Biochimica et Biophysica Acta, 603 (1980) 371—374

BBA Report

BBA 71491

DOES RANDOM COLLISIONAL CROSS-LINKING OCCUR?

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(Received August 15th, 1980)

Key words: Chemical cross-linking; Hemoglobin; Fluid membrane; Molecular collision

Summary

In fluid membranes, mobile molecules are thought to collide at high frequencies. Concern has been expressed as to whether these colliding molecules are cross-linked during the chemical cross-linking of membrane molecules, thereby creating problems in interpreting such experiments. Hemoglobin was used as a model to test this possibility. Oligomers larger than the tetramer could be cross-linked depending on factors such as hemoglobin concentration, duration of the cross-linking reaction and the type of reagent. Under certain conditions, however, such as a hemoglobin concentration less than 150 μM or a duration of cross-linking shorter than 15 min, larger oligomers were not detectable. Analysis of these data suggests that the probability of random collisional cross-links under normal conditions is insiginificant.

In fluid membranes [1], some molecules undergo rapid lateral diffusion [2] and are therefore expected to collide with one another. For example, a membrane protein of radius 2 nm and diffusion coefficient $1 \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$ is expected to undergo self-collision approximately once every second [3]. The collision frequency of lipids is approximately four orders of magnitude greater than this [4]. During these collisions, molecules can be biologically cross-linked by multivalent ligands, antibodies and lectins [5].

Chemical cross-linking has frequently been used to investigate static molecular associations in biological membranes. Because multivalent biological ligands and chemical cross-linking reagents appear to cross-link in a similar manner, however, the question arises as to whether experimentally observed cross-linked products reflect natural stable complexes or are a consequence of collisional events [6]. In previous studies [3,7,8] we have avoided this problem by milli-

second cross-linking with photosensitive heterobifunctional reagents which minimizes random collisions. These results indicate that only natural stable complexes are cross-linked in the erythrocyte membrane. The important question of the existence of random collisional cross-linking, however, remains unanswered. If the potential for such cross-links is real, then cross-linking studies can be misleading, and additional controls are necessary. On the other hand, these phenomena could be used to identify colliding molecules in fluid membranes and to investigate mechanisms of biological cross-linking of surface antigens and receptors in patch and cap formation. Solutions of hemoglobin offer a useful system for testing the possibility of random collisional cross-linking, because the structural properties and especially the diffusion coefficient of hemoglobin are well established [9]. Under normal conditions, hemoglobin exists in a form no larger than the tetramer [10]. In this communication, we report that hemoglobin in solution can be cross-linked to oligomers larger than the native tetramer depending on the hemoglobin concentration and the time period of cross-linking.

Hemoglobin was prepared as described previously [8] and cross-linked with the homobifunctional reagents, diethyladipimidate, dimethyl-3,3′-dithiobispropionimidate, and the *N*-hydroxysuccinimide ester of dithiobispropionic acid. The duration of cross-linking and concentrations of reagents and protein were varied as described below. To compensate for the rapid hydrolysis of reagents in aqueous buffer [11], they were added incrementally. Cross-linking reactions were quenched by introducing 0.1 M glycine and sodium dodecyl sulfate was immediately added to solubilize the protein. Electrophoresis was performed on 0.5% agarose—2.8% polyacrylamide gels as described previously [3].

In earlier studies, it has been demonstrated that the formation of cross-links is dependent upon the concentration, specificity, and distance between functional groups of cross-linking reagents [11]. Therefore, several types of reagents possessing different functional groups and distances were tested in varying concentrations. Diethyladipimidate (9 Å) and dimethyl-3,3′-dithiobispropionimidate (12 Å) react specifically with primary amines. The *N*-hydroxysuccinimide ester of dithiobispropionic acid (12 Å) reacts primarily with amino groups, and to a lesser extent with sulfhydryl and imidazole groups [11].

To determine the optimal concentration of reagents, hemoglobin was cross-linked at a constant concentration of 150 μM, while the concentration of cross-linking reagents was varied (not illustrated). Concentrations of diethyladipimidate and *N*-hydroxysuccinimide ester of dithiobispropionic acid greater than 0.5 mM did not increase the extent of cross-linking while 1 mM dimethyl-3,3′-dithiobispropionimidate was required for maximum cross-linking. The type of reagent influenced the extent of oligomer formation, with dimethyl-3,3′-dithiobispropionimidate the most efficient reagent. In all cases, however, no oligomer higher than the tetramer could be detected. Since collisional cross-linking should be dependent on the frequency of collisions and, therefore, on the concentration of hemoglobin, cross-linking was also carried out with different concentrations of hemoglobin at constant reagent concentrations. In addition to hemoglobin monomer, dimer, trimer and tetramer, bands of higher molecular weight (pentamer and hexamer) appear as the hemoglobin concentration is increased to 210 μM (e.g., Fig. 1). The formation of pentamer and hexamer was

