Heparinase I from *Flavobacterium heparinum*

ROLE OF POSITIVE CHARGE IN ENZYMATIC ACTIVITY*

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Heparinases are bacterial enzymes that are powerful tools to study the physiological roles of heparin-like complex polysaccharides. In addition, heparinases have significant therapeutic applications. We had proposed earlier that cysteine 135 and histidine 203 together form the catalytic domain in heparinase I. We had also identified a heparin binding domain in heparinase I containing two positively charged clusters HB-1 and HB-2 in a primary heparin binding site and other positively charged residues in the vicinity of cysteine 135. In this study, through systematic site-directed mutagenesis studies, we show that the alteration of the positive charge of the HB-1 region has a pronounced effect on heparinase I activity. More specifically, site-directed mutagenesis of K199A (contained in HB-1) results in a 15-fold reduction in catalytic activity, whereas a K198A mutation (also in HB-1) results in only a 2- to 3-fold reduction in heparinase I activity. A K132A mutation, in close proximity to cysteine 135, also resulted in reduced (8-fold) activity. Heparin affinity chromatography experiments indicated moderately lowered binding affinities for the K132A, K198A, and the K199A mutant enzymes. The above results, taken together with our previous observations, lead us to propose that the positively charged heparin binding domain provides the necessary microenvironment for the catalytic domain of heparinase I. The dominant effect of lysine 199 suggests an additional, more direct, role in catalysis for this residue.

Heparin-like glycosaminoglycans (HLGAGs) play an intricate role in the extracellular matrix, regulating a wide variety of biological functions (1, 2). HLGAGs are highly sulfated, complex, acidic polysaccharides consisting of alternating uronic acid (L-iduronic or D-glucuronic acid) and D-glucosamine residues connected through 1–4 linkages. Variations in the degree and distribution of sulfation result in a high degree of chemical heterogeneity in HLGAGs. Three enzymes that degrade HLGAGs (heparin and heparan sulfate), viz., heparinases I, II, and III from *Flavobacterium heparinum*, recognize unique sequences of sulfation and uronic acid epimerization in HLGAGs with a high degree of specificity (3–5). Heparinases have important clinical applications such as in the monitoring of heparin levels in blood (approved by the FDA) (6), neutralization of heparin in blood (in phase III clinical trials), and in production of low molecular weight heparins for use in humans. In addition, heparinases I and III are potent inhibitors of neovascularization (7). More importantly, heparinases have proven to be useful tools in understanding the important physiological roles of HLGAGs (8, 9).

Our group has cloned, sequenced, and expressed in *Escherichia coli* the genes for heparinases I, II, and III from *F. heparinum* (10–13). We subsequently carried out extensive biochemical studies to investigate structure-activity relationships of heparinase I and to understand the mechanism of heparinase degradation (14–16). Earlier, we showed that cysteine 135, in a highly positively charged environment, is catalytically active in heparinase I (14). We proposed that one possible role of the positively charged active site would be to lower the $pK_a$ of the cysteine such that it is present as a thiolate anion in the enzyme active site. As an anion, cysteine 135 could initiate catalysis by acting as a base for proton abstraction from the substrate heparin. In another study, using a combination of chemical and proteolytic digests of heparinase I in direct binding and competition assays, we identified and mapped a primary heparin binding site in heparinase I spanning residues 196–221 of the heparinase I primary sequence (15). This region contains two positively charged clusters (residues 197–204 and 207–212) as well as a calcium binding consensus motif (residues 207–220) (15). Interestingly, these positively charged clusters conform to the Cardin-Weintraub heparin binding consensus sequence (17). In a parallel study, we showed that histidine 203, contained in one of the positive clusters of the primary heparin binding site, is critical for catalytic activity in heparinase I (16). This provided compelling evidence for the positively charged heparin binding site being in close proximity to the active-site cysteine 135, and hence we proposed that cysteine 135 and histidine 203 together form part of the catalytic domain in heparinase I (16).

