Cysteine residues in human lysosomal acid lipase are involved in selective cholesteryl esterase activity

Franco PAGANI, Rajalakshmi PARIYARATH, Cristiana STUANI, Rodolfo GARCIA and Francisco E. BARALLE¹

International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34012 Trieste, Italy

Human lysosomal acid lipase (LAL) catalyses the deacylation of triacylglycerol and cholesteryl esters in the acidic lysosomal compartment. Treatment of LAL with the reducing agent dithiothreitol affected the triacylglycerol and cholesteryl esterase activities differentially, suggesting the involvement of cysteine residues in determining substrate specificity. To identify the residues involved, human LAL cDNA, under the control of the T7 promoter and tagged with a herpes simplex virus coding epitope, was specifically mutated in order to introduce single amino acid substitutions of each of the six cysteine residues of mature LAL. All Cys-227 mutants showed selectively decreased activity towards cholesteryl oleate, while preserving that towards

tri-oleylglycerol. Substitutions of Cys-236, Cys-240 and Cys-244 affected catalysis towards the two substrates to a variable degree, depending on the side chain of the amino acid introduced. The replacement of Cys-41 or Cys-188 did not result in the preferential cleavage of either one of the two substrates. These data indicate that Cys-227, Cys-236, Cys-240 and Cys-244 play a crucial role in determining LAL substrate specificity. We propose that these cysteine residues are involved in the hydrolysis of cholesteryl ester by affecting selectively the access of this substrate to the catalytic active site. In addition, the fact that the catalytic activity is never completely abolished in cysteine mutants demonstrates that LAL is not a thiol enzyme.

INTRODUCTION

Lysosomal acid lipase (LAL) is the only lysosomal hydrolase that catalyses the deacylation of both cholesteryl esters and triacylglycerols that have been internalized through the receptor-mediated endocytosis of lipoprotein particles [1,2]. In this process, the free cholesterol released regulates its endogenous synthesis and esterification, as well as the uptake of low-density lipoprotein [1]. The importance of LAL in the regulation of intracellular cholesterol flux is further supported by the fact that its altered function has been implicated in the development of atherosclerosis in the population at large [3,4].

LAL is a member of the acid lipase family, along with the enteric gastric and lingual lipases [5], and is distinct from the well characterized pancreatic, lipoprotein and hepatic neutral lipases [6]. The cloned cDNA sequence predicts a 378-amino-acid protein containing a 27-amino-acid N-terminal signal peptide and six consensus N-glycosylation sites (Asn-Xaa-Ser/Thr) [5]. Immunoelectrophoretic analysis has revealed different LAL forms, with molecular masses ranging from 41 to 54 kDa, which correspond to different levels of N-glycosylation [7,8]. LAL contains six cysteine residues at positions 41, 188, 227, 236, 240 and 244, and an additional cysteine within the 27-amino-acid N-terminal signal peptide [5]. Cys-227, Cys-236 and Cys-244 are conserved among the acid lipase family, being present in rat [9] and mouse (F. Pagani and F. E. Baralle, unpublished work) LAL. LAL has two conserved Gly-Xaa-Ser-Xaa-Gly esterase-associated amino acid motifs centred around potential serine nucleophiles at residues 99 and 153.

LAL is unique among mammalian lipases, in that it is catalytically active towards both cholesteryl esters and triacylglycerols [2,5]. A similar dual catalysis has only been reported for a hormone-sensitive lipase that shares no sequence identity with

the acid lipases [10]. Regardless of their origin or primary structure, lipases have in common a catalytic triad composed of serine, histidine and aspartate or glutamate that has been deduced from crystallographic studies [11–16]. The residues involved in the catalytic activity of LAL are not well defined. LAL can be inactivated by chemical modification of both serine and cysteine residues, and thiols are necessary for the stability of the purified enzyme [17–20]. Gastric lipase has been shown to be a thiol enzyme on the basis of chemical modification by thiol-reactive agents [21,22]. In contrast, the calf pregastric esterase has been reported to contain no functional thiol group [23]. Using a baculovirus expression system, Ser-153 has been shown to be important for the catalytic activity of LAL, even if it is not the actual catalytic nucleophile [20].

