Intemolecular Cross-linking of Monomeric Proteins and Cross-linking of Oligomeric Proteins as a Probe of Quaternary Structure

APPLICATION TO LEUCINE AMINOPEPTIDASE (BOVINE LENS)*

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SUMMARY

Reaction of leucine aminopeptidase with dimethyl suberimidate at pH 8.5 and room temperature, followed by incubation with sodium dodecyl sulfate and mercaptoethanol and then electrophoresis in 3.5% acrylamide gels resulted in six protein bands with molecular weights as integer values of 56,000. Under reaction conditions where appreciable cross-linking took place, the species corresponding to dimers, tetramers, and hexamers were much more prevalent than those corresponding to trimers and pentamers, the latter being barely detectable. Providing that the ability of the protomers to form cross-links reflects their spatial arrangement in the oligomer, the results suggest that leucine aminopeptidase is composed of six identical protomers arranged as a trimer of dimers. Of the possible arrangements for a hexamer, the planar hexamer with all heterologous interactions is incompatible with these data. Thus, cross-linking reactions may yield information on the spatial arrangement of protomers in oligomers as well as on the number and kinds of protomers in the oligomer. Individual treatment of a number of monomeric proteins with dimethyl suberimidate in the frozen state at $-10^\circ$ in the presence of sodium dodecyl sulfate and mercaptoethanol yielded intermolecular cross-linked species which could be separated into a large number of components on gel electrophoresis in sodium dodecyl sulfate. For any one cross-linked protein the plot of logarithm of molecular weight against $R_F$ values between 0.2 and 0.8. The lines described by different proteins, although generally having similar slopes, were not always superimposable. This procedure for cross-linking monomeric proteins has potential use in determining molecular weights by gel electrophoresis in sodium dodecyl sulfate.

In the past few years increasing attention has been paid to the theoretical and experimental problems associated with the quaternary structure of oligomeric proteins (1-3). Information on the number and kinds of protomers in an oligomeric protein can be obtained by several physical and chemical approaches (2) of which the cross-linking procedure of Davies and Stark (4) is especially appealing because of its simplicity in terms of equipment. Information on the arrangement in space of the protomers has been available through the physical technique of electron microscopy or x-ray crystallography (3, 5). The results presented in this report indicate that, in some instances, the cross-linking reaction of Davies and Stark (4) may yield information on the arrangement of the protomers in space as well as on the kind and number of protomers in the oligomer.

Leucine aminopeptidase (EC 3.4.1.1), isolated in crystalline form from the bovine lens (6), has a molecular weight of about 320,000 (7, 8). In view of this large size it is not surprising to find that the enzyme has a number of subunits. Kretschmer (9, 10) and Kretschmer and Hanson (11), using a variety of techniques including gel chromatography in sodium dodecyl sulfate (9), electron microscopy (10), and sedimentation velocity and diffusion studies in sodium dodecyl sulfate and urea (11), have concluded that the molecule is made up of 10 subunits with molecular weights of 32,600. On the other hand, Weber and Osborn (12), using gel electrophoresis in sodium dodecyl sulfate, and Melbye and Carpenter (8), by a variety of techniques including gel electrophoresis in sodium dodecyl sulfate and sedimentation and equilibrium studies in a number of denaturing solvents of both the crystalline enzyme and the reduced and carboxymethylated derivative, arrived at a subunit size of about 54,000, indicating a hexameric structure for the enzyme. Recent studies of Vahl and Carpenter (13) and Vahl (14) on the stoichiometry of the zinc content of the enzyme and on the replacement of zinc by magnesium and manganese are in accord with the 54,000 subunit.

The present study investigates the nature of the oligomer by the cross-linking technique of Davies and Stark (4) in which dimethyl suberimidate is used to cross link the protomers while in the oligomeric form, followed by dissociation and gel electrophoresis of the cross-linked products in sodium dodecyl sulfate. The results not only indicate the hexameric nature of the enzyme but also suggest that the protomers are arranged as a trimer of dimers.

The present report also describes procedures by which mono-
meric proteins as well as oligomeric proteins can be cross-linked to give a variety of molecular species which in some cases vary from monomer to decamer. The cross-linked proteins yield distinctive patterns on gel electrophoresis in sodium dodecyl sulfate and have potential use as standards for determining molecular weights not only of monomeric but also of oligomeric proteins by the electrophoresis procedure. The procedure for obtaining a large number of cross-linked species consists of performing the cross-linking reaction in the frozen state in the presence of sodium dodecyl sulfate and mercaptoethanol.