The above taken together raises the question as to the specific role(s) of the positively charged clusters in heparinase I. Potentially, the basic clusters in the heparin binding site could either provide the necessary charge complementarity for specific heparin binding and/or bias the active-site reactivity (15). Alternatively, given the proximity of these residues to cysteine 135 and histidine 203, specific residue(s) within the putative heparin binding site could play a more direct role in catalysis. In this study we address the role of positive charge in the primary heparin binding site in heparinase I activity using extensive site-directed mutagenesis experiments.
Role of Positive Charge in Catalysis of Heparinase I

The primary heparin binding site (residues 196–221) and tryptic peptide 4 (Td 4; residues 132–141) that were found to bind heparin in our earlier study (15) were chosen as targets for mutagenesis studies. The positive charge in the primary heparin binding site was altered in the following manner. Basic residues from the two consensus sequences, lysines 198 and 199 (HB-1) and lysines 208 and 209 (HB-2), were first jointly altered to alanines (neutral), aspartic acids (negative charge), and arginines (positive charge); lysine 211 (HB-2) was changed to an alanine, whereas the catalytically active histidine 203 (HB-1) was altered to an alanine in an earlier study (16). Since the double mutants in HB-1 affected enzyme activity, lysines 198 and 199 were also individually changed to alanines to examine the possibility of one of the residues having a dominant effect on catalytic activity. Asparagine 200 was changed to a lysine to increase the positive charge in HB-1 and to an alanine to probe the functionality of this residue. Other basic residues in the heparin binding site, viz., lysines 205 and 214, were also individually altered to alanines. Finally, to investigate the role of the two positively charged residues, lysine 132 and arginine 141, in tryptic peptide 4, they were individually altered to alanines.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**

Heparin (porcine intestinal mucosa, average molecular mass of 12 kDa and activity of 157 USP units/mg) was from Hepar (Franklin, OH). Urea, dithiothreitol, and acetonitrile were from Allied Chemicals (Deerfield, IL). Other chemicals were from Mallinckrodt Chemical Works (Chesterfield, MO). Molecular mass standards were obtained from Life Technologies. E. coli BL21(DE3) host was from Novagen, WI. Molecular biology reagents and their sources are listed in the appropriate sections below.

**Heparinase I: Protein Analyses**

Heparinase I from *F. heparinum* was purified as described previously (10, 18). The purified heparinase I was collected and lyophilized (VirTis freeze mobi model 12; VirTis Inc., NY). Heparinase I used for activity measurements was extensively desalted using a Centricon P-30 (Amicon, Beverly, MA). Protein concentration was determined using Micro BCA reagent (Pierce) relative to a bovine serum albumin standard.

**Mutagenesis, Expression, and Purification of r-heparinase I**

The recombinant and mutant heparinases I were expressed without the putative *F. heparinum* leader sequence, i.e., as a construct ([−L]) r-heparinase I) that reads Met-Glu22-Glu32 (10). To facilitate purification, the heparinase I gene was expressed using the pET-15b system (Novagen). This construct has a polyhistidine tag and a thrombin cleavage site in a 21-amino-acid N-terminal leader sequence (14).

**Kinetic constants and heparin affinities of r-heparinase I and mutant enzymes**

| Enzyme       | $k_{cat}$ | $K_m$ | NaCl concentration |
|--------------|-----------|-------|--------------------|
|              | sec⁻¹     | μM    | mM                 |
| -L           | 92        | 10.2 (±2.2) | 480 (±4)          |
| K198A/K199A  | 4.7       | 5.4 (±1.6)  | 448               |
| K198D/K199D  | 4.9       | 4.2 (±1.5)  | 445               |
| K198R/K199R  | 90        | 8.4 (±1.8)  | 475               |
| N200A        | 9.1       | 8.9 (±1.8)  | 472               |
| N200K        | ND*       | ND     | 469               |
| K198A        | 35        | 8.0 (±2.0)  | 448               |
| K199A        | 6         | 7.6 (±1.9)  | 452               |
| K208A/K209A  | 68        | 9.6 (±1.4)  | 458               |
| K208D/K209D  | 90        | 9.2 (±1.5)  | 472               |
| K208E/K209R  | 94        | 9.1 (±1.5)  | 476               |
| K132A        | 12        | 8.1 (±1.8)  | 460               |
| R141A        | 82        | 7.8 (±1.7)  | 476               |

* ND, Not determined due to very little activity.

