Residue-specific global fluorination of *Candida antarctica* lipase B in *Pichia pastoris*†‡

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We report the *in vivo* fluorination of the tryptophan, tyrosine, and phenylalanine residues in a glycosylation-deficient mutant of *Candida antarctica* lipase B, CalB N74D, expressed in the methylotrophic yeast *Pichia pastoris* and subsequently segregated into the growth medium. To achieve this, a *P. pastoris* strain auxotrophic for all three aromatic amino acids was supplemented with 5-fluoro-L-tryptophan, *meta*-fluoro-(DL)-tyrosine, or *para*-fluoro-L-phenylalanine during expression of CalB N74D. The residue-specific replacement of the canonical amino acids by their fluorinated analogs was confirmed by mass analysis. Although global fluorination induced moderate changes in the secondary structure of CalB N74D, the fluorous variant proteins were still active lipases. However, their catalytic activity was lower than that of the non-fluorinated parent protein while their resistance to proteolytic degradation by proteinase K remained unchanged. Importantly, we observed that the global fluorination prolonged the shelf life of the lipase activity, which is an especially useful feature for the storage of, *e.g.*, therapeutic proteins. Our study represents the first step on the road to the production of biotechnologically and pharmacologically relevant fluorous proteins in *P. pastoris*.

Introduction

Fluorine is a chemical element with unique properties. Its atomic radius is the second smallest after hydrogen and it is among the most chemically active nonmetallic elements. Linking fluorine with carbon, however, produces some of the most chemically inert compounds, *e.g.*, polytetrafluoroethylene (PTFE) also known as Teflon. Fluorine occurs abundantly in inorganic compounds but only a handful of organofluorines are known.[1] Being the most electronegative of the elements, it acts as a poor electron pair donor and strongly polarizes covalent bonds. C–F bonds are more polarized (with an opposite dipole moment), stronger ($\sim$14 kcal mol^{-1}), and longer ($\sim$0.4 Å) than C–H bonds.[2] Fluorocarbons are highly hydrophobic and the fluorine atoms, *e.g.* in perfluorinated model peptides, tend to self-associate in both, aqueous and organic solvents ("fluorous effect"). Therefore, globally fluorinated proteins, that contain many mono- or polyfluorinated amino acids, are anticipated to retain their structure and function in organic solvents as the fluorous effect would prevent them from unfolding. This potential could be especially interesting with regard to enzyme catalysts of organic reactions.

Fluorine's characteristics suggest that the fluorination of amino acid side chains could be used to modulate the structural and/or functional properties of proteins. Although the participation of fluorine in hydrogen bonding is still a matter of controversial discussion,[3] Samsonov *et al.* recently found that weak hydrogen bonds between water molecules and fluorinated amino acid side chains exist.[4] Similarly, we have shown that the fluorination of the proline residues in green fluorescent protein generated a network of stabilizing interactions responsible for faster folding, more efficient refolding after denaturation and an increased stability in comparison to the non-fluorinated protein.[5] It is also well documented that fluorinations improve peptide–membrane interactions and impede proteolysis. Due to the hydrophobicity of fluorocarbons, the fluorination of amino acids and other compounds such as drugs is a general tool to improve their intracellular bioavailability.[2,6] Finally, the naturally occurring isotope of fluorine ^{19}F has excellent nuclear magnetic resonance (NMR) properties.

A vital prerequisite for the availability of adequate amounts of fluorous proteins is the efficient *in vivo* incorporation of fluorinated amino acids into the amino acid sequence. Since fluoroamino acids are not encoded by the standard genetic code, specific techniques to enforce their participation in protein translation have to be applied. In an early report, Furter showed the site-specific incorporation of fluorophenylalanine in response to an amber stop codon by a misaminoacylated suppressor tRNA.[7] The method was further developed mainly by the group of Schultz and used recently to single site-specifically introduce labels for ^{19}F-NMR.[8,9] However, the generation of fluorous proteins requires multi-site or even global fluorination of a protein sequence rather than the incorporation of one fluorinated amino acid at a specific site. Early studies dealt with the global incorporation of

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monofluorinated derivatives of phenylalanine,[10–14] tyrosine,[15–18] and tryptophan[16] by supplementation of appropriate amino acid auxotrophies with the analogs. The proteins of different unicellular organisms and those of mammals[19,20] were labeled with fluorine. Later, the targeted fluorination of model proteins for mainly spectroscopic analysis purposes was reported.[21–26] By the same supplementation technique, polyfluorinated amino acids were successfully incorporated into small peptide-like proteins[27–30] as well as larger proteins.[31,32] However, some model proteins could accommodate the polyfluorinated analogs only after their naturally evolved structures had been adapted to the non-canonical side chains by directed evolution.[33,34]

So far, the targeted fluorination of proteins has been described for bacterial hosts, and most studies were performed with proteins expressed in *E. coli*. However, *E. coli* is not always the expression host of choice for biotechnologically or pharmacologically relevant proteins. It is incapable of performing post-translational modifications such as glycosylation and proteins of eukaryotic origin are often expressed in insoluble form. To circumvent this shortcut and to make fluorine with its benefits described above available to a broader spectrum of proteins, we have chosen to explore the methylotrophic yeast *P. pastoris* as a host for protein fluorination. *P. pastoris* grows to very high cell densities and at the same time provides efficient synthesis of heterologous proteins by a methanol-inducible expression system that is tightly regulated.[35–38] In an accompanying study, we have shown that *P. pastoris* can be used to incorporate methionine analogs into *Candida antarctica* lipase B (CalB).[39] The lipase is not only a suitable model protein for the incorporation of non-canonical amino acids[40] but has found a multitude of industrial applications due to its high thermostability, activity in organic solvents and broad substrate specificity.[41–43] In this study, we intended to analyze the suitability of *P. pastoris* for the incorporation of the monofluorinated aromatic amino acids shown in Fig. 1 and to study the effect of global fluorination on the structure and function of the industrially relevant lipase, CalB.

Materials and methods

Materials

Unless otherwise indicated all chemicals were purchased from Sigma (Steinheim, Germany), Fluka (Buchs, Switzerland), or Merck (Darmstadt, Germany). The amino acid analogs 5-fluoro-L-tryptophan (5FW) and *meta*-fluoro-(DL)-tyrosine (mFY) were from Sigma, and L-4-fluorophenylalanine hydrochloride (*para*-fluorophenylalanine, pFF) was from Fluka.