**EXPERIMENTAL PROCEDURE**

**Materials**

Proteins—The proteins, their molecular weight and source were as follows: horse transferrin (74,000), Nutritional Biochemicals (lot 4696); bovine serum albumin (65,000) (15), Sigma Chemical Co. (lot 9883-0450); ovalbumin (48,000) (16), Pentex (lot 10); rabbit muscle aldolase (40,000, monomer) (16), Calbiochem (lot 901488); horse apoferritin (19,000, monomer) (17), Mann Research Laboratories (lot T3577). Leucine aminopeptidase was isolated in crystalline form from bovine lenses by the procedure of Hanson et al. (6).

Chemicals—Dimethyl suberimidate was synthesized from suberonitrile (Aldrich Chemical Co.) according to the directions of Davies and Stark (4). Differences are noted on the figure legends.

**Methods**

Cross-linking of Monomeric Proteins—Proteins (0.25 to 0.5 mg) were dissolved in 50 µl of a 0.2 M triethanolamine solution at pH 8.5 containing 0.3 mg of dimethyl suberimidate dihydrochloride. To the protein solution was added 50 µl of 2% sodium dodecyl sulfate-3% mercaptoethanol in 0.2 M triethanolamine at pH 8.5. The resulting mixture was frozen in Dry Ice-isopropanol and stored at −10°C for 16 hours. The solution was then incubated at 37°C for 2 hours and subjected to electrophoresis.

Cross-linking of Leucine Aminopeptidase The reaction with dimethyl suberimidate was performed essentially as described by Davies and Stark (4). Differences are noted on the figure legends.

Sodium Dodecyl Sulfate-Gel Electrophoresis—Most of the runs were performed essentially by the Davies and Stark modified (4) of the Weber and Osborn procedure (12), on gels containing 3.5% acrylamide, 0.13% N,N'-methylenebisacrylamide, mercaptoethanol, and ammonium persulfate were products of Eastman Organic Chemicals. The source of N,N,N',N'-tetramethylethylenediamine was Matheson, Coleman, Bell; of Coomassie Brilliant Blue R250 was Colab Laboratories, Inc., and of sodium dodecyl sulfate was Sigma Chemical Co.

**RESULTS**

Cross-linking Variety of Proteins in Frozen State—A number of proteins were treated with dimethyl suberimidate at −10°C in the presence of sodium dodecyl sulfate and mercaptoethanol, incubated at 37°C, and then electrophoresed on 3.5% gels. With the exception of aldolase and apoferritin, these proteins are presumed to be monomeric during reaction with the reagent. In electrophoresis, each gave at least 5 bands of decreasing intensity with increasing molecular weight. As many as 10 bands, corresponding to the products of decamers, could be discerned in some cases. Aldolase exists normally as a tetramer (4). In agreement with this, the band representing the tetrameric form was the strongest, indicating that intramolecular cross-linking took place more readily than the intermolecular reaction. However, some pentamers, hexamers, and heptamers were also formed. Their appearance indicates that some cross-linking between oligomers also took place under these conditions.

A plot of the logarithms of the molecular weight of each cross-linked protein against the corresponding RF value in the 3.5% gels is shown in Fig. 1. The line is the best fit of all of the data by the least squares procedure. For horse transferrin, the monomer molecular weight was determined to be 74,000 from its RF value on the least square line and the cross-linked species were plotted as multiples of the 74,000 value. The points for bovine serum albumin all lie slightly above the line while the points for aldolase all lie below the line. Those for ovalbumin closely approximate the line. The average absolute relative deviation of all the points about this line is 6.2%. However, for any one

