in CD_2Cl_2 was analyzed by the GC-MS method. Upon addition of 2.5 equiv of Kagan chiral shift reagent, the ^1H NMR spectrum of the mixture was taken. The gas chromatogram showed that the retention time is longer for the (*S*)-enantiomer than for the (*R*)-enantiomer. The ^1H NMR spectrum showed that the methyl signal of the (*S*)-enantiomer is shifted upfield from that of the (*R*)-enantiomer.

Incubation of Enantiopure Sulfoxide with Microperoxidase-11. (*S*)-Methyl *p*-tolyl sulfoxide (36 μmol) was incubated with MP-11 (1.16 μmol) dissolved in 3.50 mL of sodium phosphate buffer for 50 and 100 h. The reaction mixture was worked up as usual, and the absolute configuration of sulfoxide in both cases was found by chiral chromatography to be *S*.

Results

We present our findings in this section and interpret them in the next one.

Preparation and Characterization of Materials. Immobilization of MP-11 on silica support was achieved by four methods: encapsulation into sol-gel glass,[34] covalent attachment by EDC to aminopropyl-functionalized silica gel,[35,36,38,39] and adsorption by lyophilization onto silica gel and onto aminopropyl-functionalized silica gel. In the first two cases, loading of the catalyst was determined by the elemental analysis. All of the methods of immobilization afforded lightly to darkly colored red-

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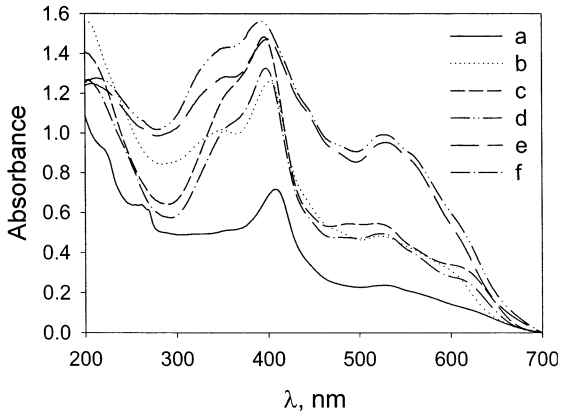

FIGURE 2. Diffuse-reflectance spectra of variously immobilized microperoxidase-11 (MP-11). This catalyst MP-11 was immobilized on silica supports by encapsulation in sol-gel silica glass (a) and by physisorption on silica gel from aqueous (b) and alcoholic (c) solutions. The catalyst was also immobilized on aminopropyl-functionalized silica supports by covalent attachment (d) and by chemisorption from aqueous solution at normal (e) and high (f) loadings. Characteristic Soret (ca. 400 nm) and β (ca. 525 nm) bands are prominent in all diffuse-reflectance spectra.

dish-brown solids. The EPR spectra of the immobilized MP-11 showed a signal with $g \approx 6.0$, which is characteristic of high-spin iron(III) porphyrins,[2,40] but no signals with $g \approx 3.2$ and $g \approx 2.1$, diagnostic of low-spin iron(III) porphyrin with imidazole and a primary amine as axial ligands,[2,40] see Figure SI 1 in Supporting Information. Figure 2 shows diffuse-reflectance UV−vis spectra of variously immobilized MP-11, with their characteristic Soret (ca. 400 nm) and β (ca. 525 nm) bands. The absorbance of encapsulated MP-11 (spectrum a) is lower than that of surface-immobilized MP-11 (spectra b−f).

The efficiency of immobilization was tested in desorption experiments. Samples of immobilized MP-11 containing the same amount of the catalyst were washed by portions of sodium phosphate buffers at pH 6.0 and pH 10.0. As Figure 3 shows, both encapsulated and covalently attached MP-11 were retained on the support even after prolonged washing, whereas chemisorbed and physisorbed MP-11 were gradually removed. Both the nature of the support and the pH of the buffer are important; desorption occurred more easily from silica gel than from aminopropyl-functionalized silica gel, and more easily at pH 10.0 than at pH 6.0.

Circular Dichroism Spectra of Dissolved Microperoxidase-11. To understand the conformation of the catalyst at reaction conditions, we recorded circular dichroism spectra of dissolved MP-11 (Figure 4). Only MP-11 in micellar solution (f) exhibits a strong negative peak around 220 nm, characteristic of α-helical peptides. In all other environments (a−e, g), MP-11 exhibits only a small shoulder around 220 nm but a strong signal around 203 nm, characteristic of randomly-coiled peptides.

