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NATIVE MOLECULAR WEIGHT OF ADENOVIRUS PROTEINS : ON THE OLIGOMERIC STRUCTURE OF THE FIBER

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Summary. Fluorescamine-modification of amino groups was used to eliminate the influence of basic charge on the final migration position of protein's in alkaline pH polyacrylamide gradient gel electrophoresis. As applied to adenovirus structural components, this type of analysis suggested the fiber to be composed of three identical subunits. The trimeric nature of both penton base and fiber therefore displaces the problem of symmetry mismatching to penton base and surrounding hexons at each vertex of the adenovirus icosahedron.

The quaternary structure of adenovirus capsid proteins has been most often deduced from the molecular weight of the constituting polypeptide subunit, as determined by SDS-polyacrylamide gel electrophoresis of SDS-denatured proteins, and/or from the physical parameters S, D, and V obtained for the isolated proteins in their native structure (reviewed in 1, 2). Electron microscopy of negatively stained samples can also confirm the subunit composition of certain virus macromolecular components. This is the case for adenovirus hexon, in which three subunits can be discerned, either in the native capsomer (3), or after cross-linking with bifunctional reagent (4). The trimeric structure of the hexon is not surprising since it has six neighbours in the virion (1,3). A three-fold symmetry is compatible with a hexavalent capsomer.

However, two recent studies have shown that the subunit composition of a virus morphological component could neither be inferred from its location on a symmetry axis, nor from the number of its nearest neighbours. X-rays crystallographic studies on polyoma virus capsid have suggested that six-coordinated capsomers can be pentamers (5). Adenovirus penton base capsomer, which is positioned on the capsid five-fold axis and is pentavalent, was thus far considered as a pentameric protein (1,6). Neutron scattering and sedimentation data have recently revealed the penton base to be composed of three polypeptide subunits (7), a result which was consistent with previous sedi-

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mentation and gel filtration analyses (2), and with the stoichiometric analysis of penton base polypeptide within the mature virion (7).

Although the molecular weight of roughly globular molecules such as hexon or penton base can be unambiguously determined by neutron scattering, major uncertainties remain for elongated or fibrous proteins such as adenovirus fiber (7). The neutron scattering diagrams obtained with the fiber were compatible with a protein molecule constituted of either two or three identical polypeptide subunits of molecular weight 62,000 (8,9). In the experiments reported here, the oligomeric structure of adenovirus type 2 fiber was re-investigated by a purely biochemical approach. It consisted of a molecular sieve electrophoresis of chemically modified fiber. The modification with fluorescamine of fiber amino groups eliminated the influence of basic charge on its mobility in polyacrylamide gradient gel electrophoresis at alkaline pH.

MATERIALS AND METHODS

. Fluorescamine labeling of proteins. Fluorescamine reaction was performed as follows. A freshly prepared solu- tion of fluorescamine (Hoffman - La Roche Inc.) in acetone at a concentration of 5 mg per ml was added to the protein sample dissolved in 0.2 M sodium bicarbonate buffer, pH 8.2, while stirring vigorously on a Vortex mixer. Usually twice 5 µl of the fluorescamine solution was added to 50 µl of protein sample at 0.5-1.0 mg/ml. Fluorescamine instantaneously reacted with primary amines. Excess reagent rapidly hydrolyzed to nonfluorescent products. Acetone was then evaporated under a current of nitrogen, and glycerol added up to 15 % final concentration, to allow layering on top of the polyacrylamide gel.

. Polyacrylamide gel electrophoresis. The alkaline gel system consisted of a polyacrylamide gradient slab gel (5-30 %) with an acrylamide to bisacrylamide ratio of 50 : 1.33, cast in 0.385 M Tris-HCl buffer, pH 9.4. The resolving gradient gel was overlaid by a spacer gel made of 5 % polyacrylamide (acrylamide : bisacrylamide ratio of 50 : 1.33) in 0.3 M Tris-HCl, pH 8.9. The tank buffer was 0.05 Tris-glycine, pH 9.4. Electrophoresis was performed at a constant voltage of 10 V/cm and arrested when the visible band of ferritin marker had migrated a distance of 1.5 - 2.0 cm from the top of the gel (usually 72-96 h). The gel was examined and photographed under UV light at 366 nm, then fixed and stained with Coomassie blue R-250.

