0014-4754/95/020121-02$1.50 + 0.20 © Birkhäuser Verlag Basel, 1995

Unexpected collagen crosslinking observed during in vitro radiolabeling of the galactosyl moiety

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Received 2 June 1994; received after revision 19 August 1994; accepted 1 September 1994

Abstract. Aldehyde groups, generated by oxidation of free primary alcohol groups of galactosyl residues of glycoproteins by galactose oxidase, can react with free amino groups on the polypeptide chain through Schiff base formation. Subsequent reduction with tritiated borohydride results in the formation of stable crosslinks instead of the expected generation of radiolabeled alcohol groups. Attempts to perform in vitro radiolabeling of collagen type I by this procedure resulted in undesirable crosslinking with profound alteration of the physical properties that rendered the resulting radiolabeled preparation unsuitable for biochemical studies.
Key words. Collagen; crosslinking; galactose; glycoproteins; radiolabeling.

In vitro radiolabeling of bio-molecules is very advantageous because it circumvents technical and ethical problems associated with in vivo labeling.

During our investigation of the effect of oxygen free radicals on corneal collagen[1,2] we found that radioisotopic methods offered a fast, simple and sensitive analytical tool to assess collagen crosslinking and degradation. Because of the inherent difficulties of radiolabeling in vivo, and of more specific problems associated with eye research such as the need to perform intraocular injection of radioactive proline and the small yield of corneal tissue obtained from rabbits, we decided to attempt in vitro radiolabeling.

The presence of galactosyl residues with unsubstituted C_6 in the prosthetic groups of collagen[3] offered what appeared to be a straightforward approach to our problem. Enzymatic oxidation of the galactosyl moiety of glycoconjugates by treatment with galactose oxidase results in the conversion of the primary alcohol in C_6 into an aldehyde[4]. By reacting this aldehyde with tritiated sodium borohydride or with ^{14}C-labeled sodium cyanide (Kiliani reaction) one obtains galactosyl moieties labeled with tritium on C_6 (ref. 4) or the corresponding ^{14}C-labeled cyanohydrin derivative[5], respectively.

We wish to report the occurrence of unexpected complications that illustrate the need to utilize this approach with caution.

Materials and methods

In order to prepare tritium labeled collagen, cold collagen type I was isolated from rabbit corneas excised from commercially available frozen rabbit eyes (Pel-Freez, Rogers, AR). The corneal extract was prepared and fractionated by the procedure described by Freeman[6]. This collagen preparation was then subjected to oxidation with galactose oxidase from Dactylium dendroides (Sigma, St. Louis, Missouri, USA), followed by reduction with tritated sodium borohydride (New England Nuclear) as previously described[4,5].

Results

Unlike the initial cold collagen preparation, or the ^{14}C-labeled collagen isolated from corneas of rabbits receiving intraocular injections of ^{14}C-labeled proline[1,2], the preparation of ^{3}H-labeled collagen obtained by this in vitro radiolabeling procedure was insoluble in phosphate buffered saline (pH 7.4). This solubility change rendered this preparation of corneal collagen unsuitable for our investigation of the formation of insoluble collagen aggregates catalyzed by autoxidative glycation[2].

In another study, while attempting to create aldehyde groups on the galactosyl residues of insoluble collagen, we encountered a similar problem. For these experiments, we utilized commercial insoluble collagen type I from bovine Achilles tendon (Sigma, St. Louis, Missouri, USA). Galactose oxidase treatment was performed at room temperature with a suspension of collagen under magnetic stirring in the presence of a small amount of toluene to prevent bacterial growth. A striking change of the physical properties of collagen was observed during this treatment. Instead of a homogenous suspension, sticky aggregates were formed with some of this material sticking tenaciously to the magnetic bar. Attempts to disperse or homogenize this material were unsuccessful. The observation that this collagen aggregation is correlated with the oxidation of the primary alcohol group of galactosyl residues to an aldehyde, suggested the hypothesis that the initial step in the formation of the

crosslinks may be the formation of Schiff bases between the ε-amino groups of lysine and hydroxylysine residues and the newly formed aldehyde groups. This would be consistent with the formation of covalent crosslinks in vivo which involves enzymatic oxidative deamination of the ε-amino groups of lysine and hydroxylysine to generate the aldehyde derivatives allysine and hydroxyallysine. These, in turn, react with remaining ε-amino groups to yield aldimines[7] which can progress to multivalent crosslinking to yield a three dimensional network of collagen fibers[7,8].

To test our hypothesis, a suspension of insoluble collagen type I from bovine Achilles tendon was subjected to citraconylation[9] in order to block the free amino groups. The citraconylated collagen was then sedimented by centrifugation and washed twice with distilled water alkalinized to pH 8.8 by addition of ammonium hydroxide. The suspension was stored overnight at 4°C and then recentrifuged, washed twice with phosphate-buffered saline (pH 7.0), and subjected to oxidation with galactose oxidase. Unlike the experiment with unprotected collagen, no collagen aggregates were observed upon incubation of citraconylated collagen with galactose oxidase. These findings indicate that free amino groups are essential for the formation of collagen crosslinks and therefore they are consistent with our hypothesis.

Discussion

Although bio-molecules radiolabeled in vitro are often more pure, more economical and with higher specific radioactivity than those obtained by metabolic labeling using radioactive precursors, special precautions must be observed to ascertain that the chemical and/or enzymatic steps utilized do not introduce changes in the composition, structure or properties of the original native molecule.

On the basis of our observations it is reasonable to conclude that during our attempts to prepare tritiated collagen by in vitro radiolabeling, the newly formed aldehyde groups generated by the action of galactose oxidase reacted with ε-amino groups of lysine and hydroxylysine to generate Schiff bases. During the subsequent treatment with tritiated sodium borohydride, reduction of the Schiff bases would result in the formation of stable covalent crosslinks and yield a radiolabeled collagen derivative substantially different from the starting material.

The possiblity of the formation of this type of artifact, particularly with proteins rich in lysyl residues, must be taken into consideration not only during radiolabeling of galactosyl residues but also during the radiolabeling of sialyl residues where exopyranosyl aldehyde groups are generated by mild periodate oxidation[10].

Citraconylation of the amino groups of glycoproteins prior to chemical or enzymatic oxidation may offer a convenient approach to preserve the aldehyde groups free and available for reduction to the radiolabeled primary alcohol. Subsequent removal of the citraconyl groups can be achieved under mild acidic conditions[9].

Acknowledgements. This research was supported in part by NIH grant EY06138, and by Research to Prevent Blindness, Inc.

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