Deletion mutagenesis of heparin cofactor II: defining the minimum size of a thrombin inhibiting serpin

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Abstract **Heparin cofactor II (HCII) is a 66 kDa plasma glycoprotein that belongs to the serpin superfamily of protease inhibitors. Its natural target is thrombin. HCII inhibits thrombin in both a progressive reaction, and in an accelerated reaction catalyzed by a glycosaminoglycan, dermatan sulphate (DS). Both modes of inhibition result in the formation of a stable, denaturation-resistant complex. Using a cDNA clone encoding rabbit HCII recently isolated and characterized in our laboratory, we have employed deletion mutagenesis to identify amino-terminal regions of the molecule which are essential to the progressive reaction. PCR was employed to produce four deletion constructs: Δ58, Δ81, Δ106, and Δ169, all in an in vitro transcription vector plasmid background. Transcription of the full-length construct, and of the four deletion constructs, followed by in vitro translation in rabbit reticulocyte lysate, was used to produce the corresponding HCII-related polypeptides. The Δ106 and Δ169 mutants failed to react with thrombin, even in the presence of DS. In contrast, the Δ58 and Δ82 mutants retained the ability to form complexes with thrombin, although the rate of complex formation was decreased for the latter mutant compared to the full-length recombinant HCII; no acceleration of complex formation in the presence of 20 μg/ml DS was noted for either truncated recombinant HCII. Alignment of the rabbit HCII primary structure with secondary structural elements found in $\alpha_1$antitrypsin and other serpins showed that the non-functional Δ106 mutant lacks helix A, while the functional Δ82 mutant contains this element. Our results suggest that helix A is an essential part of a functional serpin, and define the limits of the amino-terminal region of HCII which is not essential for thrombin inhibition.**

Key words: Heparin cofactor II; Thrombin; Serpin

1. Introduction

The serpins are members of a superfamily of serine protease inhibitors, many of which play important roles in the regulation of coagulation, fibrinolysis, or inflammation [1,2]. These related proteins share approximately 30% amino acid identity, as well as a common mechanism of action. This involves attack by target proteases at the reactive site of the inhibitor, followed by formation of a stable, 1:1 complex in which both protease and inhibitor are inactive [1–4].

Serpin regulation of thrombin is important in hemostasis, as shown by the thrombophilic tendencies of individuals genetically deficient in a serpin called antithrombin, or antithrombin III (AT, or AT-III) [5–7]. Although AT is the principal inhibitor of thrombin found in plasma, another serpin, called heparin cofactor II (HCII) also contributes to the control of thrombin [8]. While the AT-thrombin reaction is stimulated maximally by heparin [9], or heparan sulphate moieties, that of HC-II with thrombin is accelerated most effectively by dermatan sulphate [10]. The latter characteristic suggests that HC-II may play a role in the extravascular control of thrombin, because of the natural location of dermatan sulphate moieties [11].

While much has been learned concerning the structure and function of both active and inactive serpins, the molecular details of how serpins function remain to be worked out. Analysis of point mutations, both natural and engineered, has contributed to our understanding of the importance of the reactive centre region, and to other regions of serpin molecules involved in cofactor interactions (for reviews, see [12,13]). We have chosen to use deletion mutagenesis to map the minimum essential form of HCII that is sufficient for complex formation with thrombin. HCII was selected over AT because of its lack of disulphide bonding, and because we have recently demonstrated that its expression in rabbit reticulocyte lysate systems yields a more functional product than that of AT [14]. Our results suggest that the helix A structural element is an essential part of a functional serpin.

