Enzyme Mutation

DOI: 10.1002/anie.200502461

Conversion of a Carboxylesterase into a Triacylglycerol Lipase by a Random Mutation**

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Lipases and esterases are enzymes of increasing importance for classical and new industrial applications.[1,2] The most significant properties of these enzymes are that they are very stable and active, especially in organic solvents, and they possess regio- and stereospecificity.[2] Esterases (also called carboxylesterases, EC 3.1.1.1) preferentially hydrolyze water-soluble "simple" esters and usually only triglycerides bearing fatty acids shorter than C_6, whereas lipases (also known as triacylglycerol lipases, EC 3.1.1.3) prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids.[1] Despite their close relationship in sequence and structure, these enzymes differ in their profile for chain-length specificity. In fact, substrate specificity is the only completely valid criterion for distinguishing between carboxylesterases and lipases, because several exceptions exist for the other criteria used previously: the existence of a *lid*, interfacial activation, and hydrophobicity of the scissile acyl-binding site of the substrate.[1,3,4]

Lipases and esterases have already been improved by methods such as medium engineering, immobilization on suitable supports, and rational protein design. Moreover, it has been demonstrated that directed evolution can lead to enzymes with thermal stability[5] and inverted or improved enantioselectivity.[6] A few examples also showed that the chain-length specificity of lipases can be achieved.[7] Notably, in all cases the original lipase showed activity toward long-chain fatty acid esters, and laboratory evolution was used to improve and thereby modify its hydrolytic activity. Clearly, the transformation of a "true carboxylesterase" into a "true lipase" seems to be difficult, as no examples of this change have been reported. Two hypotheses may explain the supposed difficulty: 1) the active center in esterases is smaller than that in lipases, so it can only accommodate short-chain fatty acid esters;[8] or 2) the esterase requires a hydrophobic mobile *lid* to facilitate its interfacial activation, and that has only been found in lipases.[9] Whatever the case, both situations seem to be critical for a high-performance lipase. Herein, we describe the conversion of a "true esterase" into a triacylglycerol lipase by directed evolution; the significance of this study is also discussed. Enzyme R.34, retrieved from the metagenome library of bovine rumen microflora,[10] was chosen as the test enzyme.

The data in Figure 1 a and b support the notion that R.34 is a true carboxylesterase, which acts preferentially on *p*-nitrophenyl (*p*-NP) esters and the triacylglycerol of short-chain fatty acids ($\leq C_4$), and is optimal for C_3 acids (up to 240 units mg^{-1}). One round of error-prone PCR was used to create a mutant with higher activity toward long-chain fatty acid esters.[12a] Potential improved variants were identified on agar plates by using α-naphthyl laurate (αNL) and an azo dye (Fast Blue RR) that reacts with the released 2-naphthol to generate an insoluble brown product.[13] Under these conditions, *Escherichia coli* colonies expressing wild-type R.34 did not produce brown halos on αNL plates, but did produce them on α-naphthyl acetate (αNA) plates.[12b] Approximately 8200 colonies were screened in the first round, and only one clone (EL1) was identified. Such a low frequency of improvement was surprising, and may reflect the fact that long-chain esters are very inefficient substrates for R.34. Both R.34 and EL1 were produced as a fusion with a hexahistidine (His_6) tag at the C terminus,[12a] and their biochemical properties were investigated. Although the optimal pH, temperature, and

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[**] This research was supported by EC Project MERG-CT-2004-505242 "BIOMELI", Spanish CICYT Projects BIO2002-00337 and BIO2004-03773-C04-02, and the BMBF "GenoMik" initiative. M.F. thanks the European Commission for a Marie Curie postdoctoral fellowship and the Spanish Ministerio de Ciencia y Tecnología. The authors also thank ViaLactia Biosciences Ltd. (New Zealand) for financial support. K.N.T. gratefully acknowledges the generous support by the Fonds der Chemischen Industrie.