The "Background" Sulfoxidation Reaction. The oxidation of thioanisole by a 2-fold molar excess of hydrogen peroxide in the absence of MP-11 was studied

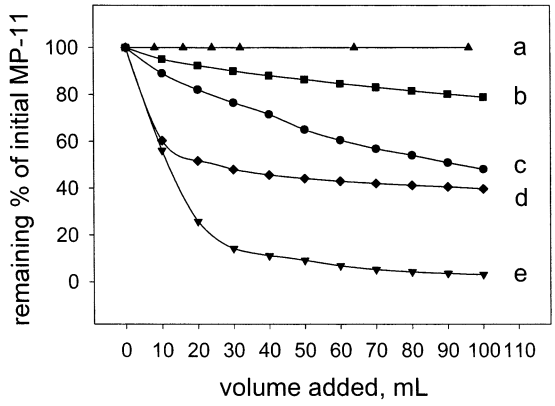

FIGURE 3. Desorption by washing with phosphate buffers of microperoxidase-11 (MP-11) immobilized in four different ways. The catalyst encapsulated in sol-gel silica or covalently attached to aminopropyl-functionalized silica gel remained on the support even after prolonged washing (a). The catalyst physisorbed on silica gel (c and e) washed off more easily than that chemisorbed on aminopropyl-functionalized silica gel (b and d). Buffer at pH 10.0 (d and e) is more effective than the buffer at pH 6.0 (b and c) in MP-11 removal.

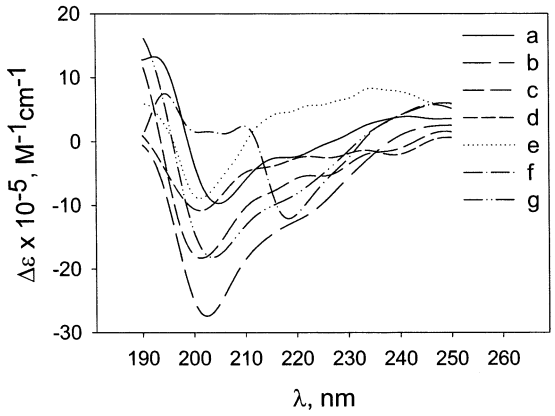

FIGURE 4. Circular dichroism (CD) spectra of dissolved microperoxidase-11 (MP-11) and N-acetylated microperoxidase-11 (*N*-Ac-MP-11) at the concentration of 331.4 μM used in sulfoxidation reaction. (a) MP-11 and (b) *N*-Ac-MP-11 in pH 6.0 buffer; (c) MP-11 and (d) *N*-Ac-MP-11 in pH 3.0 buffer; (e) MP-11 in methanol solution; (f) MP-11 in a micellar solution; (g) MP-11 in trifluoroethanol solution. For solvent details, see Table 2 footnotes.

with all solvents and all (blank) supports. In all solvents, at pH 6.0, the sulfoxide yield was <1.0%; at pH 3.0, the yield was 4 ± 1%, as a result of general acid catalysis.[31,41,42] The sulfoxide yield was 4 ± 1% in the presence of undoped sol−gel silica glass, 26 ± 5% in the presence of chromatographic quality silica gel, and 2 ± 1% when silica gel was modified with aminopropyl groups. This catalytic effect of silica on sulfoxidation reaction was recently thoroughly studied by us;[29] this effect should be taken into account when investigating and designing immobilized catalysts.

Comparison of Horseradish Peroxidase and Microperoxidase-11 as Sulfoxidation Catalysts. We

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TABLE 1. Sulfoxidation of Methyl Phenyl Sulfide by 1 Molar Equiv of Hydrogen Peroxide Catalyzed by Microperoxidase-11 (MP-11) in a 50 mM Sodium Phosphate Buffer at pH 6.0, at Ambient Temperature, for 1 h