Generation of the expression strain X33 Δ*aro*1 {pPICZαA-CalB N74D}

The expression vector pPICZαA-CALB for methanol-inducible expression of CalB[44,45] was a generous gift by Vlada Urlacher and Rolf D. Schmid. In order to avoid the naturally occurring glycosylation at asparagine in position 74, an N74D mutation was introduced into CalB on pPICZαA-CALB by site-directed mutagenesis with the mutagenesis primers CALB_N74D_fwd (5′-TTCATGCTC**GAT**GACACCCAGGTCAACA-3′) and CALB_N74D_rev (5′-CTGGGTGTC**ATC**GAGCATGAA-CGGCGGG-3′) applying standard conditions (QuikChange Site-Directed Mutagenesis, Stratagene). The mutated codon is indicated in bold. pPICZαA-CALB N74D was linearized with *Sac*I (New England Biolabs, Beverly, MA) and transformed into *P. pastoris* strain X33 Δ*aro*1 (genotype *ura*3Δ1 *aro*1::*URA*3)[46] by electroporation.[47] Transformants were screened on minimal medium plates (6.7 g L^{-1} yeast nitrogen base (with ammonium sulfate, without amino acids; BD Difco, Franklin Lakes, NJ), 150 mg L^{-1} each of L-tryptophan, L-tyrosine, and L-phenylalanine, 20 g L^{-1} glucose, 1 M sorbitol, and 20 g L^{-1} agar (Difco)) containing 125, 250, 500, or 1000 μg mL^{-1} Zeocin™ (Invitrogen GmbH, Karlsruhe, Germany). Zeocin™-resistant transformants were restreaked onto a minimal medium plate containing 250 μg mL^{-1} Zeocin™.

Small scale test expression of CalB N74D

To test CalB N74D expression in different transformants, 2 mL starter medium (1.7 g L^{-1} yeast nitrogen base (with ammonium sulfate, without amino acids; Difco), 3.7 g L^{-1} $(NH_4)_2SO_4$, 20 g L^{-1} glucose, 150 mg L^{-1} each of L-tryptophan, L-tyrosine and L-phenylalanine, 0.05 M potassium phosphate buffer pH 7) were inoculated with a single colony of a Zeocin™-resistant transformant and grown over night at 30 °C with vigorous shaking. The cells were diluted to a final volume of 5 ml with starter medium and grown again over night at 30 °C with vigorous shaking. 30 μl of these starter cultures were used to inoculate 2 ml expression medium (3.4 g L^{-1} yeast nitrogen base (with ammonium sulfate, without amino acids; Difco), 5 g L^{-1} $(NH_4)_2SO_4$, 2 ml L^{-1} methanol, 20 mg L^{-1} biotin, 150 mg L^{-1} each of L-tryptophan, L-tyrosine and L-phenylalanine, 1 ml L^{-1} PTM4 solution, 0.05 M potassium phosphate buffer pH 7). PMT4 solution contained 2 g L^{-1} $CuSO_4 \cdot 5\ H_2O$, 0.08 g L^{-1} NaI, 3 g L^{-1} $MnSO_4 \cdot H_2O$, 0.2 g L^{-1} $Na_2MoO_4 \cdot 2\ H_2O$, 0.02 g L^{-1} H_3BO_3, 0.5 g L^{-1} $CaSO_4 \cdot 2\ H_2O$, 0.5 g L^{-1} $CoCl_2$, 7 g L^{-1} $ZnCl_2$, 22 g L^{-1} $FeSO_4 \cdot 7\ H_2O$, 1 ml L^{-1} H_2SO_4 conc., and 0.2 g L^{-1} biotin. Protein expression was performed at room temperature with vigorous shaking and the cells were fed once per day with 10 μl methanol to maintain high level induction. After 72 h of induction, the culture supernatants were assayed for lipase activity as described below.

Incorporation of monofluorinated aromatic amino acid analogs into CalB N74D and protein purification

The transformant best producing CalB N74D was used for large scale protein expression in the presence of the fluorinated aromatic amino acid analogs shown in Fig. 1. For each analog and a control culture with the canonical amino acids, 20 ml starter medium were inoculated with 1 ml starter culture and incubated for 48 h at 30 °C with vigorous shaking. These cultures were used to inoculate 200 ml growth medium each (the same as the expression medium but with 20 g L^{-1} glucose instead of methanol) in which the cells were vigorously shaken at 30 °C for 24 h. The cells were harvested by low speed centrifugation (4500 × g, 20 min, room temperature) and resuspended in 200 ml starvation medium (the same as the growth medium but without supplementation of the aromatic

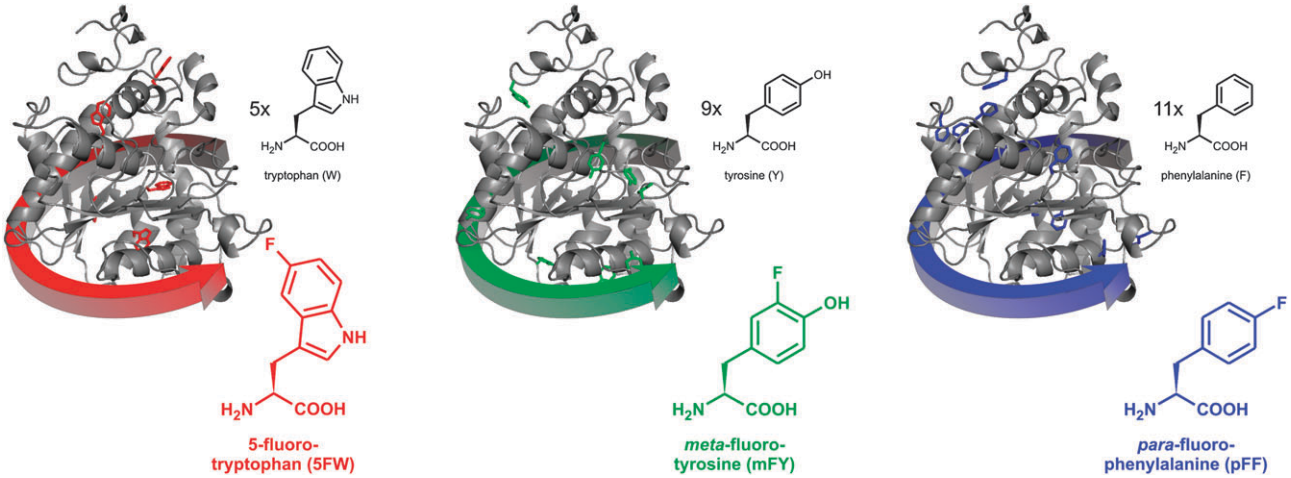

Fig. 1 Global fluorination of aromatic amino acid residues in CalB N74D. In the 3D structure of the *Candida antarctica* lipase B, the different aromatic amino acid residues are highlighted. The structures (all L-forms) of tryptophan (W), tyrosine (Y), and phenylalanine (F) and of their fluorinated analogs 5-fluorotryptophan (5FW), *meta*-fluorotyrosine (mFY), and *para*-fluorophenylalanine (pFF) are shown.

