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Apomyoglobin Mutants with Single Point Mutations at Val10 Can Form Amyloid Structures at Permissive Temperature

N. S. Katina, N. B. Ilyina, I. A. Kashparov, V. A. Balobanov, V. D. Vasiliev, and V. E. Bychkova*

Institute of Protein Research, Russian Academy of Sciences, ul. Institutskaya 4, 142290 Pushchino, Moscow Region, Russia; fax: (495) 514-0218; E-mail: bychkova@vega.protres.ru; delfinium27@rambler.ru

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Abstract—Formation of amyloid-like protein aggregates in human organs and tissues underlies many serious diseases, therefore being in the focus of numerous biochemical, medical, and molecular biological studies. So far, formation of amyloids by globular proteins has been studied mostly under conditions that strongly destabilized their native structure. Here we present our results obtained at permissive temperature by thioflavin T fluorescence, far UV CD, IR spectroscopy, and electron microscopy. We used apomyoglobin and its mutants with Ala or Phe substituted for Val10 that are structurally close to wild type apomyoglobin. It is shown that at permissive temperature the ability of the protein to form amyloids depends on the extent of its structural destabilization, but not on hydrophobicity of the substituting residue. A possible difference between amyloids formed by strongly destabilized proteins and those yielded by proteins with a slightly fluctuating native structure, as well as the stroke and infarction effect on the ability of proteins to form amyloid structures, are discussed.

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Key words: apomyoglobin, apomyoglobin mutants, amyloid structure, protein stability, cross-β-structure, aggregation kinetics

Amyloids are protein aggregates with the secondary structure of a special type called the cross-β-structure. To date, over forty diseases are known [1] to involve amyloid formation in human organs and tissues. Among them are Alzheimer's disease, Parkinson's disease, type II diabetes, and many others [1]. Besides, the presence of amyloids in the cell may affect its functions: as found, pituitary hormones are able to form amyloid-like aggregates whose conditions-dependent dissociation releases active molecules [2]. Also, it was shown that proteins and peptides incapable of *in vivo* amyloid forming gain such a capability under specially formulated conditions. This indicates that the ability to form amyloids is not a property of the unique disease-causing amino acid sequence but a common feature of polypeptide chains [3, 4].

A number of papers report that destabilization of the protein native state caused either by mutations or changed conditions (decreased pH or increased temperature) can promote formation of amyloids [4-6]. Therefore, experiments on amyloid formation were carried out under protein denaturing conditions [7-10]. As found recently, native-like proteins are able to form amyloid fibrils as well, and this requires only a slight destabilization of their structure [11].

Inasmuch as the experiments on amyloid formation so far used mostly peptides and proteins in denaturing conditions, the mechanism of aggregation of folded proteins still remains poorly studied. For example, in denaturing conditions β_2-microglobulin, acyl phosphatase, and Aβ-peptide showed enhanced ability to form amyloids, provided certain regions of their polypeptide chains had an increased hydrophobicity, but no question has been raised as to whether hydrophobicity of this or that chain region could play the key role in amyloid formation under conditions close to the native ones [12-14].

The current paper is aimed to elucidate the effect of hydrophobicity of substituting amino acids on the protein's ability to form amyloids and on the rate of this process under almost physiological conditions when the state of a folded polypeptide chain is native-like.

Sperm-whale apomyoglobin (apoMb) contains seven of eight helices typical of holoprotein; these are designated with letters from A to H (F-helix is unfolded in apo-form). ApoMb has been taken for our experiments because its structure and folding pathway, as well

Abbreviations: apoMb, apomyoglobin; EM, electron microscopy; ThT, thioflavin T; WT, wild type; [θ], molar ellipticity at a given wavelength λ.

* To whom correspondence should be addressed.

as those of some of its mutants, have been much studied [15-21].