2. Materials and methods

2.1. Materials

Human α-thrombin (>3300 NIH units/mg; >93% active) was kindly provided by Dr. J. Fenton (New York State Division of Biologicals, Albany, NY). Plasmid vector pSV3 was the generous gift of Dr. David Andrews (McMaster University, Hamilton, Ontario). All restriction, DNA modifying enzymes, RNase inhibitors, ribo- and deoxyribonucleotides, and mRNA CAP analog 7-methlyguanosylguanosine triphosphate [m^{7}G(5′)ppp(5′)G] were purchased from Promega Biotec (Madison, WI). The latter was also the source of rabbit reticulocyte lysate and plasmid pGEM5zf+. The thrombin inhibitor D-phenylalanyl-L-propyl-arginine chloromethyl ketone (PPACK) (>99% active) was purchased from Calbiochem (Mississauga, ON). T7 sequencing kits were from Pharmacia LKB Biotechnology (Baie d'Urfé, QC). Translation grade [^{35}S]methionine (>1000 Ci/mmol, 10 μCi/μl) was from NEN-DuPont (Mississauga, ON). Protein molecular weight standards were from BioRad Laboratories (Oakville, ON). Oligonucleotides were synthesized at the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario. All other chemicals and reagents not specifically listed here were of the highest quality available.

2.2. PCR and plasmid manipulations

In order to generate plasmid transcription templates encoding amino-terminal truncations of rabbit HCII, PCR was employed, using the previously described plasmid pMHC3 as template [14], and the M13 universal primer (Pharmacia) and a mutagenic HCII-specific oligonucleotide as primers. Constructs, and the truncated HCII protein they encode, are named for the number of amino acids deleted. The specific primers were as follows: for the Δ169 construct, primer 3838 was used

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(5′-TCATGACGCATCGCCTCTTCAGGA-3′); for the Δ106 construct, primer 4114 was used (5′-CCATGGCCAACGCTTTTGATAACATC-3′); and for the Δ58 construct, primer 3839 was used (5′-TCATGATCATCGACGCTGTTTCCC-3′). PCR conditions were exactly as previously described [14]. After PCR, each product was made blunt-ended with Klenow fragment, gel-purified from agarose, and introduced into the *Eco*RV site of pGEM3zf+ by standard methods of ligation and transformation [15]. The orientation of the insert was determined by restriction endonuclease digestion, and purified DNA was prepared for in vitro transcription by linearization with either *Eco*RI or *Sac*II. For the Δ81 construct, a slightly different approach was used, one that exploited the proximity of a unique BamHI site in the rabbit HCII cDNA. Two oligonucleotides, primer 4609 (5′-CATGAAAGAGCAG-3′) and primer 4610 (5′-GATCCTGCTCTT-3′) were annealed as described [16], then combined with a 1790 bp *Bam*HI–*Eco*RI restriction fragment of pMHC3 and *Nco*I–*Eco*RI digested transcription plasmid pSV3 [17] in a three-part ligation.

2.3. In vitro transcription and translation

Transcription plasmids were transcribed in vitro from phage promoters; the Δ169, 106, and 58 constructs using T_7 polymerase, and the Δ81 construct using SP_6 polymerase, as described previously [14]. Translation was performed as described [14,18], in a messenger-dependent reticulocyte lysate supplemented with [^{35}S]methionine. Following translation, products were dialyzed overnight versus Tris-buffered saline at 4°. For thrombin-complexing assays, α-thrombin was employed at a final concentration of 0.85 μM; this represents at least a 100-fold molar excess over the concentration of recombinant serpin in the reaction [18].

2.4. Miscellaneous

SDS-PAGE analysis, followed by fluorography and autoradiography, was as described [14,18]. Reactions of cell-free derived HCII and its derivatives with thrombin were also monitored as described in these references, with the exception that the different truncated product preparations were diluted with 30 mg/ml bovine hemoglobin in Tris-buffered saline to provide equivalent concentrations of radiolabeled HCII derivatives, while maintaining the same total protein concentrations between reactions. Quantitation of autoradiographic images was done using a Molecular Dynamics PhosporImager equipped with ImageQuant software.

3. Results

3.1. Cell-free synthesis of truncated HCII proteins

Recently, we described the primary sequence of rabbit HCII, and showed that cell-free derived rabbit HCII was fully reactive with human α-thrombin, in a reaction that could be accelerated by the addition of dermatan sulphate (DS). In the present study, we have applied the same experimental approach, namely synthesis in an in vitro, rabbit reticulocyte lysate translation system, to produce truncated forms of recombinant rabbit HCII.