entry	catalyst	cycle[a]	sulfoxide yield[b]	ee[c]	oligomerization by axial ligation[d]
1	MP-11 in solution	**1**	**15 ± 5**[e]	**36 ± 7**	YES[f]
2		2	12 ± 1	5 ± 3	
3		3	12 ± 3	4 ± 1	
4	MP-11 encapsulated in sol-gel silica	**1**	**55 ± 1**	**9 ± 2**	nd
5		2	9 ± 2	10 ± 2	
6		3	6 ± 1	9 ± 2	
7	MP-11 covalently attached to aminopropyl-	**1**	**92 ± 2**	**11 ± 1**	NO[h]
8	functionalized silica gel[g]	2	83 ± 2	11 ± 4	
9		3	56 ± 9	18 ± 2	
10	MP-11 physisorbed onto silica gel from	**1**	**87 ± 1**	**30 ± 6**	NO[h]
11	aqueous solution[i]	2	95 ± 1	23 ± 5	
12		3	38 ± 4	28 ± 3	
13	MP-11 physisorbed onto silica gel from	**1**	**95 ± 1**	**3 ± 1**	nd
14	alcoholic solution[i]	2	49 ± 7	3 ± 2	
15		3	46 ± 1	4 ± 6	
16	MP-11 chemisorbed onto aminopropyl-	**1**	**89 ± 2**	**17 ± 6**	NO[h]
17	functionalized silica gel[i]	2	92 ± 2	12 ± 5	
18		3	16 ± 2	20 ± 5	
19	MP-11 chemisorbed onto aminopropyl-	**1**	**93 ± 1**	**29 ± 3**	nd
20	functionalized silica gel at high loading[g]	2	91 ± 1	11 ± 3	
21		3	10 ± 2	5 ± 1	

[a] Consecutive reuse of the catalyst in three cycles; see Experimental Section for details. First cycle is in bold type. [b] Determined by ^{1}H NMR spectroscopy; see Table 1 footnote e for isolated yield. [c] Determined by a GC-MS method. The predominant enantiomer in all cases was (*S*)-PhS(O)Me. [d] Inferred from the EPR spectra; see Figure SI 1 in Supporting Information. YES means that the *g*-values are consistent with a low-spin iron(III) heme coordinated by histidine and a primary amine; NO means that the *g*-values are consistent with a high-spin iron(III) heme coordinated by histidine and water; nd means not determined. [e] Isolated yield 16 ± 2%. [f] From ref 2: $g_x = 1.46$, $g_y = 2.19$, $g_z = 3.18$. [g] High loading for covalently attached and chemisorbed MP-11 is 17.1 μmol per gram of solid. [h] $g_\perp^{eff} \approx 6.0$. [i] Usual loading for physi- and chemisorbed MP-11 is 1.93 μmol per gram of solid.

compared the activity and enantioselectivity of these two catalysts for the reaction in eq 1. With both catalysts, the maximum chemical conversion is reached after 1 h. In all experiments, the (*S*)-enantiomer of PhS(O)Me predominated. Whereas the enzyme horseradish peroxidase afforded 5 ± 1% of PhS(O)Me with an enantiomeric excess of 91 ± 3%,[29] the catalyst MP-11 afforded a somewhat higher yield but a lower enantioselectivity (Table 1, entry 1).

When the same MP-11 catalyst was used repeatedly (entries 1−3), the yields remained constant (and low), but the enantioselectivity vanished. Concomitant bleaching of the Soret band was a symptom of the oxidative degradation of the heme.[10,37,43−47] Because MP-11 achieved better yields than horseradish peroxidase, we chose it for further studies of the various means of immobilization and their effects on the catalytic activity.

Retention of Sulfoxide Configuration. Incubation of enantiopure (*S*)-TolS(O)Me with MP-11 under sulfoxidation reaction conditions, but in the absence of hydrogen peroxide, yielded only enantiopure (*S*)-TolS(O)Me, even after 100 h. Thus, MP-11 does not racemize chiral sulfoxide, once the latter is formed.

Sulfoxidation Catalyzed by Immobilized Microperoxidase-11. Variously immobilized MP-11 turned out to be more efficient as a catalyst than free MP-11 in solution. The yield of sulfoxide increased ca. 3-fold when MP-11 was encapsulated into sol-gel silica (entry 4 in Table 1). Covalently attached, physisorbed, and chemisorbed MP-11 (entries 7, 10, 16, and 19) proved to be even more effective, yielding ca. 6 times more sulfoxide than free MP-11 did. The enantioselectivity induced by the immobilized catalyst was lower than (entries 4, 7, and 16) or similar to (entries 10 and 19) that induced by the free catalyst. The catalytic effect of the matrix, which we reported for encapsulated horseradish peroxidase,[29] did not noticeably lower the enantioselectivity of MP-11 physisorbed onto silica gel from aqueous solution, because the smaller size of MP-11 and higher loadings on the surface of the silica gel resulted in a better surface coverage.

