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The lid is a structural and functional determinant of lipase activity and selectivity

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Abstract

In several lipases access to the enzyme active site is regulated by the position of a mobile structure named the lid. The role of this region in modulating lipase function is reviewed in this paper analysing the results obtained with three different recombinant lipases modified in the lid sequence: *Candida rugosa* lipase isoform 1 (CRL1), *Pseudomonas fragi* lipase (PFL) and *Bacillus subtilis* lipase A (BSLA). A CRL chimera enzyme obtained by replacing its lid with that of another *C. rugosa* lipase isoform (CRL1LID3) was found to be affected in both activity and enantioselectivity in organic solvent. Variants of the PFL protein in which three polar lid residues were replaced with amino acids strictly conserved in homologous lipases displayed altered chain length preference profile and increased thermostability. On the other hand, insertion of lid structures from structurally homologous enzymes into BSLA, a lipase that naturally does not possess such a lid structure, caused a reduction in the enzyme activity and an altered substrate specificity. These results strongly support the concept that the lid plays an important role in modulating not only activity but also specifity, enantioselectivity and stability of lipase enzymes.

Keywords: *Candida rugosa* lipase isoform 1; *Bacillus subtilis* lipase A; *Pseudomonas fragi* lipase; Specificity; Temperature stability; Enantioselectivity; Site-directed mutagenesis; Domain swapping

1. Introduction

Lipases are the enzymes with the broadest use in biocatalysis [1–6]. Their application for the preparation of chiral building blocks, especially by kinetic resolution of racemic mixtures, is of particular interest for the pharmaceutical, agrochemical and food industries. In addition, a reason that contributes to the usefulness of lipases is their high activity in non-aqueous media (organic solvents, ionic liquids, solvent-free systems), in which the synthetic reaction is favored over hydrolysis. To improve the efficiency of lipases in biocatalysis, different approaches such as medium, substrate and protein engineering have been exploited [7,8]. In this frame, the ability to recognize and modify the molecular determinants of enzyme function might provide an effective starting point towards the optimization of biocatalytic processes. From a structural point of view, besides the substrate binding region, there is evidence that suggest that the region of the lid – a mobile amphipatic structure which covers the catalytic active site of most lipases and whose length and complexity depend on the enzyme – is involved in modulating activity and selectivity of lipases [9–14]. In order to point out the importance of this structural element, herein we review our previous findings (and add some new results on the activity of BSLA and its mutant in organic solvents) on the effects of lid-related modifications and on the enzymatic properties of lipases from fungal and bacterial sources, differing from each other in sequence, presence/absence of a lid structure, molecular and biochemical features. Despite a marked divergence in sequence, all lipases feature the α/β-hydrolase fold [15,16], which makes approaches of rational mutagenesis and domain swapping possible. Targets of this comparative studies were *Candida rugosa* lipase isoform 1 (CRL1), *Pseudomonas fragi* lipase (PFL) and *Bacillus subtilis* lipase A (BSLA). Enzymes were subjected to three different approaches targeting their lids: domain exchange, sequence-based site-directed mutagenesis and domain insertion, based on the rationale discussed in the following. CRL1 belongs

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to a family of isoenzymes related in sequence but not identical in catalytic properties. The lid of CRL1 was substituted with the corresponding region of isoform 3 obtaining a chimera protein that was tested for activity and enantioselectivity [9,14]. PFL, a lipase endowed with activity at low temperature and an unusual specificity towards short-chain triglycerides, was modified by rational mutagenesis based on sequence comparison with homologous lipases showing higher temperature stability and specificity for medium- or long-chain triglycerides [17,18]. In the case of BSLA, since the wild type protein does not have a lid, the enzyme was modified by swapping lid sequences of the structurally related enzymes cutinase, acetylxylanesterase and human pancreatic lipase [19]. All modifications affected activity, specificity and, in the case of PFL, also stability of the proteins providing support to a new view of the lid structure as a key structural and functional element of lipases.