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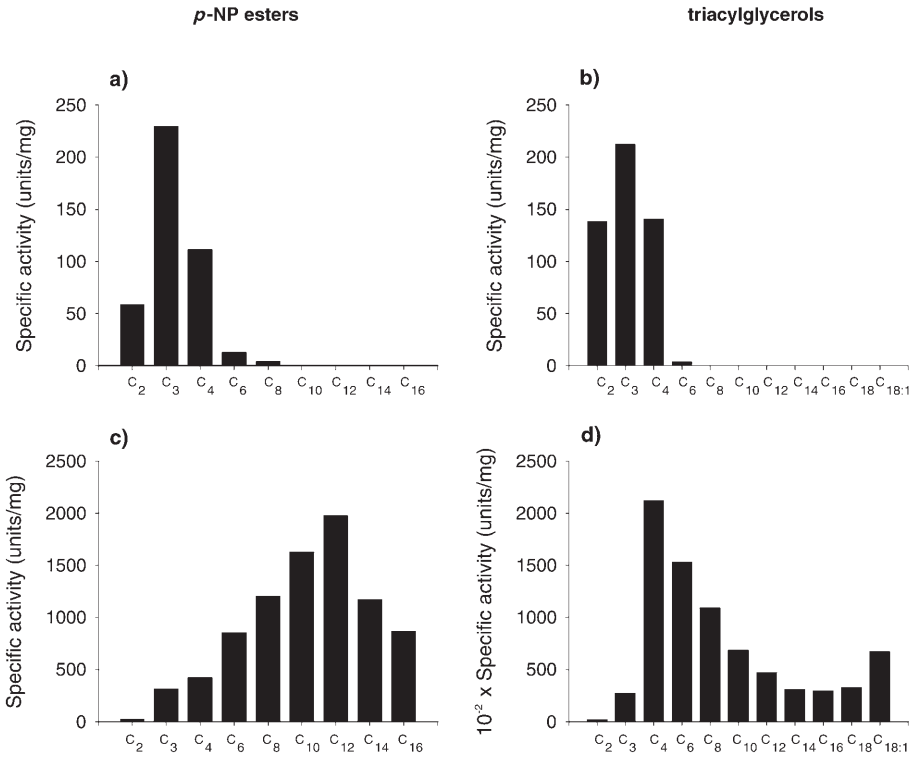

Figure 1. Relative activity of the wild-type R.34 esterase (top) and EL1 mutant (bottom) to *p*-NP esters (a and c) and triacylglycerols (b and d). Specific activities are given in units mg^{-1} pure protein. R.34 and EL1 enzymes were purified by using a Ni-Sepharose column after expression with a carboxyl-terminated His_6 tag.[12a] To exclude any influence of the His_6 tag on activity, the R.34 protein was expressed in and purified from *E. coli* XLOLR cells harboring pBKR.34 plasmid.[10] The enzyme obtained by this method did not show relevant differences in substrate specificity.

subunit composition were essentially the same for both proteins,[12c] several significant differences were found (see below).

First, the optimal acyl chain for *p*-NP esters switched to *p*-NP laurate (C_{12}) with a nearly one order of magnitude increase in specific activity ($\approx$2000 units mg^{-1}; Figure 1c). Interestingly, the specificity switched >1000-fold toward short-chain triacylglycerols (Figure 1d). Tributyrin (C_4) was the optimal substrate for EL1 (214000 units mg^{-1}), but the enzyme was also able to efficiently hydrolyze typical lipase substrates such as trilaurin, tripalmitin, and triolein (>67000 units mg^{-1}).

Encouraged by this finding, we assessed the susceptibility of EL1 to several fatty acid sulfonyl fluorides (SFs), which are potent serine-specific inhibitors. As shown in Figure 2a, R.34 was strongly inhibited by phenyl methyl sulfonyl fluoride (PMSF); however, it was partially inhibited by capryl sulfonyl fluoride (C_6SF), and was not affected by the longer lauroyl (C_{12}SF) and palmitoyl (C_{16}SF) derivatives. On the other hand, EL1 mutant was equally inhibited by all the SFs tested, which suggests that the serine residue at the active site is more accessible in this mutant compared with the wild-type enzyme.