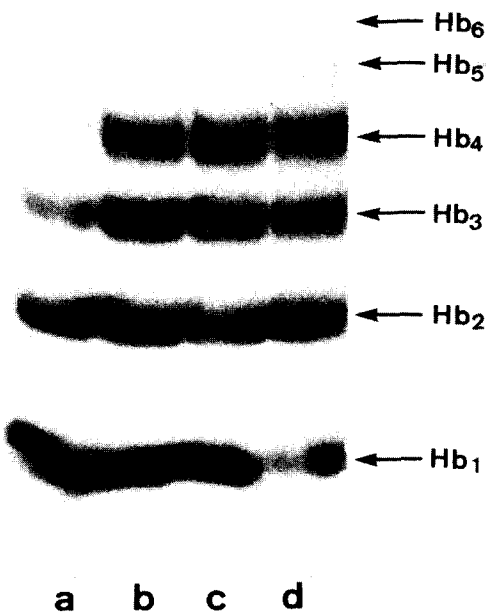

Fig. 1. Time-dependent cross-linking of human hemoglobin. Hemoglobin (210 μM) was treated for different time periods with incremental additions of dimethyl-3,3′-dithiobispropionimidate. (a) Cross-linked for 5 min with 1 mM of reagent added at 0 min. (b) Cross-linked for 30 min; 1 mM reagent was added at 0 min and 0.25 mM at 15 min. (c) Cross-linked for 60 min; 1 mM reagent was added at 0 min, 0.25 mM at 15 min and 45 min. (d) Cross-linked for 90 min; 1 mM reagent was added at 0 min, 0.25 mM at 15 min, 45 min, and 75 min.

only detectable, however, when hemoglobin was cross-linked with dimethyl-3,3′-dithiobispropionimidate. Since the number of random collisions will be dependent on the time period of cross-linking, hemoglobin at 210 μM was cross-linked with dimethyl-3,3′-dithiobispropionimidate for varying periods of time (Fig. 1). Again oligomers larger than the tetramer appeared on the gel, and after 90 min a trace of octamer was observed (Fig. 1d). These results indicate that hemoglobin in aqueous solutions can be cross-linked to oligomers larger than the tetramer. This is dependent on, among other factors, the concentration of hemoglobin as well as the duration of cross-linking and type of reagent; when the hemoglobin concentration is less than 150 μM or the duration of cross-linking shorter than 15 min, cross-linking to larger oligomers is not detectable. This suggests that random collisional cross-links are insignificant under these conditions. The diffusion coefficient of tetrameric hemoglobin is $7 \cdot 10^{-7}$

$cm^2 \cdot s^{-1}$ in aqueous buffers at 20°C [9], and the mean time between collisions of hemoglobin molecules is estimated to be 0.2 ms [3,12]. Therefore, during a cross-linking period of 15 min, each molecule is expected to collide $4.5 \cdot 10^6$ times, with the chance for a collisional cross-linking event considerably less than unity. This extremely low probability of random collisional cross-links is conceivably due to the fact that (a) the collision and the cross-linking reaction have to occur simultaneously, (b) the duration of association of randomly colliding molecules is considerably shorter than the mean time between collisions [3], and (c) a proper alignment of reactive chemical groups is necessary for successful cross-linking. The possibility also exists that the cross-linked hemoglobin oligomers larger than the tetramer represent natural aggregates of hemoglobin in solution rather than collisional complexes. This appears highly unlikely, however, since over the range of protein concentration, salt concentration and pH employed in these studies, a variety of experimental techniques show the tetramer as the only hemoglobin species present [10].

The finding that random collisional cross-linking occurs only under the extreme conditions described above supports the view that the formation of cross-links by this mechanism between membrane molecules is a rare event. This also explains the observation that the cross-linked complexes of erythrocyte membranes produced by homobifunctional reagents are similar to those produced by millisecond photochemical cross-linking (presumably natural complexes) [3,7,11]. There do exist conditions in which unnatural complexes can be cross-linked [11]; however, so caution in the interpretation of membrane cross-linking experiments must still be exercised.

This work was supported by National Science Foundation grants BMS 75-09230 and PCM 80-1193, the Research Corporation and the American Heart Association.

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