Inhibition of β-Crystallin Cross-Linking in the Ca\textsuperscript{2+}-Treated Lens

L. Lorand, S. M. Conrad, and P. T. Velasco

β-Crystallin dimers of approximately 55,000 weight are among the early products of protein cross-linking in Ca\textsuperscript{2+}-treated rabbit lens, caused by the activation of intrinsic transglutaminase; formation of the cross-linked species can be blocked by 75 mM histamine (Lorand et al, Biochemistry, 24:1525–1531, 1985). As an extension of this work, we initiated a search for more specific inhibitors of cross-linking in this system. Of the compounds tested so far, hydroxylamine, methoxyamine, bisaminoxypropane and aminoacetoni trile were particularly effective, inhibiting the generation of the cross-linked dimer in the 5 to 10 mM concentration range during a 4 hr treatment of the lens with Ca\textsuperscript{2+}-ions. Invest Ophthalmol Vis Sci 28:1218–1222, 1987

Post-translational protein modifications, in general, might play important roles in the process of lens aging and cataract formation. They could change the solubilities of crystallins, disturb their normal aggregation characteristics and alter the interactions of cytoplasmic proteins with membrane components. The isolation of N\textsuperscript{ε}-(γ-glutamyl)lysine peptides from polymers typically present only in cataractous lens\textsuperscript{1} called attention to the biochemical reactions catalyzed by transglutaminase.\textsuperscript{2}

Certain subunits of β-crystallin are highly specific substrates for transglutaminase, and the primary product is a cross-linked dimer X(β)\textsubscript{2}, with an apparent mass of about 55,000 (ie, 55K). In the rabbit material, the 55K species can be elicited either by reacting purified β-crystallin with the Ca\textsuperscript{2+}-activated enzyme\textsuperscript{3} or simply by immersing the whole lens in a Ca\textsuperscript{2+}-rich medium.\textsuperscript{4} X(β)\textsubscript{2}, though probably a heterogeneous product, by immunological cross-reactivities, is known to comprise the 29–30K\textsuperscript{4} and the 23K βBp subunits of β-crystallins.\textsuperscript{5}

We have previously shown that the formation of X(β)\textsubscript{2} could be inhibited by adding histamine (75 mM) to the incubation mixture.\textsuperscript{4} Inasmuch as the Ca\textsuperscript{2+}-treated lens might be used as an experimental cataract model,\textsuperscript{6} it was desirable to search for inhibitors which might be effective in lower concentrations than histamine and to gather insights into the conditions necessary for blocking the generation of dimeric β-crystallin.

Materials and Methods. Each rabbit lens (supplied by Pei Freez, Rogers, AK) was incubated at 37°C for 1 hr in 0.5 ml of medium buffered with 50 mM Tris-Cl, pH 7.4. The solution contained 1 mM leupeptin (gift of the U.S.-Japan Cooperative Cancer Research Program), varying concentrations of amine inhibitors and NaCl to match the concentrations of these constituents in the preincubation medium. To control specimens, 0.1 ml of 10 mM ethylenediaminetetra-acetate (EDTA) was added. At the end of the 4 hr period, lenses were cooled to −20°C and were stored
Fig. 1. The effects of histamine, 3-β-aminoethylpyrazole and 3-β-aminoethyltriazole on the generation of the ~55K cross-linked crystallin species during 4 hr of Ca\(^{2+}\)-treatment of lens. For experimental details, including SDS-electrophoresis, see Materials and Methods.

Frozen. Just prior to electrophoresis, each lens was solubilized (2 hr at 37°C) in a 4 ml solution containing 20 mM sodium phosphate, pH 7.1, 9 M urea, 40 mM dithiothreitol and 2% sodium dodecylsulfate (SDS). Insoluble material was removed by filtration through a 0.45 μm HAWP membrane (Millipore, Bedford, MA). Electrophoresis was carried out by the method of Laemmli, applying 14–20 μl material per lane, as before. Coomassie Brilliant Blue was used for staining.

Fig. 2. Inhibition of cross-linking in Ca\(^{2+}\)-treated lens by hydroxylamine and methoxyamine. Effect of Ca\(^{2+}\)-incubation in the absence of amines is shown in the gel on the right.
**Results.** Though each experiment, ie, each SDS gel, necessarily pertained to a different lens specimen, generation of the ~55K product by exposure to Ca\(^{2+}\) was remarkably reproducible, as was the dose-response for inhibiting the reaction by a given amine. Representative gel patterns are shown in Figures 1-4. For each panel, a horizontal line marks the 55K M\(_r\) position and, in order to better focus on this region of the gels, the heavily stained crystallin bands below ~33K were cut from the photographic prints.

In corroboration of earlier observations, histamine [H\(_2\)NCH\(_2\)CH\(_2\)R; R = \(\text{H}\)] was again found to be inhibitory at ≥ 20 mM concentrations, but neither the "N-dimethylated histamine [(CH\(_3\))\(_2\)NCH\(_2\)CH\(_2\)R] nor histidinol [HOCH\(_2\)CH(NH\(_2\))CH\(_2\)R] inhibited cross-linking. This indicates the importance of a primary amine group at an unbranched location in the aliphatic chain on the inhibitor.