Next, we examined the susceptibility of the EL1 variant to detergents and solvents. As illustrated in Figure 2b, the stability of the mutant was very similar to that of the wild-type enzyme upon addition of acetonitrile. This result suggests that solvent-exposed residues were, most likely, not changed by the mutation and that they were similarly exposed in both variants. However, when both enzymes were incubated at different concentrations of the ionic detergent Triton X-100, we observed that the EL1 variant was more affected than the wild-type enzyme (Figure 2c). Thus, R.34 showed maximal activity at 0.6% (w/v) detergent and retained more than 50% of the maximum activity at a 5% (w/v) concentration, whereas the EL1 mutant was strongly inhibited above 0.06% (w/v). The mutation may have induced a conformational change, whereas the catalytic residues became exposed to the large detergent micelles.

We further analyzed the secondary structure content, and found that the CD spectra for R.34 and EL1 mutant measured at 25°C were similar, with minima at 208 and 222 nm (Figure 3a), which is consistent with an α-helical protein. The thermal unfolding of each protein was determined by fitting the ellipticity at 222 nm (θ_{222}) versus temperature. As shown in Figure 3b, the melting temperature (T_m) shifted from 63.7°C (for R.34) to 51.3°C (for EL1). This result was somewhat unexpected, and indicates a lower stability for the EL1 mutant.

The creation of a lipase (EL1) prompted us to examine its positional specificity. The transesterification of the structured lipid 1,3-dipalmitoyl-2-oleoyl glycerol (POP) with ethanol[12a] was used to unambiguously identify the regiospecificity and to minimize acyl migration artifacts. When using lipase EL1 immobilized on Sepabeds EC-EP3, 2-methyl-2-butanol, and a water activity (a_w) of 0.22 (predetermined to be optimal), the reaction reached a conversion of $\approx$98% after 4 h at 30°C (Figure 4). EL1 showed a preference for the sn2 position, as deduced from the significantly higher concentrations of ethyl oleate and 1,3-dipalmitoyl glycerol over those of ethyl palmitate (Figure 4) and 1-*O*-palmitoyl- and 1(3)-palmitoyl-2-oleoyl glycerol,[12d] respectively.

The sequence analysis of EL1 mutant revealed a single amino acid substitution, N33D. The question that arises from this finding is, why does this single amino acid substitution in the EL1 mutant have such a profound effect on the substrate specificity? To understand the significant differences observed in the substrate specificity profile mediated by this substitution, we produced a three-dimensional model of the R.34 structure (Figure 5a), in which the esterase sequence was aligned with that of *Alicyclobacillus acidocaldarius*.[14]

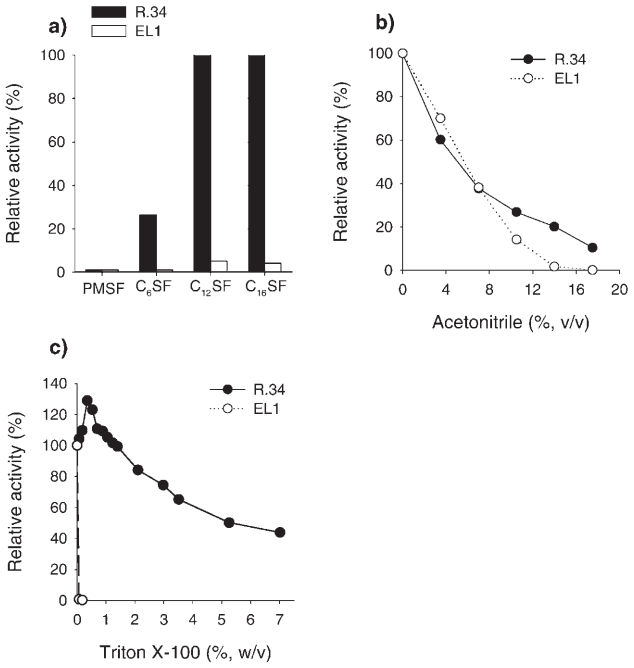

Figure 2. Parameters affecting the activities of R.34 and EL1. a) Inactivation by irreversible active-site inhibitors. Purified enzymes were incubated with 1 mM PMSF, C_6SF, C_{12}SF, and C_{16}SF. After incubation for 20 min, an aliquot was withdrawn and the hydrolytic activity was monitored. b) Effect of acetonitrile on esterase activity. c) Effect of Triton X-100 on esterase activity. Hydrolytic activity was monitored spectrophotometrically by following the increase in absorbance at 410 nm as a result of hydrolysis of *p*-NP propionate at 40 °C in HEPES buffer (100 mM, pH 7.5). The relative activity is normalized as 1.