. Molecular weight markers. Standard proteins were reacted with fluorescamine before being used as molecular weight markers (10). They were : hog thyroglobulin (669 Kd) ; horse spleen ferritin (440 Kd) bovine fibrinogen (390 Kd) ; adenovirus type 2 hexon (330 Kd, in its native state) ; beef liver catalase (232 Kd) ; beef heart lactate dehydrogenase (140 Kd) ; rabbit muscle phosphorylase B (92.5 Kd) ; aldolase (158 Kd ; composed of four subunits) ; bovine serumalbumin (67 Kd for the monomer, 134 Kd for the dimer)) ; ovalbumin (43 Kd). Ferritin, catalase, bovine serum albumin, aldolase and ovalbumin were separate components of the calibration kit Combithek II (Boehringer). Phosphorylase B was contained in the LMW protein calibration kit (Pharmacia Fine Chemicals). Thyroglobulin and lactate dehydrogenese were present in the HMW protein calibration kit (id), along with bovine serumalbumin, catalase and ferritin. Adenovirus type 2 hexon was purified as previously described (11). Bovine fibrinogen was a gift from Dr. L. Tranqui (CENG, Grenoble).

RESULTS

As shown in Fig. 1, a linear calibration curve was obtained with fluorescamine-modified standard proteins in the range of molecular weights from 80,000 to 660,000 daltons. A discrete break was visible in the curve between high and low molecular weight domains. Fluorescamine-labelled hexon migrated as a protein of molecular weight 330,000-350,000 daltons, a value consistent with the data previously reported (2) and with the amino acid sequence (12).

