Biochemical alterations in collagen IV induced by *in vitro* glycation

Hans-M. RAABE*‡, Hilke MOLSEN*, Snjezana-M. MLINARIC*, Yahya AÇIL*, Gernot H. G. SINNECKER*, Holger NOTBOHM†, Klaus KRUSE* and Peter K. MÜLLER†

*Klinik für Pädiatrie, Medizinische Universtät zu Lübeck, Kahlhorststr. 31–35, 23538 Lübeck, and †Institut für Medizinische Molekularbiologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany

Non-enzymic interactions of carbohydrates and proteins are a major feature of cumulative modification in basement membranes in the course of diabetic microvascular complications. To evaluate the significance of both glycation and glycoxidation reactions for subsequent alterations of biochemical properties, we examined the effects of *in vitro* glycation on distinct collagen IV domains under different experimental conditions. The 7 S domain and the major triple-helical domain from human placental collagen IV were incubated for various time intervals up to 14 days at 37 °C in the presence of different concentrations of either glucose or ribose under oxidative and antioxidative conditions. Carbohydrate-induced non-enzymic modification in two collagen IV domains was revealed by increased cross-linking and fluorescence. In addition, these non-enzymic modifications apparently have a major impact on molecular conformation and thermal stability of collagen IV, which in turn might influence both cell-matrix interactions and matrix assembly.

INTRODUCTION

Basement membranes are specialized layers of extracellular matrix, mainly consisting of a three-dimensional irregular lattice of collagen IV, several other glycoproteins and proteoglycans, providing mechanical support for a variety of cells [1]. In line with its unique functional importance, glycation and glycoxidation of these extracellular structures have been implicated in the cumulative development of microvascular complications observed in patients with long-term diabetes mellitus [2–4].

Although the diversity of metabolic pathways of non-enzymic modification still remains to be elucidated, there is general agreement that the early stage of the Maillard or browning reaction is initiated by the condensation of a reducing sugar with the ϵ-amino group of lysyl and/or hydroxylysyl residues [5], resulting in a Schiff base which may then rearrange into an Amadori product [6]. Such a compound is further processed into reactive α-ketoaldehydes, which subsequently may contribute to protein modification in the late stages of the Maillard reaction, causing cross-linking as well as fluorescence [7,8].

Proteins of the extracellular matrix are long-lived, so that accumulated non-enzymic modifications may alter protein configuration and properties, hence leading to those functional deficiencies which are observed in a variety of tissues exposed to reducing carbohydrates and reactive oxygen radicals [9]. Premature stiffness of arteries and joints has been suggested as a consequence of glucose-mediated covalent cross-linking of fibre-forming collagens [10,11]. For collagen II it was shown that non-enzymic modification reduced the thermal stability of the triple helix and impaired *in vitro* fibril formation [12].

Preferential glycation sites along the collagen α-chains were proposed [13], giving rise to an uneven distribution of glucose adducts. Although recent evidence suggests that the vast majority of newly formed structural components are non-fluorescent [14,15], collagen-associated fluorescence was considered to reflect the increment of protein modification with age and diabetes [16]. A further structural compound, called pentosidine, was identified as a fluorescent imidazopyridinium system derived from ribose, arginine and lysine [17]. Interestingly, metal-catalysed glycoxidative pathways were described to involve hexoses as possible precursors, in addition to pentose carbohydrates [18,19].

First evidence for effects of glycation on basement-membrane collagen was obtained by a series of *in vitro* experiments. For the NC1 domain of collagen IV a loss in the ability to interact with the triple-helical domain of collagen IV was shown [20]. The decreased interaction with heparin *in vitro*, shown for non-enzymically glycated collagen IV [21], was proposed to explain the diminished anionic charge density in the renal basement membrane [22]. Loss of size selectivity [23] was explained by clustering of basement-membrane proteins, due to increased cross-linking.

In order to investigate implications of glycation and glycoxidation on structural and functional modifications of individual collagen IV molecules, we isolated both the major triple-helical and the 7 S domain of human placental collagen IV domains by pepsin digestion and incubated the solubilized material with glucose or ribose under oxidative and antioxidative conditions.

We provide experimental evidence for carbohydrate-induced non-enzymic modification, demonstrating enhanced cross-linking and fluorescence in both collagen IV domains. Spectral analysis revealed a distinct pentosidine-like fluorescence peak and a second maximum at 405 nm. In addition, the carbohydrate-induced non-enzymic modifications described apparently have a major impact on the molecular conformation and thermal stability of collagen IV, which in turn might influence both cell-matrix interactions and matrix assembly in diabetic microvascular complications.