With the exception of the encapsulated species (entries 4−6), immobilized MP-11 fared well in the recycling experiments (entries 7−18): yields in the first and second cycles were almost always high. The enantioselectivity generally stayed constant in the successive catalyst cycles for most of the supporting matrices (entries 4−12 and 16−18).

To explain the increase in the reactivity of MP-11 upon immobilization, we hypothesized that this catalyst on various supports is aggregated less than in solution or not at all. To verify this hypothesis, we prepared monomeric MP-11 in solution and studied its catalytic effectiveness.

Sulfoxidation Catalyzed by Monomeric Microperoxidase-11 in Solution. There are several ways to preclude oligomerization of MP-11:[2] by separating the molecules,[40,48−50] changing the solvent,[5,40,49−51] or preventing the amino groups in one MP-11 molecule from coordinating to the iron atom in another.[2,3,5,37,52−57] Re-

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TABLE 2. Sulfoxidation of Thioanisole by 1 Molar Equiv of Hydrogen Peroxide Catalyzed by Dissolved Microperoxidase-11 (MP-11) at Ambient Temperature for 1 h at pH 6.0, Unless Otherwise Noted

entry	catalyst	cycle[a]	sulfoxide yield[b]	ee[c]	oligomerization by axial ligation[d]
1	MP-11 at pH 6.0	**1**	**15 ± 5**[e]	**36 ± 7**	YES[f]
2		2	12 ± 1	5 ± 3	
3		3	12 ± 3	4 ± 0	
4	N-acetylated MP-11 at pH 6.0	**1**	**56 ± 3**	**25 ± 3**	NO[g]
5		2	41 ± 2	10 ± 1	
6		3	27 ± 1	4 ± 3	
7	N-protonated MP-11 at pH 3.0[h]	**1**	**48 ± 4**	**12 ± 2**	NO[i]
8	N-acetylated MP-11 at pH 3.0[h]	**1**	**58 ± 3**	**6 ± 3**	nd
9	MP-11 in alcoholic solution[j]	**1**	**41 ± 8**[k]	**6 ± 1**	NO[l]
10		2	33 ± 3	1 ± 1	
11		3	37 ± 4	0 ± 1	
12	MP-11 in micellar solution[m]	**1**	**76 ± 3**	**34 ± 5**	NO[l]
13		2	38 ± 1	35 ± 3	
14		3	9 ± 3	46 ± 5	
15	MP-11 in TFE solution[n]	**1**	**71 ± 5**	**3 ± 3**	nd
16		2	38 ± 1	6 ± 3	
17		3	9 ± 3	4 ± 1	

[a] Consecutive reuse of the catalyst; see Experimental Section for details. First (or the only) cycle is in bold type. [b] Determined by ^{1}H NMR spectroscopy; see Table 2 footnotes e and k for isolated yields. [c] Determined by a GC-MS method. The predominant enantiomer in all cases was (*S*)-PhS(O)Me. [d] Inferred from the EPR spectra; see Figure SI 1 in Supporting Information. YES means that the *g*-values are consistent with a low-spin iron(III) heme coordinated by histidine and a primary amine; NO means that the *g*-values are consistent with a high-spin iron(III) heme coordinated by histidine and water; nd means not determined. [e] Isolated yield 16 ± 2%. [f] From ref 2: $g_x = 1.46$, $g_y = 2.19$, $g_z = 3.18$. [g] From ref 37: $g_x = g_y = 5.9$, $g_z = 2.0$. [h] Potassium hydrogen phthalate (50 mM) was used as a buffer at pH 3.0. Because of the significant absorbance of phthalate in the UV region, sodium citrate (50 mM) was used as a pH 3.0 buffer for CD measurements. [i] From ref 2: $g_\perp^{eff} = 5.70$, $g_\parallel = 2.00$. [j] Methanol/water (80:20, v/v). [k] Isolated yield 39 ± 3%. [l] From ref 40: $g_\perp^{eff} \approx 6.0$. [m] SDS (5%, w/w) in 0.1 M Me_4NBr. [n] TFE/buffer (50/50, v/v).

sults obtained by all of these methods are shown in Table 2.