**Fig. 1.** Plot of molecular weight on logarithm scale of cross-linked proteins against Rf on 3.5% gels for bovine serum albumin (○), apoferritin (●), ovalbumin (○), transferrin (●) and aldolase (△).
FIG. 2. A, reaction with dimethyl suberimidate before and after treating with sodium dodecyl sulfate and mercaptoethanol. Gel a, leucine aminopeptidase (0.25 mg per ml) was reacted at pH 8.5 in 0.2 M triethanolamine with dimethyl suberimidate (0.5 mg per ml) for 90 min at room temperature, then sodium dodecyl sulfate and mercaptoethanol were added to make 1% solutions and the resulting solutions were incubated at 37° for 2 hours, frozen at -10° overnight, and electrophoresed. Gel b, same as Gel a except that the leucine aminopeptidase was incubated with sodium dodecyl sulfate and mercaptoethanol at 37° for 2 hours, in order to disrupt the oligomeric structure of the enzyme, and then treated with dimethyl suberimidate at -10° in the frozen state. The principal component of the reaction mixture corresponds to the monomer with a slight amount of dimer also being present. These results show that very little cross-linking of monomeric proteins takes place once they have been fully denatured and enveloped in sodium dodecyl sulfate. Presumably the detergent masks the amino groups so that they can no longer react with the dimethyl suberimidate.

The effect of concentration of dimethyl suberimidate on the degree of cross-linking in the oligomer is shown in Fig. 2B where leucine aminopeptidase at 8.6 mg per ml has been reacted with the suberimidate at 6 mg per ml (Gel a) or at 0.5 mg per ml (Gel b). At the high concentration of reagent, there is very little monomer remaining and the trimer and pentamer bands are scarcely discernible. The principal species is hexamer with tetramer and dimer in reduced amounts in order. However, there is no evidence for a species of molecular weight higher than hexamer which indicates that there was little interoligomeric cross-linking.

The effect of the concentration of leucine aminopeptidase on the cross-linking reaction can be ascertained by comparison of Gel b of Fig. 2B along with the gels of Fig. 2C. The dimethyl suberimidate was kept constant at 0.5 mg per ml while the leucine aminopeptidase concentration was varied from 8.6 mg per ml (Gel b, Fig. 2B) to 0.25 mg per ml (Fig. 2C). The degree and extent of cross-linking was very similar in all cases except for a slightly decreasing diminution of the monomer form as the concentration of protein was decreased. This is the type of result that is expected of cross-linking reactions that are taking place intramolecularly in that they should be relatively insensitive to concentration of the protein.

The sizes of the various species present in the cross-linked leucine aminopeptidase were determined by comparing their RF values with those of a cross-linked preparation of ovalbumin as shown in Fig. 3. The line (—) connects the open circles (O) which are plots of the logarithm of molecular weight of various species of cross-linked ovalbumin ranging in size from monomer (45,000) to octamer (360,000) against their corresponding RF values. The cross-linked ovalbumin was prepared by reaction with dimethyl suberimidate in the frozen state. The vertical bars indicate the RF values of the various species of cross-linked leucine aminopeptidase. The molecular weights for each species along with their calculated subunit size (in parentheses) were as follows: monomer, 59,000 (59,000); dimer, 112,000 (56,000);...
trimer, 168,000 (56,000); tetramer, 220,000 (55,000); pentamer, 280,000 (56,000); and hexamer, 325,000 (54,000). The average subunit size of 56,000 is in good agreement with values previously published from this laboratory (8).

**DISCUSSION**

**Conditions for Cross-linking Monomeric Proteins**

There are two interrelated factors involved in the formation of polynucler molecules through intermolecular cross-linking of monomeric proteins with dimethyl suberimidate. These are freezing and the presence of sodium dodecyl sulfate and mercaptoethanol. Freezing alone is not sufficient nor does the presence of sodium dodecyl sulfate and mercaptoethanol in the absence of freezing have an effect. With sodium dodecyl sulfate one can visualize that the freezing process concentrates the protein and the reagent into micelles stabilized by the detergent. In this concentrated form the protein would be in a favorable condition for intermolecular cross-linking. Perhaps mercaptoethanol can operate in a similar fashion, serving as a solvent to concentrate the reagent and protein as the water is frozen out. Other addition might work as well. This point was not closely investigated but it was determined that neither the triethanolamine buffer nor the borate buffer could replace the sodium dodecyl sulfate in the frozen reaction.

The sodium dodecyl sulfate aided in the cross-linking reaction only if the solutions were frozen before incubation in the presence of detergent. If the proteins were heated in the detergent and then frozen with the dimethyl suberimidate very little cross-linking took place. Evidently denaturation and unfolding of the protein with the concomitant binding of detergent, especially to the free amino groups, effectively blocks the cross-linking reaction.

The degree of cross-linking increased as the length of time of freezing increased up to the longest time investigated (5 days). However, in most cases there was a high degree of cross-linking after freezing for 16 hours which did not increase dramatically in the next 4 days. So that for most purposes, overnight freezing should be adequate. However, if one desires to prepare cross-linked proteins containing a large variety of aggregates such as for use as standards in gel electrophoresis, it might be well to consider freezing for a prolonged period of time.

