Helical Conformation in Crystalline Inclusion Complexes of V-Amylose: A Historical Perspective

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Summary: Amylose forms V-type crystalline inclusion complexes with a large variety of small molecules. Several authors have proposed models for these complexes based on amylose single helices with 6, 7 and 8 glucosyl units per turn. However, so far, only a small number of crystal structures have been solved and several models are still hypothetical. We have recently shown that in the unit cell of such complex with isopropanol, the amylose molecules were organized in 7-fold left-handed helices, with isopropanol and water molecules located as guests both within and between the helices.

Keywords: electron crystallography; inclusion complexes; molecular modeling; single crystals; V-amylose; X-ray crystallography

Introduction

Amylose is an essentially linear homopolymer of α(1,4)-D-glucose that can be extracted from native starch.[1] This linear molecule can also be synthesized *in vitro* by amylose synthases such as amylosucrase[2] or starch phosphorylase.[3] Amylose can be recrystallized from solution by cooling or addition of precipitant. Depending on the molecular weight, concentration, temperature and nature of the solvent, different morphologies and allomorphic type are observed for the amylose crystals.[1] In the presence of a variety of inorganic and organic complexing agents,[4,5] amylose crystallizes in the so-called ''V'' form.[1,6] Due to the similarity between X-ray powder diffraction patterns of V-amylose and cyclodextrin compounds, models based on amylose single helices with 6, 7 and 8 glucose units per turn, resembling the packing of α-, β- and γ-cyclodextrins, respectively, were proposed to describe the V structures.[7] In the crystals, the complexing molecules can be entrapped between amylose helices and/or inside their hydrophobic central cavity. This important property can, for instance, find applications in pharmacology (vectorization, controlled release) and in the food industry (flavor encapsulation, texturing, etc.).[8,9]

When crystallized from dilute aqueous solutions (typically 0.1-1.0 g/L), amylose forms micrometer-sized lamellar crystals with distinct shapes and X-ray or electron diffraction patterns that depend on the complexing agent. Several generic families of V-amylose crystals have been described.[1,10,11] Whatever the degree of polymerization (DP) of amylose, the helical axis is perpendicular to the lamellae. When the chain length is much longer than the thickness of the crystal (8–10 nm), chain folding takes place at the surface, facilitated by a so-called ''flip'' between two adjacent glucosyl moieties, which enables an easy reversal of molecular trajectory.[12]

Although the knowledge of the molecular structure is important to locate the guest molecules and understand how they are entrapped within the crystal lattice, only a small number of structures have been

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resolved and several models are still hypothetical. In this paper, we have drawn a brief historical account on the study of the helical conformation of V-amylose as deduced from X-ray and electron diffraction data. In particular, we will summarize our recent results that show that amylose complexes of the $V_{isopropanol}$ family correspond to the packing of 7-fold left-handed single helices organized in an orthorhombic unit cell.[13]

Structural Studies of V-amylose Crystalline Complexes

The 6-fold Single Helix

In 1930, Katz reported on an X-ray powder diffraction pattern that was different from the A and B patterns recorded from native starch granules. The new crystal form, observed during the baking of bread, was named ''V'' (for ''*Verkleisterung*'' that means ''gelatinization'' in German).[14,15] A similar V pattern was also observed when starch pastes were precipitated with some alcohols. Bear showed that different V complexes could be obtained from whole starch depending on the precipitating agent.[16] In the study of the V forms of amylose, two types of complexes particularly had a significant historical importance: V_{iodine} and $V_{n\text{-butanol}}$.

It had been known for a long time that iodine formed a blue complex with amylose. Making an analogy with the way cyclodextrins (then called Schardinger dextrins) absorbed iodine, Freudenberg *et al.* proposed that in V_{iodine}, amylose formed 6-fold single helices and that the iodine atoms formed a column inside these helices.[17] Using X-ray powder diffraction data, Rundle *et al.* confirmed this hypothesis and proposed that the complex had a hexagonal unit cell containing helices with an external diameter of about 1.3 nm.[18,19] A more detailed structure based on an orthorhombic unit cell was proposed later on by Bluhm and Zugenmaier from X-ray diffraction data of V_{iodine} fibers.[20] More recently, Nimz *et al.* have resolved, at the atomic level, the position of the iodide ions located within the central channel of the two 6-fold helical segments occurring in a large cycloamylose consisting of 26 glucosyl units.[21] This cycloamylose stands out as a remarkable model for V-amyloses.

