JOURNAL OF VIROLOGY, Dec. 1975, p. 1678-1682
Vol. 16, No. 6
Copyright © 1975 American Society for Microbiology
Printed in U.S.A.

NOTES

Adenovirus Assembly: Self-Assembly of Partially Digested Hexons

PIERRE A. BOULANGER

Unité de Recherches n° 102 de Virologie Moléculaire de l' I. N. S. E. R. M., Lille, France

Received for publication 25 June 1975

Papainic hydrolysis of adenovirus type 2 hexon capsomers was apparently limited and induced self-aggregation of hexons in a preferred oligomeric species, hexon trimers, sedimenting at about 19S, and retaining their immunological properties. These hexon trimers were composed of two classes of trimer molecules with different electric charges. The relevance of this trimer aggregation phenomenon to the adenovirus assembly process is discussed.

The hexon capsomer is the main structural subunit of the adenovirus capsid (17) and carries both a group-specific complement-fixing antigen and type-specific antigen (12, 16, 18). Hexons are produced in large excess by the infected cell and can be isolated as soluble structural antigen (2, 3, 13, 17, 19). The molecular weight is 300,000 to 330,000 for the intact native protein, which is composed of three identical polypeptide units of 100,000 to 110,000 (4, 8, 9). The hexons have a natural tendency to self-aggregate, spontaneously or after limited proteolysis (2, 12). A study of the mode of assembly of hexon capsomers can be a valuable clue for elucidating the mechanism of adenovirus capsid assembly. The aim of the present work was to analyze the hexon aggregates occurring after a limited proteolysis of the hexon molecule; although no evidence has been reported for peptide bond cleavage of the main capsid components during assembly of adenovirus particles, this remains a possible mechanism, as demonstrated for other viruses (7, 10).

The isolation and purification of adenovirus type 2 soluble hexons have been described in detail elsewhere (3). The protease used was papain (EC 3.4.22.2), as the sodium acetate suspension (20.7 μg of protein per ml, 18 α-*N*-benzoyl-L-arginine ethyl ester units per mg) of twice-crystallized papain (Sigma Chemical Co.). Papainic digestion of hexons were conducted at 37 C for 24 h in 0.12 M potassium phosphate buffer, pH 6.8, 0.4 ionic strength, with 1% 2-mercaptoethanol, at a ratio of enzyme to substrate ranging from 1:100 to 1:10. The substrate concentration ranged from 1 to 4 mg per ml. The hexon digests were analyzed immediately after hydrolysis on nondissociating and sodium dodecyl sulfate (SDS)-containing acrylamide gels.

After papainic hydrolysis of hexons at an enzyme-to-substrate ratio of 1:50, acrylamide gel electrophoretic analysis of the papain digest revealed two major equal components of slightly different electrophoretic mobility, whose migration could correspond either to hexon breakdown products more basic than the initial protein or to hexon multimeric aggregates (Fig. 1). Denaturation of these digested hexons with SDS and subsequent analysis on dissociating SDS-containing acrylamide gels showed that papain left a hexon core of about 85,000 molecular weight, suggesting that only 25 to 30% of the hexon polypeptide subunits (110,000 in average molecular weight) was removed as low-molecular-weight peptides, visible as fast-moving bands behind the bromophenol blue marker (Fig. 2). However, the band 85 K was faintly stained in comparison to the band of control hexon, taking into account that the same amount of protein was loaded onto each gel. This could signify that papain core of hexon had a lower affinity for Coomassie blue than native hexon or that only a minor fraction of the papain cores contained a 85,000-molecular-weight polypeptide unit. It seemed more likely that most hexons had polypeptides with several nicks but retained a quasi-normal configuration because of strong hydrophobic bonds, explaining their acrylamide gel pattern under nondenaturing conditions (Fig. 1c–e), as well as their immunological properties.