amino acid relevant for substitution). Endogenous aromatic amino acid pools were depleted at 30 °C for 24 h with vigorous shaking. The cells were pelleted again by low speed centrifugation as above, washed once with 0.05 M potassium phosphate buffer pH 6 and resuspended in 100 ml expression medium without tryptophan, tyrosine, and phenylalanine. The control culture was supplemented with 1 mM each of tryptophan, tyrosine, and phenylalanine, while the remaining three cultures were supplemented with 1.5 mM 5 FW, 2 mM mFY (DL-form), or 1.5 mM pFF and 1 mM each of the appropriate canonical aromatic amino acids to supplement the aromatic auxotrophies that were not exploited for analog incorporation.

The cultures were incubated at room temperature with vigorous shaking and were fed with 0.5 ml methanol twice a day to maintain high level induction. At the end of induction after 72 h, the cells were harvested by low speed centrifugation (4500 × g, 20 min, room temperature), the cell pellets were discarded and 100 µl culture supernatant were assayed for lipase activity as described below.

The clarified culture supernatants were dialyzed (Spectra/Por® MWCO 12–14 000; Spectrum Laboratories, Inc., Rancho Dominguez, CA) against 50 mM Tris/Cl pH 8.0 and concentrated by ultrafiltration (Vivaspin 20 MWCO 10 000; Sartorius AG, Göttingen, Germany). Protein concentrations were assessed by the method of Bradford[48] with bovine serum albumin for calibration.

Liquid chromatography coupled electrospray ionization mass spectrometry (LC-ESI-MS)

20 µL aliquots of the purified variants were pre-separated on a Waters RP C4 column (300 Å pore size; 3.5 µm particle size; 100 × 2.1 mm; Waters GmbH, Eschborn, Germany) by eluting with a gradient from 20 to 90% B in A within 20 min, where eluent A was 0.05% (v/v) TFA in water and eluent B was 0.05% (v/v) TFA in acetonitrile. A flow rate of 250 µL min^{-1} was used. The masses of the eluted fractions were analyzed on a MicroTOF ESI-MS (Bruker Daltonics, Bremen, Germany).

Lipase activity assay

Lipase activity was determined by measuring the hydrolysis of *para*-nitrophenyl palmitate (*p*NPP) according to Winkler and Stuckmann.[49] The substrate solution contained 1 mM *p*NPP and 1 mg mL^{-1} gum arabic (Acros Organics, Geel, Belgium) in 25 mM Tris/Cl pH 8.0 and was emulsified using a T 25 digital Ultra-Turrax® (IKA® Werke GmbH & Co. KG, Staufen, Germany) at 20000^{-1} for 4 min at room temperature. The reaction was started by mixing 900 µl *p*NPP substrate emulsion with 100 µl lipase in 50 mM Tris/Cl pH 8.0. The contribution of autohydrolysis was addressed by including a blank of 100 µl 50 mM Tris/Cl pH 8.0 instead of enzyme. The reaction mixture was incubated at the desired temperature for 10 min with vigorous shaking. Enzymatic hydrolysis of *p*NPP was stopped by addition of 100 µl of 1 M Na_2CO_3 and the sample spun at maximum speed in a table top centrifuge for 2 min at room temperature. The absorption of the supernatant was measured at 410 nm in a spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences).

Far-UV circular dichroism (CD) spectroscopy

The far-UV CD spectra of the different CalB N74D variants were recorded on a dichrograph JASCO J-715 (JASCO International Co., Ltd., Tokyo, Japan). All spectra are averages of 4 scans, and are reported as mean residue molar ellipticity ($[\theta]_R$) in degrees × cm^2 × $dmol^{-1}$. Quartz cells (110-QS Hellma; Hellma GmbH & Co. KG, Müllheim, Germany) of 0.1 cm optical path length and protein concentrations of 0.2 mg mL^{-1} were used. Ellipticity changes were recorded between 200 and 250 nm at 20 °C in 50 mM Tris/Cl pH 8.0. Raw data were processed using the Jasco software package.

Fluorescence spectroscopy

Fluorescence emission spectra of the different CalB N74D variants were recorded at 20 °C in 50 mM Tris/Cl pH 8.0 on a LS50B spectrometer (Perkin Elmer, Überlingen, Germany). The protein concentration was 0.5 µM. The emission spectra

were recorded from 290–450 nm with an excitation wavelength of 280 nm (slit 4/4).

Proteinase K digestion

In a total volume of 20 µl 50 mM Tris/Cl pH 8.0, 4 µg of each CalB N74D variant were incubated with 4, 0.4, 0.04, 0.004, or 0.0004 µg proteinase K (Roche, Mannheim, Germany) for 35 min on ice. Proteolysis was stopped by the addition of sample buffer (2% (w/v) sodium dodecyl sulfate, 16 mM Tris/Cl pH 6.8, 2.5% (v/v) glycerol, 0.04% (w/v) bromophenol blue, 1% (v/v) 2-mercaptoethanol) and boiling at 95 °C for 10 min. The samples were analyzed by SDS-PAGE using standard procedures.[50]

Results and discussion

Expression of CalB N74D in the presence of different fluorous aromatic amino acids