MATERIALS AND METHODS

Isolation of human collagen IV

Normal human placental tissue was frozen immediately following delivery and stored at −20 °C until preparation. The triple-

‡ To whom correspondence should be addressed.

helical domain of collagen IV was prepared as described previously [24,25]. Briefly, washed and homogenized placental tissue was suspended in 0.5 M acetic acid and subjected to limited pepsin digestion (ratio 1:1000) at 10 °C for 20 h. Soluble collagen types were separated by differential salt precipitation and subsequent ion-exchange chromatography. The triple-helical region was obtained by reduction and alkylation in the native state followed by a second pepsin digestion. The 7 S domain was prepared according to the method of Weber et al. [26]. Briefly, prewashed human placental tissue was extracted with 6 M guanidine hydrochloride, digested twice with collagenase A (Boehringer, Mannheim, Germany) and passed over a Superose 12 resin (Pharmacia, Freiburg, Germany). The identity of the major triple-helical fragment and the 7 S domain were demonstrated by SDS/PAGE.

Electrophoresis

SDS/PAGE was carried out on slab gels [27], using 6% separation gels and 4% stacking gels. 10% running gels were used to analyse the 7 S domain under reducing conditions. After electrophoretic separation, gels were stained with Coomassie Blue. The relative position and staining of individual bands was determined by two-dimensional scanning and densitometry [28].

Amino acid analysis

Protein samples were hydrolysed with 6 M HCl for 24 h at 110 °C under nitrogen. Amino acid analysis was performed on a Beckmann system 6300, using ninhydrin for the post-column colour reaction. Two analyses were performed for each duplicate of any individual sample.

Hydroxylysine glycosides and hydroxylysine were purified from alkaline hydrolysates and separated on an amino acid analyser as described previously [28].

CD and transition profiles

CD spectra were recorded on a JASCO J-500 A spectropolarimeter, equipped with a temperature-controlled quartz cell of 1 cm pathlength (Gilford). The molar ellipticity was calculated on the basis of a mean residue mass of 98 Da. For the transition curves conversion into random coil corresponds to the ellipticity at 80 °C, typical for totally denatured collagen IV. Thermal transition curves were recorded at a fixed wavelength (221 nm) by raising the temperature linearly at a rate of 30 °C/h, using a Gilford temperature programmer. The sample concentration was 200 μg/ml. The sample buffer was 0.05 M phosphate, pH 7.4. A change of thermal stability due to radiation damage could not be detected.

The statistical error at 221 nm for one measurement was calculated to be 5.3% of the molar ellipticity.

Fluorometric analysis

Fluorescence measurements were performed upon excitation at 335 nm. The fluorescence was determined, running a fluorescence spectrum from 360 to 450 nm on a Perkin–Elmer Luminescence Spectrometer LS 50 B and was normalized to the protein concentration of each sample.

Incubation procedure for *in vitro* glycation

The major triple-helical segment and the 7 S domain were each incubated in PBS, pH 7.4, at 37 °C for 2 weeks in the dark. For kinetic studies, samples were withdrawn after various time intervals. Glucose and ribose were added in concentrations from 0 to 500 mM. Antioxidative conditions were generated by adding diethylenetriaminepenta-acetic acid (DTPA), EDTA and phytic acid (1 mM each), followed by bubbling the reaction buffer with nitrogen for 10 min. Sodium azide (1 mM) was added to prevent microbial growth [29,30]. Following incubation, the samples were dialysed against 0.05 M phosphate, pH 7.4, and analysed by SDS/PAGE, fluorescence analysis, CD spectroscopy and transition profile. Prior to amino acid analysis, all samples were dialysed against 0.05% acetic acid.

RESULTS

Extraction of human basement-membrane (type IV) collagen

Native collagen IV was isolated from human placental tissue by pepsin digestion. Differential salt precipitation was performed to remove type I, type III and type V collagen. Chromatography on diethylaminoethyl- and carboxymethyl-cellulose was used to separate collagen IV from heparan sulphate proteoglycan and traces of collagen I, III and V, respectively. Following reduction and alkylation under non-denaturing conditions, a second pepsin digestion liberated the major triple-helical domain, and, thereby, generated a mixture of peptides with various molecular masses ranging from intact 185 kDa α1(IV) and 175 kDa α2(IV) to the predominant 90 kDa α1(IV) and 75 kDa α2(IV) chains, respectively (Figure 1, lanes a).