We have carried out an extensive functional study of site-directed mutants of LAL in order to clarify the role of cysteine residues in substrate specificity and catalysis. Our results exclude the possibility that LAL is a thiol enzyme, and indicate essential roles for four cysteine residues in the preferential hydrolysis of cholesteryl esters.

MATERIALS AND METHODS

Chemicals

Cholesteryl [¹⁴C]oleate and tri[¹⁴C]oleylglycerol were obtained from Amersham. Restriction and modification enzymes were obtained from Boehringer Mannheim or Pharmacia Biotech. *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulphate (DOTAP) liposomes and proteinase inhibitors (Complete®) were from Boehringer Mannheim. A herpes simplex virus (HSV)-tag monoclonal antibody was from Novagen (Madison, WI, U.S.A.), and alkaline phosphatase-conjugated goat anti-mouse IgG was from DAKO. Recombinant N-Glycanase F was

Abbreviations used: LAL, lysosomal acid lipase; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulphate; HSV, herpes simplex virus; DTT, dithiothreitol.

¹ To whom correspondence should be addressed.

Figure 1 Schematic representation of LAL constructs

The open rectangle indicates the coding portion of LAL, and the black and shaded rectangles represent the glycine-rich region and the HSV tag epitope respectively. The T7 promoter is indicated by an arrow. The positions of cysteine residues, numbered according to [5], are indicated.

from BioLabs. G418 was from Life Technology (Gaithersburg, MD, U.S.A.). Recombinant vaccinia virus vTF7-3, bearing the bacteriophage T7-1 gene, was generously donated by T. Fuerst and B. Moss (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, U.S.A.) [24]. All other chemicals were from Sigma.

Construction of LAL cDNA mutants

The human LAL expression vector pLALtag [25] was used to construct all mutants. This construct contains, under the control of the T7 promoter, the entire coding portion of LAL in-frame with a five-amino-acid connecting segment (GSGGG) and an 11-amino-acid coding portion for the tag (QPELAPEDPED) derived from HSV glycoprotein D (Figure 1) [25]. All mutants were created using the PCR-based overlap extension method [26], and sequences were verified by the dideoxy chain-termination method using [α - 35 S]dATP and modified T7 DNA polymerase.

Mutagenic oligonucleotides

The sequences of the oligonucleotides used for mutagenesis are indicated below, from 5' to 3'. Changes compared with the original sequence are underlined. C41A, ATTCTGGCCCTTA-ACCGATTCTCATGG; C41S, ATTCTGTCCCTTAACCGATTCTCATGG; C41F, ATTCTGTTCTTAAACCGATTCTCATGG; C188A, GTCGCCTTCGCTACTAGCCCT; C188F, GTCGCCTTCTTACTAGCCCT; C227A, GGCTGGTACCCACGTTGCCACTCATGTC; C227S, GGCTGGTACCCACGTTTCCACTCATGTC; C227F, GGCTGGGTACCCACGTTTCACTCATGTC; C236A, AAGGAGCTCGCTGGAATCTCTG; C236S, AAGGAGCTCTCTGGAATCTCTG; C236F, AAGGAGCTCTTGGAAATCTCTG; C240A, AAGGAGCTCTGTGGAATCTCGCTTTTCTTC; C240S, AAGGAGCTCTGTGGAATCTCTCTTTTCTTC; C240F, AAGGAGCTCTGTGGAATCTCTTTTTTCTTC; C244A, AAGGAGCTCTGTGGAATCTCTGTTTCTTCTGGCTGGATT; C244S, AAGGAGCTCTGTGGAATCTCTGTTTCTTCTGTCTGGATT; C244F, AAGGAGCTCTGTGGAATCTCTGTTTCTTCTGTGTTGGATT.