When analyzed by the agar double-diffusion technique, the papainic digest of hexons elicited a normal immunological reactivity against

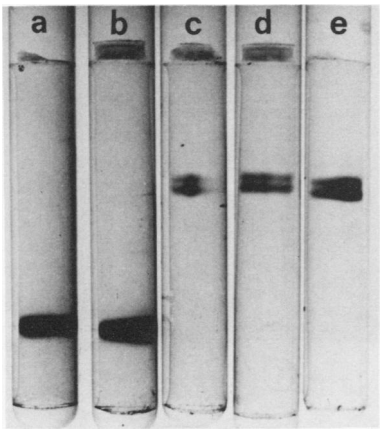

FIG. 1. *Analytical acrylamide gel electrophoresis of papain digestion products of adenovirus type 2 hexons. Hexons (4 mg/ml) were digested with papain at a ratio of enzyme to substrate of 1:50 and run on nondissociating 4.5% acrylamide gels buffered at pH 8.9 (6). Gels a and b: control hexons with respective loads of 20 and 40 μg of protein; gels c, d, e: papain digestion hexons with respective loads of 20, 40, and 80 μg of protein. Two neighbouring components were visible on these three gels. Anode is at the bottom.*

a rabbit anti-hexon immune serum (Fig. 3a). In contrast, after papainic digestion at high enzyme concentration ratio of enzyme to substrate of 1:10, the papainic-digested hexons gave a reaction of partial identity with both control undigested hexons and papain-digested hexons at a lower enzyme-to-substrate ratio of 1:50, with a spur indicating the loss of an antigenic determinant (Fig. 3a). The immunodiffusion test using adenovirus type 5 hexons as control indicated that adenovirus type 2 hexons digested by papain at high enzyme concentration had lost the group-specific antigenic determinants (Fig. 3b), confirming previous studies on digestion of hexons with chymotrypsin, subtilisin, or papain at relatively high enzyme concentration (12).

The papainic digest of hexons was analyzed by analytical ultracentrifugation. A unique homogeneous peak was observed, corresponding to components of one single class in molecular weight. The sedimentation coefficient of these components was found to be 19.3*S*. The sedimentation coefficient of control undigested hexons centrifuged in the same buffer conditions and at the same protein concentration was found to be 10.4*S*. This value was somewhat lower than that expected for native type 2 hexons, which has been found to sediment at 10.6 to 12.2*S* (14, 18), but no extrapolation to zero in protein concentration was made here. If it was assumed

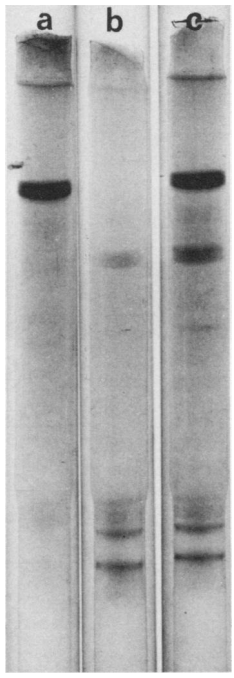

FIG. 2. *Dissociating SDS-polyacrylamide gel analysis of the papain digestion products of hexons. Papain digestion products of hexons presented in Fig. 1c–e were heated at 100 C for 1 min in 2% SDS-5% mercaptoethanol and analyzed on SDS-containing gels in the discontinuous buffer system of Laemmli (10). The gels were composite gels, made of a lower gel of 18% in acrylamide concentration, 5 cm in height to retain the low-molecular-weight products of papain cleavage, overlayered by a 10% acrylamide gel, 10 cm in height. (a) Control undigested hexons; load, 100 μg of protein; (b) hexon papainic digestion products; load, 100 μg of protein; (c) mixture of 100 μg of control hexons and 100 μg of hexon papain digest. The gels were run for 15 h at 3 V per cm. Anode is at the bottom. Low-molecular-weight peptides were visible in the highly concentrated acrylamide gel; the remaining hexon core was about 85,000 molecular weight as indicated by comparative migration on gel slab with adenovirus polypeptide markers.*

that papain degraded the hexon polypeptide unit to a size of 85,000, the value of 19.3*S* was compatible with the sedimentation coefficient value for trimers of these partially digested hexons, since the Schachman relation (15), which neglects shape factors, gave theoretical values of 21.6*S* for trimers of intact hexons (330,000 molecular weight; 10.4*S*) and 18.2*S* for trimers of trimeric papain cores of hexons 255,000 molecular weight; 8.75*S*). Thus the two neighboring bands visible in acrylamide gel pattern (Fig. 1c–e) corresponded to components