While Meyer *et al.* had shown that native starch could be fractionated into two components (linear amylose and branched amylopectin) in hot water,[22] a more efficient route for extracting linear amylose, based on the selective precipitation/crystallization of amylose with *n*-butanol, was described by Schoch[23] and Kerr *et al.*[24,25] Alternative protocols to extract linear amylose, based on a complexation with thymol and cyclohexanol[26] or *l*-menthone[27] were proposed later on. In addition to *l*-menthone, Kuge and Takeo tested more than 100 organic substances for their ability to form insoluble complexes with amylose.[27] Out of these, 45 could potentially be used for amylose fractionation.

Using X-ray diffraction, Rundle and Edwards studied wet (*i.e.*, in water saturated with butanol), partially hydrated (V_H) and anhydrous (V_a) samples prepared from $V_{n\text{-butanol}}$ crystalline powders. These authors proposed that, in the first two cases, 6-fold helices with a 1.37 nm diameter and a helical repeat of 0.8 nm were closely packed in orthorhombic unit cells. In the wet complex, butanol was located inside the helical cavity.[28] The unit cell of V_a crystals was hexagonal with a helix diameter of 1.3 nm.[19] Comparing the powder X-ray diffraction patterns of V complexes prepared with methanol, ethanol and 1-propanol, Valletta *et al.* discussed the origin of the 1.3 and 1.37 nm 6-fold helices.[29] Later on, the $V_{n\text{-butanol}}$, V_H and V_a structures were described in more details by several authors using higher resolution X-ray diffraction data collected from oriented fibers.[30–33]

The left-handed 6-fold helical system has also been proposed for V-amylose complexed with fatty acids,[34] dimethylsulfoxide[35] and ethylenediamine.[36] The structure of the V_{lipid} complex resembles that of V_{iodine} in the sense that, in most

cases, the unit cell is hexagonal.[37,38] Moreover, it was shown that the aliphatic part of the guest molecules was located inside the helices while the polar head remained outside.[39] The structures proposed for V_{DMSO} and $V_{ethylenediamine}$ are very similar, consisting of tetragonal lattices. However, the helices are no longer close-packed since, in addition to the intrahelical guests, DMSO and ethylenediamine molecules are in interstitial positions between the helices.[40,36]

The 7-fold Single Helix

Bear was the first to point out that two types of V complexes were formed depending on whether the complexing alcohols were linear or branched. He suggested that bulkier guest molecules should be accommodated in a helix with a larger cross-section, *i.e.*, with more residues per turn.[41] By comparing the volume of the pseudo-hexagonal unit cells of the partially dried complexes formed with *tert*-butanol and *n*-butanol, Zaslow obtained a ratio of about 7:6 in favor of the existence of a 7-fold helix for the $V_{tert\text{-butanol}}$ complex.[42] This observation was confirmed by Yamashita and Hirai who prepared V-amylose single crystals with several branched alcohols larger than *n*-butanol. They analyzed their electron diffraction patterns and proposed that the orthorhombic unit cells accommodated four 7-fold helices with a diameter of 1.5 nm and a repeat distance of 0.79 nm.[7] They also mentioned the fact that the 7-fold helix could be readily converted to a 6-fold one when the crystals were dispersed in methanol, the transition occurring without dissolution since the shape of the crystals was unchanged. 7-fold helices were also reported to occur, for instance, in V_{DMSO} prepared under dry conditions[43] and in complexes prepared with *n*-butyric and *n*-valeric fatty acids,[38] cyclohexanol,[44] salicylic acid[45] and 2-naphthol.[46]

The Contribution of Transmission Electron Microscopy and Electron Diffraction

Before the 1960's, very few images revealing the morphology of V-amylose complexes had been published. The optical micrographs of Wiegel showed spherulitic and flowerlike aggregates of starch precipitated with isobutyl alcohol and isopropanol, respectively.[47] While the $V_{n\text{-butanol}}$ complexes seen in the same paper had ill-defined shapes, the images made by Kerr and Severson revealed rectangular platelet crystals[25] and those of Schoch, recorded after staining the crystals with iodine, showed flowerlike planar aggregates.[23]