2. Lid sequence in wtCRL1, wtPFL, wtBSLA and their mutants

CRL isoenzymes 1 and 3 are related by 89% sequence identity on the overall peptide chain and the sequence of their lids differs for six amino acids as shown in Fig. 1a. Swapping of the CRL3 lid on the CRL1 scaffold therefore produces a chimera differing from the wtCRL1 in only 6 out of 534 residues. Fig. 1b compares the sequence of the PFL lid with those of other lipases from *Pseudomonas*/*Burkholderia* species related to each other and to PFL by approximately 40% sequence identity but differing from each other in temperature stability and substrate preference profile. Residues strictly conserved in the reference lipases at positions 137, 138 and 141 (valine, asparagines and glycine) are substituted in PFL by threonine (137 and 138) and serine (141).

The BSLA naturally does not possess a lid structure. In computer-based studies, three lipolytic enzymes that do exhibit a lid or lid-like structure, cutinase from *Fusarium solani pisi* [20], acetylxylanesterase from *Penicillium purpurogenum* [21] and the human pancreatic lipase [22], were chosen based on their structural homology to BSLA (Fig. 1c). The surroundings of the active sites were compared in silico. The lids and lid-like structures of these three enzymes were modeled into the structure of BSLA indicating experimental options to engineer these lids into BSLA without disturbing the core of the α/β-hydrolase fold. The resulting variants were named CUTIlip, AXElip and HPlip, respectively.

3. Activity and enantioselectivity of wtCRL1 and CRL1LID3

Activity of the chimeric enzyme in aqueous medium was studied in a previous work, where it was shown that lid swapping conferred to CRL1 the ability to hydrolyze cholesterol esters typical of isoform 3. Moreover, wt and chimeric lipases displayed a different sensitivity to non-ionic detergents in the reaction mixture [9]. Activity and selectivity was further investigated in organic solvents [14]. Table 1 shows the results obtained in a model reaction, the alcoholysis of chloro ethyl 2-hydroxy hexanoate with methanol in hexane and isooctane. Specific activity was higher in the case of wtCRL1 at any tested water activity value. Highest transesterification activity was obtained with both enzymes at $a_w = 0.53$, thus ruling out effects related to different

Table 1
Transesterification rate of wtCRL1 and CRL1LID3 in organic solvents at different a_w-values

Enzyme	Rate (μmol/h per mg of lipase)					
	Hexane			Isooctane		
	a_w 0.06	a_w 0.53	a_w 0.84	a_w 0.06	a_w 0.53	a_w 0.84
wtCRL1	0.06	1	0.09	0.08	1.49	0.1
CRL1LID3	0.05	0.4	0.05	0.05	0.52	0.05

Transesterification was carried out with 0.5 mg of lipase, using as model reaction the alcoholysis of chloro ethyl 2-hydroxy hexanoate (0.013 M) with methanol (0.25 M). The mixture was shaken at 150 rpm and at 25 °C. The reaction progress was monitored by GLC (column: dimethylpentyl, β-cyclodextrin 25 m, 0.25 mm ID, MEGA) with an oven temperature from 90 to 130 °C with heating rate 2.5 °C/min.