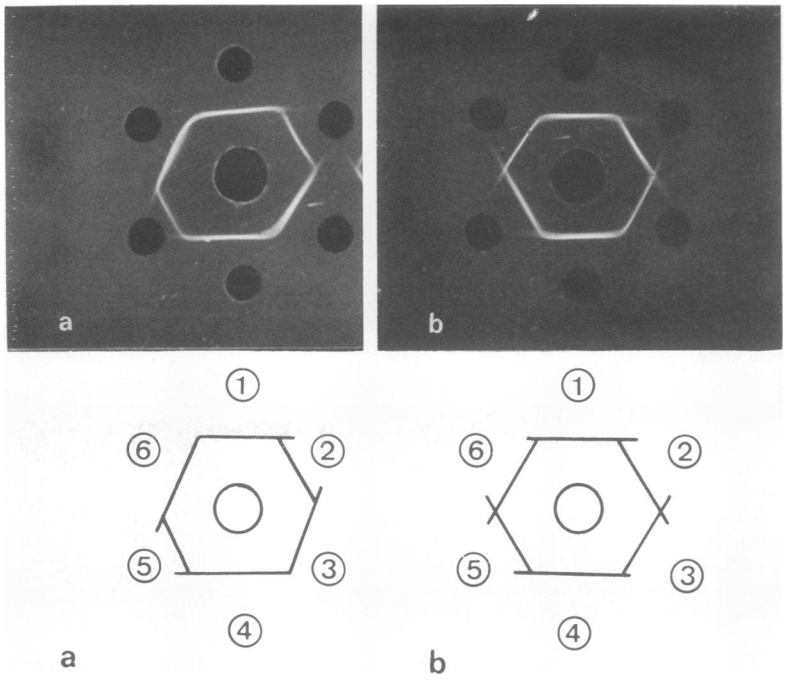

FIG. 3. *Immunodiffusion tests. Control and papain digested hexons were analyzed by the double-diffusion technique against a rabbit anti-adenovirus type 2 hexon immune serum (central wells). (a) Wells 1 and 4, control type 2 hexons; wells 2 and 5, type 2 hexons digested with papain at a ratio of enzyme to substrate of 1:10; wells 3 and 6: type 2 hexons digested with papain at a ratio of enzyme to substrate of 1:50. A pattern of partial identity, evidenced by the spur, was observed between control hexons and hexons digested at high enzyme concentration. A pattern of complete identity was demonstrated between control hexons and hexons digested with low concentration of papain. (b) Wells 1 and 4, control type 2 hexons; wells 2 and 5, type 2 hexons digested with papain at a ratio of enzyme to substrate of 1:10; wells 3 and 6, control adenovirus type 5 hexons. A pattern of partial identity was demonstrated between control type 2 and type 5 hexons and between control type 2 hexons and papain-digested type 2 hexons. A reaction of complete antigenic nonidentity was evidenced between adenovirus type 5 hexons and papain-digested type 2 hexons, demonstrating the loss of common group-specific antigenic determinants from papain-digested hexons. The faint spur between control type 2 and control type 5 hexons corresponded to the type 2 type-specific determinants, and the spur between control type 2 and papain-digested type 2 hexons corresponded to the group-specific antigenic determinants. The precipitation lines of wells 2 and 5 thus represented the remaining type-specific determinants of the papain-digested type 2 hexons.*

having the same molecular weight and different electric charges.