Wildtype CalB contains an N-glycosylation site at N74,[51,52] but glycosylation is not essential for the catalytic activity of the lipase.[53] In order to avoid glycosylation, which is usually heterogeneous[54,55] and complicates mass analysis, we introduced an N74D mutation into CALB. We expressed the N-glycosylation deficient mutant CalB N74D in the presence of the aromatic amino acid analogs (Fig. 1), 5-fluorotryptophan (5FW), *meta*-fluorotyrosine (mFY), and *para*-fluorophenylalanine (pFF), as well as with canonical tryptophan (W), tyrosine (Y), and phenylalanine (F) (refer to the Materials and methods section for experimental details). As the expression host, we chose *P. pastoris* strain X33 Δ*aro*1[46] that is auxotrophic for all three aromatic amino acids. The methanol-inducible CalB N74D expression construct carries an N-terminal fusion to the *S. cerevisiae* alpha-mating factor pre–pro leader sequence that drives secretion of the lipase into the growth medium (ESI, Fig. S1).‡ The combination of an aromatic amino acid auxotrophic strain and a strongly inducible yet tightly controllable expression system is crucial for successful translation of the fluorinated amino acids. The amino acid auxotrophy facilitates the control of intracellular amino acid pools. A major condition for the successful translational incorporation of a non-canonical amino acid in general is that the analog functions as a substrate for one of the host aminoacyl-tRNA synthetases (AARS). Due to their natural substrate tolerance, AARS can activate and charge non-canonical analogs, though much worse than the canonical counterparts. Consequently, for an efficient aminoacylation of an analog by an AARS, its intracellular concentration must be substantially higher than that of the canonical amino acid. Usually, analogs do not support cell growth though high cell densities are desired for good target protein yields. The incorporation protocol must thus be designed to solve the dilemma that the canonical amino acids are necessary for growth but at the same time impede efficient translation of their analogs. In order to avoid the competitive incorporation of, *e.g.*, W, Y, or F and the fluorinated analogs, it is necessary to deplete the endogenous pools of these amino acids before the expression of CalB N74D with the analogs is induced. Basically, two different strategies can be followed for the depletion of endogenous amino acid pools in auxotrophic expression strains. Either, the host is grown to the desired growth phase, *e.g.* mid-log, in the presence of a limiting amount of a specific canonical amino acid. Alternatively, the cells are cultivated with standard supplementation of the amino acid, then harvested and washed, and finally starved in unsupplemented medium. In both cases, the expression of the target protein is afterwards induced in the presence of the analog. We successfully incorporated methionine analogs into CalB N74D by the first method.[39] Here, however, we applied the latter strategy, especially since incorporation of deuterated tyrosine by an analogous approach was reported with the expression host X33 Δ*aro*1.[46]

In order to analyze the extracellular expression of CalB N74D in the presence of the canonical aromatic amino acids or their fluorinated derivatives, we assayed the supernatants of the yeast cultures for lipase activity. They all contained highly active lipase (data not shown), indicating successful expression in the presence of the canonical as well as the fluorinated aromatic amino acids. Apparently, none of the analogs interfered with expression or secretion of active CalB N74D.

We dialyzed the culture supernatants and concentrated them by ultrafiltration to purify the CalB N74D variants. SDS-PAGE (Fig. 2) revealed high purity of all preparations. The electrophoretic behavior was equal for all lipase variants and they migrated with the 34 kDa band of the molecular weight standard. However, the preparation of CalB N74D expressed with mFY contained a faint side band running slightly lower than the main band.

We obtained roughly 34, 26, and 17 mg of purified CalB N74D per litre culture when the expression medium was supplemented with 5FW, mFY, or pFF, respectively. The yield of parent lipase containing exclusively W, Y, and F was 53 mg L^{-1}.

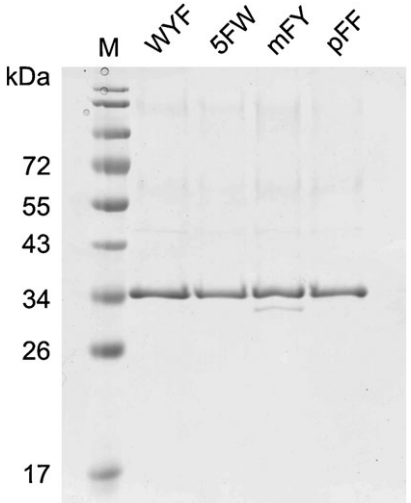

Fig. 2 Protein preparations of CalB N74D expressed in the presence of the amino acids indicated on the top of the gel image: tryptophan, tyrosine, phenylalanine (WYF); 5-fluorotryptophan (5FW), *meta*-fluorotyrosine (mFY), and *para*-fluorophenylalanine (pFF). The bands of the molecular weight marker (M) are indicated on the left margin; equal amounts of protein were loaded onto each lane. The calculated molecular weight of CalB N74D is 33 kDa.

Analysis of the incorporation efficiency of 5FW, mFY, and pFF

We assessed the incorporation efficiency of 5FW, mFY, and pFF by LC-ESI-MS analysis of the CalB N74D variant preparations. Correctly processed CalB N74D consists of 319 amino acids with a calculated mass of 33299.4101 Da. It contains 5 tryptophan, 9 tyrosine, and 11 phenylalanine residues (ESI, Fig. S2).‡ During segregation, the EKREAEA peptide of the alpha-mating factor pre–pro leader sequence is first cleaved by Kex2 protease (Kex2p) between R and E (EKR↓EAEA), then the *STE*13 gene product further trims the N-terminal EA repeats.[54] Accurately processed CalB N74D contains a non-native EF dipeptide extension at its N-terminus, which is encoded by the *Eco*RI site on the expression plasmid (ESI, Fig. S1, Fig. S2).‡

The mass spectrum of the parent WYF-CalB N74D is shown in Fig. 3a and the mass peaks are evaluated in Table 1. We did not observe a mass peak corresponding to correctly processed WYF-CalB N74D, instead, we found masses of protein species cleaved incompletely by Ste13p (EAEF and EAEAEF N-termini) and incorrectly by Kex2p (N-terminal extension REAEAEF). Incorrect Kex2p processing was reported previously for a CalB N74S mutant.[56] Possibly, the intracellular levels of Ste13p and Kex2p are insufficient to correctly process the large amounts of recombinantly produced CalB N74D.[57] In addition to the incorrectly processed protein species, we found two mass peaks shifted by +162 Da each (ESI, Fig. S3a; Table S1),‡ a mass shift typical for attached hexose sugar residues.[58] Obviously, the N74D mutation did not completely abolish glycosylation. However, as this would have gone beyond the scope of this study, we did not further analyze the glycosylation status of CalB N74D, and the mass shift might originate from another, unidentified protein modification. We noticed a comparable mass shift and differential proteolytic processing in our parallel study on the incorporation of methionine analogs into CalB N74D.[39]