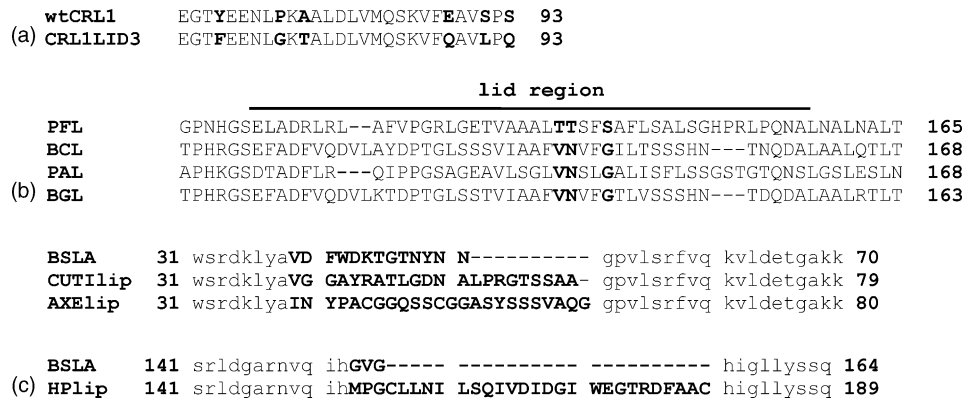

Fig. 1. (a) Lid sequence of lipases from wtCRL1 and CRL1LID3, where residues differing in the two enzymes are highlighted in bold. (b) Comparison of the sequences of the lids of lipases from *Pseudomonas fragi* (PFL), *Burkholderia cepacia* (BCL), *Pseudomonas aeruginosa* (PAL) and *Burkholderia glumae* (BGL). Aminoacids subjected to mutagenesis in PFL are in bold. (c) Alignment of the structurally homologous sequences of *Bacillus subtilis* lipase A (BSLA) with the lid regions of its variants, generated by insertion of the lid sequences of *Fusarium solani pisi* cutinase (CUTIlip), *Penicillium purpurogenum* acetylxylane esterase (AXElip) and human pancreatic lipase (HPlip). The lid regions of CUTIlip and AXElip as well as the corresponding amino acids in BSLA are indicated in capital bold characters. In the same manner, the corresponding amino acids of BSLA and HPlip are indicated.

Table 2
Enhancement of activity of CRL1 and CRL1LID3 after bioimprinting with *N*-octyl-β-D-glucopyranoside

Enzyme	Enhancement factor[a]
wtCRL1	11.4
CRL1LID3	2.4

[a]Transesterification activity was determined at $a_w = 0.53$ using chloro ethyl 2-hydroxy hexanoate as the substrate, methanol as the nucleophile, petroleum ether as the solvent, under reaction conditions identical to those employed for the non-treated enzyme. For the bioimprinting procedure, see Ref. [14].

hydration states of the two proteins and pointing to the lid as involved in the lower catalytic activity of the chimera.

Several authors have attributed the lower activity of lipases in organic solvents than in aqueous media to the possibility that the enzymes are in the conformation with the lid closed (inactive). According to this hypothesis, the lower activity of CRL1LID3 could be ascribed to a smaller fraction of enzyme molecules trapped in the open (active) conformation. This point was investigated by applying bioimprinting [23], an approach that relies on treatment of lipase molecules with detergents that favor the open enzyme conformation. Such conformation is retained upon lyophilization giving rise to a population of activated enzymes, when the preparation is resuspended in organic solvent. The lower enhancement factor observed for CRL1LID3 (Table 2) imprinted with *N*-octyl-β-D-glucopyranoside, is likely to be a consequence of a lower propensity of the chimera enzyme to shift from the closed to the open form.

Besides activity, also enantioselectivity was found to be affected by lid replacement when tested with the racemic substrate choro ethyl 2-hydroxy hexanoate in both isooctane and hexane at different a_w-values. In particular, CRL1LID3 showed a lower *E*-value than wtCRL1 in all tested conditions (Fig. 2). Interestingly, Colton et al. [24] have suggested that the increase of enantioselectivity of *C. rugosa* lipase observed upon treatment with 2-propanol might be due to the conversion of the enzyme conformation from the closed to the open form. This agrees with our hypothesis of a higher fraction of enzyme molecules in the open form in the case of CRL1 (that also shows higher *E*-values) compared to CRL1LID3.

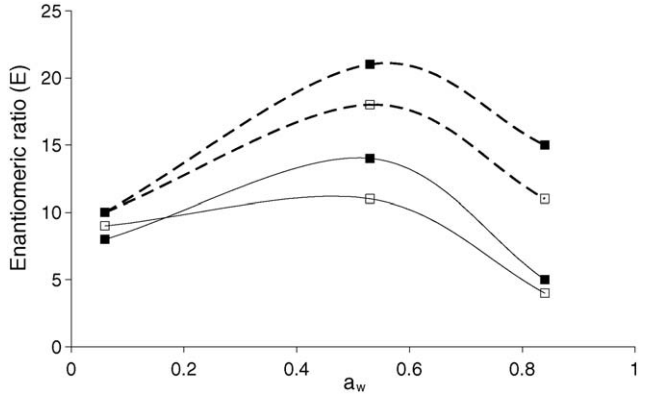

Fig. 2. Effect of water activity on the enantiomeric ratio (*E*) of wtCRL1 (dashed line) and CRL1LID3 in the kinetic resolution of a racemic mixture of 2-hydroxycaproic acid chloroethylester with methanol as nucleophile, in isooctane (□) and hexane (■). For reaction conditions, see Table 1. The *E*-values were calculated according to Ref. [25]. The conversion degree and the enantiomeric excess for *E* calculation were measured by GLC chromatography using the same conditions described in Table 1 legend.