Transient expression of LAL mutants in HeLa cells, and determination of cholesteryl esterase and triacylglycerol lipase activities

Expression of LAL mutants and determination of acid lipase activity were performed as described previously [8,25]. HeLa cells were grown in Dulbecco's modified Eagle's medium con-

taining 10% (v/v) foetal bovine serum and 500 μ g/ml gentamycin. Cells at 80% confluence were infected with vTF7-3 and transfected with each mutated LAL plasmid DNA complexed with cationic DOTAP liposomes. Cells were harvested at 16–24 h after infection/transfection. In each experiment, controls consisting of vaccinia-infected cells transfected with plasmid pBS and of non-infected cells were included. After being harvested, cells were resuspended in 100 mM sodium acetate buffer (pH 4.8) containing the protease inhibitors leupeptin (15 μ g/ml), aprotinin (15 μ g/ml), pepstatin (5 μ g/ml), PMSF (0.25 mM) and EGTA (0.7 mM), at 0 °C. Cell extracts were prepared by sonication for 10 s at an amplitude of 10 μ m (Soniprep 150; MSE), with the subsequent addition of Triton X-100 [0.2% (w/v) final concentration].

HeLa cell extracts (30–50 μ g of protein), normalized for the amount of material immunoreactive to the HSV tag (see below), were incubated in 100 mM sodium acetate buffer (pH 4.8), 0.2% (w/v) Triton X-100, 0.2 mM dithiothreitol (DTT) and either cholesteryl [14 C]oleate (0.2 mM; 5 μ Ci/ml) or tri[14 C]oleylglycerol (0.3 mM; 5 μ Ci/ml) in a total volume of 0.1 ml. After incubation at 37 °C for 30 min, the reaction product ([14 C]oleate) was separated by extraction of the reaction mixtures with benzene/chloroform/methanol (10:5:12, by vol.) containing 0.3 mM oleic acid, as described by Pittman et al. [27]. The aqueous upper phases containing the partitioned [14 C]oleate were counted for β -radioactivity. Cholesteryl oleate esterase and trioleylglycerol hydrolase activities (pmol of oleate released/h per μ g of protein) of mutant enzymes were expressed as percentages of those of wild-type LAL.

The effects of the reducing agent DTT on cholesteryl esterase and triacylglycerol lipase activities were analysed by incubating total cell extracts with concentrations of the dithiol up to 100 mM. The choice of such a wide concentration range was based on the fact that, on one hand, LAL has been reported to be stabilized by 1 mM DTT [17,28] and, on the other, the cleavage of disulphide bridges of a number of enzymes requires high concentrations of DTT [29]. Cell extracts were resuspended in PBS containing 0.2% (w/v) Triton X-100 in the presence of protease inhibitors, and sonicated for 2 $\times$ 5 s at 6 μ m amplitude in ice. After incubation with DTT for 1 h at room temperature, 0.1 vol. of 1 M sodium acetate buffer (pH 4.8) was added, and cholesteryl esterase and triacylglycerol lipase activities were determined. Results were expressed as percentages of the LAL activity in cell extracts incubated without DTT. In order to exclude possible interference with the lipid substrates, DTT at concentrations of up to 100 mM was included in the standard incubation mixtures (pH 4.8). No significant effect on the activities was observed.

Immunoblotting and endoglycosidase treatment

Extracts of HeLa cells (10 μ g of protein) expressing wild-type or mutant LALs were resuspended in denaturing sample buffer containing 4% SDS and 3% DTT, heated at 100 °C for 5 min, subjected to electrophoresis on 12% polyacrylamide gels, and Western blotted. Tag antigens were detected using a HSV-tag monoclonal antibody followed by an alkaline phosphatase-conjugated goat anti-mouse IgG.

For endoglycosidase treatment, cell extracts (30–50 μ g of protein) were resuspended in 100 mM sodium acetate buffer (pH 4.8), 0.2% (w/v) Triton X-100 and 0.2 mM DTT, and incubated at 37 °C with 2500 units of N-Glycanase F for 12 h.