Fig. 1 summarizes the forms of rabbit HCII which were produced and synthesized in this study. As previously demonstrated, the principal product of cell-free translation of the template encoding the entire mature HCII is a 50.8 kDa polypeptide that most likely arises due to translational initiation at Met-15 (Upper panel, lane 1). No difference in the rate of either progressive or DS-accelerated thrombin inhibition is seen when the 52.4 kDa full-length product is compared to the more prominent Met-15 product [14]. The products of the four truncated transcription templates that were generated are also shown in Fig. 1. The major products are of expected size: Δ169, 33.4 kDa; Δ106, 40.4 kDa; Δ81, 43.4 kDa; and Δ58, 45.7 kDa. Other minor products of more rapid mobility than the major product are also seen, varying in abundance between transcription templates. For instance, a 39 kDa polypeptide product is found along with all synthesis reactions except for the Δ169

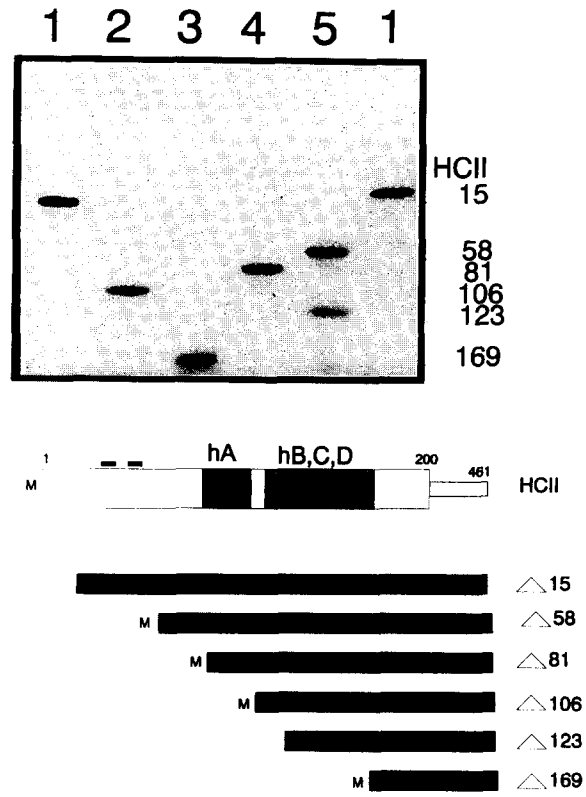

Fig. 1. Gel analysis of cell-free derived recombinant rabbit HCII and derivatives. Upper panel: in vitro translation products were electrophoresed on a 10% SDS-polyacrylamide gel, and radiolabeled proteins detected following fluorography and autoradiography. Lane 1, full length recombinant rabbit HCII (showing the prominent Δ15 internal product); lane 2, Δ106; lane 3, Δ169; lane 4, Δ81 (showing the Δ123 internal product); lane 5, Δ58. The positions of the major protein species produced are indicated at right. The lower panel shows a schematic diagram of rabbit HCII derivatives and predicted structural elements. Uppermost line diagram shows a linear representation of full length recombinant rabbit HCII, engineered to contain an N-terminal Met for cell free-synthesis (M). Diagram is to scale for residues 1–200 (exploded) and not to scale for residues 201–461. Helix A, hA; helices B, C, and D, hB,C,D. The two large negative signs highlight the position of the N-terminal hirudin-like acidic repeats. Solid bars, below, delineate the extent of the different N-terminally truncated molecules generated in this study, named at right. Deliberately engineered constructs contain an added N-terminal Met residue; products generated by internal initiation lack this extra amino acid.

reaction, and is particularly prominent in the Δ58 reaction. This profile suggests that it is the product of internal initiation at a Met residue amino-terminal to residue 169; given that Met residues are found only at positions 15 and 123, this product most likely arises from internal initiation at Met-123.