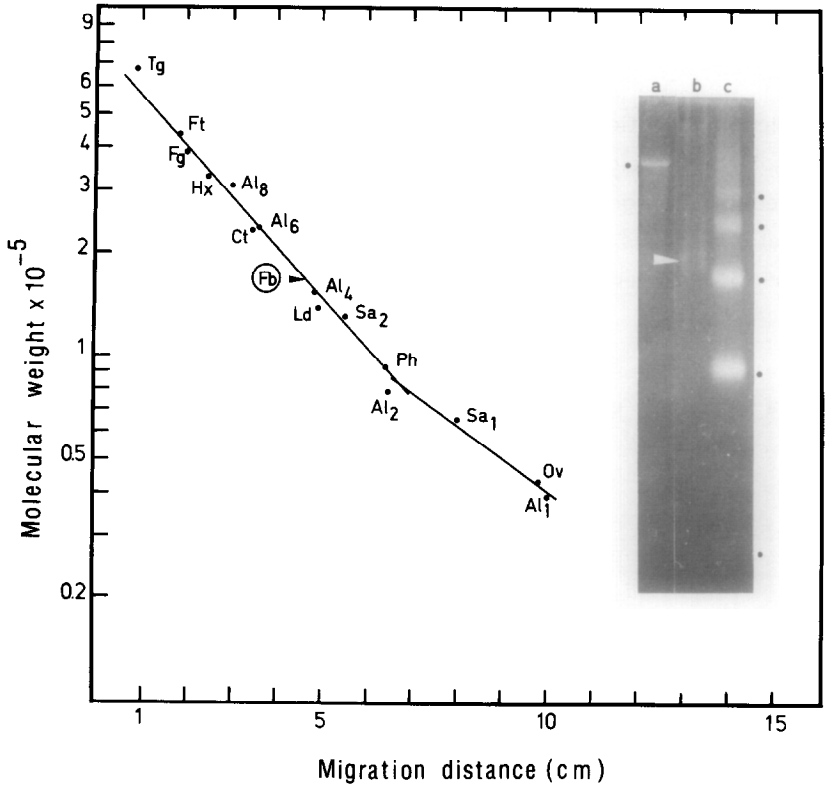

Figure 1 : Calibration curve of proteins electrophoresed in a 5-30 % polyacrylamide gradient gel run in alkaline pH. Standard proteins were : thyroglobulin (Tg, 670 Kd) ; ferritin (Ft, 440 Kd) bovine fibrinogen (Fg, 390 Kd) ; adenovirus 2 hexon capsomer (Hx, 330 Kd) ; catalase (Ct, 232 Kd) ; aldolase (Al, 158 Kd) ; lactate dehydrogenase (Ld, 140 Kd) ; phosphorylase b (Ph, 92.5 Kd) ; serumalbumin (Sa, 67 Kd) ; ovalbumin (Ov, 43 Kd). Sa-1 corresponded to the serumalbumin monomer, Sa-2 to its dimeric form. Aldolase, which is normally composed of 4 subunits of 39 Kd (Al-4) dissociated into isolated subunits (Al-1) and pairs of subunits (Al-2) and reaggregated into multiple of subunit pairs (hexamers, Al-6, and octamers, Al-8). Note the curve break at about 80,000 between high molecular weight and low molecular weight domains. The arrowhead indicates the migration position of adenovirus 2 fiber (Fb) in its native form. Inset : gradient gel photographed under UV light at 366 nm showing fluorescamine-labeled adenovirus 2 hexon (a), fiber (b) and the series of oligomers of aldolase subunits (c). Note the presence of fiber aggregates on top of slot (b), a phenomenon which has been often observed (1).

After fluorescamine modification and prolonged electrophoresis in an alkaline gradient gel, no significant difference in electrophoretic migration was observed between globular and fibrous proteins : fibrinogen was positioned on the curve at its theoretical molecular weight of 390 K.

Fluorescamine-modified adenovirus fiber migrated slightly slower than aldolase in its native enzymatic form (i.e. tetrameric association of 40 Kd subunit) and significantly faster than (232 Kd) catalase and aldolase subunit hexamer (240 Kd). The calibration curve gave fiber a molecular weight of 165,000 daltons (Fig. 1), a value compatible with a trimer, and too high for a dimer of a 62 Kd subunit.

DISCUSSION

The electrophoretic mobility of a non-denatured protein sample depends upon its molecular weight and its net electric charge. The influence of the intrinsic electric charge is far from being negligible. This is examplified by electrophoretic analysis of adenovirus capsid proteins : the fiber migrates in non-denaturing homogeneous acrylamide gel slower than the penton base and