The type of oligomeric species occurring during and after papainic hydrolysis of hexons was examined under the electron microscope. Figure 4 shows that the trimerization was the prevalent mode of association of the hexon capsomers. Beside a number of hexon trimers, many irregular aggregates were also seen, which likely represented a secondary association of hexon trimers, since these aggregates were neither observed in ultracentrifugation nor in the top of low-concentrated acrylamide gels. Although the morphology of the digested hexons was somewhat altered by the proteolysis (Fig. 4, inset a) when compared to native hexons, these hexon trimers were reminiscent of the groups of three hexons arranged in threefold rotational symmetry to form groups of nine obtained upon disruption of the adenovirus capsid (Fig. 4, inset b).

The hexon papainic digest containing the 19.3*S* multimeric components was chromatographed onto Sephadex G-75 to eliminate the low-molecular-weight peptides released by papain from the hexon molecule. The peak eluting in the void volume of the column was controlled in electron microscopy and was found to contain the hexon trimer aggregates. Amino acid composition of these trimer aggregates of hexons, compared with the control hexons, showed no significant modification but an enrichment in basic amino acids and particularly in lysine (Table 1). This drastic increase in lysine content (about 70%) could not be explained if papain digestion was limited, leaving a hexon core of 85,000 molecular weight, and confirmed the hypothesis of multiple nicks pro-