**Heparinase I Activity Assays**

**Heparin-POROS Chromatography—** About 30–40 μg of -L r-heparinase I and the various mutant enzymes were injected into a heparin-POROS (4.6 × 100 mm) column (PerSeptive BioSystems, Framingham, MA) connected to a BioCAD system (PerSeptive BioSystems). Proteins were eluted using a linear gradient of 0–1 M NaCl in 10 min (10 mM Tris, 1 mM EDTA, pH 7.0) and monitored at 210 nm. EDTA was added to chelate any calcium ions that may be present in the buffers.

**Strategy for Site-directed Mutagenesis Studies**

**FIG. 1. Schematic representation of the various heparinase I mutations undertaken for this study.** The primary heparin binding site (residues 196–221) and tryptic peptide 4 (Td 4; residues 132–141) that were found to bind heparin in our earlier study (15) were chosen as targets for mutagenesis studies. The positive charge in the primary heparin binding site was altered in the following manner. Basic residues from the two consensus sequences, lysines 198 and 199 (HB-1) and lysines 208 and 209 (HB-2), were first jointly altered to alanines (neutral), aspartic acids (negative charge), and arginines (positive charge); lysine 211 (HB-2) was changed to an alanine, whereas the catalytically active histidine 203 (HB-1) was altered to an alanine in an earlier study (16). Since the double mutants in HB-1 affected enzyme activity, lysines 198 and 199 were also individually changed to alanines to examine the possibility of one of the residues having a dominant effect on catalytic activity. Asparagine 200 was changed to a lysine to increase the positive charge in HB-1 and to an alanine to probe the functionality of this residue. Other basic residues in the heparin binding site, viz., lysines 205 and 214, were also individually altered to alanines. Finally, to investigate the role of the two positively charged residues, lysine 132 and arginine 141, in tryptic peptide 4, they were individually altered to alanines.
All mutant r-heparinases were constructed in the pET-15b expression system (Novagen, WI) and expressed in the BL21(DE3) host as described previously (14). The level of protein expression for all the r-heparinases was identical in the BL21(DE3) host (data not shown).

**RESULTS AND DISCUSSION**

**HB-1 Mutagenesis**—Table I lists the kinetic parameters obtained for wild-type r-heparinase I and all the mutant enzymes. When lysine 198 and lysine 199 from HB-1 of the heparin binding site were jointly altered to alanines (creating a double mutant K198A/K199A), the enzyme activity was drastically affected, with a 20-fold reduction in $k_{\text{cat}}$. A similar effect was observed for the K198D/K199D double mutant enzyme. Interestingly, when the lysines 198 and 199 were jointly changed to arginines (K198R/K199R), the enzyme activity remained unaltered, as observed by the $k_{\text{cat}}$ value (Table I), suggesting that the positive charge of these residues is important for heparinase I activity. When the heparin degradation reactions of the double mutants K198A/K199A and K198D/K199D were allowed to go to completion and the products were resolved using anion-exchange HPLC, the product profiles were altered when compared with the control r-heparinase I product profile (Fig. 2, a–c); a significant fraction of higher order fragments were observed. The heparin degradation product profile of the K198R/K199R double mutant (Fig. 2d) was similar to the wild-type enzyme.