In 1963-64, Hirai *et al.*,[48] Yamashita[49] and Manley[50] simultaneously published the first transmission electron microscopy (TEM) images of 8-10 nm-thick lamellar single crystals of $V_{n\text{-butanol}}$ and their corresponding electron diffraction patterns. It was concluded that the helices were oriented perpendicular to the lamellar plane and that, considering the crystal thickness and average DP of amylose, chain folding was involved, like in single crystals of linear synthetic polymers. Patterns recorded with the beam perpendicular to the crystal plane thus contained crucial information on the organization of amylose helices packed in the (*a,b*) base plane. During the following years, numerous images and base-plane electron diffraction patterns of V-amylose single crystals prepared with various complexing agents were published.[7,44,51–53]

One of the main difficulties to unambiguously characterize the structure of V-amylose complexes was that, although it was known that the X-ray diffraction patterns of wet and dry specimens were often different, the electron diffraction diagrams were recorded from crystals that had lost part or all of the volatile guest molecules in the vacuum of the microscope. One typical paradox was that, at room temperature, hexagonal electron diffraction patterns were recorded from rectangular $V_{n\text{-butanol}}$ crystals.[50,51] A major progress was achieved by Booy *et al.* who, for the first time, probed wet $V_{n\text{-butanol}}$ lamellar crystals quench-frozen in liquid nitrogen.[54] The electron diffraction patterns recorded at low temperature unambiguously corresponded to an orthorhombic

unit cell. Once dried *in vacuo* at room temperature, the crystals lost the included butanol molecules and became converted to the close-packed hexagonal V_H structure observed by Yamashita[51] and Manley.[50] Helbert and Chanzy confirmed this behavior and studied the desolvation process in more details for $V_{n\text{-butanol}}$ and $V_{n\text{-pentanol}}$ single crystals.[55] A model was proposed where the guest molecules were located both inside and between the 6-fold helices.

Using the same approach, high-resolution electron diffraction patterns were recorded from frozen-wet $V_{isopropanol}$ and $V_{acetone}$ crystals.[56] Contrary to the previous assumption of a close-packed arrangement of 7-fold helices,[57] it was proposed that the diffraction data could as well be accounted for by a system of 6-fold amylose helices separated by guest molecules. Such model would explain the easy conversion to V_H upon desolvation.[56] Many other complexing agents (thymol,[10] fenchone, menthone, geraniol,[58] linalool and terpineol,[11] to name but a few) were shown to form rectangular single crystals which yielded electron diffraction patterns similar to that of $V_{isopropanol}$, with only minor variations in spot intensities.

The molecular structure of V_H amylose was determined by combining a stereochemical refinement procedure with electron diffraction intensities recorded from frozen-wet hexagonal crystals. The structure was refined following a model of randomly distributed up and down left-handed 6-fold helices, organized in a close-packed manner, within the hexagonal unit cell. In addition, there were water molecules located inside and between helices.[59] Finally, $V_{glycerol}$ square crystals isomorphous to those of V_{DMSO}[35] and $V_{ethylene\text{-}diamine}$[36] were prepared under dry conditions. Their base-plane electron diffraction pattern suggested an orthorhombic unit cell containing pairs of antiparallel left-handed 6-fold helices with glycerol molecules probably located inside and between helices.[60] Again, desolvation in the solid state promoted the conversion to V_H amylose.

The 8-fold Amylose Single Helix

Very few complexing agents have been proposed to favor the formation of 8-fold amylose helices. As proposed by Yamashita and Monobe[53] and Helbert,[10] the tetragonal unit cell of V-amylose complexed with α-naphthol or quinoline would contain 8-fold helices with an external diameter of 1.62 nm. The helical repeat is still around 0.8 nm, identical to the one measured for 6- and 7-fold V-helices. Oguchi *et al.* also reported on the formation of 8-fold helices when amylose was crystallized with salicylic acid.[45] So far, the strongest evidence of an 8-fold helix has been provided by Cardoso *et al.* who published high-resolution lattice images, showing a 8 subunit projection along the helical axis.[61] A brief mention of a 9-fold helix in $V_{\alpha\text{-naphthol}}$ crystals can be found in a paper by Jane and Robyt but the authors admit that their data are insufficient to support this hypothesis.[62]