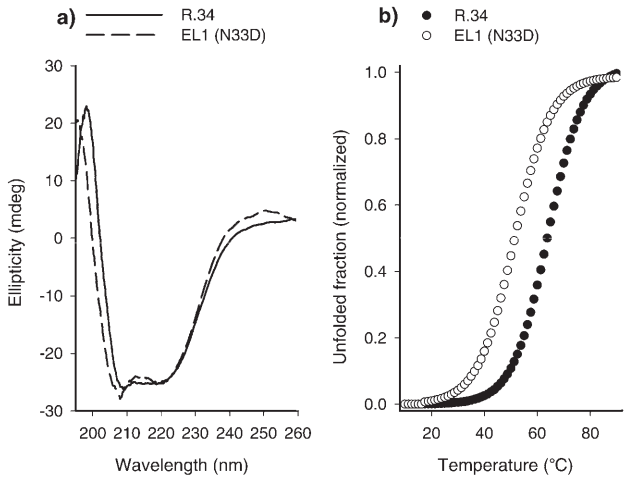

Figure 3. Circular dichroism studies. a) Far-UV CD spectra of R.34 and the EL1 mutant. b) Unfolding profiles of proteins. The samples were heated from 15 to 90 °C at 1 °C min^{-1} and the ellipticity was recorded at 222 nm. The T_m values were calculated by a nonlinear least-squares fit of the transition temperatures.

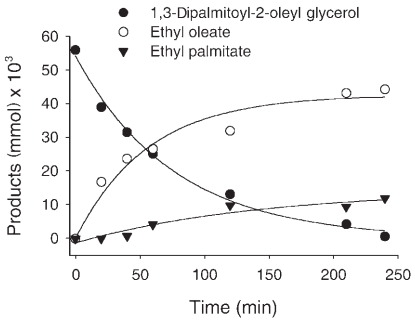

Figure 4. Time course of the transesterification reaction of POP with ethanol in 2-methyl-2-butanol using immobilized EL1 lipase (as determined by GC).[12a] Initial conditions: POP (0.056 mmol), ethanol (0.58 mmol), immobilized EL1 lipase (a_w = 0.22, 5 mg), 30 °C, 2-methyl-2-butanol (a_w = 0.22, 1 mL). Conditions as described in Ref. [12a]. The data are not fitted to any model; each point represents the mean of three experiments.

