Distribution of non-enzymatically bound glucose in in vivo and in vitro glycosylated type I collagen molecules

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Non-enzymatic glycosylation of collagen occurs both in vivo during diabetes and in vitro after incubation with glucose. Glycosylated collagen exhibits altered physicochemical and biological properties which could explain some of the complications of diabetes. To provide a mechanistic explanation of this modification the localization of bound glucose was investigated using $NaB[^3H]H_4$ reduction and CNBr cleavage. Glucose fixation is distributed mainly on the α_1CB6 peptide after in vitro glycosylation whereas this distribution occurs less specifically during diabetes. It is concluded that fibrillogenesis alteration of in vitro glycosylated collagen is related to glucose fixation on free ϵNH_2 sites normally implied in intermolecular interactions.

Collagen *Nonenzymatic glycosylation* *Diabetes* *Glucose localization* *CNBr cleavage* *Fluorography*

1. INTRODUCTION

The non-enzymatic glycosylation of proteins demonstrated for hemoglobin [1], lens crystallin [2], erythrocyte membrane proteins [3], collagen [4,5] and others [6] is a post-translational modification inducing changes in physicochemical properties and thus may play a role in the development of the late complications of diabetes. Investigations on the effect of this process on the physicochemical properties of collagen are, however, inconclusive due to the occurrence of associated changes such as increased cross-linking [7,8].

In an attempt to investigate the precise effect of non-enzymatic glycosylation, in vitro glycosylated collagen has been used. In vitro glycosylation slows the rate of fibril formation and subsequently reduces stabilization via cross-linking [9]. However, this effect cannot be observed with collagen isolated from diabetic rats [7] but both in vitro and in vivo glycosylated collagens exhibit increased potency of inducing platelet aggregation [10,11]. These findings led us to determine the distribution of non-enzymatically bound glucose in the CNBr peptides of in vitro and in vivo glycosylated type I collagen. Significant differences were found, possibly related to the observed dissimilarities in fibrillogenesis and physicochemical properties.

2. MATERIALS AND METHODS

2.1. *Induction of experimental diabetes in rats and collagen preparation*

One-month-old male Sprague Dawley rats (Charles River CD strain, France) were made diabetic by intravenous injection of 65 mg streptozotocin (Calbiochem, A grade) per kg body wt in 0.1 M sodium citrate buffer (pH 4.0). The animals were killed 5 months later, at which time the mean blood glucose value was 385 ± 72 mg/100 ml and glucosuria 8–10 g/l; only rats with bilateral cataract were selected. Acid-soluble collagen was extracted from tail tendons as in [12].

2.2. *Preparation of in vitro glycosylated collagen*

Acid-soluble collagen obtained from 6-month-

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old control rats was adjusted to 1 mg/ml in phosphate-buffered saline (PBS, pH 7.8). The preparation was incubated at 29°C for 25 days after addition of glucose to a final concentration of 0.17 M. Penicillin (100 IU/ml) and streptomycin (100 μg/ml) were added to prevent bacterial contamination.

A second acidic extraction was carried out on the reprecipitated polymeric collagen which appeared during incubation. Insoluble residue was removed by centrifugation at 10000 × *g* for 20 min.

2.3. *Labelling and preparation of cyanogen bromide peptides*

Forty mg of each sample (control, diabetic and in vitro glycosylated) at a concentration of 2 mg/ml in ice-cold 0.15 M NaCl, 0.1 M sodium phosphate buffer (pH 7.4) were reduced by addition of 10 mg $NaBH_4$ containing 5.5×10^7 Bq $NaB[^3H]H_4$ (Radiochemical Centre, Amersham) for 1 h as in [13]. The reaction was stopped by addition of 8 N acetic acid to lower the pH to 4.0, and samples were dialyzed against running deionized water cooled to 4°C for 3 days. Freeze-dried reduced samples were adjusted to 10 mg/ml and cleaved for 4 h at 30°C using a 70% formic acid solution containing 10 mg/ml CNBr. Each preparation was then diluted 10-times in deionized water and deaerated under vacuum for 3 h prior to freeze-drying.

2.4. *SDS-polyacrylamide gel electrophoresis and fluorography*

Samples were taken up in SDS buffer [14] and submitted to SDS-polyacrylamide gel electrophoresis as in [15] with a 12% acrylamide (w/v) gel.

