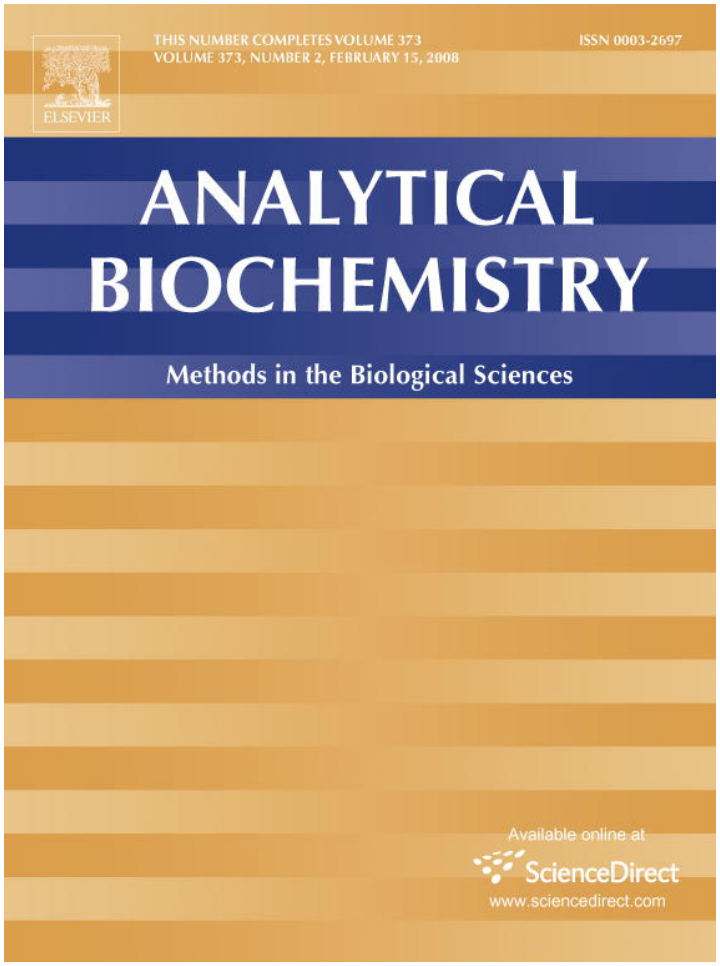

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Notes & Tips

Determination of protein oligomerization state: Two approaches based on glutaraldehyde crosslinking

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Abstract

Many biochemical and biophysical methods can be used to characterize the oligomerization state of proteins. One of the most widely used is glutaraldehyde crosslinking, mainly because of the minimum equipment and reagents required. However, the crosslinking procedures currently in use are impaired by the low specificity of the reagent, which can chemically bond any two amino groups that are close in space. Thus, extensive and time-consuming investigation of the reaction conditions is usually required. Here we describe two approaches based on glutaraldehyde that readily give reliable results.

Crosslinking by glutaraldehyde is often used to obtain preliminary information on the quaternary association of proteins (for instance, see Refs. [1,2]). The simplicity of the procedure, which requires only the mixing of glutaraldehyde with the protein solution [3–5], the availability of glutaraldehyde as a common reagent easily found in a biochemical laboratory, and the direct detection of crosslinked products by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) have led to the wide application of this method. However, the results are often ambiguous and uninformative. The major limitation of the technique arises from the nonspecificity of the reagent, which can react with all the nitrogens of a protein [6] and mainly with lysines, tyrosines, histidines, and arginines [7,8]. Intra- and intermolecular links are formed that could connect atoms of neighboring but not interacting molecules, yielding artificial protein oligomers that lack biological significance. To eliminate the possibility of artificial interactions, the proper reaction conditions must be established through a detailed and often time-consuming investigation of the influence of many parameters such as protein concentration, reagent concentration, temperature, and time of reaction [8]. Here we propose two protocols for readily identifying the oligomerization state of a protein without an exhaustive examination of reaction conditions. This is achieved by avoiding the direct mixing of protein with glutaraldehyde solution, which is the main deviation from the conventional "in-solution" approaches. The procedures described below expand on previously reported ideas [9,10], and they have been adapted for investigating the quaternary association of a protein in native conditions, optimized for giving rapid and reliable results, and tested to ensure a "ready-to-use" protocol for general application.