When primary amino groups of MP-11 are acetylated (entry 4) or protonated (entry 7), the yield of sulfoxide increases 2−3 times over that achieved with oligomeric MP-11[51] (entry 1). Acetylation does not markedly affect the enantioselectivity (entries 4 vs 1); protonation lowers it (entries 7 vs 1 and 8 vs 4). In successive cycles, the yield gradually decreases, while the enantioselectivity vanishes (entries 4−6).

When MP-11 is made monomeric in alcoholic solution (entry 9), the yield of sulfoxide again increases 2−3 times in comparison with oligomeric MP-11 (entry 1). Enantiomeric excesses are very low in the first cycles and nonexistent in subsequent ones, but the yield stays approximately constant in successive cycles (entries 9−11).

When molecules of MP-11 are separated by entrapment in the detergent micelles (entry 12), the yield of sulfoxide is ca. 5 times higher than when these molecules are allowed to aggregate (entry 1). In the subsequent cycles (entries 13 and 14), the yield drops, but the enantioselectivity remains approximately the same. A shortcoming of this method is that the micellar components (SDS and Me_4NBr) are partially leached into the organic phase during extraction, as ^{1}H NMR spectra of the organic phase showed.

We checked the importance of maintaining the secondary structure of microperoxidase-11. A solution of 2,2,2-trifluoroethanol (TFE) stabilizes the α-helices in peptides and proteins.[58,59] Yields and their recycling trend for MP-11 in TFE-containing solutions (entries 15−17) are the same as for MP-11 in micellar solution (entries 12−14), but the enantioselectivity is as low as that obtained in methanol solution (entries 9−11).

Discussion

Adsorption of Microperoxidase-11 on Silica and Modified Silica Surfaces. The strength of MP-11 adsorption depends on the nature of the support and the pH of the buffer. The overall charge, estimated on the basis of the known pK_a values for conversions between different states of MP-11,[2] is −2 at pH 6.0 and −3 at pH 10.0. At both pH values, the surface of silica gel is negatively charged owing to deprotonation of the hydroxyl groups. The surface of aminopropyl-functionalized silica gel is positively charged at pH 6.0 and approximately neutral at pH 10.0. The greater retention at pH 6.0 and on the aminopropyl-modified surface, evident in Figure 2, is caused by electrostatic interactions. The

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repulsion between MP-11 and the silica gel surface is stronger at higher pH, which facilitates the removal of microperoxidase-11. The effect of pH is less pronounced for aminopropyl-functionalized silica gel, since its surface is neutral or even positively charged.

Diffuse-Reflectance UV−vis Spectra of Immobilized Microperoxidase-11. Since our materials are opaque, colored powders, transmittance spectroscopy could not be done. We chose the diffuse-reflectance method to record at the UV−vis spectra of immobilized MP-11. Since the process of diffuse reflectance is nonlinear,[60] the bandwidth observed in such spectra depends on the particle size and on the nature of the material. Characteristic Soret (ca. 400 nm) and β (ca. 525 nm) bands are present in all spectra of immobilized MP-11 (Figure 2). The majority of our materials (spectra a−c and e−f) are silica particles with MP-11 layered on the surface via covalent attachment, chemisorption or physisorption. The spectra of these materials have similar intensities; the highest intensity is observed for the material with the highest loading of MP-11 on the surface (spectrum a). In another type of material, MP-11 encapsulated in sol-gel silica glass, microperoxidase-11 molecules are distributed through the volume of each particle. Therefore, a smaller number of these molecule are located closer to the particle surface, where they can be detected by diffuse-reflectance spectroscopy. Thus, a lower overall intensity is observed for encapsulated MP-11 (spectrum d).

Conformation of Dissolved MP-11. In the molecule of MP-11, undecapeptide 11−21 of cytochrome *c* is tethered to the heme via three residues: Cys 14, Cys 17, and His 18; see Figure 1. Therefore, the pentapeptide segment 14−18 is confomationally rigid; NMR[63,64] and resonance Raman[63] spectra consistently indicate one turn of the α-helix. This α-helical motif is stabilized by intramolecular hydrogen bonds,[62] which are especially favored by the hydrophobic environment in the micelle.[63]

The rest of the peptide chain is very flexible. Circular dichroism spectra of MP-11 in aqueous buffer at pH 9.0[64] or in 90% TFE solution at pH 7.0[65] show that, at these conditions, the peptide moiety in MP-11 is essentially a random coil. Calculation of the helical content from the relative intensities of the CD peak at ca. 203 (random coil) and the small shoulder at ca. 220 nm (α-helix) indicates that only a small fraction of the undecapeptide is α-helical in aqueous buffer.[64]