Previously, apoMb was shown to aggregate into amyloid fibrils (protofibrils) under strongly destabilizing conditions, e.g. at high temperature, and this ability increased with increasing proportion of the unfolded protein chain [6, 22]. Also, evidence has been reported that the N-terminal sequence is of importance for this process. Specifically, it was demonstrated that substitutions within this sequence influenced the rate of amyloid formation, and that a peptide identical to the apoMb N-terminal sequence could form amyloid structures [23, 24]. Moreover, when in a native-like conformation in the presence of 5-15% trifluoroethanol, horse apoMb with the double substitution Trp7PheTrp14Phe (W7FW14F) within its A-helix formed fibrils at as low as room temperature during very long incubation initiating this process [25, 26]. This suggested that the enhanced ability of apoMb to form amyloids is underlain by either increased hydrophobicity of its N-terminal sequence or its elevated capacity for making the β-structure [27].

Taking into account the importance of apoMb A-helix for amyloid formation and the presumable effect of substituting residue hydrophobicity, we substituted Phe, a more hydrophobic residue [28], and Ala, a less hydrophobic one, for Val10 located within the A-helix; in other words, we constructed mutants Val10Phe and Val10Ala, respectively. The properties of aggregates formed by wild-type (WT) apoMb and by its mutants, as well as kinetics of this process, were studied using light scattering, IR spectroscopy, far UV CD, thioflavin T (ThT) fluorescence, and electron microscopy (EM). Stability of monomeric apoMb was assessed by far UV CD and scanning microcalorimetry from urea-induced unfolding and heat denaturation data.

It was shown that unlike WT apoMb, the mutants with substitutions at Val10 were able to form amyloid-like aggregates under conditions close to native when the protein native state is still preserved. As found, the major effect on amyloid formation was caused by the substitution-dependent destabilization of the protein structure. Hydrophobicity of the substituting residue at this position had no noticeable effect either on the fibril forming capacity of the protein or on the rate of this process. Here, a possible difference between aggregating of a strongly destabilized protein and that of a protein with a native-like structure, as well as the stroke- or infarction-caused effect on the process, are discussed.

MATERIALS AND METHODS

Protein expression and isolation. WT apoMb and its mutants (Val10Ala, Val10Phe) were isolated from inclusion bodies after expression of the corresponding plasmids in *E. coli* BL21 (DE3) cells [29, 30]. The inclusion bodies were subjected to ultrasonic destruction under denaturing conditions. The proteins were purified by reversed-phase chromatography using a C18 column. The protein was eluted in acetonitrile ranging from 10 to 100%. The purity of the resultant protein was monitored by 15% polyacrylamide gel electrophoresis in the presence of SDS. The pure protein was collected and lyophilized.

Measuring conditions. Lyophilized proteins were diluted to a final concentration of 5 mg/ml in 10 mM of deionized water-based Na-phosphate buffer, pH 5.5. A Thermo Orion 720 digital pH meter (USA) was used to take pH measurements.

To have the already available aggregates precipitated, prior to the aggregation experiments, the samples of protein solution were subjected to centrifugation at 70,000 rpm for 30 min at 4°C using a Beckman 100 ultracentrifuge (Beckman, USA) with a TLA 100 rotor (Beckman). The process of amyloid formation was studied by incubating the protein samples at 40°C for various time periods.

Concentration of the protein was determined spectrophotometrically (Cary 100, Australia) at 280 nm. The extinction coefficient was found by the nitrogen micromethod [31] as $A_{1cm}^{1mg/ml} = 0.88$ both for WT apoMb and its mutants with substitutions Val10Ala and Val10Phe [29].

Electron microscopy. The samples were negatively stained with 1% aqueous uranyl acetate using the single-layer carbon technique [32]. Carbon films (2-3 nm thick) on the surface of freshly cleaved mica were prepared using an electron beam evaporator [33]. Electron micrographs were taken using a JEM-100C electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV and magnification of 40,000. The protein concentration was 0.1-0.05 mg/ml.

IR spectroscopy. A Nicolet 6700 Fourier transform spectrometer (Thermo Scientific, USA) was used to obtain IR spectra at 27°C. The protein concentration was 5 mg/ml. The samples were sandwiched between plates made of CaF_2; the optical path length was 5.8 μm. Each spectrum, an average of 256 scanning results, was taken at a resolution of 4 cm^{-1}; in the course of measurement water steam spectra were automatically suppressed. Variation of the β-structure content from aggregate to aggregate was monitored by the ratio between absorption spectrum intensity at 1620 cm^{-1} (the band typical of cross-β-structure) and that at 1650 cm^{-1} (the band typical of α-helical structure) [34].