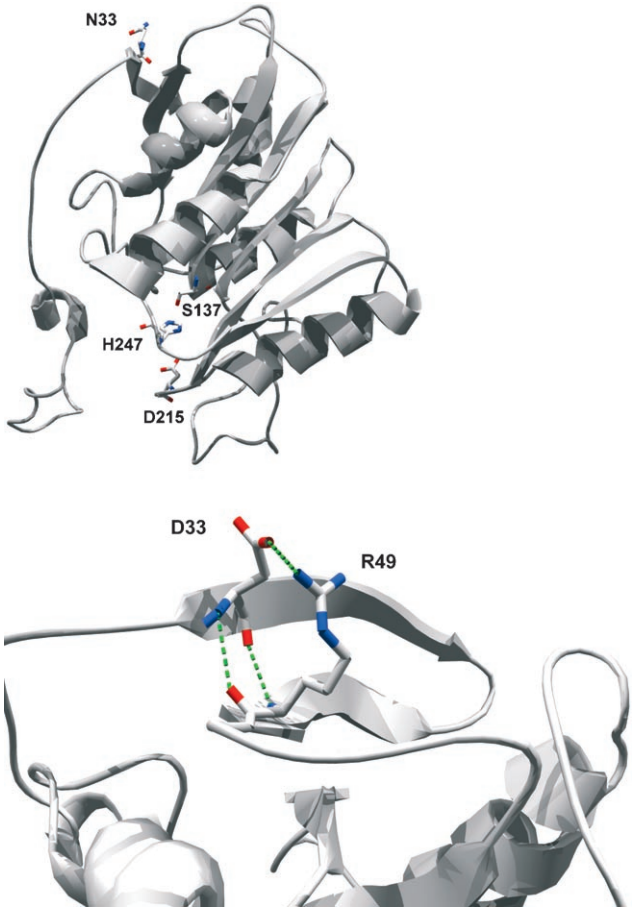

Figure 5. a) Overall three-dimensional structure of R.34, as obtained by homology modeling. Residues belonging to the catalytic triad and N33 are explicitly shown. b) Schematic representation of the putative salt-bridge binding residues D33 and R49 in the EL1 mutant.

The analysis of the *A. acidocaldarius* EST2 esterase structure indicates the existence of an ion pair between the two residues (Glu and Arg) present at positions equivalent to those of Asn33 and Arg49 in the R.34 sequence. This finding prompted us to suggest that the substitution of Asn33 by Asp leads to the formation of a salt bridge between the newly introduced Asp33 and Arg49 (Figure 5b). Most likely, this may cause a distortion of the enzyme structure (proof of which awaits 3D structure determination), which would make

the catalytic site more accessible to larger substrates, but also more labile. This argument was supported experimentally by the analysis of the substrate-specificity profile (Figure 1), in which it was clear that the active site accommodated longer fatty acid esters, and by the higher susceptibility of EL1 to chemical inactivation and denaturation (Figure 2). In addition, the midpoint of unfolding was 12 °C lower. To prove the supposed interaction between D33 and R49, and the fact that this interaction affects the catalytic activity of the mutant enzyme toward triacylglycerols, single R49D and R49N mutant variants of the enzyme were generated by site-directed mutagenesis. Mutations at R49 produced variants with no activity on αNL plates, but they did hydrolyze αNA.[12e] Furthermore, we also introduced a reversed mutation N33R-R49D in R.34 and obtained the lipase phenotype in both αNL[12f] and rhodamine–triolein plates.[12g] These results unambiguously confirm that the interaction between residues 33 and 49 exists, and that it is essential for the substrate preference of the R.34 enzyme.

In summary, we have provided clear proof that the substrate specificity of a true carboxylesterase can be modified toward insoluble substrates, that is, turned into a true triacylglycerol lipase, without modification of the shape, size, or hydrophobicity of the substrate-binding sites that are considered to be essential for chain-length specificity.[1,7,8] Moreover, minimal changes in the structure are sufficient for enhancing the acyl chain-length preference of esterases. More significantly, compared with other lipases,[17,18] the EL1 mutant may constitute an important step toward the synthesis of structured lipids[19] and may have other lipase applications, which are under investigation.

Received: July 14, 2005
Revised: September 26, 2005
Published online: October 27, 2005

Keywords: enzymes · esterases · lipases · mutagenesis · regiospecificity

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[12] a) Detailed experimental procedures are available in the Supporting Information; b) The esterase–lipase phenotype of the EL1 improved variant and wild-type R.34 is shown in the Supporting Information; c) The optimal pH (7.5), temperature (50 °C), and subunit composition (monomer of ≈ 34 kDa) were essentially the same for both R.34 and EL1 enzymes (see the Supporting Information); d) HPLC chromatograms of the reaction products (from mono- to triglycerides) are shown in the Supporting Information; e) The esterase–lipase phenotype of EL1 variants containing R49N and R49D mutations is shown in the Supporting Information; f) The esterase–lipase phenotype of R.34 variant containing a reverse mutation N33R-R49D is shown in the Supporting Information; g) The lipase phenotype of R.34, EL1, and mutant variants on rhodamine–triolein plates is shown in the Supporting Information.[15]
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