4. Activity and stability of PLF and its lid mutants

The lipase from *P. fragi* is a cold-active enzyme as it retains 59% of its activity at 10 °C. At the same time, it is unstable at moderate temperatures with a half life of ca. 4 h at 29 °C. A further peculiar feature of this enzyme is a marked selectivity for short chain substrates [17]. Targets for mutagenesis were selected in the lid region based on the comparison with the sequences of homologous lipases differing from PFL both in thermostability and substrate preference. The alignment shown in Fig. 1b suggested to address residues conserved in the reference lipases, in particular at positions 137 and 138 where in PFL a polar threonine residue substitutes valine and asparagine, respectively, and position 141, where serine substitutes a glycine residue conserved in other lipases. Substrate specificity of PFL variants was assayed on triglycerides of growing chain length, i.e. tributyrin (C4), tricaprylin (C8) and trilaurin (C12). We noticed that substitutions T137V and T138N increased the relative activity on C8 substrates whereas no specific effects were produced by the substitution S141G [18]. The same proteins were further assayed for stability at 29 and 37 °C. Cold-adapted enzymes in fact often display high conformational flexibility as a molecular adaptation allowing them to counteract the decrease of catalytic efficiency at low temperature through low-energy cost interactions between the enzyme active site and the substrates [25]. Such flexibility causes low temperature stability.

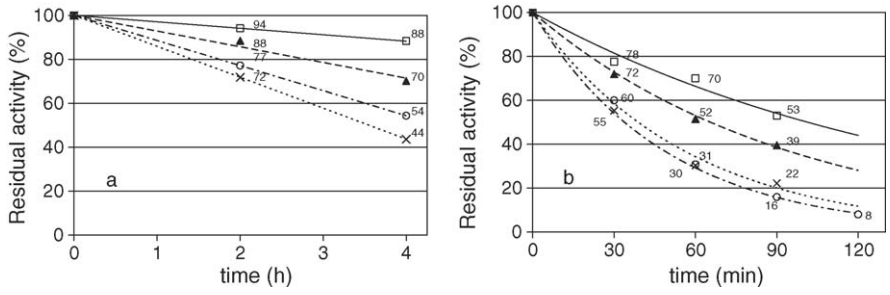

Fig. 3. Temperature-dependent loss of activity of wtPFL and mutants as measured in the hydrolysis of tricaprylin at 29 °C (a) and 37 °C (b). (◯) wt; (□) T137V; (▲) T138N; (×) S141G.

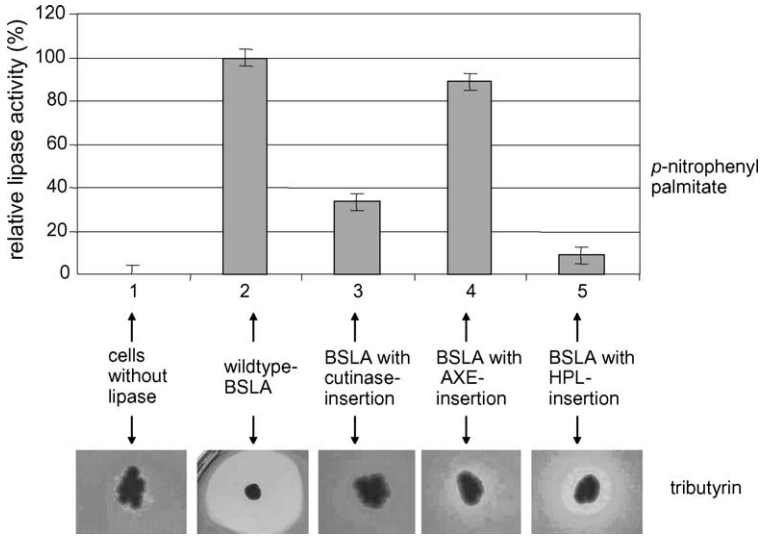

Fig. 4. Activity of BSLA and its lid-variants with the substrates *p*-nitrophenyl palmitate and tributyrin [17].

wtPFL and mutants were preincubated at either 29 or 37 °C and then assayed for activity at 29 °C, the T_{opt} of the enzyme. After 4 h incubation at 29 °C activity of wtPFL on tricaprylin was reduced by 50%, whereas mutants T137V and T138N retained 90 and 70% activity, respectively. At the less permissive temperature of 37 °C, differences become still more obvious (Fig. 3). Substitution S141G is destabilizing, possibly because it increases protein flexibility.