3.2. Reaction of HCII derivatives with thrombin

Fig. 2 shows the results of an experiment in which the ability of the full-length HCII to form complexes with thrombin was compared with that of the two most truncated deletion mutants, Δ169 and Δ106. After 10 minutes of reaction, thrombin-dependent species of 89 and 87 kDa were prominently visible, and the input full-length and the internal Δ15 species concordantly diminished (lanes 2 and 3), either in the presence (lane 2 or absence (lane 3) of DS. Densitometry revealed that 64% of the input HCII had been consumed after 10 minutes of

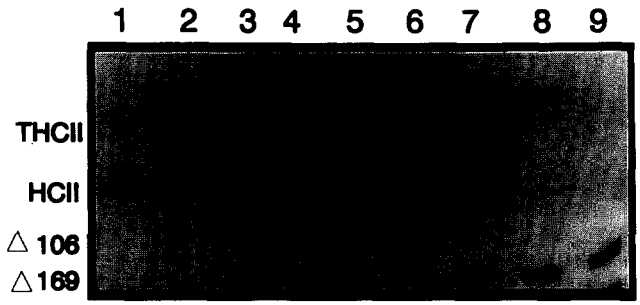

Fig. 2. Reaction of thrombin with cell-free derived rabbit HCII, Δ106, and Δ169. In vitro translation products were reacted with thrombin for 0 (lanes 1, 4, and 7), or 10 minutes, in the absence (lanes 2, 5, and 8) or presence (lanes 3, 6 and 9) of dermatan sulfate. Lanes 1–3, full-length HCII; lanes 4–6, Δ106; lanes 7–9, Δ169.

reaction in the absence of DS, while in 1 minute 82% of input HCII was consumed in its presence. In contrast, neither any higher molecular weight products of lesser mobility than the input HCII species, nor any dimunition of input band intensities is observable in the case of either Δ169 or Δ106 (Fig. 2, lanes 5 and 6, and 8 and 9), even upon prolonged overexposure of the autoradiogram (not shown).

In contrast to the inactive status of the most truncated deletion mutants, two deletion mutants with lesser truncations retained some measure of thrombin reactivity. As shown in Fig. 3, the Δ58 mutant formed complexes with thrombin with similar kinetics to the full-length or Δ15 forms in the absence of DS; quantitation showed that 59.4% of input Δ58 was consumed within 10 minutes of thrombin addition, as compared to 64.8% of input full-length recombinant HCII. In the presence of 20 μg/ml DS, a concentration which we have determined is sufficient to give the maximal response in this system (not shown), no acceleration of complex formation was seen. In contrast, the full-length HCII was largely consumed in less than 1 minute when this level of DS was provided in the reaction. Similar results were observed when the Δ82 mutant was analyzed in the same way as the Δ58 mutant, in that complex formation was observable, and that this complexing was not accelerated by DS. However, the extent of complex formation was somewhat reduced, when compared to that observed with the full-length rHCII, in that only 35.1% of input Δ81 was consumed by thrombin in 10 minutes, as compared to 63.8% of the input full-length rHCII. The lack of reactivity of the Δ123 HCII species, which is a prominent side-product of the Δ58 reaction, is consistent with the inactivity of the Δ169 and Δ106 products.

3.3. *Alignment of deletion mutants with known and predicted structural elements*

The schematic diagram shown in the lower panel of Fig. 1 summarizes the amino-terminal truncations produced in this study, either deliberately through DNA manipulations, or serendipitously, via internal initiation. While Δ82 retained progressive thrombin inhibitory capacity, Δ106 was devoid of activity. This suggests that an essential structural or functional element lies between these two residues on HCII. Comparison to serpin secondary structural elements mapped onto a linear display of the rabbit HCII (Fig. 1) highlights the fact that helix A is found at this position.

4. Discussion

Our objective in this study was to define the minimum domain(s) of rabbit HCII required for complex formation with thrombin. Our approach, that of generating a series of amino-terminal deletions of the protein, was determined by several factors. Firstly, the greatest degree of divergence between human and animal HCII primary structures is seen in this region [14], a difference that suggested that this region could be altered without abrograting the function of the molecule. Secondly, we had previously demonstrated [14] that an internally initiated cell-free synthesis product lacking the first 15 amino acid residues of the protein was just as active as the full-length product. Thirdly, Tollefsen and colleagues had previously shown, using recombinant forms of the highly homologous human AT expressed in bacteria, that the amino-terminus of the protein was important for glycosaminoglycan-accelerated thrombin inhibition. The proximity of the reactive centre of the molecule (Leu425–Ser426) to the carboxyl terminus, and the abundance of naturally occuring mutants that abrogate serpin function found in this area [12], was also a consideration in choosing to perform deletion analysis on the amino-terminal end of the molecule.

