THE RESTRICTED REACTIVITY OF THE SIDE-CHAIN AMINO GROUPS OF COLLAGEN TOWARD FORMALDEHYDE

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1. Introduction

The side-chain functional groups in proteins exert a dominant influence upon molecular conformation and biologic function. In collagen, the side-chain amino groups seem to be especially important. During collagen synthesis and maturation, lysyl side chains undergo hydroxylation and subsequently some serve as attachment sites for hexoses [1, 2]. Certain strategic lysyl side chains undergo enzymatic oxidative deamination and give rise to the intramolecular covalent crosslinks [3], and recent data indicate that the amino groups of both lysyl and hydroxylysyl side chains may participate in intermolecular crosslinking via Schiff-base formation [4, 5].

In several cases it has been possible, by the use of group-specific reagents, to distinguish between those functional groups which are available for biologic reactions and those buried within the molecule [6]. We have examined the location and configuration of the side-chain amino groups in collagen using various reagents that react with primary amines. We wish to report the presence in native soluble collagen of one site per alpha-chain which exhibits a high propensity for Schiff-base formation when formaldehyde is used as the reagent.

2. Experimental

Collagen was extracted from the skins of normal and lathyritic rats in 1 M NaCl, purified, and lyophylized using procedures previously described [7]. Collagen was placed in solution at a concentration of 4 mg/ml by stirring overnight at 4° in a buffer containing 0.5 M NaCl with 0.05 M disodium phosphate adjusted to pH 7.4 with HCl. Using the same buffer ^{14}C-formaldehyde containing 12 μCi/μmole (Lot 345-78, New England Nuclear Corp., Boston, Mass.) was adjusted to a concentration of 16 μmoles/ml as determined by assay as by Nash [8].

The reaction of formaldehyde with collagen was carried out in 10 ml beakers at 4° for a period of 30 min while stirring. Each beaker contained 1 ml of collagen solution, 0.08 to 32 μmoles of formaldehyde and buffer to a final volume of 4 ml. At the end of the reaction period the mixture was reduced with sodium borohydride, dialyzed exhaustively against 0.1 M acetic acid, lyophylized and stored in a desiccator over NaOH pellets. The degree of formaldehyde binding to each sample was determined by counting the radioactivity present using standard techniques. One mg of the lyophylized material, along with a control sample which was not treated with formaldehyde, was dissolved in 1 ml of hyamine X-10 in potassium-free scintillation vials by warming. This was followed by addition of 10 ml of scintillation fluid (15.1 g of PPO, 1.89 g of dimethyl POPOP, and 1 gallon of toluene). The vials were cooled to 4° in the dark and the radioactivity counted in a Packard model 3375 liquid scintillation spectrometer with an efficiency of 81%. Each sample was counted in triplicate. A molecular weight of 100,000 was assumed for the alpha-chain for calculation of aldehyde groups bound per chain.

3. Results

The binding curve for the reaction of formaldehyde with native collagen is shown in fig. 1. The curve exhibits two distinct phases. A curve which is almost

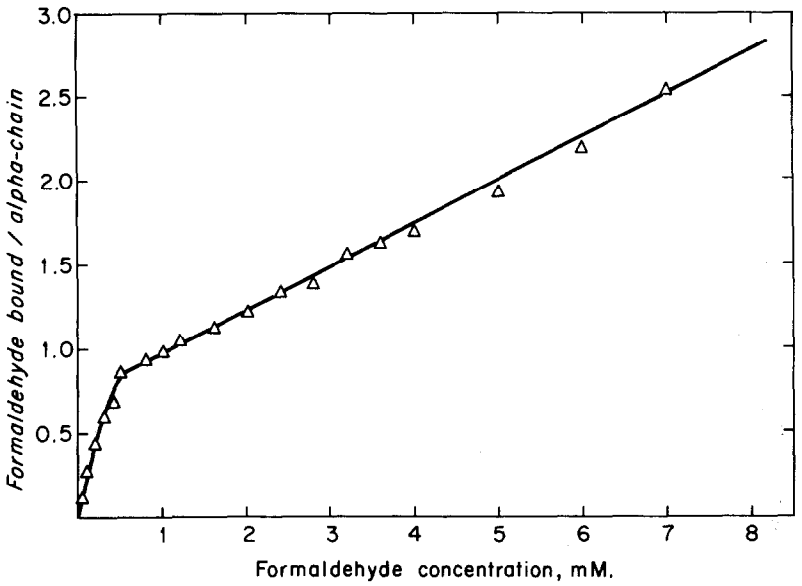

Fig. 1. Binding curve for the reaction of formaldehyde with salt-soluble native collagen. For details see Experimental section.

linear and exhibiting a steep slope is seen at reagent concentrations up to 0.5 mM. At this point 0.75 aldehyde groups per alpha-chain are bound. The curve then levels off significantly and continues apparently linearly to the highest concentrations of reagent tested.

In order to determine the number and reactivity of the binding sites and the influence of native helical structure upon them, binding curve data were obtained for both native tropocollagen and heat-denatured collagen and treated in the form of Scatchard plots [9]. As shown in the uppermost curve of fig. 2, native tropocollagen exhibits 1.3 binding sites per alpha-chain with a binding constant of 2.8×10^3. A second set of binding sites is indicated by the second phase of the curve. However, the slope of this portion of the curve approaches zero, indicating that it only slowly, if ever, intercepts the abscissa. Thus, the second set of binding sites appears to have a very low affinity constant and, for the reagent concentration range tested, they appear to be infinite in number. The Scatchard plot for heat-denatured collagen is seen in the lower curve in fig. 2. In contrast to our expectation, and to our previous findings with pyridoxal phosphate binding to collagen [10, 11], there appear to be 0.3 sites per alpha-chain which exhibit accentuated reactivity in denatured collagen.