Production of a stable clone expressing human LAL

The entire coding region of human LAL tagged with the HSV epitope derived from pLALtag [25] was subcloned in the ex-

pression vector pKG5 under the control of the simian virus 40 promoter. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum and 500 $\mu\text{g/ml}$ gentamycin, and transfected by the calcium phosphate method [30]. G418 was used for selection at a concentration of 800 $\mu\text{g/ml}$. Clones were obtained by limiting dilution, and recombinant human LAL production was assayed by immunoblot analysis with anti-(HSV tag) antibody and by determining acid lipase activity.

RESULTS

Since LAL is ubiquitously expressed in mammalian cells, the recombinant proteins tested in the present study were distinguished from the endogenous ones using a convenient epitope tagging, as described previously (Figure 1) [25]. In order to characterize the substrate specificity of LAL, we generated a stable line with a high level of activity. Normal LAL tagged at the C-terminus with an HSV epitope was cloned under the control of the simian virus 40 promoter and transfected into COS-7 cells. The cholesteryl esterase and triacylglycerol lipase activities of the stable clone were approx. 70 times higher than those in mock-transfected cells (1005 compared with 14.8 pmol/h per μg for triacylglycerol lipase, and 296 compared with 4.4 pmol/h per μg for cholesteryl esterase).

Effect of the reducing agent DTT on substrate specificity

Treatment of COS cell extracts stably expressing LAL with the reducing agent DTT over a wide range of concentrations showed that the hydrolytic activities towards the two substrates (cholesteryl ester and triacylglycerol) could be differentially affected (Figure 2). This suggested that cysteine residues are involved in substrate specificity. Concentrations of DTT of up to 2.5 mM did not change the substrate specificity of the enzyme significantly, and only a modest decrease in triacylglycerol lipase activity was observed between 0.2 and 1 mM DTT. This is in agreement with previous reports showing that 1 mM DTT stabilizes LAL [7,28]. In contrast, incubation of recombinant LAL with concentrations of DTT higher than 5 mM produced a

Figure 2 Effect of DTT on the lipolytic activity of recombinant LAL

Cellular extracts from COS cells expressing human LAL were incubated with different concentrations of DTT. The enzymic activity was determined with [^{14}C]cholesteryl oleate (●) or [^{14}C]trioleilglycerol (■) as substrate. Results are expressed as percentages of the activity of samples incubated without DTT, and are means $\pm$ S.D. of three experiments.

Figure 3 Cysteine LAL mutants expressed in HeLa cells

(A) Hydrolysis of trioleylglycerol (■) and cholesteryl oleate (□) by extracts of T7-vaccinia-infected HeLa cells expressing wild-type or cysteine-mutated LALs. Activities are expressed as percentages of those of wild-type LAL, and are means $\pm$ S.D. of at least three independent experiments. (B) Immunoblots of selected cysteine mutants. Extracts from T7-vaccinia-infected HeLa cells were subjected to 12%-PAGE. The resulting immunoblots were probed with anti-(HSV tag) monoclonal antibody. (C) Immunoblots of the C240S mutant. HeLa cell extracts were digested or not with N-Glycanase F (PNGase F), immunoblotted and probed with anti-(HSV tag) monoclonal antibody. The estimated molecular masses (kDa) of the different forms detected are indicated.

significant, selective inhibition of cholesteryl esterase activity, whereas the triacylglycerol lipase activity increased slightly. The same effect of DTT was observed on the acid lipase activity of extracts from non-transfected COS and HeLa cells (results not shown).

Effect of cysteine mutations on substrate specificity

In order to investigate the role of thiol groups in the preferential substrate specificity of LAL, we generated mutant enzymes in which the cysteine residues at positions 41, 188, 227, 236, 240 and 244 were altered. These variants were transiently expressed in HeLa cells using the T7 expression system and assayed for the presence of HSV-tag immunoreactive material, as determined by Western blotting, and for catalytic activity towards trioleylglycerol and cholesteryl oleate.