The location of tritiated peptides was done by fluorography as in [16] using Enhance (New England Nuclear) as a liquid scintillator and preflashed Fuji X-ray films. Tritium quantitation was carried out after excision of stained bands from the gel, homogenization in a Potter device, digestion at 60°C in Soluene 350 (Packard) then counting in Unisolve (Koch light) liquid scintillator.

3. RESULTS

Fig.1 shows a typical electrophoretic pattern of

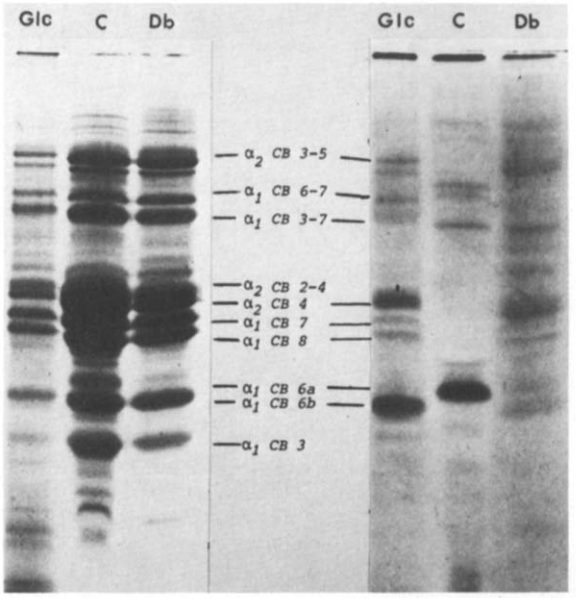

Fig.1. Fluorographic detection (right) of labelled CB peptides separated by SDS-polyacrylamide gel electrophoresis (left) from $NaB[^3H]H_4$-reduced collagen: in vitro glycosylated (Glc), control (C), diabetic (Db).

the major cyanogen bromide (CB) peptides of acid-soluble type I collagen. This appears unaffected by either in vivo or in vitro glycosylation. α_1CB6a and α_1CB3-5 peptides which both contain tyrosyl residues were identified on the acrylamide gels by ^{125}I labelling prior to CNBr cleavage. As described in [17] α_1CB6 runs as a doublet. ^{125}I-labelled α_1CB6a corresponds to the intact peptide and has a higher M_r. Unlabelled α_1CB6b results from partial hydrolysis during the experimental procedure and may appear as the major component after electrophoresis (fig.1).

Fluorography (fig.1) and tritium quantitation of $NaB[^3H]H_4$-reduced samples (table 1) demonstrate significant differences in tritium partition on the different peptides. To achieve maximal fluorographic resolution, the same amount of radioactivity was applied on the acrylamide gel so that the peptide content differed greatly from one sample to another because of changes in total specific activity (specific activity for normal = 1300 Bq/mg, diabetic = 2500 Bq/mg, in vitro glycosylated = 3400 Bq/mg).

Control collagen contains highly labelled α_1CB6a (30% of the sample radioactivity);

Table 1

Quantitation of the specific activities of CB peptides obtained from reduced collagens

Collagen	Peptide									
	α_2CB3-5	α_1CB6-7	α_1CB3-7	α_1CB2-4	α_2CB4	α_1CB7	α_1CB8	α_1CB6a	α_1CB6b	α_1CB3
Control	2.6	2.0	5.0	2.2	1.9	1.5	2.0	8.1	1.4	0.9
Diabetic	6.1	3.3	7.0	4.3	13.2	8.8	3.7	6.8	5.9	1.2
In vitro glycosylated	13.4	7.6	20	12.8	29.8	18.4	11.3	8.9	17.7	2.6

Values are expressed in Bq

α_1CB3-7 and to a less extent α_1CB6-7 also exhibits some radioactivity.

In the sample from diabetics, α_1CB6a was poorly labelled. The radioactivity was mainly distributed on fractions α_2CB4 (20%), α_1CB3-7 and α_1CB7.

In vitro glycosylated collagen also contains several labelled peptides including α_1CB6b (19%), α_2CB4 (21%), α_1CB3-7 (14%) and to a smaller extent α_1CB7 and α_1CB8.

