SCHIFF BASE FORMATION BY THE LYSYL AND HYDROXYLYSYL SIDE CHAINS OF COLLAGEN

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Recent evidence suggests that lysyl residues in collagen serve a dominant role in determining the biologic characteristics of the molecule. Certain strategic lysyl side chains undergo oxidative deamination and give rise to the intramolecular crosslink (Bornstein and Piez, 1966); lysyl side chains subsequent to hydroxylation serve as a site of attachment of hexoses (Butler and Cunningham, 1966); and recent data indicate that lysyl side chains may also participate in intermolecular crosslinking via Schiff base formation (Bailey, 1968; Tanzer, 1968). Thus, collagen might be expected to contain a small family of unusually accessible and reactive side chains, the characteristics of which are dependent upon native conformation. The ability to mark these residues would permit the determination of their location and characteristics.

Pyridoxal phosphate seems to be a particularly suitable reagent with which to mark these side chains. This compound reacts with lysyl side chains in both enzymatic and non-enzymatic proteins by Schiff base formation (Fischer et al, 1958; Dempsey and Christensen, 1962). It may be present at the active site of the enzyme which normally oxidizes the crosslinking lysyl residues and therefore might be expected to have a high affinity for them (Hill and Kim, 1967; Pinnell, et al, 1968; Page,

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1968). Very small amounts of pyridoxal phosphate can be detected in protein as a result of the characteristic absorption and fluorescence spectra.

Experimental

Collagen was extracted from the skins of growing rats in 1M NaCl, purified as previously described (Page and Benditt, 1968) and lyophylized. Collagen solutions for reaction with pyridoxal phosphate (PLP) were prepared in 0.5M NaCl with 0.05M phosphate pH 7.4 at a concentration of 4 mg/ml. Pyridoxal phosphate (Calbiochem, Grade A) was prepared in the same buffer at a concentration of 16 uM/ml. Reduction was carried out using a solution of sodium borohydride of 10 mg/ml made up in water. The reaction was carried out on mixtures containing 2 ml of collagen solution, 0.16 to 32 uMoles of PLP, and buffer to make a final volume of 4 ml in 10 ml beakers at 4° with stirring. After completion of the reaction, the mixture was reduced by the addition of 0.15 ml of borohydride solution; stirring was continued, and additional portions of 0.15 ml of borohydride solution were added after 15 and 30 minutes. The mixtures were then dialyzed against 0.1N acetic acid until pyridoxyl absorbance reached a stable minimum, heated at 45° for 20 minutes, and centrifuged at 100,000 x g for 15 minutes. Collagen concentration was determined after centrifugation by measurement of the hydroxyproline content (Page and Benditt, 1966). The pyridoxyl-collagen preparations were then scanned in a spectrophotometer against a reduced collagen blank. The molar absorbance of ε-N-pyridoxyllysine was taken as 5,800 (Dempsey and Christensen, 1962), and the molecular weight of the alpha-chain of collagen as 100,000.

Results

A typical absorption spectrum obtained from the reduced pyridoxyl-collagen is shown in Figure 1. There is an absorption maximum at 323mu; this is characteristic of ε-N-pyridoxyllysine.* In reaction mixtures which

* Standard ε-N-pyridoxyllysine was the generous gift of Drs. Edmond Fischer and Paul Strausberg.

were not reduced prior to dialysis, the 323 mu absorbance could not be detected, indicating a high degree of reversibility and a bond of low stability. In reaction mixtures carried out using pyridoxamine and pyridoxine phosphates, no binding could be demonstrated. Thus, the aldehyde function of pyridoxal phosphate does react with collagen to form a bond with the characteristics of a Schiff base. Studies of the time course of this reaction showed that equilibrium is reached within 30 minutes, and all subsequent experiments were done using this time period.

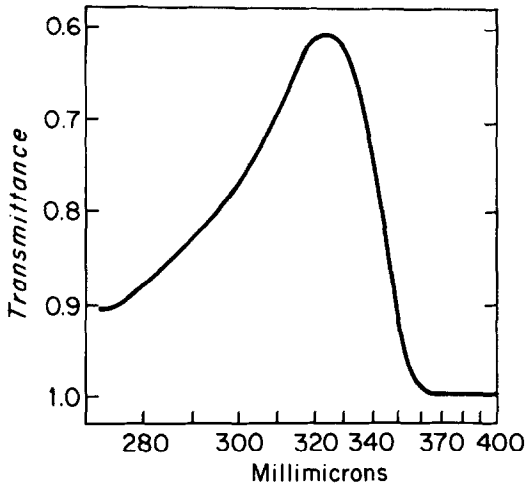

Fig. 1. The difference spectrum of pyridoxyl-collagen and reduced collagen; 4 mg/ml in 0.1N acetic acid.

The binding curves for the reaction of pyridoxal phosphate with native and heat-denatured collagens are shown in Figure 2. Native collagen binds approximately six PLP groups per alpha-chain while heat-denatured collagen binds considerably more. These data suggest that the presence of native helical structure influences the number and reactivity of the binding sites. This conclusion is supported by the fact that the binding curve for denatured collagen is relatively smooth and symmetric, suggesting similarity of the binding sites, while the curve for native collagen exhibits two or three plateaus, suggesting the presence of sets of binding sites with differing affinities for the reagent.