The first procedure includes an affinity chromatography step, is applicable to proteins that carry an affinity tag, and has been refined and presented here specifically for His-tagged proteins [11], which today have wide application. It has been demonstrated that immobilization of a protein onto an affinity matrix at low density could be critical to applications including bifunctional reagents [9,12]. Our protocol requires amounts of protein as small as 500 μg and employs a Ni–NTA column (Qiagen). The sample is diluted to a final volume of 4 ml with 10 mM imidazole,

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50 mM NaCl, 50 mM phosphate buffer, pH 8.0 (buffer A). It is better to avoid buffers containing amino groups such as Tris because the reactivity of these groups with glutaraldehyde could decrease the yield of the desired reaction (between protein and glutaraldehyde). A final volume of 0.8 ml of Ni–NTA agarose resin (Qiagen) is packed into a column (0.8 × 4 cm) and equilibrated with buffer A. The sample is slowly (at approximately 60 ml/h) applied to the column. Alternatively, the resin could first bind the protein in a batch mode and then be packed into a column. Two washing steps follow: the first consists of 8 ml (10 bed vol) buffer A, and the second, 8 ml (10 bed vol) of 50 mM NaCl, 50 mM phosphate buffer, pH 8.0 (buffer B). Glutaraldehyde is diluted with buffer B in a final concentration of 0.05% (v/v), and 2.5 ml of this solution (about 3 bed vol) is added on the column and left to pass through the resin by gravity. The excess reagent is washed out with 8 ml of 0.5 M Tris/HCl, pH 8.0, which also serves to quench the reaction. The bound protein is eluted with 300 mM imidazole, 50 mM NaCl, 50 mM phosphate buffer, pH 8.0, and the covalent oligomers are detected by SDS–PAGE. All steps are carried out at room temperature. To eliminate artificial interactions, that is, the formation of bridges between different quaternary entities, the protein is spread out on the matrix in a low protein/matrix ratio (0.6 mg/ml). Moreover, an even distribution of the protein on the resin is ensured by the presence of a low concentration of imidazole in the loading buffer. This requirement might be better achieved by the alternative procedure, in which the resin first binds the protein in a batch mode and then is packed into a column. Fig. 1A and B illustrate application of the protocol to the proteins Rop and *Bc*ZBP, respectively. SDS–PAGE analysis of the crosslinked products clearly indicates the dimeric association of the former and the hexameric association of the latter, results that are consistent with previous studies [13–15].

The second protocol also requires small amounts of sample, has wider application than the first protocol, and is even milder because the protein is not directly mixed with the glutaraldehyde but the volatility of the reagent is exploited. A related procedure has been previously described for crosslinking of protein crystals for cryocrystallography [10]. The experimental setup includes siliconized coverslips (microscope coverglasses of 22 mm, Molecular Dimensions Ltd.), a Linbro-like cell culture plate (XRL 24 well plate, Molecular Dimensions Ltd), vacuum grease sealant (Dow Corning high-vacuum grease, Hampton Research), microbridges (Hampton Research), and a water bath for temperature control. The experimental arrangement, which is illustrated in Fig. 2 is similar to a hanging drop crystallization experiment and proceeds as follows: A microbridge is filled with 40 µl of 25% (v/v) glutaraldehyde acidified with 1 µl of 5 N HCl and is placed on the bottom of the well. Alternatively, the glutaraldehyde solution could be placed directly on the bottom of the well without the microbridge. In that case, the only deviation from the procedure described below would be the greater