These results were confirmed by CNBr cleavage and fluorography of collagen previously incubated with [U-^{14}C]glucose then reduced with unlabelled $NaBH_4$. In this case, the distribution was similar to fluorography performed after $NaBH_4$ reduction; however, we observed an additional radioactive band between α_1CB3-7 and α_2CB2-4 peptides (not shown). This radioactive fraction was further identified as a contaminant in labelled glucose reported in [18].

4. DISCUSSION

In control collagen a high level of tritium activity is found on the α_1CB6a peptide, i.e., the minor peptidic fraction of the α_1CB6 doublet. α_1CB6a differs from α_1CB6b only by an additive peptide identified as the carboxytelopeptidic end of α_1 chains. The negligible amount of radioactivity associated with α_1CB6b demonstrates that no glucose fixation occurs in this part of the molecule despite the presence of 5 potentially glycosylatable ϵ-amino groups [19]. Therefore the telopeptide preferentially binds tritium although it does not contain any glycosylatable group, but it does contain the reducible allysine 1044 normally implied in the intermolecular dehydrohydroxylysinonorleucine cross-link [20].

The low amount of tritium observed in α_1CB3-7 and α_1CB6-7 must be related to the slight glucose fixation previously reported during aging in physiological conditions [21], since no reducible precursors are present in this part of the α_1 chains.

In samples from diabetics the labelling is distributed mainly on the domain which contains 7 lysyl and four hydroxylysyl residues. The low activity observed in α_1CB6a could be related to the lesser amount of this peptide sampled in the gel as compared to control collagen. In other CNBr peptides including α_1CB6b, the distribution of the radioactivity appears in relation with the density of free ϵ-NH_2 groups, probably due to the non-enzymatic glucose fixation.

After in vitro non-enzymatic glycosylation specific activity increases by 250% as referred to control collagen and tritium is mainly found on α_2CB4 and α_1CB6. As described above for collagen from diabetics, the high content in glycosylatable ϵNH_2 groups would entail both in vivo and in vitro glucose fixation via the reducible glycosylamines in α_2CB4.

In α_1CB6 radioactivity is essentially located in α_1CB6b, pointing out the occurrence of reducible glycosylamines. Even when the labelling intensity is referred to the potentially glycosylatable ϵ-amino groups (fig.2) this peptide appears to be a privileged target for in vitro glucose fixation. A mechanistic explanation would imply the HYL930 residue which is known to react with allysine 9 leading to the dehydrohydroxylysinonorleucine cross-link. Acetic acid extraction of collagen prior to incubation with glucose results in the recovery of hydroxylysine 930 and the reactivity of this residue towards glucose should be markedly in-

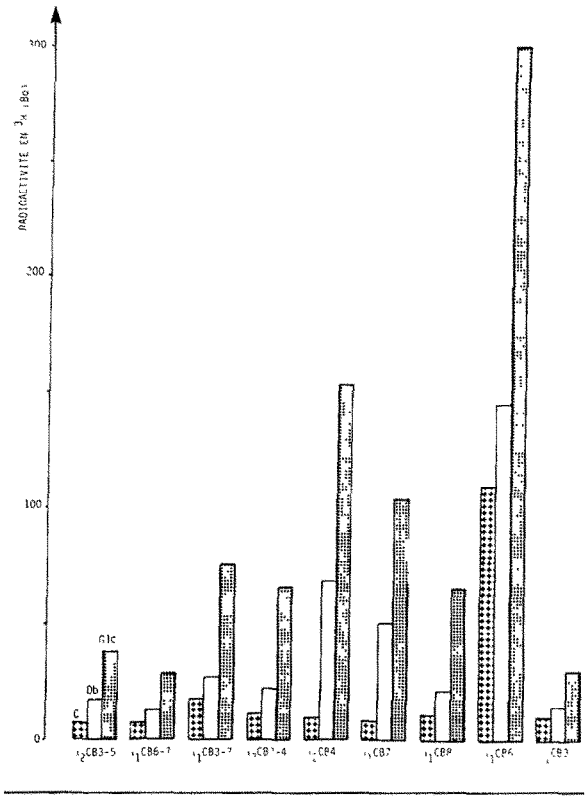

Fig.2. Distribution of tritium activity in the CB peptides of NaB[^{3}H]H$_4$-reduced collagen: control (C), in vitro glycosylated (Glc), diabetic (Db). Data are expressed as the specific activities referred to the potentially glycosylatable ϵ-amino groups.

creased due to the similarity between the ϵ-*N*-glycosylation and cross-linking processes (fig.3). Such an hypothesis is supported by the low glucose fixation observed in α_1CB6b from diabetic collagen, probably as a consequence of the preestablished cross-links.