Fluorescence. A Varian Cary Eclipse (Australia) spectrofluorimeter was used for thioflavin T (ThT) fluorescence and light scattering registration. ThT fluorescence spectra were recorded with 450 nm excitation wavelength and 20°C over a range of 460-600 nm. In the studied samples the protein concentration was 0.5 mg/ml, and that of dye was 4 μM. The kinetic curves were plotted with ThT fluorescence intensity recorded at 480 nm.

In light scattering experiments the excitation wavelength was 600 nm, the recorded spectra were within the 590-610 nm range, and the protein concentration was 0.1 mg/ml.

Circular dichroism. A JASCO J-600 (Japan) spectropolarimeter with 0.1-mm path length cells was used to obtain CD spectra and melting curves. The protein concentration was 1 mg/ml. The molar ellipticity parameter [θ] was derived from $[\theta] = \theta \cdot MRW/l \cdot c$, where θ is its measured value (deg, 10^{-3}), MRW is the average residue molecular weight calculated from the amino acid sequence, l is the path length (mm), and c is the protein concentration (mg/ml). The melting curves were monitored through ellipticity change at 222 nm caused by increasing temperature.

Differential scanning microcalorimetry. Heat denaturation of apoMb was studied using a SCAL-1 (Scal Co. Ltd., Russia) differential scanning calorimeter [35] with 0.34 ml cylindrical glass cells. The heating rate was 1 K/min, temperature accuracy was ±0.3°C, and protein concentration was 1 mg/ml. Prior to the experiments, all samples were equilibrated by dialysis against 10 mM Na-phosphate buffer overnight.

Aggregation kinetics assay. The kinetics of aggregate formation was studied on the basis of varying ThT fluorescence data, IR spectra, and EM-monitored particle growth. In kinetic experiments on ThT fluorescence the final protein concentration was 5 mg/ml, and the final ThT concentration was 40 μM. For the initial aggregation stage, the rate constants were calculated from the combined Sigma Plot approximation of three kinetics curves from three independent experiments.

RESULTS

Protein selection and mutagenesis. The studied proteins were two apoMb mutants with a single substitution at Val10 belonging to A-helix within the N-terminal part of the chain. Val10 was replaced by Ala to preserve helicity, or by Phe theoretically predicted to enhance the amyloid forming ability of apoMb [28]. The plasmids were constructed by D. A. Dolgikh [29] and expressed in *E. coli* BL21 (DE3) cells to give inclusion bodies that were used to isolate the required proteins, whose purity was checked electrophoretically with SDS in 15% polyacrylamide gel.

Stability of WT apoMb and its mutants. Stability of apoMb and its mutants was studied through their heat denaturation using scanning microcalorimetry and far UV CD. Figure 1 shows the CD curves that illustrate varying molar ellipticity of the three proteins at 222 nm. As seen, the mutants differ from WT apoMb only slightly. Apparently, these mutations have made no significant change in the protein secondary structure. However, the mutants show a shift of the heat mid-transition towards lower temperature, thereby demonstrating destabilization of their native (folded) state relative to their heat-denatured state, as compared with WT apoMb.

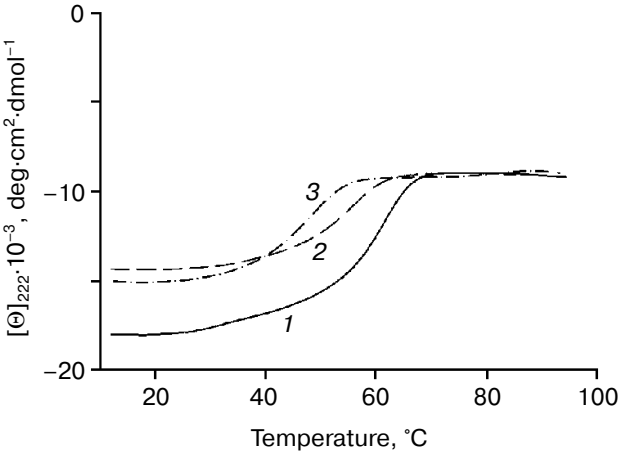

Fig. 1. Far UV CD spectra for heat denaturation of the studied proteins taken at 222 nm: *1*) WT apoMb; *2*) Val10Ala; *3*) Val10Phe.