We recorded the CD spectra of MP-11 and its N-acetylated derivative in all solvents and at the same concentration that we used in the sulfoxidation reactions; see Figure 4. Only in a micellar solution (spectrum f) is the peptide in MP-11 significantly α-helical. In all other solvents (spectra a−e and g), the peptide moiety in MP-11 and *N*-Ac-MP-11 is randomly coiled. This catalyst is aggregated via axial ligation of primary amino groups (at the N-terminus and in lysine side chains) in one molecule of MP-11 to the iron atom in another. At physiological pH, the major species are pentamers and hexamers.[2] The presence (spectrum a) or absence (spectra b−g) of oligomerization, caused by axial ligation, is not reflected in the positions and intensity of CD peaks.

As shown in Tables 1 and 2, MP-11 catalyst was tested and recycled under a range of conditions. Comparisons of the results for the first cycle under various conditions allow us to judge the reactivity and selectivity of the catalyst. Comparisons of the results for consecutive cycles under the same conditions reveal the recyclability of the catalyst.

Effect of Immobilization on the Oxygenase Activity of Microperoxidase-11. The yields in Table 1 show that the oxygen-atom transfer from the ferryl form of MP-11 to the substrate becomes more efficient when MP-11 is immobilized. Breakup of the aforementioned oligomers upon immobilization presumably makes more heme groups available for catalysis, and the yield increases.

We tested this hypothesis in two ways. First, EPR spectra of our immobilized MP-11 contain signals characteristic of the monomer[37,40] and lack signals characteristic of the oligomers.[2,40] Second, since several methods for deoligomerization of microperoxidases in solution are known, we applied them in this study of the reaction in eq 1.

Protonation or acetylation prevents primary amino groups (at the N-terminus and in lysine side chains) in one molecule of MP-11 from coordinating to the iron atom in another.[2,3,5,37,52−57] The sulfoxidation yields obtained upon blocking the amino groups in these two ways are similar (Table 2, entries 4 and 7), but lower than the yields obtained with some of the immobilized catalysts (Table 1, entries 7, 10, 16, and 19).

Microperoxidases are deoligomerized in alcoholic solution.[5,40,49−51,65,66] With the consequent greater exposure of the heme groups, the sulfoxide yield increases, as entries 9 and 1 in Table 2 show.

Because MP-11 is monomeric in micellar solution,[40,48−50] the yield of sulfoxidation achieved by the catalyst under these conditions is high (entry 12 in Table 2). Since the concentration of trifluoroethanol (TFE) was relatively high,[58,59] we had to consider the possible effects of the alcohol group and the TFE hydrophobicity; these effects are expected to favor the monomeric form of MP-11. The catalysts in TFE solution (entries 15−17) and in micellar solution (entries 12−14) afford similarly high yields of sulfoxide.

Enantioselectivity of the Sulfoxidation Reaction Catalyzed by Variously Immobilized and Dissolved MP-11. The enantioselectivity of the reaction is induced by the chiral environment around the active site in the catalyst. If the heme is exposed but the peptide conformation is appreciably changed, the yield may be relatively high, but the enantioselectivity may be relatively low. Indeed, Tables 1 and 2 show that the yield of sulfoxide and the enantioselectivity in its formation are not necessarily correlated.

We correlated the enantioselectivity with the concentration of MP-11 on the support surface and with the nature of the support. Using the atomic coordinates for the heme and the amino acid residues 11−21 of cyto-

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chrome *c* in the RASMOL program,[67] we estimated the cross-sectional "footprint" of MP-11 to be 130−140 Å^2. Knowing the loading of MP-11 per gram of silica gel, whose surface area is 550 m^2/g, we estimated the ratio of the surface area and the MP-11 "footprint". This ratio was 26−38 for MP-11 that was covalently attached or chemisorbed at high loading and 490−530 for MP-11 physisorbed or chemisorbed at normal loading. In all cases, MP-11 molecules can be sufficiently far apart not to interact with one another. Indeed, the EPR spectra showed that axial ligation does not occur in any of these four samples, containing immobilized MP-11. However, intermolecular interactions of other types (i.e., hydrophobic forces or interpeptide hydrogen bonding) are still conceivable. The average number of surface aminopropyl groups per footprint of MP-11 molecule can be estimated from the known concentration of these groups on the surface, which is 1.12 mmol/g. This number is zero for physisorption on silica gel and ca. 2 for chemisorption on the aminopropyl-functionalized silica gel or covalent attachment to aminopropyl-functionalized silica gel.