5. Activity and enantioselectivity of wtBSLA and its lid mutants

The BSLA-variants CUTIlip, AXElip and HPlip (Fig. 1c) were first assayed for enzymatic activity with the substrates *p*-nitrophenyl palmitate and tributyrin (Fig. 4). All of them showed enzymatic activity, at least against one of the substrates, although the variants containing artificial lid-structures exhibited a lower activity than the wild type [19].

BSLA is fairly active in organic solvent (petroleum ether). Instead the mutants have a modest activity in the transesterification reaction between sulcatol and vinylacetate and are inactive in the alcoholysis of chloro ethyl 2-hydroxy hexanoate with methanol (Table 3).

In the transesterification reaction between sulcatol and vinylacetate, carried out with the enzymes co-lyophilized with PEG (Table 3), after 260 h the degree of conversion (product formation) in the case of BSLA was about 30% and the enatiomeric ratio (*E*-value) was 10.2. The highest conversion degrees obtained in the case of BSLA lid-mutants were 14, 14 and 10% for CUTIlip, AXElip and HPlip, respectively; the negative control was 4%. The different conversion yields observed after 260 h should depend on different enzyme activities and stabilities. The results obtained with BSLA mutants with the substrate sulcatol are at least in part due to the mutated enzymes since both the reaction rates (Table 3) and the final conversion degree were higher than that observed in the case of the negative control, where an endogenous hydrolase activity might be present in the host microorganism. Because of the interference of the transesterification activity of the host microorganism and of the low activity values, a precise estimation of the BSLA lid-mutants enantioselectivity was not possible. It is interesting to note that the BSLA is more active in the alcoholysis of chloro ethyl 2-hydroxy hexanoate (the *E*-value with this substrate was 4.0) than in the transesterification reaction with sulcatol. This is just the opposite of what was observed with the mutants. Co-lyophilization of all enzyme preparations with methoxy poly(ethylene glycol) (PEG), an additive usually used to preserve the protein native structure during freeze-drying, led to a substantial increase of activity (Table 3) [26].

Table 3
Transesterification rate in petroleum ether of wtBSLA and BSLA lid-mutants

Enzyme	Rate (nmol/min)	
	Sulcatol[a]	Chloro ethyl 2-hydroxy hexanoate[b]
BSLA	122 (19)	566
HPlip	14 (3)	2
AXElip	16 (3)	3
CUTIlip	18 (0.5)	2
Negative control	9 (0.4)	3

Forty milligrams of enzyme were lyophilized with 5 mg of PEG and added to a final reaction volume of 1 ml. The reaction mixture was shaken at 150 rpm and at 25 °C. The reaction progress was monitored by GLC using the same conditions described in Table 1 legend. In parentheses, the rate obtained using the same amount of enzyme lyophilized without PEG.

[a] Rate of transesterification at $a_w = 0.11$ of sulcatol (0.013 M) with vinyl acetate (0.11 M) as acyl donor.

[b] Rate of transesterification at $a_w = 0.11$ of chloro ethyl 2-hydroxy hexanoate (0.013 M) with methanol (0.25 M).

6. Conclusions

The data we present in this work indicate that the lid is involved in the modulation of lipase's functional properties as all changes introduced affected the behaviour of the target

enzymes. Activity and enantioselectivity of wtCRL1 in organic solvents were reduced by mutations in the lid sequence. On the other hand, the chimeric enzyme displayed novel specificities in aqueous solution as, for example, the ability of hydrolysing cholesterol esters. In the case of PFL, substitutions at T137 and T138 altered the chain length preference profile and increased the temperature stability of the enzyme. Insertion of lid fragments into BSLA yielded variants active in aqueous media and also in organic solvent although the activity in the latter was only 4–7% of the wild type enzyme. These results showed that the insertion of different lids into the same core-protein indeed result in different properties of the variants concerning substrate specificity. The overall decrease in activity of the variants compared to wtBSLA can be accounted by the fact that the foreign lid-structures are not as adapted to the core-protein of BSLA as they are to their host-protein structure.

In conclusion, the lid appears to play a very complex role in lipases activity, specificity and conformational stability. We have shown that protein engineering of lid structures might provide enzymes with new properties but often causes a reduction in the enzyme activity. This effect might be counteracted by adapting the modified lid to the protein context, i.e. through an approach of directed evolution.