The 7 S domain was separated by two consecutive collagenase digests of guanidine hydrochloride-extracted human placental basement membrane, followed by molecular sieve chromatography on a Superose 12 resin. In SDS/PAGE under reducing

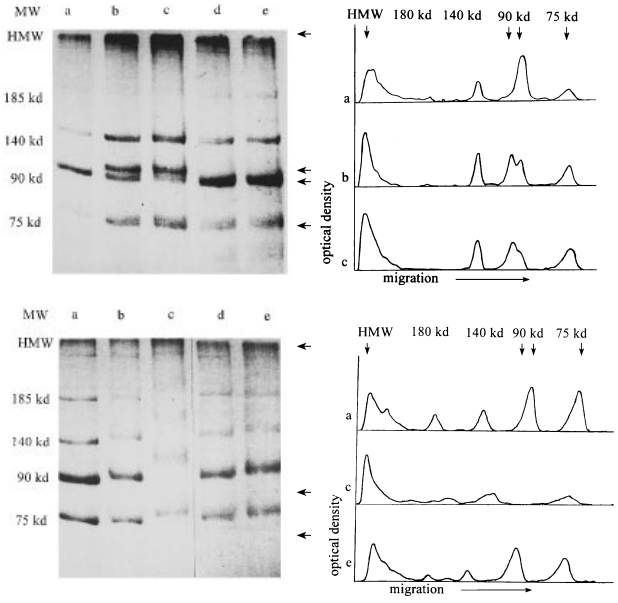

Figure 1 Electropherogram of the major triple-helical domain of human placental collagen IV incubated with glucose (upper panels) and with ribose (lower panels)

Upper panels: Control (a), 200 mM gluocse (b), 500 mM glucose (c) under oxidative, and 200 mM glucose (d), 500 mM glucose (e) under antioxidative conditions. The right-hand panel shows two-dimensional scanning of lanes a, b and c. Lower panels: Control (a), 200 mM ribose (b), 500 mM ribose (c) under oxidative, and 200 mM ribose (d), 500 mM ribose (e) under antioxidative conditions. The right-hand panel shows two-dimensional scanning of lanes a, c and e.

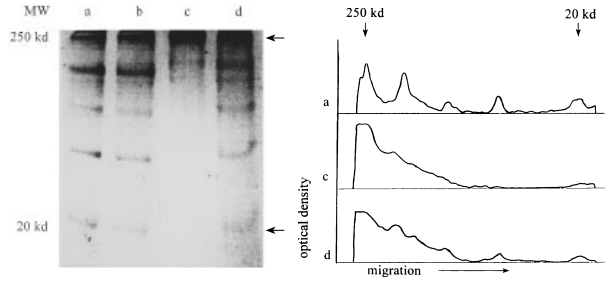

Figure 2 Electropherogram of the 7 S domain incubated with glucose and with ribose

Left-hand panel: Control (a), 500 mM glucose (b), 500 mM ribose (c) under oxidative conditions, and 500 mM ribose under antioxidative conditions (d). The right-hand panel shows two-dimensional scanning of lanes a, c and d.

Table 1 Relative percentage increase of high-molecular-mass components in the major triple-helical domain (mj. triple-helix) and the 7 S domain of human placental collagen IV following incubation with glucose or ribose under oxidative (a) and antioxidative (b) conditions

Values represent mean of three independent measurements ± S.D. Abbreviation: n.d., not detected.

		Glucose		Ribose	
	Carbohydrate concn. (mM)	mj. triple helix	7 S domain	mj. triple helix	7 S domain
a	200	1.1 ± 1.6	n.d.	29.1 ± 0.5	n.d.
	500	9.6 ± 2.3	6.4 ± 1.2	57.5 ± 0.7	20.4 ± 3.2
b	200	0.8 ± 0.9	n.d.	0.5 ± 1.8	n.d.
	500	0.2 ± 1.9	0.0 ± 0.8	22.2 ± 0.7	8.1 ± 0.3

conditions, a mixture of peptides ranging from monomer (20 kDa) up to the dodecameric polymer of approximately 250 kDa, could be seen (Figure 2, lane a).

Cross-linking of collagen IV by glucose and ribose under oxidative and antioxidative conditions

The major triple-helical domain and the 7 S domain of human placental collagen IV were incubated *in vitro* for 2 weeks at 37 °C in the presence of 200 and 500 mM D-glucose and ribose under oxidative or antioxidative conditions.