In the same work, we found that the methionine residues of CalB N74D were stochastically substituted by their analogs, yielding highly complex mass spectra. Here, we observed similarly complex spectra suggesting a mixture of fluorinated protein species (ESI, Fig. S3b–d).‡ For facilitated evaluation of the incorporation efficiency, selected mass windows spanning the range relevant for CalB N74D species with an EAEF N-terminus are shown in Fig. 3b–d. Full range mass spectra are shown in the ESI, Fig. S3b–d,‡ and the corresponding mass peaks are interpreted in the ESI, Table S1.‡ All five tryptophans were substituted with 5FW in the 5FW-CalB N74D preparation (Fig. 3b; Table 1), yet the mass peak corresponding to two substitutions dominated. The mass range shown in Fig. 3b refers to unglycosylated 5FW-CalB N74D, nevertheless, differentially processed and glycosylated protein species were also fluorinated at the tryptophan positions (ESI, Fig. S3b; Table S1).‡ Similarly, full substitution of the nine tyrosines with mFY was achieved (Fig. 3c; Table 1). However, the mass corresponding to CalB N74D with nine mFY (33818.2024 Da) may as well be interpreted as WYF with two hexose molecules attached (*vide infra* and ESI, Fig. S3c, Table S1).‡ The spectrum in Fig. 3c shows differentially fluorinated protein species with a single hexose sugar attached. Given that the differentially fluorinated proteins are equally ionized by electrospray ionization, species with three or four substitutions prevail as they yield the highest peak intensities (Fig. 3c; Table 1). The replacement by pFF of eight out of eleven phenylalanines could be unequivocally identified (Fig. 3d; Table 1). The identification of CalB N74D species containing 9, 10, or 11 pFF is ambiguous: Fluorination adds 18 Da to the protein mass and 9 fluorinations sum up to +162 Da. Thus, the peaks of heavily fluorinated protein species overlap with those of less fluorinated yet glycosylated species in the pFF-CalB N74D spectrum (Fig. 3d; Table 1 and ESI, Fig. S3d; Table S1),‡ allowing two alternative interpretations of the same mass peak. Especially since the intensities of the relevant peaks (Fig. 3d; 33655.8672 Da, 33673.9492 Da, 33692.3116 Da) are noticeably higher than those of the other fluorinated protein species (Fig. 3d; bold black mass indications), the presence of fully substituted pFF-CalB N74D in the preparation appears likely. However, the comparison of the peak intensities presumes similar ionization of the protein species, *e.g.*, non-glycosylated *vs.* glycosylated.

Taken together, the incorporation into CalB N74D of the different fluorinated aromatic analogs shown in Fig. 1 was successful. As can be deduced from the full range spectra in the ESI Fig. S3b–d‡ and the accompanying mass evaluation Table S1,‡ substitutions occurred independent of processing or glycosylation. This is the first indication that fluorinated aromatic amino acids can be introduced at multiple sites into a protein expressed in *P. pastoris*. The incorporation, however, is stochastic and yields a mixture of differentially fluorinated proteins. This phenomenon of stochastic incorporation was observed previously with methionine analogs in *P. pastoris*[39] and *S. cerevisiae*.[59] It also occurred with trifluorinated methionine and leucine analogs in *E. coli*,[60] though the expression conditions can be tightly controlled in the bacterial host so that usually full or at least high level substitution is achieved.[61] A major issue in this respect is the intracellular availability of the non-canonical analog relative to its canonical counterpart. In contrast to *E. coli*, protein expression in the yeasts is performed not only for several hours but days. During this long period intracellular levels of canonical and non-canonical amino acids might change due to protein turnover, or metabolic conversion, respectively. As outlined above, the intracellular level of the analog must be noticeably higher than that of the canonical amino acid it replaces in order to avoid competitive incorporation. To date, the knowledge of the intracellular fate of non-canonical amino acid analogs in *E. coli* and, more so, in eukaryotic cells is scarce. Nevertheless, this issue deserves in-depth analysis especially in *P. pastoris* to improve the analog incorporation efficiencies.

Characterization of the fluorinated CalB N74D variants

In order to assess the effects of fluorination on CalB N74D secondary structure and function, we performed circular dichroism (CD) spectroscopy in the far-UV range, fluorescence spectroscopy, and lipase assays, respectively.

The dichroic profile of WYF-CalB N74D presents two characteristic minima at 208 and 222 nm (Fig. 4a, solid line). It reflects the alpha/beta hydrolase fold of CalB,[52] and its loop