The specific activities of the six site-directed cysteine mutant LAL enzymes towards triacylglycerol and cholesteryl oleate are compared in Figure 3. Differing abilities to hydrolyse triacylglycerol as compared with cholesteryl ester were observed for some of the mutants. Changing Cys-227 to Ser, Phe or Ala resulted in recombinant enzymes with a cholesteryl esterase activity that was significantly decreased compared with the hydrolysis of triolein. Changing Cys-236 to Ala or Phe resulted again in a decrease of mainly cholesteryl esterase activity; this

was more evident for mutant C236A. In contrast, mutant C236S showed proportional decreases in the two activities. Two out of three Cys-240 mutants, i.e. C240A and C240S, showed a preferential loss of cholesteryl oleate hydrolase activity. They retained 19% and 5% respectively of activity towards cholesteryl oleate, whereas triacylglycerol hydrolase activity was 95% and 18% respectively of that of wild-type LAL. In contrast, replacement of Cys-240 with Phe resulted in an overall decrease in enzyme activity, with a preferential inhibition of triacylglycerol lipase. In this particular case the enzyme was 4 times more active towards cholesteryl ester than with the triolein. The substitution of Phe for Cys at position 244 resulted in a dramatic decrease in cholesteryl oleate activity, while the hydrolysis of triacylglycerol was 50% of that of the wild type. Changing Cys-244 to Ser or Ala affected both activities proportionally, without modifying the substrate specificity. Amino acid substitutions of the other two cysteine residues, i.e. Cys-41 and Cys-188, did not change the substrate specificity; some of these mutants (C188A, C188F and C41F) showed proportional decreases of both triacylglycerol lipase and cholesteryl esterase activities, whereas others (C41S and C41A) had activities similar to those of the wild-type enzyme. The effect of DTT on cholesteryl esterase and triacylglycerol lipase activities was analysed in mutants C41A, C227A, C240A and C244F. C41A showed a selective inhibition by DTT of the cholesteryl esterase activity, as expected from the fact that this mutant is indistinguishable from normal LAL (Figure 3). In contrast, substrate specificity was not affected significantly by DTT treatment of the mutants with low cholesteryl esterase activity (results not shown).

Changes in the electrophoretic pattern of HSV-tagged immunoreactive material were observed only for the mutant C240S, indicating that the site-directed mutagenesis of Cys-227, Cys-236 and Cys-244 did not affect either N-glycosylation or other post-translational processing (Figure 3B). C240S showed a higher-molecular-mass form of 56 kDa (Figure 3C). This Cys → Ser substitution creates an additional N-glycosylation site at Asn-238 (Asn-Leu-Cys to Asn-Leu-Ser) which is effectively occupied, as shown by digestion with N-Glycanase F (Figure 3C). This treatment produced the usual unglycosylated 42 kDa form (Figure 3C), confirming that N-glycosylation at an additional site was responsible for the 56 kDa form of the C240S mutant. In order to evaluate the effect of the additional N-glycosylation on enzymic activity, we analysed the cholesteryl esterase and triacylglycerol lipase activities of deglycosylated C240S. Deglycosylation reduced both enzymic activities by 50% (results not shown), as is the case for wild-type LAL [8], and the selective loss of cholesteryl esterase activity was maintained. This suggests that the changes in substrate specificity of mutant C240S are not due to the presence of an additional N-glycosylation site.

DISCUSSION

In the acidic lysosomal compartment, the hydrolysis of both the relatively small and flexible triacylglycerol molecule and the more rigid structure of the cholesteryl ester is performed exclusively by LAL [2]. In order to assess how modifications in protein structure may affect substrate specificity, recombinant LAL expressed in COS cells was treated with DTT, a thiol-reactive agent that reduces disulphide bridges. We found that the triacylglycerol lipase and cholesteryl esterase activities were differentially affected by DTT (Figure 2), indicating that the stability of disulphide bonds is important for substrate specificity. In order to identify the residues involved, we changed each of the cysteines present in LAL by site-directed mutagenesis. The results obtained show that four of these six residues (Cys-227, Cys-236,

Cys-240 and Cys-244) are involved in conferring specificity towards the two classes of lipid substrates (Figure 3). In the case of Cys-227 and Cys-240, all mutants analysed showed changed substrate specificity. At positions 236 and 244, not only the presence of cysteine but also the nature of the replacing amino acid seems to determine substrate specificity. The fact that both DTT treatment and site-directed mutation modify the substrate specificity of LAL raises the possibility of the formation of disulphide bridges involving Cys-227, Cys-236, Cys-240 and Cys-244.