SDS/PAGE and densitometric analysis of the major triple-helical fragment revealed significantly increased amounts of high-molecular-mass material following incubation with 500 mM glucose (Figure 1, upper panel, lane c; Table 1). No significant cross-linking was found following incubation with 200 mM glucose (Figure 1, upper panel, lane b).

Whereas in fibre-forming collagen larger polymers of heterogenous size were observed, which did not penetrate the stacking gel [9,29], cross-linked material of the major triple-helical domain of collagen IV remained soluble and revealed a distinct molecular mass, which did not exceed the molecular mass of already existing material (Figure 1). Furthermore, with increasing concentrations of carbohydrate, increasing amounts of the 90 kDa α1(IV) chain of the major triple-helical domain showed a diminished migration, which was virtually inhibited under antioxidative conditions (Figure 1, upper panel, lanes d and e).

In the 7 S domain, glucose-induced cross-linking resulted in the formation of significantly increased amounts of a distinct protein band of approximately 250 kDa (Figure 2, lanes a and b; Table 1), which co-migrated with the native dodecameric form of this domain, shown by SDS/PAGE under non-reducing conditions (results not shown). Although there is apparently more material cross-linked in the major triple-helical fragment of collagen IV, when compared with the 7 S domain (Figure 1, upper panels, lanes a–c; Figure 2, lanes a and b), the difference was of no statistic significance (Table 1).

Furthermore, cross-link formation was inhibited to baseline levels in the presence of chelating agents in a nitrogen atmosphere (Figure 1, upper panel, lanes d and e; Table 1).

In the presence of ribose, all observations, including a diminished migration, appeared to be more thorough (Figure 1, lower panel, lanes a–c; Figure 2; Table 1), and could not be inhibited completely when antioxidative conditions were applied (Figure 1, lower panel, lanes d and e; Figure 2, lane d; Table 1). Densitometric analysis revealed significantly greater amounts of cross-linked material in the major triple-helical fragment of collagen IV, when compared with the 7 S domain (Table 1).

Kinetic studies of cross-linking in the major triple-helical domain of collagen IV clearly revealed a time-dependent hyperbolic function on carbohydrate-induced high-molecular-mass material formation (Figure 3). Obviously, increased cross-linking was found in correlation to the amount of carbohydrate applied.

Cross-link formation was reduced in the presence of chelating agents in a nitrogen atmosphere. Complete inhibition in the presence of 200 mM ribose, but partial inhibition in the presence of 500 mM ribose, provides evidence that inhibition of cross-link formation under antioxidative conditions is dependent on the concentration of carbohydrate applied.

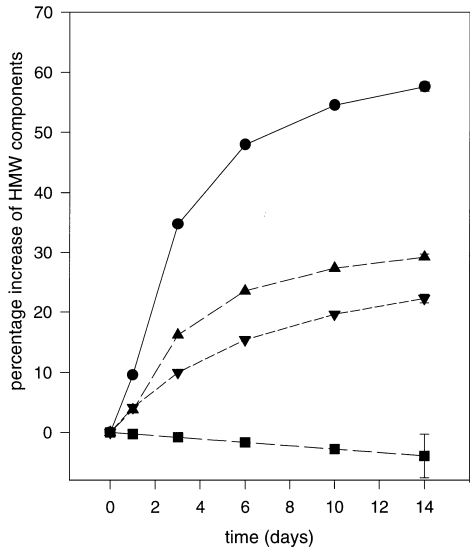

Figure 3 Kinetics of cross-link formation in the major triple-helical domain of human placental collagen IV by ribose under oxidative and antioxidative conditions

Values obtained by densitometry of SDS/PAGE are expressed as percentage increase of high-molecular-mass material per lane. 500 mM Ribose [oxidative (●), antioxidative (▼)], 200 mM ribose [oxidative (▲), antioxidative (■)].

Amino acid analysis

Following acid hydrolysis (Table 2a), the amino acid analysis of the major triple-helical domain showed the expected content of hydroxyproline and hydroxylysine in accordance with published

Table 2 Partial amino acid composition of pepsin-solubilized human collagen IV (Co) following incubation with 500 mM glucose (Gl) and 500 mM ribose (Rb)

Amino acid analysis of acid- (a) and alkaline-hydrolysed (b) major triple-helical domain.