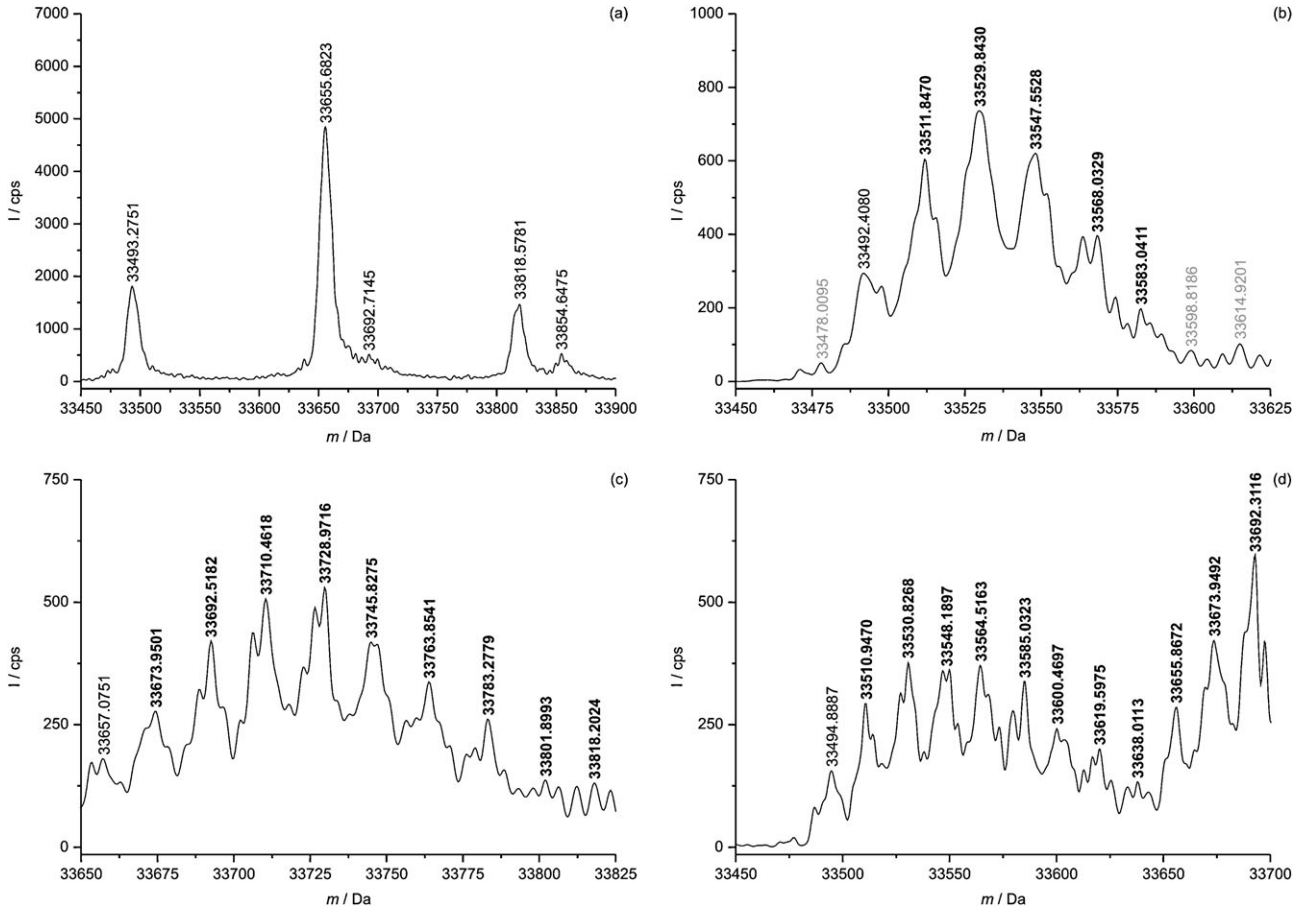

Fig. 3 ESI-mass spectra of the different CalB N74D variant preparations. Peaks assigned to fluorinated CalB N74D species are shown in bold black. Peaks corresponding to canonical CalB N74D are shown in black and undefined peaks are indicated in gray. Representative mass ranges are shown each. (a) WYF-CalB N74D; the shown mass range covers that of the spectra in panels (b)–(d); (b) 5FW-CalB N74D; (c) mFY-CalB N74D; (d) pFF-CalB N74D. For interpretation of the mass peaks refer to Table 1. I, intensity; cps, counts per second; m, mass. For full range mass spectra refer to ESI, Fig. S3.‡

structures contribute to the stronger intensity around the minimum at 208 nm. This secondary structure profile remains intact after substitution of the eleven phenylalanine residues by pFF (Fig. 4a, dash-dot line). Yet, the intensities of both minima are lowered indicating a destabilization relative to the canonical lipase. This effect is more pronounced upon the fluorination of the five tryptophans in CalB N74D (Fig. 4a, broken line). A closer inspection of the 3D structure of the lipase (PDB ID 1TCC[52]) revealed that half of the phenylalanine residues are placed in loop structures. Most probably, these positions play a minor role for the structural integrity of the lipase as their fluorination is well tolerated. In contrast, all five tryptophan residues are integral parts of alpha-helices (W52, W113, W155) or beta-sheets (W65, W104). Their fluorination might induce perturbing effects in the local microenvironments, which in turn contribute to the observed high degree of structural destabilization.

Like the phenylalanines, the majority of the tyrosine residues are present in loop structures of CalB. In contrast to the former, however, their fluorination provoked a pronounced increase in random coil content as indicated by a noticeably altered dichroic profile of mFY-CalB N74D. The first minimum shifted towards 205 nm and the second minimum at 222 nm virtually disappeared (Fig. 4a, dotted line). This finding indicates an enormously important role of the tyrosine residues for the structural integrity of the lipase which is also reflected in the activity of mFY-CalB N74D (*vide infra*). Our observation is not unexpected, since it is well known that some fluorinated amino acids have conformational preferences different to those of their canonical counterparts. For example, hexafluoroleucine destabilizes an alpha helical peptide but stabilizes beta-sheets.[62,63] Although comparable studies with monofluorinated amino acids are lacking, it cannot be ruled out that they have similar effects, especially in multi-site fluorinated proteins such as the CalB N74D variants described here.

We expected the increase in random coils to have an effect on the tertiary structure of mFY-CalB N74D as well. Indeed, the fluorescence spectrum of mFY-CalB N74D is clearly discernible with respect to intensity and profile from the spectra of the remaining variants (Fig. 4b, dotted line). As we excited the protein samples at 280 nm, the emission spectra are dominated by the fluorescence of tryptophan residues, which is extremely sensitive to its local microenvironment. All protein variants showed an emission profile with two fluorescence maxima at 316 and 328 nm (Fig. 4b). While the maxima of WYF-CalB N74D were nearly equally intensive (Fig. 4b, solid

Table 1 Interpretation of the mass peaks in Fig. 3. The EKR↓EA*EA*EF peptide in the alpha-mating factor pre–pro leader sequence is cleaved by Kex2p (↓) and Ste13p (*), EF originates from cloning of the CalB N74D coding sequence into the *Eco*RI site on plasmid pPICZαA. Masses were calculated assuming reduced cysteine residues; Δ*m* denotes the mass difference between found mass and calculated mass; Hex, attached hexose sugar residue of about 162 Da;[58] Kex2p(i), incorrect Kex2p digestion; S–S, disulfide bridge. Mass peaks allocated to CalB N74D variants containing an analog are indicated in bold, peaks corresponding to parent proteins are in standard print and unidentified peaks are designated as such. Alternative interpretations of the same peak are possible.