The Structure of Amylose $V_{isopropanol}$ Crystals

In order to propose a conclusive three-dimensional model for the complexes of the $V_{isopropanol}$ family, we have prepared $V_{isopropanol}$ single crystals and recorded their electron diffraction patterns. The data were used for a structure refinement procedure, based on conformational analysis, exhaustive search of packing and classical crystalline polymer refinement methods. As described in details by Nishiyama *et al.*,[13] 0.5 g/L aqueous solutions of DP 100 synthetic amylose were heated at 150°C during 30 min and cooled down to 90°C. 35% (v/v) preheated isopropanol was added and the solution was allowed to slowly cool in a Dewar bottle. The crystals were deposited on carbon-coated TEM grids and observed with a Philips CM200 'Cryo' microscope. Rectangular lamellar crystals ($4 \times 1\ \mu m^2$) organized in planar rosettes were observed (Figure 1a). Base-plane electron diffraction patterns were recorded at low temperature from frozen-wet preparations, on Fujifilm imaging plates. The patterns were indexed with a rectangular $P2_12_12_1$ unit cell with

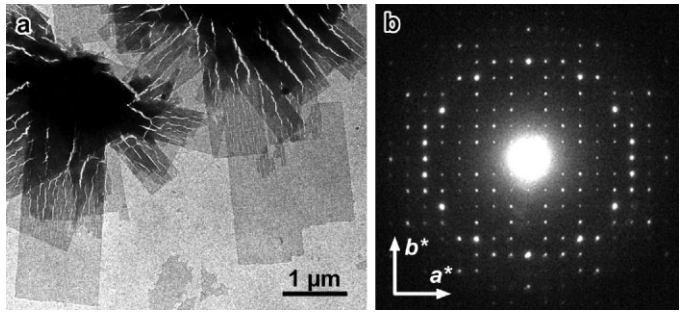

Figure 1.
a) TEM image of amylose $V_{isopropanol}$ lamellar single crystals; b) corresponding low temperature base-plane electron diffraction pattern recorded from a frozen-wet crystal.

$a = 2.83$ nm and $b = 2.95$ nm (Figure 1b). The c parameter was taken as 0.8 nm, as previously determined by Buléon *et al.*[56] and Helbert.[10] The intensity of the diffraction spots was measured semi-automatically using a tailor-made program. Considering the symmetries of the patterns, the intensities of *hk*0, -*hk*0, *h*-*k*0 and -*h*-*k*0 reflections were averaged, resulting in a final set of 108 independent diffracted intensities for reflections up to a *d*-spacing of 0.2 nm.

The first step of the structure determination consisted in building a set of right- and left-handed (R and L, respectively) 6-, 7- and 8-fold helices with hydroxymethyl moieties in gauche-gauche (*gg*), trans-gauche (*tg*) or gauche-trans (*gt*) conformation.[13] The axial projections of the 18 resulting regular amylose helices, built and energy-minimized with the Cerius program,[63] are shown in Figure 2.

In a second step, the rigid helices were packed in the $P2_12_12_1$ unit cell. In order to find their best position, they were systematically translated in the (*a,b*) plane. At each position, they were translated along and rotated about their helical axis, and the model with the lowest packing energy was selected. A reliability factor (R-factor) evaluating the agreement between the calculated and experimental diffraction intensities was calculated at each (*a,b*) position. In a first approximation, the guest molecules located inside and between helices were implicitly taken into account by using a modified atomic scattering factor which assumed a continuous background with a 0.8 g/cm^3 density (*i.e.*, that of isopropanol).[13]

The comparison of the packing energy and R-factor maps determined for the models built from the 18 helices led to a few plausible models for further refinement. The 8-fold helices were rapidly ruled out since the corresponding models exhibited significant overlap leading to very high packing energy. In the case of 6- and 7-fold helices, only four models stood out as being the best in term of low packing energy and R-factor, namely those built from L7-*gg*, L7-*gt*, R7-*gg* and R7-*tg* helices. They were further refined with the SHELX-L program that adjusted the conformation of the helices.[64] The lowest R-factor was obtained for the model built from L7-*gg* helices (0.28 *vs* about 0.45 for the three other models). For this model, we used an additional intensity scaling procedure, which consists in refining a scale parameter applied to circular shells of reflections corresponding to different ranges of scattering angles.[13] The use of this scaling parameter resulted in a decrease of the R-factor to 0.23.