		Sample			
	Amino acid	Co	Gl	Co	Rb
(a)	Hyp	113.1	115.5	108.8	109.1
	Pro	87.5	88.4	87.3	93
	Gly	327.3	335.6	340	357.8
	Hyl	59.6	49.9	58	33.1
	Lys	12.1	11.4	13.7	7.6
	Arg	32.2	33.4	36.4	38.2
	Hyl/Hyp	0.528	0.432	0.477	0.304
(b)	Glu-Gal-Hyl	42.4	37.2	42.5	27.1
	Gal-Hyl	4.5	3.7	3.3	2.3
	Hyl	13.3	9.1	12.1	7.4

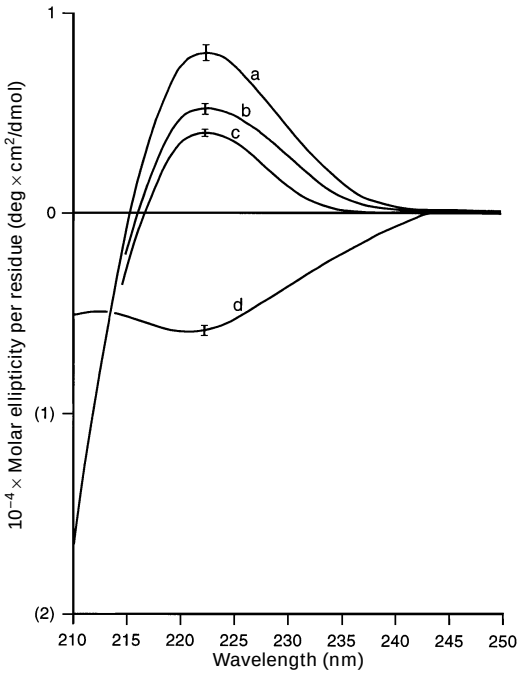

Figure 4 CD spectra of the major triple-helical domain of human placental collagen IV at 20 °C (a), at 96 °C (d), incubated with 500 mM glucose (b) and with 500 mM ribose (c) under oxidative conditions

Error bars represent the statistical error at 221 nm, which was calculated to be 5.3% of the molar ellipticity. Values for the percentage decrease in helicity of individual samples are given in the text.

data [24,25]. The hydroxylysine content of the triple-helical fragment showed a decline, which was more pronounced after ribose incubation. Analysis of alkaline hydrolysates (Table 2b) demonstrates the reduced content of enzymically glycosylated hydroxylysyl derivatives, suggesting that these amino acid residues were targets for glycation irrespective of their individual degree of post-translational modification.

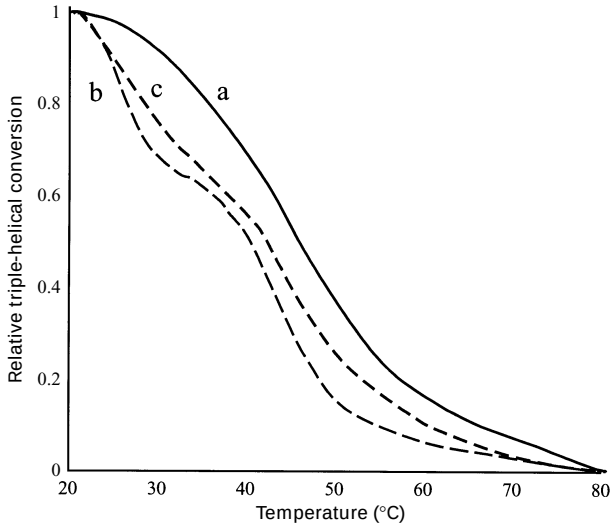

Figure 5 Melting profiles of the glycated major triple-helical region of human placental collagen IV

Control (a), 500 mM glucose under oxidative conditions (b) and 500 mM glucose under antioxidative conditions (c).

Glycation-induced fluorescence in collagen IV

Fluorometric analysis of the major triple-helical domain and the 7 S domain of collagen IV revealed increased fluorescence at 378 nm upon excitation at 335 nm following *in vitro* glycation. In correlation to the results obtained for cross-link formation, fluorescence of the major triple-helical domain was more pronounced, when compared with the 7 S domain, and could be inhibited to baseline levels under antioxidative conditions in glucose incubations, whereas ribose-induced fluorescence was found to be inhibited only in part in the absence of oxygen and transition metals.

Conformation and thermal stability

To assess the effect of glycation on triple-helical conformation, the collagenous domain of collagen IV was examined by CD spectroscopy. For the major triple-helical segment, a CD spectrum in agreement with that reported previously [31] was observed (Figure 4). In comparison with control (Figure 4, line a), the signal at 221 nm decreased significantly with increasing concentrations of glucose. When incubated with 500 mM glucose, (Figure 4, line b), the decrease in helicity accounted for 23 % compared with 33 % on incubation with 500 mM ribose (Figure 4, line c), thereby approximating the CD spectrum of collagen IV after total thermal denaturation (Figure 4, line d). Whereas this effect was completely inhibited in glucose incubations under antioxidative conditions, triple-helical conversion was reduced only partially under antioxidative conditions in the presence of 500 mM ribose (26 % decrease in helicity).