The initial mutant that we generated was Δ169. This deletion was essentially selected as a random deletion of the amino-terminal third of the molecule, and was planned to establish a benchmark on the HCII polypeptide chain. Functional Δ169 would indicate that more extensive deletions were required; non-functional Δ169 would suggest that less extensive deletions should next be attempted. As shown in Fig. 2, Δ169 failed to form complexes with thrombin. Inspection of HCII aligned with secondary structural elements found in $\alpha_1$antitrypsin and other serpins (Fig. 1) revealed that helices A through D as well as intervening β-stranded regions had been largely removed from the molecule. Accordingly, these regions were 'added back' in subsequent deletions.

Two acidic sequence motifs are found in the first 58 amino

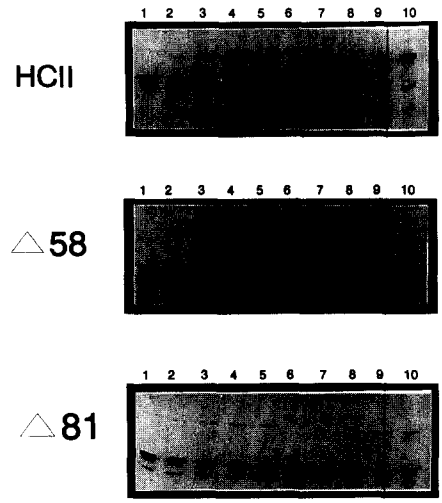

Fig. 3. Reaction of thrombin with cell-free derived rabbit HCII, Δ58, and Δ81. In vitro translation products were reacted with thrombin for 0 (lanes 1 and 6), 1 (lanes 2 and 7), 2 (lanes 3 and 8), 5 (lanes 4 and 9), or 10 minutes (lanes 5 and 10) in the absence (lanes 1–5) or presence (lanes 6–10) of dermatan sulphate. Top panel, reaction of thrombin with full-length HCII; middle panel, reaction of thrombin with Δ58; bottom panel, reaction of thrombin with Δ81.

acids of rabbit HCII (corresponding to the first 74 amino acids of the human molecule), both containing a Glu-Asp-Asp-Asp-Tyr pentapeptide. It has been shown previously [19,20] that deletion of the first 74 amino acids of human HCII expressed in bacteria resulted in an HCII protein with greatly reduced DS-accelerated inhibitory activity. We made the corresponding deletion in rabbit HCII, and expressed the mutant Δ58 polypeptide. As shown in Fig. 3, Δ58 retained the ability to react with thrombin in the progressive reaction, but lost the ability to react in a fashion accelerated by DS. Our results are therefore entirely consistent with the more quantitative analysis performed on the recombinant human HCII.

Provision of helices B through D and connecting domains in mutant Δ106 failed to restore antithrombin activity (Fig. 3). However, provision of the additional 24 amino acids predicted to comprise helix A, in mutant Δ81, restored the thrombin inhibitory capacity of the molecule. The properties of Δ81 ressemble those of Δ58, in that no effect of DS was seen; however, the extent and speed of complex formation of this polypeptide was somewhat decreased compared to the full-length rHCII.

Is the observed requirement for helix A structural or mechanistic? While neither HCII nor $\alpha_1$antitrypsin have been crystallized in intact form, the latter serpin has been crystallized as a cleaved form [21], and the crystallization of plakalbumin [22], ovalbumin [23], the latent form of PAI-1 [24], and most recently a dimer of AT consisting of one active molecule linked to an inactive one [25], have yielded good structural models of intact serpins. Inspection of schematic representations of a1AT and AT show that helix A traverses the molecule, underpinning the primarily β-sheeted structures above it. Helix A appears to stabilize helices G and H [21,25]. This interpretation is supported by a naturally occuring mutation of α_1AT, the I variant, that maps to helix A; in this variant Arg-39 (corresponding to Arg-101 in rabbit HCII) is altered to Cys, eliminating the interhelical contacts, and giving rise to a dysfunctional, partially destabilized antitrypsin [26]. Alterations to Arg-47 of the A helix of antithrombin are associated with decreased heparin affinity [27,28]; these point mutations would be expected to create only localized distortions of the extreme N-terminal portions of the helix, as opposed to the more critical alteration seen in α_1AT I. Taken together with the results presented in this report, these structural data and interpretations support the contention that helix A is an essential part of a functional serpin, and suggest that its contribution is of importance chiefly in supporting the structural integrity of serpin molecules.