Since the joint alteration of lysines 198 and 199 affected heparinase I activity, we investigated the effect of individually altering these residues to alanine to examine whether one mutation had a more pronounced effect than the other on heparinase I activity. When lysine 198 was altered to alanine, the activity ($k_{\text{cat}}$) was reduced only by about 2–3-fold (Table I), and the product profile was similar to the wild-type enzyme (Fig. 3b). However the K199A mutation resulted in an approximately 15-fold drop in activity ($k_{\text{cat}}$) when compared with wild-type r-heparinase I (Fig. 3c). This suggests that lysine 199 in HB-1 plays a dominant role in modulating the enzymatic activity of heparinase I and raises the question of a potential role of this residue in catalysis (see below).

Since the positive charge of lysines 198 and 199 were important, the effect of increasing the overall charge in HB-1 (KKNIAH) was investigated by creating a N200K mutation. Furthermore, it must be pointed out that HB-1 does not strictly conform to the Cardin-Weintraub heparin binding consensus sequence. Hence an N200K mutation would result in this region (HB-1) conforming to the ideal Cardin-Weintraub heparin binding consensus sequence and would also result in increased overall positive charge density in the region. The N200K mutation resulted in enzyme activity being diminished to below the sensitivity of our assay (Fig. 4b). Importantly, an N200A mutation resulted in unchanged enzyme activity, suggesting a nonfunctional role for asparagine 200 (Fig. 4c). These results,
coupled with the results of heparin affinity chromatography presented below, indicate that the inability of N200K to perform catalysis is not due to the fact that it binds heparin more tightly, inhibiting turnover. A likely explanation for the N200K result is that the added positive charge topologically interferes with the activities of the neighboring residues or with catalysis.

**HB-2 and Other Positive Charge Mutagenesis**—When lysines 208 and 209 were jointly changed to alanines, the enzyme activity ($k_{cat}$) was reduced by less than 1.5-fold (Table I), and the product profile was similar to wild-type heparinase I (Fig. 5). However, neither the K208R/K209R nor the K208D/K209D double mutations altered the activity and product profiles of the mutant enzymes relative to wild-type heparinase I (Fig. 5). Further, the K211A mutation (HB-2) did not affect the enzyme activity and product profile (Fig. 3d). Thus, compared with HB-1, the positive charge in HB-2 of the heparin binding site, within the limits of this study, does not seem to play a significant role in heparinase I activity. However, it must be pointed out that other residues of HB-2 are part of the putative calcium binding site in heparinase I (15). We are currently investigating the effect of this region in chelating calcium and exploring the role of different residues from the calcium binding consensus sequence in modulating heparinase I activity.

Earlier we had shown that the tryptic peptide containing cysteine 135 (tryptic peptide 4) bound heparin, and we had postulated that the heparin binding site (residues 195–221) and the basic residues (lysine 132 and arginine 141) close to cysteine 135 together constitute a heparin binding domain in heparinase I (15). Hence we sought to investigate the role of two positively charged residues, lysine 132 and arginine 141, in enzyme activity. Although the R141A mutation had no effect, the K132A mutation resulted in lowered enzyme activity (approximately 8-fold reduction in $k_{cat}$) (Table I; Fig. 6, b and c), suggesting that lysine 132 could also be important in heparinase I activity. More specifically, lysine 132, being in close proximity to cysteine 135, could be integral to enzymatic activity through heparin binding or by activating catalytic residues (see below).

The effect of the other basic residue (lysine 205) in the primary heparin binding site in the vicinity of basic cluster HB-1 was also investigated. Mutation K205A (Fig. 6d) did not affect the activity of the enzyme. In addition, a K333A/K334A mutation in the C-terminal Cardin-Weintraub consensus sequence (HB-3) also resulted in the enzyme activity and product profile being unchanged, suggesting that this region does not play a role in enzyme activity (Fig. 4d).