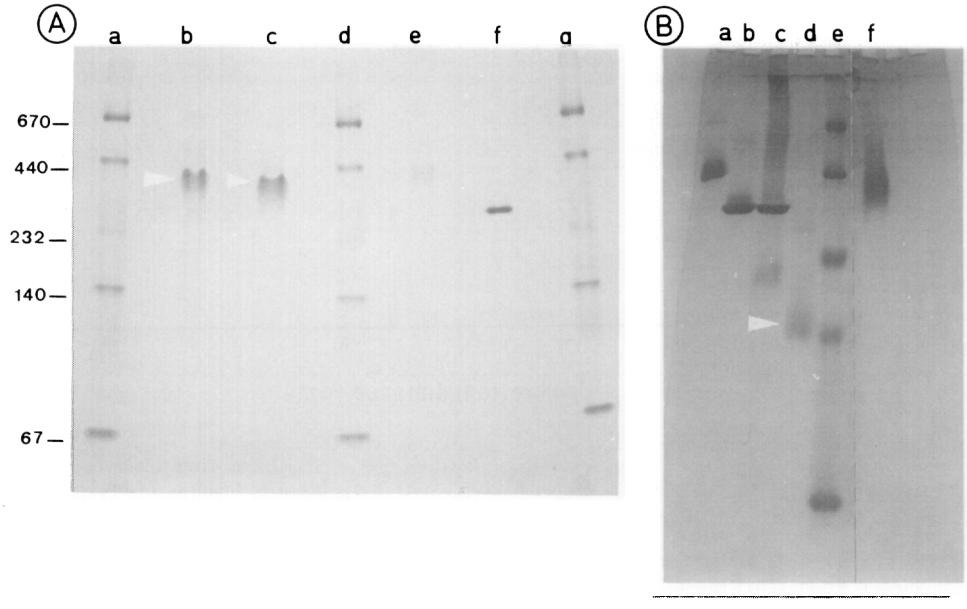

Figure 2 : Comparative migration of unmodified (A) and fluorescamine-modified (B) proteins in polyacrylamide gradient gel. Gel (A) ; (a,d,g) : HMW calibration kit proteins (thyroglobulin, ferritin, catalase, lactate dehydrogenase, and albumin) ; (b,c) : adenovirus 2 fiber ; (e) : adenovirus 2 penton ; (f) : adenovirus 2 hexon. Gel (B) ; (a) ferritin ; (b,c) adenovirus 2 hexon ; (d) adenovirus 2 fiber ; (e) HMW calibration kit markers ; (f) bovine fibrinogen. Both gels, made of 5-30 % polyacrylamide, were dried and stained with Coomassie blue R-250. The molecular weights of protein markers are given in kilodaltons. Arrowheads indicate the position of the fiber.

hexon of higher molecular weights (6,11). This major drawback has been partly overcome by electrophoresis in acrylamide gradient gel. Prolonged electrophoresis in a pore gradient gel minimizes the influence of the proteins's charge on their final migration position (13). However, this was not the case for the adenovirus structural proteins. As shown in Fig. 2A, native fiber migrated slower than hexon, even in gradient gel. This is why it appeared necessary to eliminate the electric charge parameter from the electrophoretic analysis.

The modification of proteins amino groups will increase their intrinsic negative charge and at alkaline pH all the peptides and proteins will migrate towards the anode. Their final migration will then only depend upon their molecular weight. Fluorescamine has been largely used for fluorimetric assay of amino acids (14) or for fluorescent labeling of peptides and proteins (15,16). The use of fluorescamine offers at least two advantages : (i) it renders the proteins visible under UV light during and after migration in gel ; (ii) it blocks the basic amino groups. After fluorescamine-labeling, the native hexon and fiber migrated in alkaline gradient gel as proteins of 330,000 and 165,000 daltons, respectively (Fig. 2B).

The quaternary structure of the fiber remained a matter of controversy. Cross-linking of protein samples with a variety of bifunctional reagents and subsequent analysis in SDS-polyacrylamide gel, a method profitably applied to the determination of the hexon oligomeric structure (4), was found to be unsuccessful in the case of the fibre : no discrete band of preferred oligomeric species was displayed, even when electrophoresis was performed in gels of low acrylamide concentration and/or of low degree of bisacrylamide cross-linking (unpublished data). It has been recently shown that in chemical cross-linking of proteins, cooperative cross-linking events can occur, resulting in mixtures of covalently bound aggregates (17,18). This probably explains why most of the cross-linked fiber material remained on top of the acrylamide gel, whatever the experimental condition, viz. when fiber was reacted at low concentration to minimize the intermolecular cross-links, or when low reagent concentration was used to decrease the cooperativity phenomenon (not shown).

The results of electrophoretic analysis of fluorescamine-modified adenovirus fiber analyzed in alkaline polyacrylamide gradient gel were thus in good agreement with those obtained with conventional biophysical methods suggesting a trimeric structure for adenovirus fiber (2,19). The trimeric nature of both fiber and penton base therefore displaces the symmetry-matching problem to the penton base and surrounding hexons at each apex of the adenovirus capsid.

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