The scanning microcalorimetry experiments revealed that WT apoMb and its mutants had a heat absorption peak, which is indicative of a rigid packing of side chains in all the studied proteins under given conditions (data not shown). According to the microcalorimetry results, the melting temperature of WT apoMb was 64.5°C, while for the Ala- and Phe-mutants it was 60.3 and 53.5°C, respectively.

Our previous studies and data reported by Griko et al. [16] were the basis for 40°C to be taken as a condition of apoMb aggregation. This temperature allowed only such fluctuations of the protein structure that could cause no heat denaturation. A possibility of occurrence of structural fluctuations capable of promoting amyloid formation by other proteins was described previously [11].

Properties of aggregates formed by WT apoMb and by its mutants. To compare the abilities of WT apoMb and its mutants Val10Ala and Val10Phe to form amyloids, we studied the structure of the resultant aggregates by light scattering, ThT fluorescence, electron microscopy (EM), far UV CD, and IR spectroscopy.

The proteins were incubated with 10 mM Na-phosphate buffer, pH 5.5, at 40°C. These conditions are permissible for a cell and insufficient for apoMb denaturation. Also, they accelerate the aggregation process in comparison with 37°C, thereby facilitating its experimental studies. Structurally, 40°C and pH 5.5 allow a conformational state of apoMb and its mutants that is still close to their native state. Under these conditions the protein properties remain identical to those in the native state corresponding to the basic lines of their pH and temperature plots [16], and neither temperature- nor pH-dependent protein denaturation is observed yet [20].

Aggregate properties after 24 h incubation. ThT fluorescence, a technique widely used in amyloid formation studies, provides specific information about the presence of cross-β-structure in aggregates. Figure 2a presents ThT fluorescence spectra of apoMb recorded before and after the incubation, and its inset shows the light scattering curves. As seen, light scattering increases with time, thereby indicating appearance of large particles. A similar increase of the light scattering intensity was also observed for both mutants. However, the absence of considerable variability of WT apoMb fluorescence intensity shows a low level of dye interaction with the aggregates. This is indicative of minor or zero quantity of cross-β-structure in the formed aggregates.

The secondary structure of the proteins and their aggregates were studied by far UV CD and IR spectroscopy. The shape of CD spectra of WT apoMb before and after the incubation remained almost unchanged (Fig. 2b), with two minima at 208 and 222 nm typical of α-helical proteins. EM experiments demonstrated that after 24 h incubation WT apoMb formed aggregates the majority of which had a globular morphology, although Fig. 2c shows the presence of some protofibrillar structures as well.

The IR spectra of the proteins had several absorption bands. The amide II band (1480-1570 cm^{-1}) reflected turns in the N–H bond and stretching fluctuations of the C–N bond, being only slightly sensitive to the protein conformation. The amide I band (1600-1690 cm^{-1}) was interpreted as a result of stretching fluctuations of the C=O bond and presented the most complete information about the secondary structure elements [34]. Figure 2d shows IR spectra of WT apoMb and its aggregates formed after 24 h incubation at 40°C. The spectra are seen to have at 1650 cm^{-1} virtually one pronounced maximum typical of α-helical structure. Similarity of these spectra is indicative of only slight changes occurring in the protein secondary structure in the course of aggregate formation,