As pointed out earlier (14), the positive cluster around the catalytic site of heparinase I could play a role either in biasing the reactivity of the active-site residues or in binding to the anionic substrate heparin through charge interactions. To further delineate the role of the positive charge, we performed heparin affinity chromatography experiments with the various mutants as discussed below.

**Heparin Affinity Chromatography**—We have shown previously that, in the absence of calcium, native heparinase I from *F. hepaticum* binds a heparin-POROS column and can be
eluted at a salt concentration of about 500 mM (15). It should be pointed out that compared with other heparin-binding proteins, such as lipoprotein lipase or fibroblast growth factor, which elute at much higher salt concentrations of about 0.9–1.5 M NaCl (22, 23), heparinase has a lower affinity for heparin (15). We used heparin-POROS chromatography to investigate whether the mutations affected heparin binding and hence altered the elution profile. As shown in Table I, wild-type r-heparinase I elutes at a salt concentration of about 480 mM. The K198A/K199A and the K198D/K199D double mutants eluted at lower salt concentrations of about 448 and 445 mM, respectively. The individual mutants K198A and K199A also eluted at lower salt concentrations of about 448 and 452 mM, respectively. Furthermore, the K132A mutation eluted at a moderately lower salt concentration of about 460 mM. The K198/199R double mutant, however, eluted at about 475 mM, similar to wild-type enzyme. The HB-2 mutant enzymes (K208/209A, K208/209D, and K208/209R) also eluted at salt concentrations comparable to wild-type r-heparinase I (Table I). Increasing the positive charge in HB-1 (N200K) did not result in tighter heparin binding, with the mutant eluting at about 469 mM salt concentration.

Fig. 4. Anion-exchange HPLC separation of oligosaccharides. Heparin (2 mg/ml) was incubated with -L and mutant heparinases I in 100 mM MOPS buffer, 5 mM calcium acetate, pH 7.0, for 18 h as described under “Experimental Procedures.” The reaction was then subjected to anion-exchange HPLC using a POROS Q/M column with a salt gradient of 0–2 M NaCl in 10 min and monitored at 232 nm. A shows the product profile of heparin degradation by wild-type r-heparinase I, B shows the product profile of heparin degradation by N200K, C shows the product profile of heparin degradation by N200A, D shows the product profile of heparin degradation by K333A/K334A.
Heparin binding to heparinase is a relatively weak interaction, and the observed effects on heparin binding upon mutagenesis also is not dramatic. Under the experimental conditions tested, relative to wild-type heparinase I, the respective differences in heparin binding observed for the K132A, K198A, and K199A mutations are not as substantial as the differences observed in their respective catalytic activities. This suggests that the observed differences in catalytic activity cannot be explained by lowered heparin binding alone. It is possible that in addition to heparin binding, these residues play a role in maintaining the overall positively charged nature of the environment, which is essential to the catalytic domain.

The Heparin Binding Domain of Heparinase I—The data presented in this study leads us to postulate that lysine 199 is important for catalysis and raises interesting possibilities for the role of lysine 199 in catalysis. It has been proposed as a general mechanism for polysaccharide lyases (24) that an important step in the catalytic mechanism is the neutralization of the negative charge on the C-6 carboxylate anion of the uronate in heparin. This function is most likely performed by a lysine residue by the formation of a salt bridge. Based on the above and the data presented here, it is possible that lysine 199 acts...
as an acid to stabilize the negative charge developing on the carboxylate of the uronate in heparin.

We previously showed that the active-site environment around cysteine 135 is positively charged and hypothesized that the role of the positively charged residues could be to activate the thiol group for catalysis by lowering its $pK_a$ (14). In addition, it is interesting to note that for protein-tyrosine phosphatases (25), which, like heparinases, cleave highly polyanionic substrates, the active-site cysteine is stabilized as a thiolate anion by surrounding positively charged residues. The positively charged environment causes a dramatic reduction in $pK_a$ of the active-site cysteine residue (26). It is possible that lysine 199 could be one of the residues in heparinase I involved in stabilizing the thiol group of cysteine 135, as observed for the protein-tyrosine phosphatases.