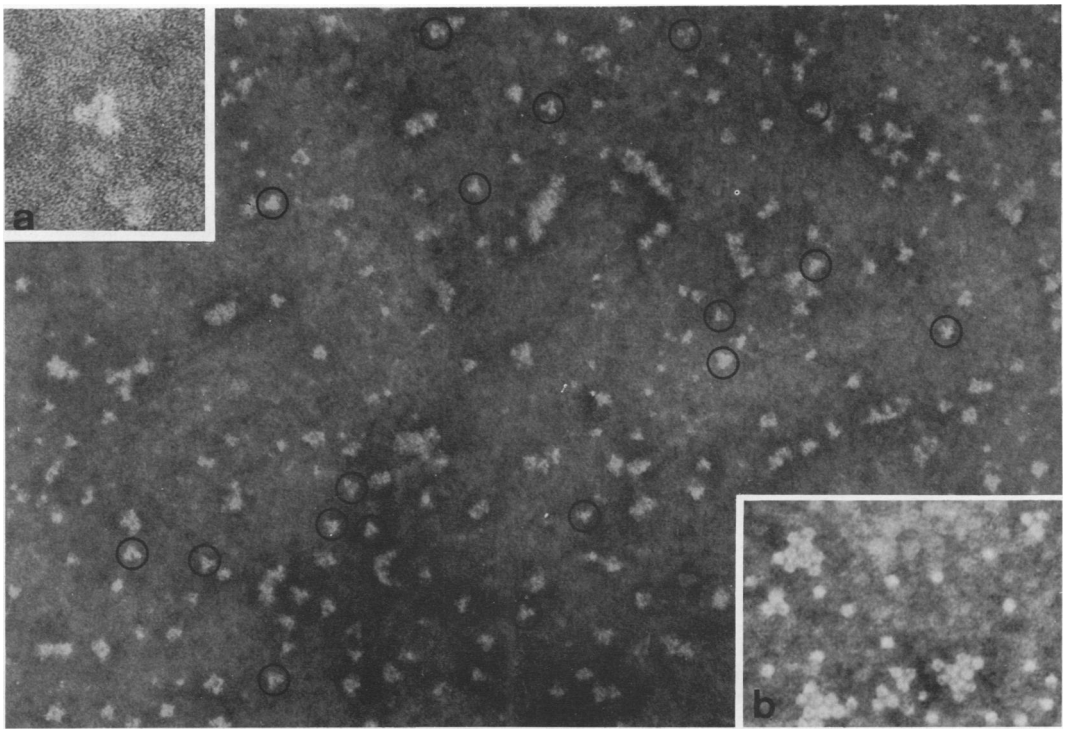

FIG. 4. *Electron microscopic control of papain-digestion products of hexons. The hexon papain digest, analyzed on acrylamide gel (Fig. 1c–e) and ultracentrifugation (19.3S), was negatively stained with 1.5% potassium phosphotungstate and examined in the electron microscope. A number of hexon trimer aggregates were visible (circles). Irregular aggregates were also present, which could result from a secondary aggregation of preformed trimers (× 180,000). (Inset a) "Papain-generated trimers" of hexons at higher magnification (× 500,000). (Inset b) Isolated hexons; groups of three and groups of nine hexons obtained after disruption of adenovirus capsids (× 270,000).*

voked by an enzyme with a broad specificity of peptide bond cleavage on the hexon polypeptide chain, as suggested by the SDS-acrylamide gel pattern (Fig. 2b).

Thus, the proteolysis of native hexons with papain at the usual ratio of enzyme to substrate of 1:100 to 1:50 was apparently limited, with no detectable alteration of either group- or type-specific antigenic determinants. The quasi-integrity of these partially digested hexon capsomers in electron microscopy, and the absence of stainable peptides migrating with the buffer front in the nondissociating acrylamide gels, suggested that strong hydrophobic bonds maintained the cleaved peptides generated by papain hydrolysis associated altogether, thus preserving the immunological properties of the hexon antigen. A minor fraction of the hexon papain cores contained a large polypeptide unit of 85,000 molecular weight, the smaller cleaved peptides belonging mainly to the hexon moiety involved in the group-specific determinants, as indicated by the results of a more extensive papainic degradation at higher enzyme concentrations. This apparently limited proteolysis of the hexon capsomers, although not greatly modifying the whole amino acid composition of the remaining protein molecules, their immunological properties, and their morphology, was nevertheless sufficient to provoke some physicochemical modifications which promote their polymerization in a preferred type of association, viz., hexon trimerization. Two classes of trimer molecules were thus obtained, slightly differing in their electric charges.

Although no higher-molecular-weight precursor for hexon polypeptide unit has been yet demonstrated in vivo (1), the results of the present study in vitro of the self-assembly patterns of the papain proteolysis products from adenovirus hexon capsomers substantiates in a certain way the hypothesis that, within the cell, assembly of preformed hexon capsomers into viral capsids could proceed through some limited cleavage of externally located critical peptides by cellular or virus-induced protease(s). This would unmask the trimer bonding sites of the hexons (4, 5, 20) and provoke their assembly

TABLE 1. *Amino acid composition of control hexons and of papain-generated hexon trimers*[a]

Amino acid	Control hexon		Papain-generated hexon trimers	
	(a)	(b)	(a)	(b)
Asp	14.0	26.4	14.1	25.2
Thr	6.8	12.8	6.7	11.9
Ser	7.4	13.9	6.7	11.9
Glu	8.3	15.6	7.8	13.9
Pro	6.0	11.3	6.2	11.1
Gly	7.6	14.3	7.8	13.9
Ala	7.7	14.5	6.5	11.6
Cys	traces		traces	
Val	5.3	*10.0*	5.6	*10.0*
Met	2.2	4.1	2.1	3.7
Ile	3.2	6.0	3.6	6.4
Leu	7.8	14.7	7.2	12.8
Tyr	5.0	9.4	5.7	10.2
Phe	4.5	8.5	4.1	7.3
Lys	4.7	8.8	8.0	14.2
His	2.0	3.8	1.7	3.0
Arg	6.3	11.9	5.4	9.6
Trp	1.2	2.2	0.8	1.4

[a] Samples of 300 to 600 μg of protein were hydrolyzed for 24 h at 110 C in 5.6 N HCl in sealed tubes under nitrogen with the addition of 1 mg of tryptamin per ml to prevent tryptophan degradation (11). The results obtained from the average of three different analyses are expressed as moles per 100 moles of amino acid (a) and in number of moles per 10 moles of valine (b).

into trimers which in turn assemble into highly specific groups of nine with a threefold rotational symmetry. Alternatively, hexons could be in a metastable state, and some peptide bond cleavage would provide energy for a self-assembly process. However, the application to conditions in vivo remains to be demonstrated.