	Mass				
CalB N74D variant	Found (Da)	Calculated (Da)	Δ*m* (Da)	Interpretation	N-terminus
WYF (Fig. 3a)	33493.2751	33499.6024	−6	WYF, 3 S–S	EAEF
	33655.6823	33661.7432	−6	WYF, 3 S–S, 1 Hex	EAEF
	33692.7145	33699.7947	−7	WYF, 3 S–S	EAEAEF
	33818.5781	33823.8841	−5	WYF, 3 S–S, 2 Hex	EAEF
	33854.6475	33855.9807	−1	WYF, Kex2p(i)	REAEAEF
5FW (Fig. 3b)	33478.0095	—		unidentified	
	33492.4080	33499.6024	−7	WYF, 3 S–S	EAEF
	33511.8470	**33517.5928**	**−6**	**1 5FW, 3 S–S**	**EAEF**
	33529.8430	**33535.5833**	**−6**	**2 5FW, 3 S–S**	**EAEF**
	33547.5528	**33553.5738**	**−6**	**3 5FW, 3 S–S**	**EAEF**
	33568.0329	**33571.5642**	**−4**	**4 5FW, 2 S–S**	**EAEF**
	33583.0411	**33589.5547**	**−7**	**5 5FW, 3 S–S**	**EAEF**
	33598.8186	—		unidentified	
	33614.9201	—		unidentified	
mFY (Fig. 3c)	33657.0751	33661.7432	−5	WFY, 3 S–S, 1 Hex	EAEF
	33673.9501	**33679.7337**	**−6**	**1 mFY, 3 S–S, 1 Hex**	**EAEF**
	33692.5182	**33697.7242**	**−5**	**2 mFY, 3 S–S, 1 Hex**	**EAEF**
	33710.4618	**33715.7146**	**−5**	**3 mFY, 3 S–S, 1 Hex**	**EAEF**
	33728.9716	**33733.7051**	**−5**	**4 mFY, 3 S–S, 1 Hex**	**EAEF**
	33745.8275	**33751.6955**	**−6**	**5 mFY, 3 S–S, 1 Hex**	**EAEF**
	33763.8541	**33769.6860**	**−6**	**6 mFY, 3 S–S, 1 Hex**	**EAEF**
	33783.2779	**33787.6765**	**−4**	**7 mFY, 2 S–S, 1 Hex**	**EAEF**
	33801.8993	**33805.6669**	**−4**	**8 mFY, 2 S–S, 1 Hex**	**EAEF**
	33818.2024	**33823.6574**	**−5**	**9 mFY, 3 S–S, 1 Hex**	**EAEF**
	33818.2024	33823.8841	−6	WYF, 3 S–S, 2 Hex	EAEF
pFF (Fig. 3d)	33494.8887	33499.6024	−5	WYF, 3 S–S	EAEF
	33510.9470	**33517.5928**	**−7**	**1 pFF, 3 S–S**	**EAEF**
	33530.8268	**33535.5833**	**−5**	**2 pFF, 3 S–S**	**EAEF**
	33548.1897	**33553.5738**	**−5**	**3 pFF, 3 S–S**	**EAEF**
	33564.5163	**33571.5642**	**−7**	**4 pFF, 3 S–S**	**EAEF**
	33585.0323	**33589.5547**	**−5**	**5 pFF, 3 S–S**	**EAEF**
	33600.4697	**33607.5452**	**−7**	**6 pFF, 3 S–S**	**EAEF**
	33619.5975	**33625.5356**	**−6**	**7 pFF, 3 S–S**	**EAEF**
	33638.0113	**33643.5261**	**−6**	**8 pFF, 3 S–S**	**EAEF**
	33655.8672	**33661.5165**	**−6**	**9 pFF, 3 S–S**	**EAEF**
	33655.8672	33661.7432	−6	WYF, 3 S–S, 1 Hex	EAEF
	33673.9492	**33679.5070**	**−6**	**10 pFF, 3 S–S**	**EAEF**
	33673.9492	**33679.7337**	**−6**	**1 pFF, 3 S–S, 1 Hex**	**EAEF**
	33692.3116	**33697.4975**	**−5**	**11 pFF, 3 S–S**	**EAEF**
	33692.3116	**33697.7242**	**−5**	**2 pFF, 3 S–S, 1 Hex**	**EAEF**

line), the longer wavelength maxima of 5FW-, mFY-, and pFF-CalB N74D were more intensive than the shorter wavelength maxima (Fig. 4b, broken, dotted, and dash-dot line, respectively), indicating a higher number of solvent exposed tryptophan residues.[64] 5FW-CalB N74D displayed a shoulder at 316 nm rather than a maximum (Fig. 4b, broken line). Taken together, the fluorinations induced perturbations in the variant protein tertiary structures whereby the substitution of the tyrosines with mFY caused the most dramatic effect.

Next, we assessed the impact of the fluorination of the tryptophan, tyrosine, and phenylalanine residues of CalB N74D on the activity of the lipase. CalB is a highly thermostable enzyme that shows maximal activity between 60 and 80 °C.[65,66] The 5FW-, mFY-, and pFF variants were optimally active in the same temperature range. Yet, their activity was lower than that of the parent protein and decreased in the order pFF > mFY > 5FW (Fig. 5a). Thus, fluorination of tryptophan, tyrosine, and phenylalanine residues in CalB does not obliterate lipase activity though the catalytic activity is lowered. This is in contrast to our observation with the methionine analog, norleucine whose incorporation improved the activity of CalB N74D.[39] Schoffelen *et al.* reported a similar activity loss when CalB was expressed with the methionine analog azidohomoalanine in *E. coli*.[40] However, after refrigerated storage of the variant proteins for several months, we again assayed the lipase activity at 60 °C. Surprisingly, we found that the fluorinated variants were equally (5FW-CalB N74D) or slightly more active (mFY-, and pFF-CalB N74D) than the non-fluorinated parent protein (Fig. 5b). Thus, fluorination appears to prolong the shelf life of CalB activity.