For the 7 S domain a CD spectrum was recorded, which was not influenced by any incubation conditions applied (results not shown).

To evaluate the effect of glycation and glycoxidation on the molecular stability of collagen IV, transition profiles of the collagen IV triple helix were determined. The melting point at 48 °C (Figure 5, profile a) was in agreement with values described previously for the major triple-helical segment of collagen IV [31].

After incubation with glucose, the transition profile of the collagen IV triple helix revealed a shift towards lower temperatures. For the major triple-helical domain, glycated under antioxidative conditions, the shift of the melting point was less pronounced (Figure 5, profile c). No effect of glycation on the thermal stability of the 7 S domain was observed, independent of the experimental conditions applied.

DISCUSSION

Both glycation and glycoxidation have been implicated in non-enzymic modification of protein, reflecting the metabolic situation of tissues exposed to reducing carbohydrate moieties and reactive oxygen species [3,14,17,29]. Since most proteins of the extracellular matrix are long-lived, advanced modification in those proteins might be of particular importance for subsequent structural and functional changes, due to accumulative protein damage.

In order to obtain detailed information on the role of glycation and glycoxidation for basement-membrane alterations observed in the course of diabetic microvascular complications, non-enzymic modification of the collagen IV lattice has been investigated by several authors [23,32,33].

In contrast to this approach, we investigated the effects of carbohydrate-induced browning reactions on two distinct domains of collagen IV, since glycation and hence non-enzymic modification of the particular collagen IV triple helix might have important implications for triple-helical conformation and thermal stability, which in turn might influence both matrix assembly and cell-matrix interactions.

Non-enzymic modification of both the 7 S domain and the major triple-helical domain of collagen IV induced by *in vitro* glycation and glycoxidation was demonstrated by different lines of experimental evidence.

In agreement with data published previously, increased amounts of fluorescence revealed fluorophore formation as a result of protein carbohydrate interactions in the course of the Maillard reaction [15,17,29,34].

A second line of evidence for glycation was obtained by SDS/PAGE, revealing cross-link formation in both the 7 S domain and the major triple-helical domain. In agreement with previous observations for collagen I, the formation of high-molecular-mass material in collagen IV could be inhibited under antioxidative conditions hence emphasizing the significance of oxidation chemistry for carbohydrate-induced protein cross-linking [14,19]. While carbohydrate-induced intermolecular cross-linking is described for fibre-forming collagens [10,14,29] and for the laterally aligned triple helix of collagen IV in basement membranes [1,32], our experiments on extracted collagen IV provide evidence for intra- rather than inter-molecular cross-linking in the major triple-helical domain of collagen IV, as glycation had a minor impact on the molecular mass and the modified protein remained soluble.

A different situation holds for the 7 S domain. In this fragment, enhanced formation of a 250 kDa protein band, representing the complete dodecameric form, suggests both intra- and intermolecular cross-linking. In addition to the data obtained by fluorometric analysis, the 7 S domain seems to be less susceptible to non-enzymic modification than the triple-helical domain, presumably due to obvious differences in amino acid sequence and/or conformation. In accordance with results described previously [9,35], kinetics of cross-link formation in the major triple-helical domain of collagen IV under oxidative and antioxidative conditions provide evidence that both oxidative and non-oxidative pathways might well contribute to the protein modifications observed.

Partial inhibition of cross-link formation in incubations under antioxidative conditions with 500 mM ribose, but complete inhibition in incubations with 200 mM ribose, might be explained by the finding that inhibition by chelating agents correlates to monosaccharide concentration [36], due to a slow removal of transition metals from monosaccharides.

A reduced migration of the 90 kDa α1(IV) collagen chain following incubation under oxidative conditions, which was partially reversed under antioxidative conditions, implies oxygen-requiring, rather than oxygen-independent, reactions involved in this modification as well.

Amino acid analysis demonstrates a turnover of hydroxylysine in glucose incubations and more distinctly in ribose incubations. Furthermore, we provide initial evidence that in collagen IV not only free hydroxylysyl residues, but also their enzymically glycosylated derivatives are involved in protein–carbohydrate interactions. Although fluorescence spectra imply pentosidine formation [17], amino acid analysis was not sensitive enough to demonstrate a turnover of arginine residues. This might be explained by the relatively low formation rate of pentosidine, representing only 1% of carbohydrate-derived cross-links in collagens [14].