Actually, even slight changes in the imidazole moiety of the histamine molecule were deleterious to inhibition. As shown in Figure 1, two of the histamine analogues, 3-β-aminoethylpyrazole and 3-β-aminoethyltriazole, had no effect, emphasizing the significance of side chain substituents for specificity.

The inhibitory potencies of cystamine [H\(_2\)NCH\(_2\)-CH\(_2\)SSCH\(_2\)CH\(_2\)NH\(_2\)] and glycine methylester [H\(_2\)NCH\(_2\)COOCH\(_3\)] were similar to that of histamine. However, the N-methylated derivative of glycine methylester, sarcosine methylester, had no effect on cross-linking.

Hydroxylamine [H\(_2\)NOH] and methoxyamine [H\(_2\)NOCH\(_3\)] were more powerful as inhibitors than any of the compounds mentioned above. Figure 2 shows that 10 mM hydroxylamine essentially blocked the formation of the 55K dimer. The bifunctional, O-substituted derivative, bisaminoxy propane [H\(_2\)NOCH\(_2\)CH\(_2\)CH\(_2\)ONH\(_2\)], appeared to be even more potent (Fig. 3). Interestingly, aminoxypropionate [H\(_2\)NOCH\(_2\)CH\(_2\)COOH] was not inhibitory at all, suggesting that the negatively-charged anion in the side chain was undesirable.

As in the experiments with Ca\(^{2+}\)-loaded human erythrocytes, protein cross-linking in lens could be prevented by relatively low concentrations of aminoacetonitrile [H\(_2\)NCH\(_2\)CN; cyanomethylamine], as shown in Figure 4.

**Discussion.** By virtue of their inhibitory effects, primary amines of certain types play a prominent role in strategies of probing transglutaminase-mediated cross-linking phenomena in biological systems. The rationale for the inhibition is that the enzyme (E) can catalyze not only the cross-linking of proteins, (illustrated as P and P' in scheme 1):

\[
P + \text{CONH} + \text{H}_2\text{N} \xrightarrow{E} \text{P} \xrightarrow{\text{Ca}^{2+}} 
P' + \text{NH}_3 \quad (1)
\]

but also the incorporation of small naturally occurring or synthetic amines (H\(_2\)NR) into the reactive \(\gamma\)-glutamine-containing protein substrate (P in scheme 2):

\[
P + \text{CONH} + \text{H}_2\text{NR} \xrightarrow{E} \text{P} \xrightarrow{\text{Ca}^{2+}} 
P' + \text{NH}_3 \quad (2)
\]
In a biochemical sense, both schemes are oversimplified, not showing either the Michaelis complex or the covalent acylenzyme intermediate: $P \rightleftharpoons \text{COS} - E$. As first discussed for the selective inhibition of fibrin cross-linking during blood coagulation,$^8$ there is a competition between the e-lysine nucleophile of the donor substrate $P$ of scheme (1) and $\text{RNH}_2$ of scheme (2) for the aminolytic partitioning of this acylenzyme intermediate. The pathway of deacylation also involves a Michaelis complexation step for the binding of the amine to the acylenzyme.

Transglutaminase shows marked specificities towards amines. In experiments with the purified enzyme acting on synthetic substrates, amine specificity can be expressed by well-defined kinetic parameters, comprising the affinity of the amine for the acylenzyme intermediate and the deacylation rate constant.$^2$ The first was shown to be greatly influenced by chemical nature, such as the length and composition of the $R$ substituent, and the second seems to depend on the nucleophilic character of the amino group in $\text{H}_2\text{NR}$.

When protein cross-linking occurs in an extracellular environment (such as in vertebrate and invertebrate blood coagulation, or in the clotting of seminal fluid),$^2$ relative inhibitory potencies of amines correlate rather well with their affinities for the enzyme and with their nucleophilicities. The situation, however, is quite different when intra-cellular cross-linking phenomena are investigated. In such instances, additional factors such as the penetration of the inhibitor into the cell, associations and secondary chemical reactions of the amine may become of overriding significance and, in practice, every cell type has to be evaluated on its own merit. Aminoxy compounds, for example, which could not be used with human erythrocytes because of the problem of forming coordination complexes with the iron in hemoglobin, were among the best for inhibiting the cross-linking reaction in the lens (Figs. 2 and 3). Aminoacetonitrile or cyanomethylamine (Fig. 4) was also very effective in blocking the formation of the 55K product during Ca$^{2+}$-treatment of the lens. Perhaps the relatively small bulk together with the low $pK_a$s of the ammonium ions in these compounds allow for easy access to the cross-linking sites in the packed crystallin array.

All the amines shown to be effective in this study are thought to act as competitive inhibitors of protein cross-linking. If conditions for treatment of the lens with Ca$^{2+}$ were to be different, the actual inhibitory potencies of these compounds might change. In addition, cystamine could also function through a disulfide exchange mechanism, blocking transglutaminase directly. It is noteworthy in this regard that iodoacetate itself (2 mM) was found to inhibit the production of the $\sim$55K species.

Key words: cataract, transglutaminase, $\beta$-crystallin dimer, inhibition of cross-linking

From the Department of Biochemistry, Molecular and Cell Biology, Northwestern University, Evanston, Illinois. Supported by USPHS Career Award HL-03512 and by Grant EY-03942 from the
National Institutes of Health. Submitted for publication: January 22, 1987. Reprint requests: Laszlo Lorand, Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 Sheridan Road, Evanston, IL 60201.

References