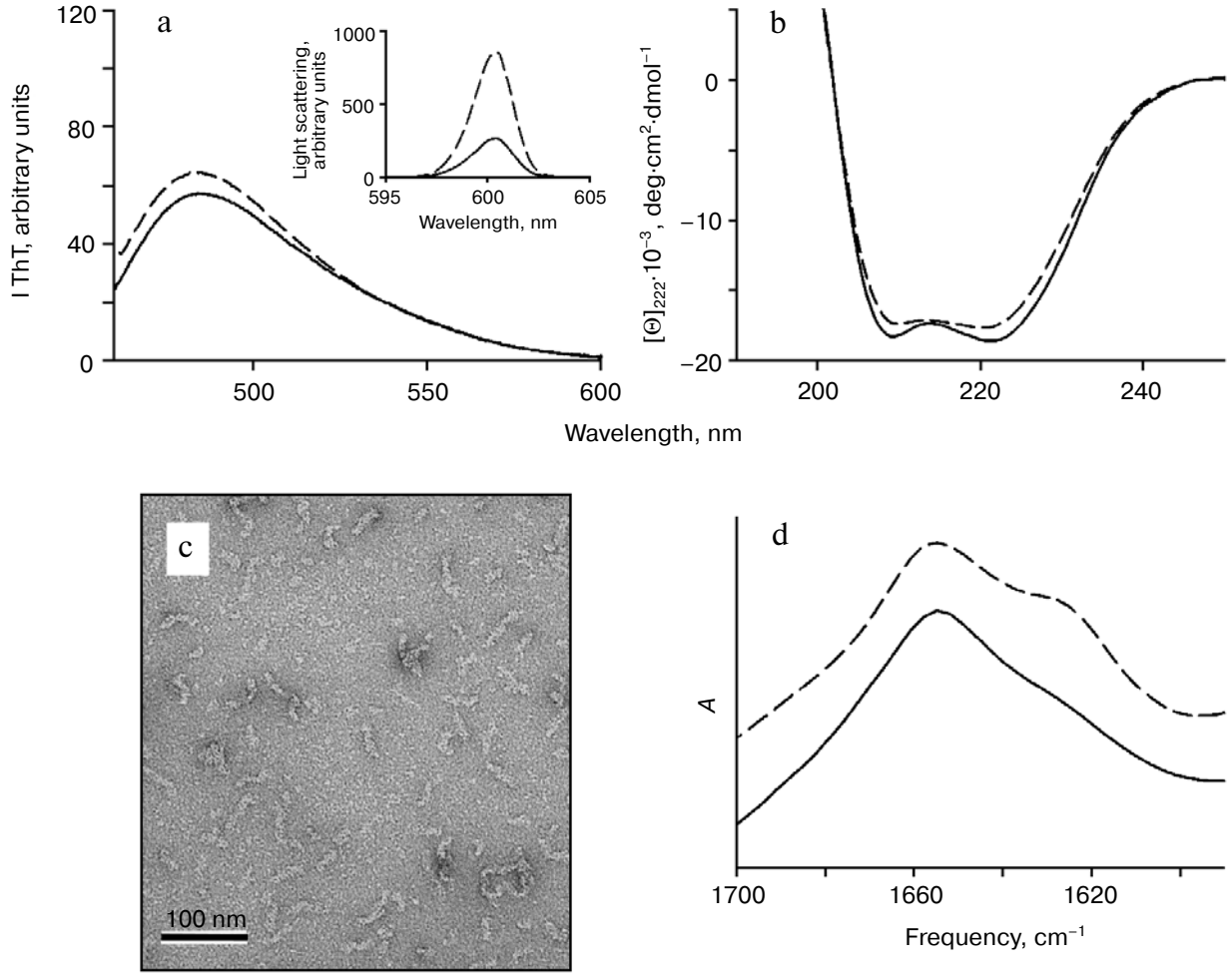

Fig. 2. Comparison of monomeric WT apoMb with its aggregates formed after 24 h incubation at 40°C. a) ThT fluorescence spectra (inset shows light scattering spectra at 600 nm); b) far UV CD spectra; c) EM image of aggregates; d) IR spectra. Monomer spectra are shown as solid lines, and aggregate spectra as dashed lines.

which is confirmed by CD spectra as well (Fig. 2b). Thus, the results obtained by both techniques are in agreement and indicate that WT apoMb aggregates are formed without considerable changes in the protein secondary structure. Together, the data provided by a variety of techniques show that under the chosen conditions WT apoMb form virtually no aggregates with amyloid-like properties.