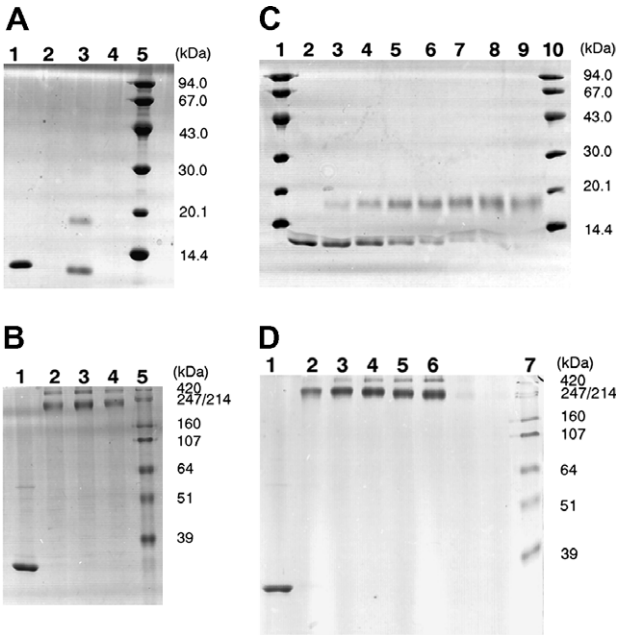

Fig. 1. Glutaraldehyde crosslinking of proteins for the identification of quaternary protein structure. (A) SDS–PAGE, 15% Laemmli of the His-tagged Rop protein treated according to the first protocol. Lane 1: Sample loaded onto the Ni–NTA agarose matrix (the molecular weight of monomers is approximately 7 kDa). Lanes 2–4: First, second, and third elution volumes from the column of His-tagged protein after glutaraldehyde treatment. A second population of molecular weight <20 kDa has appeared, clearly indicating the presence of dimers. Lane 5: Low-molecular-weight marker. (B) SDS–PAGE, 12% Laemmli of the His-tagged *Bc*ZBP protein treated according to the first protocol. Lane 1: Sample loaded onto the Ni–NTA agarose matrix. Monomers run as a band with apparent molecular weight of about 30 kDa. Lanes 2–4: first, second, and third elution volumes from the column of His-tagged protein after glutaraldehyde treatment. Most of the sample runs as a population with apparent molecular weight <200 kDa, indicating the presence of hexamers. Lane 5: High-molecular-weight marker. (C) SDS–PAGE, 15% Laemmli of the Rop protein treated according to the second protocol. Lanes 1 and 10: Low-molecular-weight marker. Lane 2: Original sample not treated with glutaraldehyde. Lanes 3–9: Influence of glutaraldehyde vapor on the protein solution at different time intervals: 10, 20, 30, 40, 60, 120, and 180 min. A second population of molecular weight corresponding to that of dimers has appeared, consistent with results obtained from the first protocol (A). (D) SDS–PAGE, 12% Laemmli of the *Bc*ZBP protein treated according to the second protocol. Lane 1: Original sample not treated with glutaraldehyde. Lanes 2–6: Influence of glutaraldehyde vapor on the protein solution for different time intervals: 10, 20, 30, 40, and 60 min. Most of the sample runs as a population with an apparent molecular weight corresponding to that of hexamers, consistent with results obtained from application of the first protocol (B). Lane 7: High-molecular-weight marker.

time needed for reagent evaporation and crosslinking. Ten to fifteen microliters of approximately 0.5 mg/ml protein solution is placed onto the coverslip which is then used to seal the well, with the protein solution left to form a hanging drop inside the well. A small amount of grease applied to the rim of the well ensures isolation of the system from the environment. The entire arrangement can be placed in a water bath for temperature control. Many time intervals can be checked simultaneously. Application of the protocol is exemplified for Rop and *Bc*ZBP proteins, and

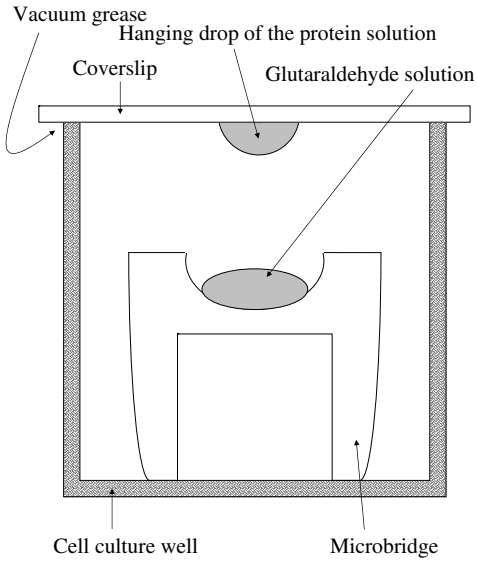

Fig. 2. Schematic diagram illustrating the entire experimental setup, used in the second protocol.

the results are demonstrated in Fig. 1C and D. SDS–PAGE analysis of the crosslinked products of Rop (Fig. 1C) reveals slow conversion from monomers to dimers. The time intervals are 10, 20, 30, 40, 60, 120, and 180 min while the temperature is 30 °C. SDS–PAGE analysis of the crosslinked *Bc*ZBP protein, which is illustrated in Fig. 1D, reveals different behavior. The conversion from monomers to hexamers is almost completed in the first 20 min of the experiment. The time intervals are 10, 20, 30, 40, and 60 min and the temperature is maintained at 30 °C.

In conclusion, we have described two procedures for glutaraldehyde-based crosslinking of proteins that allow the efficient and dependable determination of their oligomerization state. Our results suggest that these procedures are generally applicable to all globular proteins.

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