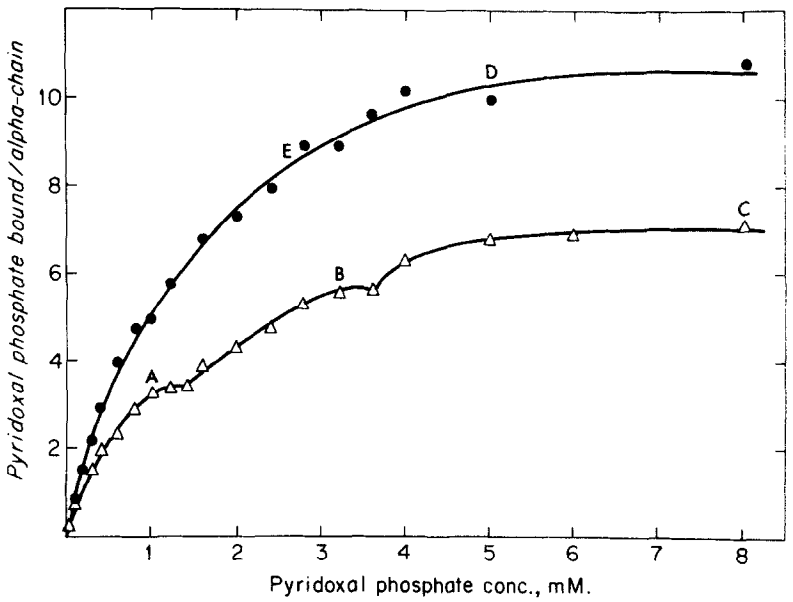

Fig. 2. Binding curves for the reaction of pyridoxal phosphate with native (-Δ-Δ-Δ-) and heat-denatured (-●-●-●-) salt-soluble collagen. The letters (A-E) denote samples used for the chromatographic analysis reported in Table I.

In order to determine the nature of the binding sites, several samples of native and denatured collagen containing different amounts of bound pyridoxyl were prepared, hydrolyzed, and analyzed chromatographically using the 21-hour Technicon amino acid procedure. Standard ε-N-pyridoxyl-lysine added to hydrolysates of collagen or to standard amino acid mixtures, eluted as a well-resolved symmetric peak between histidine and arginine. Chromatograms of pyridoxyl-collagen exhibited a peak in this same position. Addition of standard ε-N-pyridoxyllysine to these hydrolysates accentuated the size of this peak, but did not alter its symmetry. In addition to the ε-N-pyridoxyllysine peak, a small amount of material eluted as a shoulder on histidine and subsequent to arginine. As indicated below, this material

most likely represents ε-N-pyridoxylhydroxylysine or its breakdown product.

The chromatographic data are summarized in Table I. Comparing the number of ε-N-pyridoxyllysine residues found by the chromatographic analyses with the observed reduction in the number of lysine residues, we find excellent agreement; however, these values do not account for all of the pyridoxyl bound to the original sample as measured spectrophotometrically. In addition to the missing lysine residues, the number of hydroxylysine residues in both native and heat-denatured collagen was reduced; the total missing residues, allowing for some destruction during hydrolysis, corresponds well with the amount of pyridoxyl known to be present in the sample. Thus, the aldehyde function of pyridoxal phosphate reacts specifically with the epsilon amino group of a small number of lysyl and hydroxylysyl side chains by a bond exhibiting the characteristics of a Schiff base; all of the pyridoxal bound can be accounted for by this reaction.

TABLE I. CHROMATOGRAPHIC ANALYSIS OF PYRIDOXYL-COLLAGEN*

Sample	Native			Denatured	
	A**	B	C	D	E
Pyridoxyl content***	3.2	5.5	6.0	8.5	10.0
PLP-Lys content	1.0	2.5	3.3	3.8	6.2
Lys residues missing	1.0	2.1	3.2	3.8	6.6
Hyl residues missing	1.9	2.3	2.1	3.4	3.3
Total missing residues	2.9	4.4	5.3	7.2	9.9

*Reported as residues per 1000 amino acid residues

**Refers to samples noted in Fig. 1

***Pyridoxyl content was measured spectrophotometrically; PLP-lys, lys, and hyl content were measured on duplicate amino acid chromatograms using standard techniques.

In native collagen reacted with very low concentrations of pyridoxal phosphate (a 3.3 fold molar excess was used to obtain data in Column A, Table 1), only one lysyl and two hydroxylysyl side chains per alpha-chain react. The remaining hydroxylysyl groups in native collagen will not react even at extremely high reagent concentrations; however, heat denaturation makes one additional hydroxylysyl group available for reaction. Increasing the reagent concentration causes two additional lysyl side chains in native collagen to react and heat-denaturation makes three or four more available to the reagent. The collagen used for these experiments contained 27.5 lysyl and 7.1 hydroxylysyl residues per alpha-chain by triplicate amino acid analysis. Of these 35 groups, only one lysyl and two hydroxyllysyl side chains exhibit a high degree of availability for Schiff base formation.

These data demonstrate in collagen the presence of a small family of lysyl and hydroxylysyl side chains exhibiting the reactivity which might be expected of residues which participate in molecular aggregation and fibril maturation.

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