Finally, explicit isopropanol molecules were inserted in this refined model, inside and between helices. Water molecules were then added to reach the experimental crystal density of 1.42 g/cm^3.[56] The guest molecules were positioned manually within the cavities calculated with a Connolly algorithm in Cerius and further refined

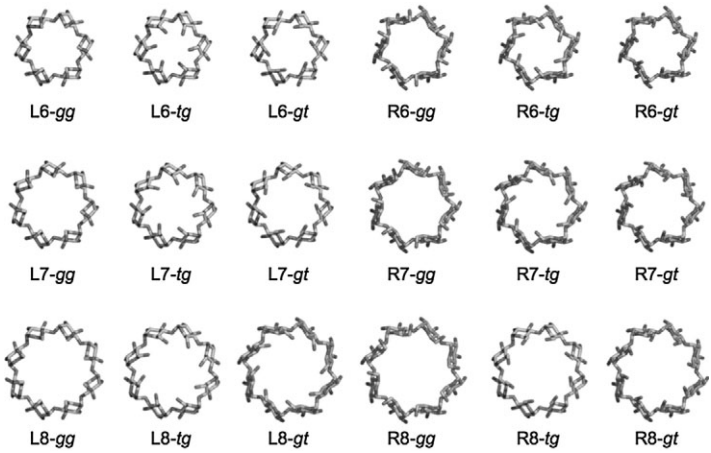

Figure 2.
Axial projections of the 18 helices defined by their left (L) or right (R) helicity, the number of glucose residues per turn (6, 7 or 8) and conformation of the hydroxymethyl group (*gg*, *tg* or *gt*). For clarity, the hydrogen atoms have been omitted.

against the experimental diffraction data using SHELX. The axial projection of the final structure is shown in Figure 3a. This model holds 2 isopropanol and 3 water molecules within the helical cavity together with 2 isopropanol and 4 water molecules between the helices. The excellent agreement between the experimental and calculated electron diffraction patterns (R-factor of 0.10) can be visualized in Figure 3b.

As previously mentioned, several authors had suggested that the complexes of amylose with molecules larger than *n*-butanol, which also occur as rectangular lamellae,[7] were built from 7-fold helices.[41,42] Takeo and Kuge suggested that these helices were organized in a close-packed hexagonal system[57] but Zaslow had previously ruled out this possibility by stating that a hexagonal close packing of 7-fold helices was not possible.[42] The

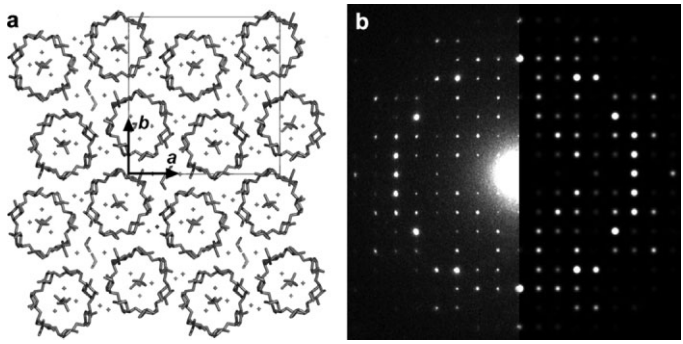

Figure 3.
a) Axial projection of the molecular model of the amylose $V_{isopropanol}$ complex. For clarity, the hydrogen atoms have been omitted. The isopropanol guest molecules are located inside and between the 7-fold amylose helices. The isolated crosses correspond to the oxygen atoms of the water molecules. b) Comparison of the experimental electron diffraction pattern (left) with that calculated from the model in a (right).

structure that we propose is thus original in the sense that the organization of the helical skeleton is a compromise between the opposing constraints of packing symmetry and helicity. The packing of the helices can be described using two regularly alternating motifs: one is nearly tetragonal and holds a cavity filled with isopropanol and water molecules, whereas the other corresponds to 4 close-packed helices with only water molecules inserted in the interhelical spaces (Figure 3a).[13] However, as only base-plane diffracted intensities were used in our study, the proposed $V_{isopropanol}$ three-dimensional structure remains to be validated by collecting electron diffraction data from titled crystals and comparing them to the calculated ones.