References

[1] K. Drauz, H. Waldmann, Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook, vols. I–III, second ed., Wiley–VCH, Weinheim, 2002.
[2] K.E. Jaeger, T. Eggert, Curr. Opin. Biotechnol. 13 (2002) 390–397.
[3] M.T. Reetz, Curr. Opin. Chem. Biol. 6 (2002) 145–150.
[4] K.E. Jaeger, S. Ransac, B.W. Dijkstra, C. Colson, M. van Heuvel, O. Misset, FEMS Microbiol. Rev. 15 (1994) 29–63.
[5] R.D. Schmid, R. Verger, Angew. Chem. Int. Ed. Engl. 37 (1998) 1608–1633.
[6] R.J. Kazlauskas, U.T. Bornscheuer, in: H.J. Rehm, G. Reed, A. Pühler, P.J.W. Stadler, D.R. Kelly (Eds.), Biotechnology, Biotransformations with Lipases, vol. 8a, Wiley–VCH, Weinheim, 1998, pp. 37–191.
[7] A. Svendsen, Biochim. Biophys. Acta 1543 (2000) 223–238.
[8] P. Villeneuve, J.M. Muderhwa, J. Graille, M.J. Haas, J. Mol. Catal. B: Enzym. 9 (2000) 113–148.
[9] S. Brocca, F. Secundo, M. Ossola, L. Alberghina, G. Carrea, M. Lotti, Protein Sci. 12 (2003), 2312–2319 (and references therein).
[10] K.A. Dugi, H.L. Dichek, S. Santamarina-Fojo, J. Biol. Chem. 270 (1995) 25396–25401.
[11] M. Holmquist, I.G. Clausen, S. Patkar, A. Svendsen, K. Hult, J. Protein Chem. 14 (1995) 217–224.
[12] M. Holmquist, M. Martinelle, P. Berglund, I.G. Clausen, S. Patkar, A. Svendsen, K. Hult, J. Protein Chem. 12 (1995) 749–757.
[13] M. Martinelle, M. Holmquist, I.G. Clausen, S. Patkar, A. Svendsen, K. Hult, Protein Eng. 9 (1996) 519–524.
[14] F. Secundo, G. Carrea, C. Tarabiono, S. Brocca, M. Lotti, Biotechnol. Bioeng. 86 (2004) 236–240.
[15] D.L. Ollis, E. Cheah, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verscheuren, A. Goldman, Protein Eng. 5 (1992) 197–211.
[16] M. Nardini, B.W. Dijkstra, Curr. Opin. Struct. Biol. 9 (1999) 732–737.
[17] C. Alquati, L. DeGioia, G. Santarossa, L. Alberghina, P. Fantucci, M. Lotti, Eur. J. Biochem. 269 (2002) 3321–3328.
[18] G. Santarossa, P. Gatti Lafranconi, C. Alquati, L. De Gioia, L. Alberghina, P. Fantucci, M. Lotti, FEBS Lett. 579 (2005) 2383–2386.
[19] T. Eggert, C. Leggewie, M. Puls, W. Streit, G. Van Pouderoyen, B.W. Dijkstra, K.-E. Jaeger, Biocatal. Biotransform. 22 (2004) 139–144.
[20] C. Martinez, P. De Geus, M. Lauwereys, G. Matthyssens, C. Cambillau, Nature 356 (1992) 615–618.
[21] D. Ghosh, M. Erman, M. Sawicki, L. Puloma, D.R. Weeks, L. Naiyin, W. Pangborn, D.J. Thiel, R. Gutiérrez, J. Eyzaguirre, Acta Crystallogr. D 55 (1999) 779–784.
[22] F.K. Winkler, A. D'Arcy, W. Hunziker, Nature 343 (1990) 771–774.
[23] H. Gonzalez-Navarro, M.C. Bano, C. Abad, Biochemistry 40 (2001) 3174–3183.
[24] I.J. Colton, N. Ahmed, R.J. Kazlauskas, J. Org. Chem. 60 (1995) 212–217.
[25] G. Feller, CMLS Cell. Mol. Life Sci. 60 (2003) 648–662.
[26] F. Secundo, G. Carrea, Chem. Eur. J. 9 (2003) 3194–3199.