The involvement of a cysteine and histidine in the heparinase I active site resembles other enzymatic depolymerization reactions for which the enzymology is much more established. Serine proteases such as chymotrypsin, trypsin, and subtilisin (acting on peptide bonds) have been shown to be members of a super gene family whose activity depends on a so-called "charge relay system" involving a catalytic triad of serine, histidine, and glutamic acid.

**Fig. 6. Anion-exchange HPLC separation of oligosaccharides.** Heparin (2 mg/ml) was incubated with -L and mutant heparinases I in 100 mM MOPS buffer, 5 mM calcium acetate, pH 7.0, for 18 h as described under "Experimental Procedures." The reaction was then subjected to anion-exchange HPLC using a POROS Q/M column with a salt gradient of 0–2 M NaCl in 10 min and monitored at 232 nm. A shows the product profile of heparin degradation by wild-type r-heparinase I, B shows the product profile of heparin degradation by K132A, C shows the product profile of heparin degradation by R141A, D shows the product profile of heparin degradation by K205A.
and aspartic acid (27). Cysteine proteases rely on a similar charge relay system with cysteine, histidine, and asparagine or aspartate (28). Thus the mechanism involving a catalytic triad has evolved as a common theme for hydrolases (which catalyze reactions accompanied with the addition of a water molecule), often supported by x-ray crystallographic structure determination. Lysases, on the other hand, catalyze elimination reactions involving removal of a group from or addition of a group to a double bond. Gacesa (24) proposed a general mechanism for polysaccharide lyases involving three steps: (i) removal of the negative charge on the carboxylate anion, (ii) a general base-catalyzed abstraction of the C-5 proton from the uronate, (iii) β-elimination of the glycosidic bond and protonation of the leaving group. Three different amino acids are proposed to participate in each of the above three steps. The above taken together and the results presented in this study suggest that cysteine 135, histidine 203, and presumably lysine 199 form the active site in heparinase I.

We have shown that the positive charge of the heparin binding site is important for heparinase I activity. Importantly, alteration of lysines 199, 198 (HB-1), and lysine 132 has a more dramatic effect compared with the positive charge of HB-2. Results from heparin affinity chromatography experiments are consistent with our earlier heparin competition experiments (15). It has been observed that in addition to linear heparin binding, heparinase I also controls heparin binding (29). In the case of lactoferrin, alteration of lysines 199, 198 (HB-1), and lysine 132 has a more dramatic effect compared with the positive charge of HB-2. Results from heparin affinity chromatography experiments are consistent with our earlier heparin competition experiments (15).

In conclusion, we postulate that the positively charged heparin binding domain of heparinase I provides the appropriate microenvironment for activating the catalytic residues (Fig. 7). The dominant effect of lysine 199 in heparinase I activity suggests an additional, more direct, role in catalysis for this residue. We propose that lysine 199 could play a role either in stabilizing the negative charge of the uronate of substrate heparin or in stabilizing the thiol group of cysteine 135. We are currently investigating the role of calcium in the catalytic mechanism of heparinase I. Thus, as in hydrolases, heparinase I could also possess a catalytic triad, and hence, this work now lays the foundation to investigate and propose for the first time a catalytic mechanism for heparinase I, in specific, and for polysaccharide degrading lyases in general.