**Determination of Molecular Weights**

Since the introduction by Shapiro et al. (18) of the sodium dodecyl sulfate-gel electrophoresis procedure for estimating molecular weights of proteins, the technique has been applied extensively, especially by Weber and Osborn (12), to a variety of proteins. According to Reynolds and Tanford (19), the electrophoretic mobility of proteins in gels can be a function of molecular weight when (a) the charge per unit mass is constant and (b) the hydrodynamic properties are a function of molecular length only. Plotting the logarithm of the molecular weight for the monomer, dimer, etc. of serum albumin (Fig. 1) against gave a very good straight line, especially for values between 0.2 and 0.8. Similarly each of the other proteins shown in Fig. 1 yielded excellent straight lines. Thus for any one protein there is good correlation between size and . However, the lines for each protein, although generally having similar slopes, do not always coincide. This difference may be due in part to the better control of conditions when cross-linked proteins in one gel are compared rather than when different proteins in different gels are compared. Also the location of the line depends on the value selected for the monomer molecular weight. Using a slightly higher value for aldolase (42,000 rather than 40,000) and a slightly lower value for bovine serum albumin (63,000 rather than 65,000) would make these two proteins closely approximate the least square line.

Griffith (20) has recently reported that some cross-linked oligomers formed by reaction of proteins with glutaraldehyde migrated at a faster rate in sodium dodecyl sulfate electrophoresis than would be expected from their calculated molecular weights. Although we did not make a careful study of this possibility, the results shown in Fig. 1 do not reveal a consistent trend of this nature. Presumably both glutaraldehyde and dimethyl suberimidate form cross links primarily through the epsilon amino groups of lysine. However, they differ in that (a) the charge is retained in the reaction with dimethyl suberimidate while it is largely lost on reaction with the dialdehyde and (b) the length and flexibility of the connection formed with the dimethyl suberimidate is somewhat greater than that formed with the dialdehyde. These differences in the nature of the cross-linking reagent may account for the differences observed on electrophoresis of the two types of branched oligomers. Also in Griffith's work, the cross-linked oligomers were compared with monomers which did not contain any intramolecular cross links. In our work, the monomeric species could very well contain a number of intramolecular cross links. Such intramolecular cross links might constrain the molecule so as to affect the ratio of length to molecular weight in the denatured molecule.

The above results have some implications on the use of the sodium dodecyl sulfate-gel electrophoresis for determining the molecular weights of unknown proteins. (a) Cross-linking standard proteins makes it possible to cover a very wide range of molecular weight.
molecular weights. This will be useful especially in studies on oligomeric proteins. (b) Comparable cross-linking of an unknown protein will yield a number of points for comparison rather than just one point. (c) If the proteins selected here are representative cross-section of the behavior one can expect to find in nature, then cross-linked ovalbumin would serve as the best single marker protein for proteins above 40,000 daltons in that its regression line closely approximates the least square line for all points. Consequently, cross-linked ovalbumin was used as a standard in the following studies on leucine aminopeptidase.

Leucine Aminopeptidase

All Protomers of Same Size—Several factors indicate that the protomers of leucine aminopeptidase are all of the same size which suggests that they may be identical. Upon disruption in sodium dodecyl sulfate and electrophoresis, predominantly one species was obtained. If the oligomer had contained species of different size they should have been detected as equally prominent bands on electrophoresis. Upon disruption of intragligomeric cross-linked leucine aminopeptidase and electrophoresis, the sizes of the various species bore a whole number relationship to one another. This would not be possible if the oligomer contained protomers of different size except in the event that one protomer was exactly one-half of the size of the other protomer.

Number of Protomers Is Six—Disruption of the cross-linked oligomer, followed by electrophoresis, yielded five readily discernible bands and a sixth band, corresponding to pentamer, which was weak but detectable in preparations that had not been too heavily cross-linked. The six bands indicate a hexameric structure for the protein. In preparations which have been heavily cross-linked the hexamer was the most prominent species and there were no bands corresponding in size to anything greater than the hexamer. The molecular weight of the slowest moving band in cross-linked and dissociated leucine aminopeptidase agreed well in size (325,000) with that found by hydrodynamic methods (8) for the native oligomer (326,000). When this is coupled with the fact that the smallest unit detected by a variety of techniques, including sodium dodecyl sulfate-gel electrophoresis of noncross-linked, cross-linked, and carboxamidomethylated leucine aminopeptidase and equilibrium centrifugation in dissociating solvents such as urea and guanidinium chloride (8), has a molecular weight of 54,000 ± 4,000, then the weight ratio of oligomer to monomer is 6.