CD spectra indicate a loss of triple-helical conformation in the glycated collagen IV triple helix, in accordance with conformational changes in collagen IV, observed for tanned basement membranes [37]. Since both conformational changes and cross-linking were found to be inhibited completely when antioxidative conditions were applied, it is tempting to speculate that cross-linking might be a reasonable molecular mechanism explaining the conformational loss observed.

Melting profiles indicate a decreased thermal stability for the glycated collagen IV triple helix, an observation reported earlier for browned collagen II from intervertebral disc of the elderly [12]. The reversal of this effect, which was found to be only partial when antioxidative conditions were applied, might indicate additional mechanisms apart from cross-link formation. Whether these observations were due to the introduction of hydrophilic monosaccharides by the attachment of carbohydrate, as was dicussed for glycated rat tail tendon [38], remains to be elucidated.

Taking into account data published previously [32], we propose that glycated basement membranes mainly consist of a collagen IV network functionally impaired by increased intermolecular cross-linking, thereby generating a stiff and brittle matrix. Non-enzymic modification of the collagen IV molecule might cause conformational changes and thermal instability of the particular collagen IV triple helix.

Accordingly, our data may explain the altered cell-matrix interactions, observed for endothelial cells on a non-enzymically modified matrix [30]. This hypothesis is based on studies [39,40] which indicate that cell-matrix interactions are dependent on an intact triple-helical conformation. Since matrix interactions like nidogen-mediated laminin and heparan sulphate-proteoglycan binding are dependent on an intact triple helix of collagen IV [41–43] the reduced glycosaminoglycan content of diabetic basement membrane [44], the basis for microalbuminuria in incipient diabetic nephropathy [45], might be explained by the described loss of triple-helical conformation as well.

In summary, increased cross-linking and fluorescence formation, as well as the turnover of predisposed amino acids, provide experimental evidence for non-enzymic modification of human placental collagen IV.

In addition, the carbohydrate-induced modifications observed

apparently have a major impact on molecular conformation and thermal stability of the collagen IV molecule, which might be the molecular basis for pathophysiological changes in both cell-matrix interactions and matrix assembly which are observed for basement membranes in diabetic microvascular complications.

We thank C. Sesselmann and I. Fleischhauer for excellent technical assistance. This work was supported by the Deutsche Forschunsgemeinschaft (SFB 367-A5), Bonn.