This work was supported by contract CRL 74-5043-03 from the Institut National de la Recherche Médicale, the ERA 225 from the Centre National de la Recherche Scientifique and a contract from the Université du Droit et de la Santé de Lille.

I acknowledge with thanks the help of M. Dautrevaux in amino acid analysis, of M. Cogniez in the Laboratoire de Biochimie de la Faculté des Sciences de Lille for analytical ultracentrifugation, and of A. Petitprez for his electron microscopic controls. The expert secretarial aid of M. C. Bonningue is also gratefully acknowledged.

LITERATURE CITED

1. Anderson, C. W., P. R. Baum, and R. F. Gesteland. 1973. Processing of adenovirus 2-induced proteins. J. Virol. 12:241–252.
2. Boulanger, P. A., P. Flamencourt, and G. Biserte. 1969. Isolation and comparative chemical study of structural proteins of the adenoviruses 2 and 5: hexon and fiber antigens. Eur. J. Biochem. 10:116–131.
3. Boulanger, P. A., and F. Puvion. 1973. Large-scale preparation of adenovirus soluble antigens in a highly purified form. Eur. J. Biochem. 39:37–42.
4. Boulanger, P. A., and F. Puvion. 1974. Adenovirus assembly. Cross-linking of adenovirus type 2 hexons in vitro. Eur. J. Biochem. 43:465–470.
5. Crowther, R. A., and R. M. Franklin. 1972. The structure of the groups of nine hexons from adenovirus. J. Mol. Biol. 68:181–184.
6. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404–427.
7. Eiserling, F. A., and R. C. Dickson. 1972. Assembly of viruses. Annu. Rev. Biochem. 41:467–502.
8. Grütter, M., and R. M. Franklin. 1974. Study on the molecular weight of the adenovirus type 2 hexon and its subunit. J. Mol. Biol. 89:163–178.
9. Jörnvall, H., U. Pettersson, and L. Philipson. 1974. Structural study of adenovirus type 2 hexon protein Eur. J. Biochem. 48:179–192.
10. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (London) 227:680–685.
11. Liu, T. Y., and Y. H. Chang. 1971. Hydrolysis of proteins with p. toluene sulfonic acid. Determination of tryptophan. J. Biol. Chem. 246:2842–2848.
12. Pettersson, U. 1971. Structural proteins of adenoviruses. VI. On the antigenic determinants of the hexon. Virology 43:123–136.
13. Pettersson, U., L. Philipson, and S. Höglund. 1967. Structural proteins of adenoviruses. I. Purification and characterization of the adenovirus type 2 hexon antigen. Virology 33:575–590.
14. Philipson, L., and U. Pettersson. 1973. Structure and function of virion proteins of adenoviruses. Prog. Exp. Tumor Res. 18:1–55.
15. Schachman, H. K. (ed.). 1959. *In* Ultracentrifugation in biochemistry, p. 215–247. Academic Press Inc., New York.
16. Stinski, M., and H. S. Ginsberg. 1975. Hexon peptides of type 2, 3, and 5 adenoviruses and their relationship to hexon structure J. Virol. 15:898–905.
17. Valentine, R. C., and H. G. Pereira. 1965. Antigens and the structure of adenovirus. J. Mol. Biol. 13:13–20.
18. Wadell, G. 1970. Habilitation thesis. David Broberg's Printing, Stockholm.
19. Wilcox, W. C., and H. S. Ginsberg. 1963. Structure of type 5 adenovirus. I. Antigenic relationship of virus structural proteins to virus specific soluble antigens from infected cells. J. Exp. Med. 118:295–306.
20. Wrigley, N. G., and H. G. Pereira. 1974. In vitro reconstitution, hexon bonding and handedness of incomplete adenovirus capsid. J. Mol. Biol. 85:617–631.