Arrangement of Protomers in Oligomer—The finding that the dimer, tetramer, and hexamer were the predominant species formed in the intragligomeric cross-linking reaction can be explained in two ways: (a) "spatial reactivity," meaning that the ability of a reagent to form cross links depends solely on the spatial arrangement of the protomers and their reactive groups; or (b) "induced reactivity," meaning that the reactive groups on the protomers are so arranged that when a cross link has been formed between any two protomers, the reactivity of the remaining groups is changed (in this case, the reactivity is diminished or the groups are masked). Although the experimental evidence does not allow a differentiation between these two explanations, the first is the most attractive because of its implications for use in determination of quaternary structure. When the interfaces between one protomer and any two or more adjacent protomers are not identical, one can expect this difference to be reflected in the ability of the reactive groups to form cross links. In turn this difference in ability to form cross links may be reflected in the distribution of cross-linked species in a partial reaction. For leucine aminopeptidase, the distribution of cross-linked species can arise from a spatial arrangement consisting of a trimer of dimers where the rates of formation of intradimer cross links are not the same as the rates for interdimer cross links.

Fig. 4 represents two planar arrangements of six protomers to give a hexamer. Cornish-Bowden and Koshland (3) have recently discussed the theory of quaternary structure of proteins composed of identical subunits for oligomers composed of dimers, trimers, and tetramers. In their terms of reference, Fig. 4A represents a planar hexamer composed of all heterologous binding domains (ppq). The lower diagram (Fig. 4B) represents a case where the hexamer is formed by all isologous interactions (pp, qqq, + - - + + -). In the theoretical treatment developed by Cornish-Bowden and Koshland (3), all possible interactions were allowed, i.e. pp, qqq, and pq, in the same oligomer. For the planar hexamer this treatment gives rise to a number of asymmetric forms between the extremes of the all heterologous and all isologous (Fig. 4) both of which are symmetrical. For simplicity the present discussion will be confined to those cases where all interactions are not allowed, i.e. an isologous interaction (pp) excludes a heterologous interaction (pq) and vice versa. With this limitation for the planar hexamer, only the two forms depicted in Fig. 4 exist. Of these two forms, the upper (Fig. 4A), consisting of all heterologous interactions, is incompatible with the cross-linking results on leucine aminopeptidase in that it should yield, depending upon the degree of cross-linking, either
increasing or decreasing amounts of all of the species from monomer to hexamer. On the other hand, the all isologous structure (Fig. 4B) is compatible with the cross-linking results on leucine aminopeptidase. If one assumes that cross-linking reactions take place more readily between pp interfaces than between qq (or vice versa), then in a partially cross-linked product reactions take place more readily between pp interfaces than on leucine aminopeptidase. If one assumes that cross-linking structure (Fig. 4B) is compatible with the cross-linking results given at the start of this section. Consequently there could exist enough differences in behavior between the four structures depicted here to allow their complete resolution by a comparison of experimental data with theoretical yields. The data on leucine aminopeptidase was not obtained in sufficient detail or accuracy to warrant such an attempt. At present the only structure which can be definitely excluded is the planar hexamer with all heterologous interactions. Even this conclusion is subject to some ambiguity because of the two explanations for the cross-linking results given at the start of this section. Nevertheless the cross-linking reactions deserve further consideration both theoretically and experimentally as a method for elucidating quaternary structure.

In this connection it should be noted that Kohlhaw and Boatman (21) observed a preponderance of dimers and tetramers over trimers in cross-linking studies performed on the tetrameric form of isopropylmalate synthetase with dimethyl suberimidate. This result is incompatible with an all heterologous planar structure (pq) for the tetramer. Either of the other two potential structures for a tetramer, the isologous planar tetramer (pp, qq, + + +) or the tetrahedron (pp, qq, rr) (3), could yield this cross-linking result. However, the fact that under some conditions the enzyme exists as a dimer (21) would favor the isologous planar structure for the tetramer.

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REFERENCES
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