The bond formed between formaldehyde and collagen is of a weak covalent nature. In reaction mixtures which were not reduced prior to dialysis only insignificant amounts of label could be found. Thus, the bond is unstable in dilute acid, but can be stabilized by reduction under very mild conditions. These are characteristics expected of Schiff-base compounds.

In order to determine the nature of the formaldehyde-binding sites, hydrolysates of collagens containing various amounts of covalently bonded ^{14}C-formaldehyde were prepared and examined on a Technicon amino acid analyzer using the 21-hr analysis procedure with monitoring of radioactivity and ninhydrin color. In addition, collagen labeled *in vivo* with uniformly labeled ^{14}C-lysine was reacted with non-labeled formaldehyde, and hydrolysates of this material analyzed in the same manner. Hydrolysates of collagen samples reacted with low concentrations of formaldehyde (up to 0.5 mM) exhibited a single new radioactive ninhydrin-positive maximum which eluted as a leading shoulder on hydroxylysine. This substance was present in ^{14}C-lysylcollagen reacted with non-labeled formaldehyde and in non-labeled collagen reacted with ^{14}C-formaldehyde. The unknown substance co-chromatographs with standard epsilon-*N*-methylhydroxylysine (Cyclo Chemical Co., Los Angles, Calif.).

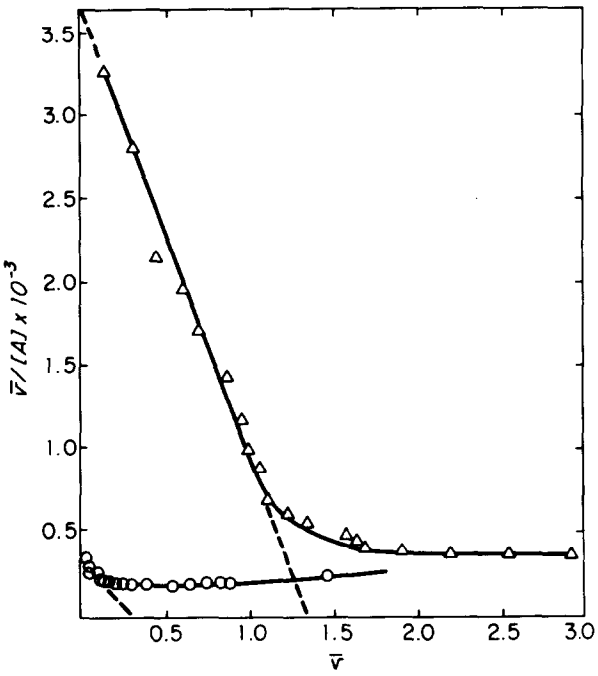

Fig. 2. Scatchard plot for the binding of formaldehyde to native salt-soluble collagen. The symbol $\bar{\nu}$ indicates the number of binding sites occupied per alpha-chain of collagen and (A) is the molar concentration of formaldehyde in the reaction mixture. The intercept on the abscissa shows the number of binding sites present and the intercept on the ordinate is the product of the number of binding sites and the affinity constant. △—△, Native tropocollagen; ⊙—○, heat denatured collagen.

Hydrolysates of material reacted with concentrations of formaldehyde in the range 1–2 mM exhibited the chromatographic peak described above, and in addition a second ninhydrin-positive radioactive substance which eluted in the position of standard epsilon-*N*-methyl-lysine was seen. Hydrolysates of material prepared with very high concentrations of formaldehyde exhibit a large number of ninhydrin-positive substances which contain label and which were not further identified.

4. Discussion

Since collagen is a stiff, rod-like molecule which is only 12–15Å in diameter, it has been considered likely that all of the functional side-chain groups are exposed on the surface of the molecule. However, the biologic properties of collagen suggest that there may be great variation in the environment of various functional side chains and thus in their availability for reaction. For example, only a very few side chains appear to be available to serve as substrate for lysyl oxidase [12] and lysyl hydroxylase, and an even smaller number serve as the attachment site for hexoses [1]. These considerations have led us to use small molecules as agents with which to probe the surface of collagen molecules, particularly with regard to the availability and reactivity of lysyl and hydroxylysyl side chains.

In previous experiments we have shown the presence in native collagen of 15 binding sites per tropocollagen molecule exhibiting a high propensity for Schiff-base formation with pyridoxal phosphate [10, 11]. The magnitude of the affinity constants of these sites was dependent upon native conformation of the molecule. Six of these sites were shown to be hydroxylysyl side chains and 9 were lysyl side chains. The hydroxylysyl side chains were blocked at the very lowest concentration of reagent tested [11], suggesting a high affinity for the reagent. With formaldehyde as the molecular probe, it appears that there is a set of 4 binding sites per tropocollagen molecule and these are most likely all hydroxylysyl side chains. Thus, while pyridoxal phosphate labels a family of reactive epsilon amino groups comprised of both lysyl and hydroxylysyl side chains, formaldehyde appears to be more specific. It labels fewer side chains and it may provide a means whereby the reactive hydroxylysyl side chains may be differentiated from the reactive lysyl side chains.

It is somewhat puzzling that under the conditions used over 95% of the total epsilon amino groups in native soluble collagen are prevented from reacting with formaldehyde, or they do so only with a sluggish affinity. This observation, and the data obtained using pyridoxal phosphate as reagent, suggest that even though all of the functional side-chain groups in collagen may be located on the surface of the molecule, there may be side-chain interactions and side chain-solvent interactions leading to significant variation in the availability and reactivity of the side-chain functional groups. Collagen molecules may therefore have a far more complex surface structure than has previously been suspected.

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