Consider enantioselectivities for the following pairs of entries in Table 1: 16 vs 19 (first cycles) and 17 vs 20 (second cycles). Evidently, an increase in the MP-11 concentration on the aminopropyl-modified surface (entries 19 and 20) does not affect the enantioselectivity of sulfoxidation; the average number of surface aminopropyl groups per adsorbed MP-11 molecule is the same. Comparing enantioselectivities for the catalysts with the same MP-11 loading on different supports (aminopropyl-functionalized silica gel in entries 7−9, 16−18, and 19−21 vs silica gel in entries 10−12), we conclude that at the same loading of MP-11 on the surface, the presence of aminopropyl groups lowers the enantioselectivity in sulfoxidation reaction. On the other hand, our desorption studies (see above) showed that the presence of the surface aminopropyl groups strengthens the interactions between the support and microperoxidase-11. We presume that these stronger interactions between the surface and the catalyst cause a conformation change in the catalyst, which lowers the enantioselectivity.

The absence of enantioselectivity of MP-11 dissolved in methanol (Table 2, entries 9−11) suggests that the conformation of the catalyst differs from that in water. To further test the effect of an alcoholic environment on the enantioselectivity, MP-11 was physisorbed onto silica gel not only from aqueous solution (Table 1, entries 10−12) but also from methanol solution (entries 13−15). Interestingly, MP-11 physisorbed onto silica gel from methanol solution delivers the same low levels of enantioselectivity as MP-11 in methanol (Table 2, entries 9−11) and not the higher levels observed for MP-11 physisorbed onto silica gel from aqueous solution. Thus, these experiments show that both the amine-rich and alcohol-rich environments lead to a greatly diminished chiral induction by microperoxidase-11. It is now plausible to conclude that the low enantioselectivity of encapsulated MP-11 (Table 1, entries 4−6) is caused by methanol (byproduct of hydrolysis of tetramethoxysilane) in the silica sol prior to entrapment of the catalyst.

The low enantioselectivity observed for MP-11 in trifluoroethanol solution (entries 15−17) mirrors that in methanol solution (entries 9−11), again a consequence of the alcohol functionality (as shown above for methanol). Circular dichroism measurements by us (Figure 4, spectrum g) and others[65] consistently show a vanishingly low α-helicity of MP-11 dissolved in trifluoroethanol solution.

N-Acetylation of MP-11 does not change the enantioselectivity of sulfoxidation reaction (Table 2, entry 4 vs entry 1). A decrease in enantioselectivity as the pH is lowered from 6.0 to 3.0 (entries 1 vs 7 and 4 vs 8) is attributable to the enhanced "background" reaction, which does not involve microperoxidase-11.

The incipient α-helical motif of residues 14−18 is stabilized by intramolecular hydrogen bonds,[62] which in turn are favored by the hydrophobic environment within the micelle.[63] The good enantioselectivity in entries 12−14 may be due to this α-helicity. Whereas in simple solution the enantioselectivity is nearly lost in the repeated cycles, in the micelle, it is completely preserved. As spectrum f in Figure 4 shows, the micellar solution is the only one in which MP-11 exhibits CD peaks characteristic of a significant α-helical content. The enantioselectivity of the catalyst indeed seems to arise from its conformation.

However, in monomeric MP-11 molecule (Figure 1), most of the chiral groups are located on the side of heme opposite from that participating in oxygen transfer. Although MP-11 in micellar solutions is not oligomerized via axial ligation,[40,48−50] it may still be aggregated via intermolecular interactions (i.e., hydrophobic forces or interpeptide hydrogen bonding). MP-11 physisorbed onto silica gel from aqueous solution is not oligomerized via axial ligation (Table 1); the relatively higher enantioselectivity of this catalyst (Table 1, entries 10−12) can also be induced by noncovalent aggregation. In these aggregates, a chiral environment around the exposed heme side (the active site) will make the catalyst enantioselective.