In addition, the activity of the site-directed mutants indicates that the thiol groups of LAL are not directly involved in catalysis. In fact, the substitution of cysteines with residues such as alanine or phenylalanine does not result in a completely inactive enzyme. It is interesting to note that a similar absence of functional thiol groups has been observed recently in the calf pregastric esterase [23], a member of the acid lipase family, whereas dog, rabbit and human gastric lipases have been shown to be thiol enzymes [21,22].

Altogether, our results clearly indicate that cysteine residues, although not directly involved in catalysis, play a crucial role in determining lipase substrate specificity in LAL. This is the first time that a selective alteration of cholesteryl esterase activity has been reported for a lipase. In the absence of both information on the three-dimensional structure of acid lipases and sequence similarity to other lipases, it is not possible to reach conclusions about the possible structural basis of substrate specificity. In the case of human hepatic and lipoprotein lipases, a loop covering the catalytic site (the lid) has been shown to be critical for both the interaction with lipid substrates [31] and a preferential activity towards triacylglycerols or phospholipids [32,33]. The binding of some lipases to the water/oil interface of substrate micelles has been postulated to result in the movement of the lid, exposing a hydrophobic surface through which access to the catalytic pocket is gained [11,12,31]. Changes in substrate specificity may be the result of modifications of the lid structure or of the hydrophobic area. In the well characterized lipoprotein and hepatic lipases, the lid, composed of 22 amino acids, is stabilized by a disulphide bond. The four cysteines involved in substrate specificity that we have identified in LAL could stabilize similar structures through the formation of one or more disulphide bridge(s). The disappearance of these putative bridges, either through the action of DTT or by mutagenesis of cysteine residues, could change the protein conformation, modifying the loop or the hydrophobic area close to the catalytic site. Access of the substrate to the active site would be affected to a greater extent in the case of the rigid structure of cholesteryl ester than in the case of the more flexible triacylglycerol.

On the other hand, the direct participation of free cysteines in specific lipid binding cannot be excluded. In two unrelated proteins, the glucocorticoid receptor and 3 α -hydroxysteroid dehydrogenase, a single cysteine residue has been identified as mediating steroid binding [34,35], suggesting that cysteine residues may be essential constituents of a common steroid binding cavity present in different proteins. Three-dimensional structural data will be necessary in order to evaluate the role of LAL cysteines in substrate binding or in allowing access to the catalytic site.

REFERENCES

- 1 Brown, M. S. and Goldstein, J. L. (1986) *Science* **232**, 34–47
- 2 Assman, G. and Seedorf, U. (1995) in *The Metabolic Basis of Inherited Diseases*, vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 2563–2587, McGraw-Hill, New York
- 3 Coates, P. M., Langer, T. and Cortner, J. A. (1986) *Atherosclerosis* **62**, 11–20