Perspectives

The existence of a left-handed 6-fold helix has previously been demonstrated for V_{DSMO},[40] V_{iodine},[20] and V_H/V_{lipid}[59,39] amylose crystals, and our results unambiguously showed that the crystals of the $V_{isopropanol}$ family were based on the packing of left-handed 7-fold helices.[13] While strong experimental evidence of an 8-fold helix has been provided by studying amylose $V_{\alpha\text{-naphthol}}$ crystals,[61] the electron diffraction data have not been quantitatively analyzed yet and a conclusive molecular model is still to be proposed.

Work is in progress in our group to transpose the approach described in this paper to the $V_{n\text{-butanol}}$ structure that all authors have described as a packing of 6-fold helices.[18,51] The $V_{n\text{-butanol}}$ single crystals are rectangular like the $V_{isopropanol}$ ones, but their base-plane electron diffraction pattern is clearly different. Considering the number of papers published early on in relation to the fractionation of starch using *n*-butanol, solving the $V_{n\text{-butanol}}$ crystal structure appears to have a particular historical importance.

The quantitative analysis of electron diffraction data for structure determination is limited by two factors. First, even though sharp patterns can be recorded from tilted crystals,[61] the intensities of all patterns are difficult to normalize and merge. Second, the diffracted intensities are often (if not always) affected by minor crystal tilts and/or by secondary diffraction. This effect increases with increasing crystal thickness (Figure 4) and particularly affects the low-intensity high-resolution spots.

Two approaches can be considered in order to reduce the effect of secondary diffraction and collect more reliable datasets. First, significant progress is expected by the use of the so-called ''precession'' electron diffraction, although the method remains to be tested on polymer single crystals. This recent technique consists in precessing the incident electron beam in a hollow cone around a centered zone axis of the crystal while the patterns are recorded, resulting in a reduction of the number of reflections excited simultaneously in the off-zone condition.[65,66]

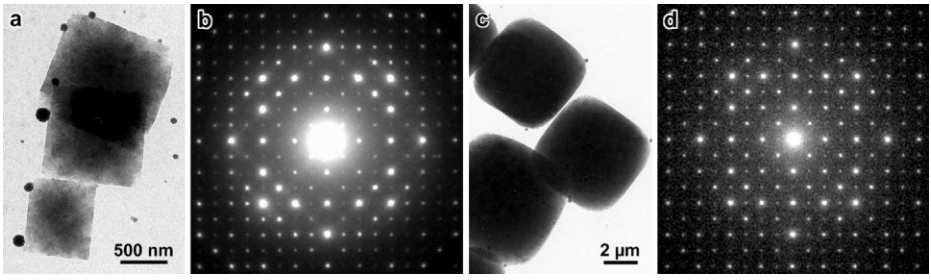

Figure 4.
Small / thin (a) and large / thick (c) lamellar single crystals of amylose $V_{\alpha\text{-naphthol}}$ (TEM images); b and d) corresponding base-plane electron diffraction diagrams illustrating the significant increase of secondary diffraction with crystal thickness.

Secondary diffraction will also be reduced if the crystals can be probed by a small beam of synchrotron X-rays. While it is highly difficult to grow large polymer single crystals, fractions of short synthetic amylose chains have successfully been used to prepare crystals in the 10 μm range. In particular, spindle-like A-type single crystals have recently been grown from dilute aqueous solutions in the presence of acetone vapors.[67] Popov *et al*. have probed such crystals with an X-ray microbeam on the ID13 beamline at ESRF (Grenoble, France), keeping the sample at low temperature during the experiment using a nitrogen cryoflow.[68] For the first time on such small polymer crystals, X-ray single crystal methods have been applied to the resulting dataset and a revised model of the A-type structure was produced. Provided that crystallization procedures are optimized to grow larger V-type single crystals (such as the $V_{\alpha\text{-naphthol}}$ crystals shown in Figure 4c[11]), and taking advantage of the constant progress in goniometer instrumentation and beam size reduction,[69] a similar approach can certainly be successfully used to solve the structure of several amylose inclusion complexes.

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