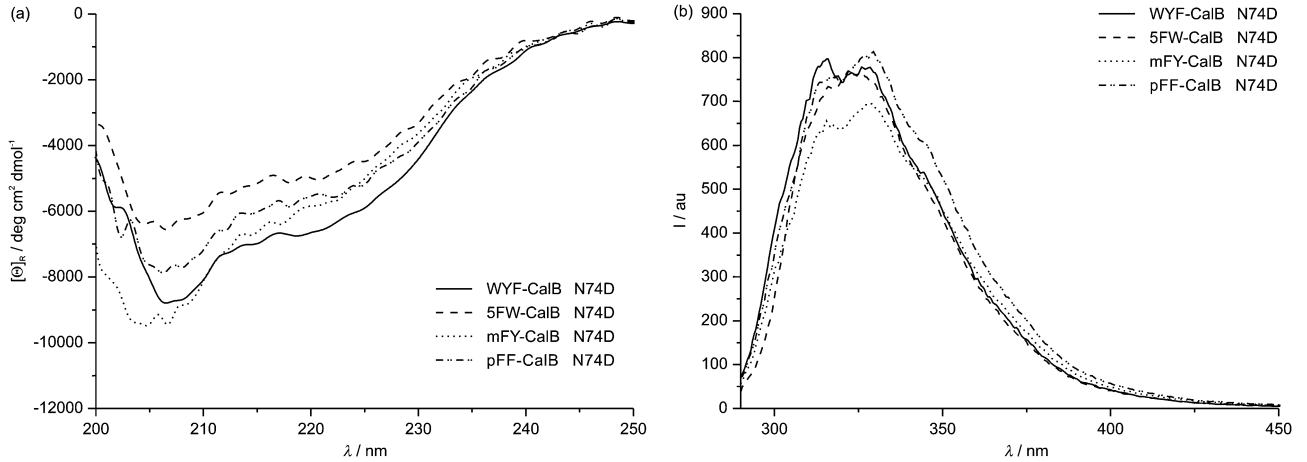

Fig. 4 (a) Far-UV CD spectra and (b) fluorescence spectra of the fluorinated CalB N74D variants at 20 °C. The excitation wavelength was 280 nm. $[\theta]_R$, mean residue molar ellipticity; I, intensity; au, arbitrary units.

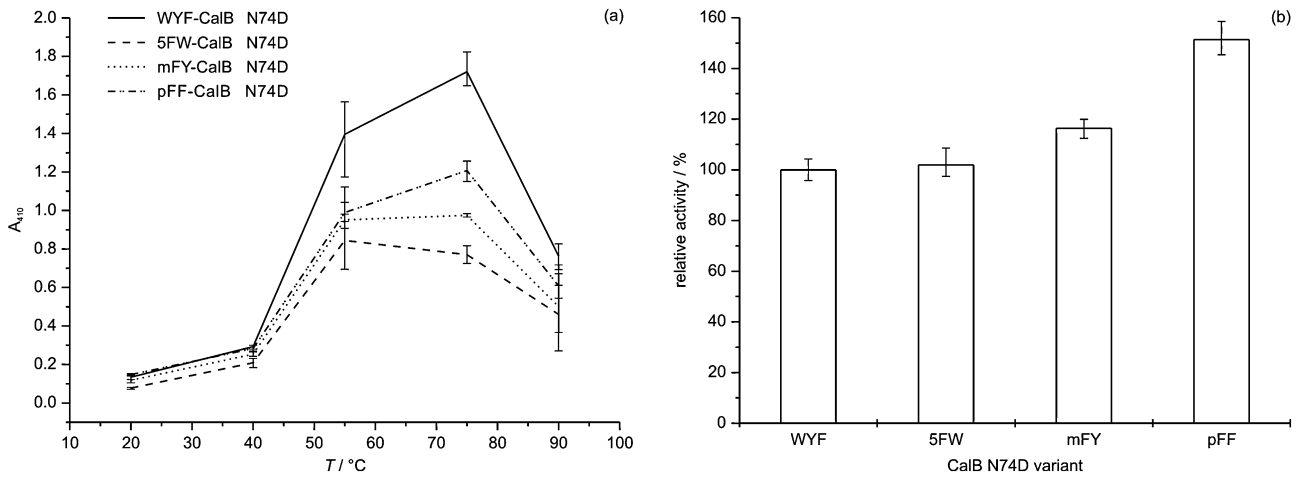

Fig. 5 (a) Temperature optimum of the different CalB N74D variants. (b) Catalytic activity of the parent CalB N74D (WYF; set as 100%) relative to the 5FW-, mFY-, and pFF variants at 60 °C after several months of storage at 4 °C. Mean values of three replicates are shown and the error bars denote the maximal and minimal measured activities.

Recently, we observed a similar effect with a globally fluorinated superfolder green fluorescent protein.[5]

Finally, we analyzed the susceptibility of the different CalB N74D variants towards proteolytic digestion with proteinase K. Proteinase K cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids.[67] The variants were incubated with different amounts of proteinase K and the cleavage products analyzed by SDS-PAGE. WYF-CalB N74D was completely digested by 4 and 0.4 μg proteinase K (ESI, Fig. S4a),‡ lower amounts left the protein intact, though. We observed the same cleavage pattern with the fluorinated CalB N74D variants, which were all essentially impervious to digestion by low amounts of proteinase K (ESI, Fig. S4b–d).‡ Thus, fluorination does neither inhibit proteolytic digestion by proteinase K nor are the variants containing fluorinated aromatic amino acids more or less prone to proteolysis. Obviously, the structural alterations inflicted by the different fluorinated aromatic analogs (Fig. 4a) were insufficient to provide the protease with better access to its cleavage sites in comparison to the non-fluorinated parent protein.

The experimental data presented here clearly demonstrate that multi-site fluorination of proteins expressed in the yeast *P. pastoris* is feasible. Moreover, the fluorinated proteins were efficiently segregated into the medium, which is an important advantage for the large-scale production of fluorinated therapeutic proteins. We achieved high-level substitution of tryptophan, tyrosine, and phenylalanine residues in CalB N74D by the non-canonical analogs 5FW, mFY, and pFF. The fluorinated allolipases behaved equally towards proteinase K treatment as did the non-fluorinated parent protein. The variant proteins showed alterations in their secondary structures but lipase activity was not obliterated yet lowered under standard conditions. In agreement with a previous observation,[5] we found that fluorination preserved lipase activity upon storage. These compliant findings with two structurally and functionally different proteins, *i.e.* green

fluorescent protein and CalB, indicate that structurally innocuous, global fluorination of proteins might represent a general way to preserve or even enhance their biological activity over prolonged periods of time. Fluorination has already proved to be an efficient tool to enhance the bioavailability and activity of many low-molecular weight drugs, as well as peptides. In addition, we have shown that the multisite introduction of fluorine into proteins prolongs their shelf life. We are convinced that *P. pastoris* will be a valuable host for the production of biotechnologically and pharmacologically important fluoroproteins in the future.

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