- 4 Yatsu, F. M., Hagemenas, F. C., Manauha, L. C. and Galambos, T. (1980) *Lipids* **15**, 1019–1022
- 5 Anderson, R. A. and Sando, G. N. (1991) *J. Biol. Chem.* **266**, 22479–22484
- 6 Hide, W. A., Chan, L. and Li, W. H. (1992) *J. Lipid Res.* **33**, 167–178
- 7 Sando, G. N. and Henke, V. L. (1982) *J. Lipid Res.* **23**, 114–123
- 8 Pariyarath, R., Pagani, F., Stuaní, C., Garcia, R. and Baralle, F. E. (1996) *FEBS Lett.* **397**, 79–82
- 9 Nakagawa, H., Matsubara, S., Kuriyama, M., Yoshidome, H., Fujiyama, J., Yoshida, H. and Osame, M. (1995) *J. Lipid Res.* **36**, 2212–2218
- 10 Langin, D., Laurell, H., Holst, L. S., Belfrage, P. and Holm, C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4897–4901
- 11 Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Høge-Jensen, B., Patkar, S. A. and Thim, L. (1991) *Nature (London)* **351**, 491–494
- 12 Brady, L., Brzozowski, A. M., Derewenda, Z. S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J. P., Christiansen, L., Høge-Jensen, B., Nørskov, L. et al. (1990) *Nature (London)* **343**, 767–770
- 13 Winkler, F. K., D'Arcy, A. and Hunziker, W. (1990) *Nature (London)* **343**, 771–774
- 14 Schrag, J. D., Li, Y., Wu, S. and Cygler, M. (1991) *Nature (London)* **351**, 761–764
- 15 Lowe, M. E. (1992) *J. Biol. Chem.* **267**, 17069–17073
- 16 Beer, H. D., Wohlfahrt, G., McCarthy, J. E. G., Schomburg, D. and Schmid, R. D. (1996) *Protein Eng.* **9**, 507–517
- 17 Sando, G. N. and Rosenbaum, L. M. (1985) *J. Biol. Chem.* **260**, 15186–15193
- 18 Rindler-Ludwig, R., Patsch, W., Sailer, S. and Braunsteiner, H. (1977) *Biochim. Biophys. Acta* **488**, 294–304
- 19 Burton, B. K. and Mueller, H. W. (1980) *Biochim. Biophys. Acta* **618**, 449–460
- 20 Sheriff, S., Du, H. and Grabowski, G. A. (1995) *J. Biol. Chem.* **270**, 27766–27772
- 21 Gargouri, Y., Moreau, H., Pieroni, G. and Verger, R. (1988) *J. Biol. Chem.* **263**, 2159–2162
- 22 Gargouri, Y., Moreau, H., Pieroni, G. and Verger, R. (1989) *Eur. J. Biochem.* **180**, 367–371
- 23 Timmermans, M. Y. J., Reekmans, G. and Kupers, L. P. M. (1996) *Biochem. J.* **314**, 931–936
- 24 Fuerst, T. R., Earl, P. L. and Moss, B. (1987) *Mol. Cell. Biol.* **7**, 2538–2544
- 25 Pagani, F., Garcia, R., Pariyarath, R., Stuaní, C., Gridelli, B., Paone, G. and Baralle, F. E. (1996) *Hum. Mol. Genet.* **10**, 1611–1617
- 26 Higuchi, R., Krummel, B. and Saiki, R. K. (1988) *Nucleic Acids Res.* **16**, 7351–7367
- 27 Pittman, R. C., Khoo, J. C. and Steinberg, D. (1975) *J. Biol. Chem.* **250**, 4505–4511
- 28 Ameis, D., Merkel, M., Eckerskorn, C. and Greten, H. (1994) *Eur. J. Biochem.* **219**, 905–914
- 29 Creighton, T. E. (1990) *Protein Structure: A Practical Approach*, pp. 155–167, IRL Press, Oxford
- 30 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 31 Dugi, K. A., Dichek, H. L., Talley, G. D., Brewer, B. H. and Santamaria-Fojo, S. (1992) *J. Biol. Chem.* **267**, 25086–25091
- 32 Dugi, K. A., Dichek, H. L. and Santamaria-Fojo, S. (1995) *J. Biol. Chem.* **270**, 25396–25401
- 33 Kobayashi, J., Applebaum-Bowden, D., Dugi, K. A., Brown, D. R., Kashyap, V. S., Parrott, C., Duarte, C., Maeda, N. and Santamarina-Fojo, S. (1996) *J. Biol. Chem.* **271**, 26296–26301
- 34 Pawlowski, J. E. and Penning, T. M. (1994) *J. Biol. Chem.* **269**, 13502–13510
- 35 Chen, D. and Stallcup, M. R. (1994) *J. Biol. Chem.* **269**, 7914–7918