REFERENCES

1 Yurchenko, P. D. and Schittny, J. C. (1990) FASEB J. **4**, 1577–1590
2 Baynes, J. W. and Monnier, K. M. (1989) The Maillard Reaction in Aging, Diabetes and Nutrition, A. Liss, New York
3 Wolff, S. P., Jiang, Z. Y. and Hunt, J. V. (1991) Free Radicals Biol. Med. **10**, 339–352
4 Lapolla, A., Fedele, D. and Traldi, P. (1994) Amino Acids **6**, 65–96
5 Robins, S. P. and Bailey, A. J. (1972) Biochem. Biophys. Res. Commun. **48**, 76–84
6 Reynolds, T. M. (1963) Adv. Food Res. **12**, 1–52
7 Monnier, V. M. and Cerami, A. (1983) Am. Chem. Soc. Symp. Ser. **215**, 413–419
8 Ledl, F., Fritsch, G., Hiebel, J., Parchmayr, O. and Severin, T. (1986) in Aminocarbonyl Reactions in Food and Biological Systems (Fujimaki, M., Namiki, H. and Kato, H., eds.), pp.173–282, Elsevier, Amsterdam
9 Fu, M., Wells-Knecht, K. J., Blackledge, J. A., Lyons, T. J., Thorpe, S. R. and Baynes, J. W. (1994) Diabetes **43**, 676–683
10 Kent, M. J. C., Light, N. D. and Bailey, A. J. (1985) Biochem. J. **225**, 745–752
11 Ricard-Blum, S. and Ville, R. (1988) Cell. Mol. Biol. **34**, 581–590
12 Yang, C., Notbohm, H., Acil, Y., Haifeng, R., Bierbaum, S. and Müller, P. K. (1995) Biochem. J. **306**, 871–875
13 Reiser, K. M., Amigable, M. A. and Last, J. A. (1992) J. Biol. Chem. **267**, 24207–24216
14 Baynes, J. W. (1991) Diabetes **40**, 405–412
15 Bailey, A. J., Sims, T. J., Avery, N. C. and Halligan, E. P. (1995) Biochem. J. **305**, 385–390
16 Monnier, K. M., Kohn, R. R. and Cerami, A. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 583–587
17 Sell, D. R. and Monnier, K. M. (1989) J. Biol. Chem. **264**, 21597–21602
18 Chace, K. V., Carubelli, R. and Nordquist, R. E. (1991) Arch. Biochem. Biophys. **288**, 473–480
19 Dyer, D. G., Blackledge, J. A., Thorpe, S. R. and Baynes, J. W. (1991) J. Biol. Chem. **266**, 11654–11660
20 Tsilibary, E. C., Charonis, A. S., Reger, L. A., Wohlhueter, R. M. and Furcht, L. T. (1988) J. Biol. Chem. **263**, 4302–4308
21 Tarsio, J. F., Reger, L. A. and Furcht, L. T. (1987) Biochemistry **26**, 1014–1020
22 Shimomura, H. and Spiro, R. G. (1987) Diabetes **36**, 374–381
23 Anderson, S. S., Tsilibary, E. C. and Charonis, A. S. (1993) J. Clin. Invest. **92**, 3045–3052
24 Glanville, R. W., Rauter, A. and Fietzek, P. P. (1979) Eur. J. Biochem. **95**, 383–389
25 MacWright, R. S., Benson, V. A., Lovello, K. T., van der Rest, M. and Fietzek, P. P. (1983) Biochemistry **22**, 4940–4948
26 Weber, S., Engel, J., Wiedemann, H., Glanville, R. W. and Timpl, R. (1984) Eur. J. Biochem. **139**, 401–410
27 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
28 Yang, C., Rui, H., Mosler, S., Notbohm, H., Sawaryn, A. and Müller, P. K. (1993) Eur. J. Biochem. **213**, 1297–1302
29 Fu, M., Knecht, K. J., Thorpe, S. R. and Baynes, J. W. (1992) Diabetes **41**, 42–48
30 Hasegawa, C., Hunter, A. J. and Charonis, A. S. (1995) J. Biol. Chem. **270**, 3278–3283
31 Risteli, J., Bächinger, H. P., Engel, J., Furthmayr, H. and Timpl, R. (1980) Eur. J. Biochem. **108**, 239–250
32 Bailey, A. J., Sims, T. J., Avery, N. C. and Miles, C. A. (1993) Biochem. J. **296**, 489–496
33 Charonis, A. S. and Tsilibary, E. C. (1992) Diabetes **41**, 49–51
34 Shaw, S. M. and Crabbe, J. M. C. (1994) Int. J. Peptide Prot. Res. **44**, 594–602
35 Wolff, S. P. and Dean, R. T. (1987) Biochem. J. **245**, 243–250
36 Aruoma, O. I., Chandahary, S. S., Grootveld, M. and Halliwell, B. (1989) J. Inorg. Biochem. **35**, 149–155
37 Fisher, R. F. and Hayes, B. P. (1987) J. Mol. Biol. **198**, 263–279
38 Tanaka, S., Avigad, G., Eikenberry, E. F. and Brodsky, B. (1988) J. Mol. Biol. **203**, 495–505
39 Aumailley, M. and Timpl, R. J. (1986) J. Cell Biol. **103**, 1569–1575
40 Eble, J. A., Golbik, R., Mann, K. and Kühn, K. (1993) EMBO J. **12**, 4795–4802
41 Woodley, D. T., Rao, C. N., Hassel, J. R., Liotta, L. A., Martin, G. R. and Kleinman, H. K. (1983) Biochim. Biophys. Acta **761**, 278–285
42 Laurie, G. W., Bing, J. T., Kleinman, H. K., Hassell, J. R., Aumailley, M., Martin, G. R. and Feldmann, J. R. (1986) J. Mol. Biol. **189**, 205–216
43 Timpl, R. (1989) Eur. J. Biochem. **180**, 487–502
44 Parthasarathy, N. and Spiro, R. G. (1982) Diabetes **31**, 738–741
45 Mogensen, C. E., Chachati, A., Christensen, C. K., Close, C. F., Deckert, T., Hommel, E., Kastrup, J., Lefevre, P., Mathiesen, E. R., Feldt-Rasmussen, B., Schmitz, A. and Viberti, G. C. (1986) Uremia Investigation **9**, 85–95

Received 7 May 1996/27 June 1996; accepted 5 July 1996