The same experimental techniques were used to characterize structures formed by the mutants Val10Ala and Val10Phe (see Fig. 3). As it appeared, these aggregates showed a considerable level of thioflavin binding, which was indicative of the presence of cross-β-structure, a characteristic feature of amyloids (Fig. 3, a and b). Besides, unlike WT apoMb, both mutants were able to form aggregates characterized by fibrillar morphology as seen in their EM micrographs after 24 h incubation (Fig. 3, c and d). Figure 3 (e and f) shows that CD spectra of the mutants before incubation differ from those after incubation: the latter lack the minima at 208 and 222 nm and have an extended minimum within the 210-215 nm range, which is indicative of the presence of β-structure. Before incubation, IR spectra of the mutants were similar to the WT apoMb spectrum, with one maximum at 1650 cm^{-1} typical of α-helical structure. But after 24 h incubation their IR spectra demonstrated increased absorption intensity and a shoulder within the 1617-1630 cm^{-1} range, which is characteristic for β-structure (Fig. 3, g and h). Thus, IR and CD provide evidence for considerable structural changes and appearance of β-structure in the aggregates as a result of the incubation.

The data described above indicated that the apoMb mutants form aggregates with amyloid properties. Also, as follows from comparison of WT apoMb with its mutants, a single substitution can provoke amyloid formation at permissive temperature, i.e. under native-like conditions. However, morphology and the secondary structure of aggregates formed by Val10Ala showed no significant difference from those of Val10Phe.

Kinetics of amyloid formation by apoMb mutants. IR spectroscopy, ThT fluorescence, light scattering intensity technique (data not shown), and EM were used to study kinetics of amyloid formation by the apoMb mutants. IR spectroscopy monitored changes in the aggregate secondary structure, while ThT fluorescence registered the increasing content of cross-β-structure, a typical feature of amyloids. Additionally, EM provided information on structural changes in the aggregates that accompanied their morphologic alteration in the course of amyloid formation.

The varying content of cross-β-structure was monitored by continuous ThT fluorescence with a dye added to the protein solution (Fig. 4a). The kinetic results on thioflavin binding could be approximated by the double-exponential approximation only, which is indicative of the presence of at least two steps in apoMb aggregating. As seen from the figure, the kinetics curves for the apoMb mutants are virtually identical. This means that during aggregate formation their secondary structures change with the same rate.

To monitor the varying secondary structure of the mutants by IR spectroscopy, we took their IR spectra after various incubation periods and then plotted incubation time against the 1620 cm^{-1}/1650 cm^{-1} absorption ratio (Fig. 4b). This technique showed changes in mutant spectra without regard to absorption intensity that could vary due to experimental errors. The curves obtained by the above two techniques showed similar shapes, thereby indicating that the β-structure content started increasing right at the moment of initiation of the aggregation process by a temperature of 40°C and involved no lag-phase.

Electron microscopy revealed changes occurring in the aggregate morphology during the process of amyloid formation. Figure 5 shows kinetics of this process for the Val10Phe mutant. EM images taken after 2 h incubation demonstrate mostly moderate globular aggregates with a diameter of about 10 nm (Fig. 5a). Since the size of an apoMb molecule is about 4.5 × 3.5 × 2.5 nm, a globular aggregate can comprise, by a rough estimate, approximately 10-20 monomeric protein molecules. As follows from the EM images, in the course of incubation the aggregates changed in size and shape. Gradually, these small globular aggregates (of about 10 nm) transformed first into elongated and then fibril-like structures that were 10 nm wide and up to 100-200 nm long, although all images show the presence of a certain number of globular aggregates as well. As long as 10-day incubation under the given conditions did not yield aggregates dramatically different in size and shape from those after 24 h incubation.

DISCUSSION

As it has been amply reported, amyloid formation is promoted by protein destabilization. Therefore, mostly, strongly destabilizing conditions were used in aggregation experiments, i.e. extreme pH values, added organic solvents or salts, high temperature [4-6]. However, in the cell, the protein structure can be slightly destabilized by a single point mutation, moderately low pH or permissive temperature. Recently, globular proteins were shown to be able to form amyloid aggregates when their folded state is still preserved and no considerable chain unfolding has occurred [11]. But the mechanism of aggregation of a protein in this state is still poorly understood.