To test the aggregation explanation, we studied the effect of the concentration of N-acetylated MP-11 on the enantioselectivity of this catalyst. In N-Ac-MP-11, oligomerization via axial ligation is now impossible, since all primary amino groups are blocked. Solutions of *N*-acetyl-MP-11 obey Beer's law at concentrations as high as 20−60 μM;[37,57] above this limit, "noncovalent aggregation of heme units" was suggested.[2,57] We examined the concentration range from ca. 331 μM (our usual reaction conditions) to 16.6 μM (5% of this value). The increase in sulfoxide yield upon this 20-fold dilution (Table 3, entry 4 vs entry 1) can be a consequence of partial blocking of the access to the heme groups in the noncovalent aggregates. Importantly, the data in Table 3 show that the enantioselectivity is diminished upon dilution, reaching practically zero at the catalyst concentration of 33.1 μM. Evidently, in noncovalent aggregates, which exist at higher concentrations of *N*-acetyl-MP-11 (entries 1 and 2), a greater chirality of the active site environment is reflected in higher enantioselectivities. Breakup of these aggregates upon dilution is reflected in very low enantioselectivities (entries 3 and 4).

These results again show that the catalytic activity of MP-11 depends on its aggregation state and consequent

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TABLE 3. Sulfoxidation of Thioanisole by 1 Molar Equiv of Hydrogen Peroxide Catalyzed by N-Acetylated Microperoxidase-11 (*N*-Ac-MP-11) at Ambient Temperature and pH 6.0 for 1 h, with a *N*-Ac-MP-11:PhSMe Molar Ratio of 1:31[a]

entry	[*N*-Ac-MP-11], μM	dilution	sulfoxide yield[b]	ee[c]
1	331	none	56 ± 3	25 ± 3
2	110	1:3	74 ± 4	15 ± 7
3	33.1	1:10	93 ± 1	4 ± 3
4	16.6	1:20	92 ± 7	5 ± 2

[a] Only the first cycle is reported; see Experimental Section for details. Each experiment was run in triplicate. [b] Determined by ^{1}H NMR spectroscopy. [c] Determined by a GC-MS method. The predominant enantiomer in all cases was (*S*)-PhS(O)Me.

properties. An understanding of this dependence allowed us to control the yield and enantioselectivity of oxidation of organic sulfides by hydrogen peroxide.

Conclusion

The product yield in the oxygenase-like reactions of MP-11 increases when the heme is made more accessible by prevention of oligomerization of the catalyst caused by axial ligation. This can be achieved by chemisorption and physisorption of MP-11 onto silica gel or aminopropyl-functionalized silica gel. Methods for similar prevention of oligomerization by axial ligation ("monomerization") in microperoxidases, which were previously used in spectroscopic studies,[2,3,37,40,48,50,52,53,56,57] are successfully applied in this study for controlling the oxygenase-like function of microperoxidase.

The effects of the medium and of the oligomerization state on the enantioselectivity of the catalyst are subtler and more intricate than their effects on the reaction yield. The enantioselectivity is minimal when the hydrogen-bonding groups present on the support (NH_2) or in the medium (OH) interact with the MP-11 molecule and presumably alter its conformation. If during the preparation of the catalyst alcohol is present in large concentrations (as is the case during encapsulation into sol-gel silica glass or physisorption onto silica gel from methanolic solution), the detrimental effect of alcoholic environment remains even after the immobilized MP-11 is resuspended in the aqueous buffer for the reaction.

In the absence of effects caused by these functional groups, aggregation of small chiral monomers of MP-11 or its N-acetylated derivative actually preserves the high levels of enantioselectivity. This aggregation can occur via axial ligation (MP-11 in aqueous buffer) or via noncovalent interactions (MP-11 physisorbed on silica gel from aqueous solution, MP-11 in micellar solution, and N-acetylated MP-11 in aqueous buffer). Using the most selective catalysts such as MP-11 physisorbed on silica gel from aqueous solution and MP-11 in micellar solution allows an enantioselectivity of ca. 40% to be achieved and sustained in repeated uses of the same catalyst.

Some of our catalysts, such as MP-11 covalently attached to aminopropyl-functionalized silica, are active and robust, but are less enantioselective than MP-11 in aqueous solution. Other catalysts, such as MP-11 in micellar solution, are both active and enantioselective, but cannot be recycled efficiently. A compromise among these desirable qualities was achieved by adjusting the oligomerization state of the catalyst and the reaction medium.

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Supporting Information Available: EPR spectra of variously immobilized microperoxidase-11. This material is available free of charge via the Internet at http://pubs.acs.org.

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