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References

[1] Hunt, L.T. and Dayhoff, M.O. (1980) Biochem. Biophys. Res. Commun. 95, 864–871.
[2] Huber, R. and Carrell, R.W. (1989) Biochemistry 28, 8951–8966.
[3] Bode, W. and Huber, R. (1992) Eur. J. Biochem. 204, 433–451.
[4] Carrell, R.W. and Evans, D.L.I. (1992) Curr. Opp. Struc. Biol. 2, 438–446.
[5] Egeberg, O. (1965) Thromb. Diath. Haemorrh. 13, 516–530.
[6] Thaler, E. and Lechner, K. (1981) Clin. Haematol. 10, 369–390.
[7] Demers, C., Ginsberg, J.S., Hirsh. J., Henderson, P. and Blajchman, M.A. (1992) Ann. Int. Med. 116, 754–761.
[8] Tollefsen, D.M., Majerus, D.W. and Blank, M.K. (1982) J. Biol. Chem. 257, 2162–2169.
[9] Rosenberg, R.D. and Damus, P.S. (1973) J. Biol. Chem. 248, 6490–6505.
[10] Tollefsen, D.M., Pestka, C.A. and Monafo, W.J. (1983) J. Biol. Chem. 258, 6713–6716.
[11] McGuire, E.A. and Tollefsen, DM. (1987) J. Biol. Chem. 262, 169–175.
[12] Blajchman, M.A., Austin, R.C., Fernandez-Rachubinski, F. and Sheffield, W.P. (1992) Blood 80, 2159–2171.
[13] Sheffield, W.P., Wu, Y.I. and Blajchman, M.A. Marcel Dekker, NY, in press.
[14] Sheffield, W.P., Schuyler, P.D. and Blajchman, M.A. (1994) Thromb. Haemostas. 71, 778–782.
[15] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology, vol. 1, pp. 3.5.1–3.16.10, Wiley-Interscience, NY.
[16] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (1987) in: Current Protocols in Molecular Biology, vol. 2, pp. 8.2.8, Wiley-Interscience, NY.
[17] Falcone, D. and Andrews, D.W. (1991) Mol. Cell. Biol. 11, 2656–2664.
[18] Sheffield, W.P., Brothers, A.B., Wells, M.J., Hatton, M.W.C., Clarke, B.J. and Blajchman, M.A. (1992) Blood 79, 2330–2339.
[19] Van Deerlin, V.M.D. and Tollefsen, D.M. (1991) J. Biol. Chem. 266, 20223–20231.
[20] Ragg, H., Ulshofer, T. and Gerewitz, J. (1990) J. Biol. Chem. 265, 5211–5218.
[21] Loeberman, H., Tokuoka, R., Deisenhofer, J. and Huber, R. (1984) J. Mol. Biol. 177, 531–556.
[22] Wright, H.T., Qian, H.X. and Huber, R. (1990) J. Mol. Biol. 213, 513–528.
[23] Stein, P.E., Leslie, A.G.W., Finch, J.T., Turnell, W.G., McLaughlin, P.J. and Carrell, RW. (1990) Nature 347, 99–102.
[24] Mottonen, J., Strand, A., Symersky, J., Sweet, R.M., Danley, D.E., Geoghegan, K.F., Gerard, R.C. and Goldsmith, E.J. (1992) Nature 355, 270–273.
[25] Carrell, R.W., Stein, P.E., Fermi, G. and Wardell, M.R. (1994) Structure 2, 257–270.
[26] Graham, A., Kalsheker, N.A., Newton, C., Bamforth, F.J., Powell, S.J. and Markham, A.F. (1989) Hum. Genet. 84, 55–58.
[27] Owen, M.C., Borg, J.Y., Soria, C., Soria, J., Caen, J. and Carrell, R.W. (1987) Blood 69, 1275–1279.
[28] Borg, J.Y., Owen, M.C., Soria, C., Caen, J. and Carrell, R.W. (1988) J. Clin. Invest. 81, 1292–1296.