A recent report [22] claimed that in vitro glycosylation would occur along the entire length of the collagen chains without site specificity. This disagrees with our results which give evidence for an ordered glucose fixation which differs from in vitro to in vivo glycosylated collagen. Such an orientated glycosylation could be related to the observed dissimilarities in the physicochemical properties of these two types of glycosylated collagen [7,9]. For example, the directed fixation of glucose on sites of in vitro glycosylated collagen normally implied in cross-linkage may be proposed

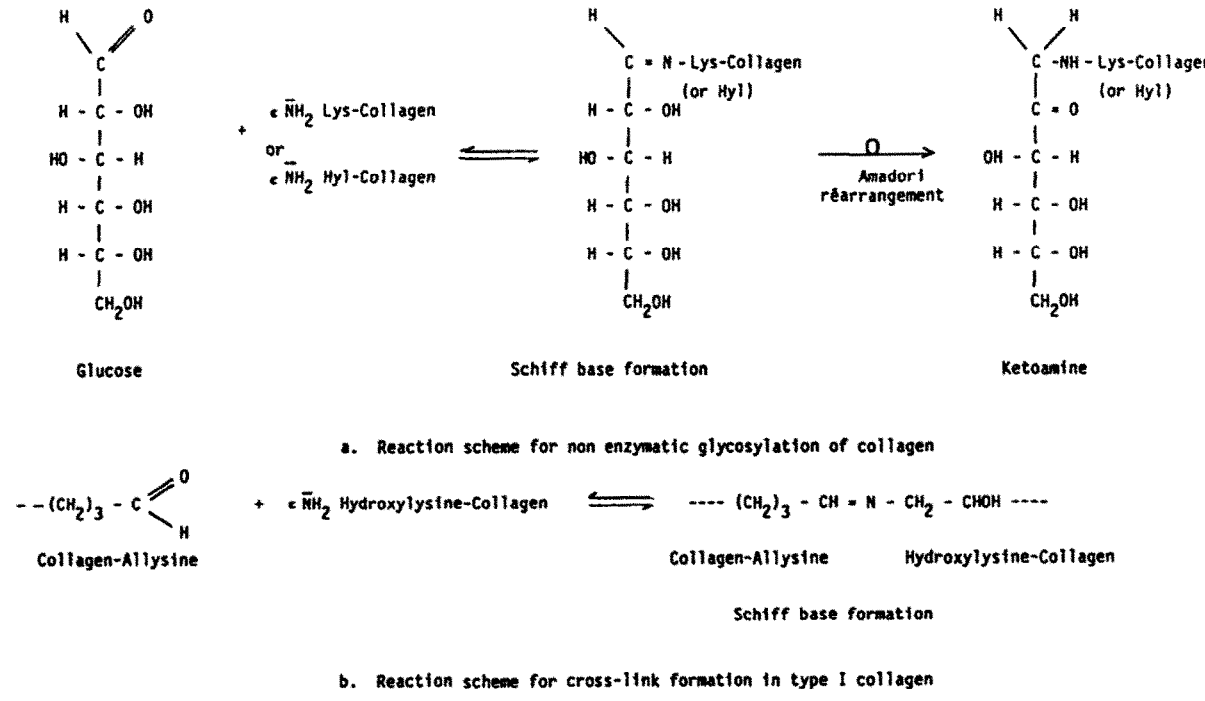

Fig.3. Comparative reaction scheme for ϵ-*N*-glycosylation (a) and cross-link formation (b).

as an explanation for the previously observed alterations of fibrillogenesis parameters and for the absence of fiber stabilization via cross-linking of in vitro glycosylated collagen [9].

The use of in vitro glycosylation, since it eliminates metabolic consequences of diabetes [23], allowed us to demonstrate the direct effect of glucose fixation in the increased platelet aggregating potency of collagen observed in diabetes [10]. Platelet adhesion and aggregation to collagen are closely dependent on the state of polymerization of collagen fibrils [24] and this cell–matrix interaction is known to imply specific domains of the molecule [25,26]. This localization of the bound glucose both in vivo and in vitro would thus permit a better understanding of the altered biological properties of glycosylated collagen which represents an important thrombotic risk during diabetes.

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