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REFERENCES

1. Jackson, R. L., Busch, S. J. & Cardin, A. D. (1991) Physiol. Rev. 71, 481–539
2. Lindahl, U., Lätholt, K., Spilmann, D. & Kjellén, L. (1994) Thromb. Res. 75, 1–32
3. Lohse, D. L. & Linhardt, R. J. (1992) J. Biol. Chem. 267, 24347–24355
4. Linhardt, R. J., Turnbull, J. E., Wang, H. M., Loganathan, D. & Gallagher, J. T. (1990) Biochimica et Biophysica Acta 1009, 2611–2617
5. Desai, U. R., Wang, H. & Linhardt, R. J. (1993) Arch. Biochem. Biophys. 306, 461–468
6. Tejidor, L., Oman, D., Zimmermann, J., Russo, A., Rose, M., Borzhemskaya, L. & Pelzer, H. (1993) Thromb. Hemostasis 70, 866
7. Sasisekharan, R., Moses, M. A., Nugent, M. A., Cooney, C. L. & Langer, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1524–1528
8. Ernst, S. E., Langer, R. S., Cooney, C. L. & Sasisekharan, R., (1995) Crit. Rev. Biochem. Mol. Biol. 30, 387–444
9. Binari, R., Stavelve, B., Johnson, W., Godavarti, R., Sasisekharan, R. & Manoukian, A. (1997) Development 124, 2623–2632
10. Sasisekharan, R., Bulmer, M., Moremen, K. W., Cooney, C. L. & Langer, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3660–3664
11. Ernst, S. E., Venkataraman, G., Winkler, S., Godavarti, R., Langer, R., Cooney, C. L. & Sasisekharan, R. (1996) Biochem. J. 315, 589–597
12. Godavarti, R., Davis, M., Venkataraman, G., Cooney, C. L. & Langer, R. (1996) Biochem. Biophys. Res. Commun. 225, 751–758
13. Godavarti, R. & Sasisekharan, R. (1996) Biochem. Biophys. Res. Commun. 239, 770–777
14. Sasisekharan, R., Leckhand, D., Godavarti, R., Venkataraman, G., Cooney, C. L. & Langer, R. (1995) Biochemistry 34, 14441–14448
15. Sasisekharan, R., Venkataraman, G., Godavarti, R., Ernst, S. E., Cooney, C. L. & Langer, R. (1996) J. Biol. Chem. 271, 3124–3131
16. Godavarti, R., Cooney, C. L., Langer, R. & Sasisekharan, R. (1996) Biochemistry 35, 6846–6852
17. Cardin, A. D. & Weintraub, H. J. R. (1989) Arteriosclerosis 9, 21–32
18. Yang, V. C., Linhardt, R. J., Bernstein, H., Cooney, C. L. & Langer, R. (1985) J. Biol. Chem. 260, 1849–1857
19. Higuchi, R. (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, Inc., San Diego, CA
20. Bernhardt, H., Yang, V. C., Cooney, C. L. & Langer, R. (1988) Methods Enzymol. 137, 515–529
21. Cardin, A. D., Demeter, D. A., Weintraub, H. J. R. & Jackson, R. L. (1991) Methods Enzymol. 203, 556–583
22. Klagshorn, M. & Baird, A. (1991) Cell 67, 229–231
23. Hata, A., Rüdinger, D., Sutherland, S., Eimi, M., Shuhua, Z., Myers, R., Ren, K., Cheng, T., Ioune, I., Wilson, D., Iverius, P. & Lalouel, J. (1993) J. Biol. Chem. 268, 8447–8457
24. Gacesa, P. (1987) FEBS Lett. 212, 199–202
25. Zhang, Z. & Dixon, J. (1993) Biochemistry 32, 9340–9345
26. Stuckey, J., Schubert, F., Fauman, E., Zhang, Z., Dixon, J. & Saper, M. (1994) Nature 370, 571–575
27. DiPersio, L., Fontaine, R. N. & Hui, D. Y. (1993) J. Biol. Chem. 268, 4033–4036
28. Lewis, S. D., Johnson, F. A. & Shafer, J. A. (1981) Biochemistry 20, 48–51
29. San Antonio, J. D., Slover, J., Lawler, J., Karnovsky, M. J. & Lander, A. D. (1993) Biochemistry 32, 4746–4755
30. Wu, H., Monroe, D. & Church, F. (1995) Arch. Biochem. Biophys. 317, 85–92