The current study is focused on amyloid formation by apoMb mutants with a single point mutation under conditions corresponding to the base lines of the apoMb native state upon its pH- and heat denaturation. Such conditions keep a protein folded, and no signs of denaturation, except increased fluctuations of the folded chain, can be expected. As revealed by incubation, in these conditions the apoMb mutants were able to form amyloid

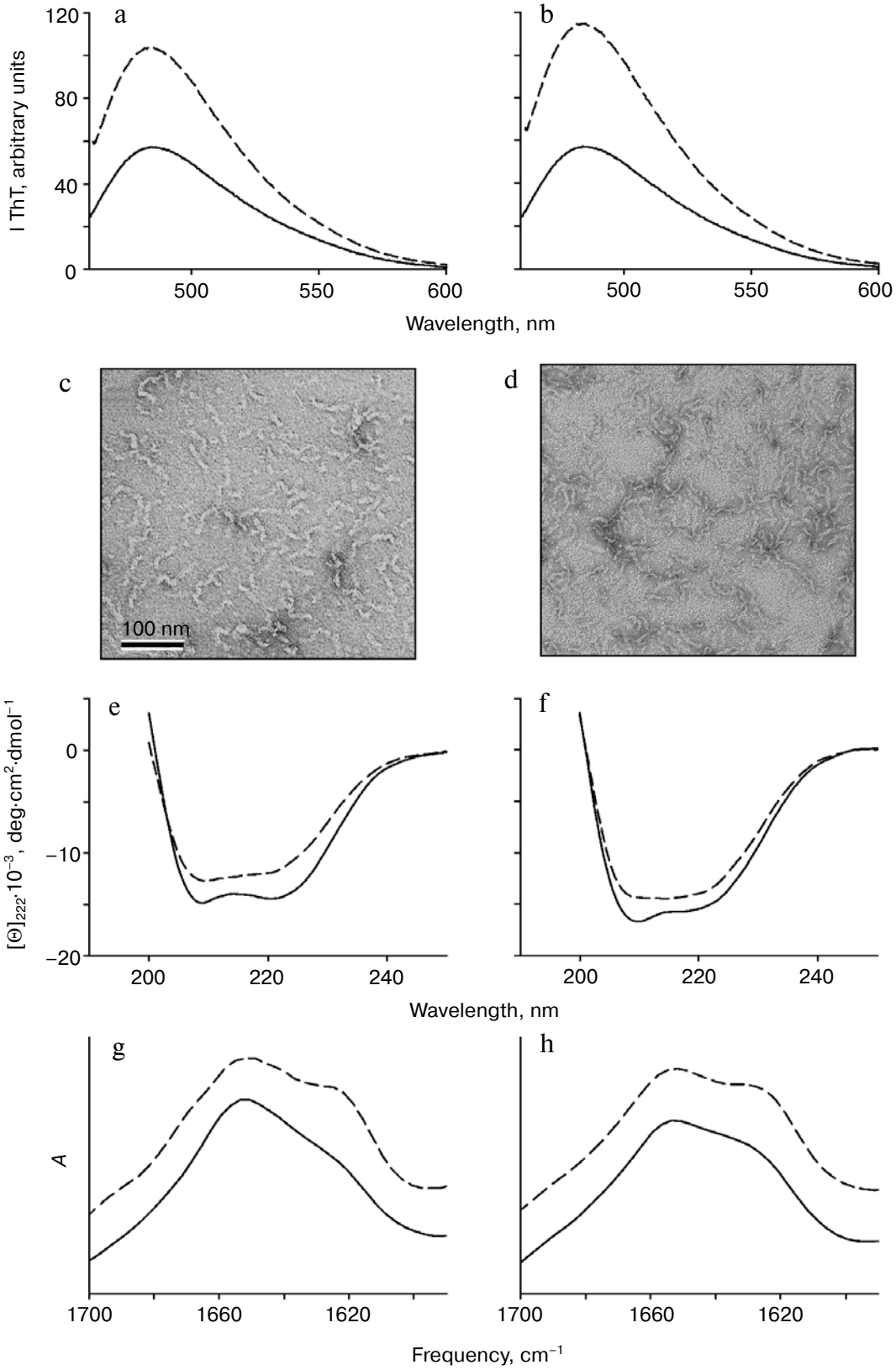

Fig. 3. Comparison of the apoMb mutants Val10Ala (a, c, e, g) and Val10Phe (b, d, f, h) with their aggregates formed after 24 h incubation at 40°C. Monomer spectra are shown as solid lines, and aggregate spectra as dashed lines. a, b) ThT fluorescence spectra; c, d) EM images; e, f) far UV CD spectra; g, h) IR spectra.

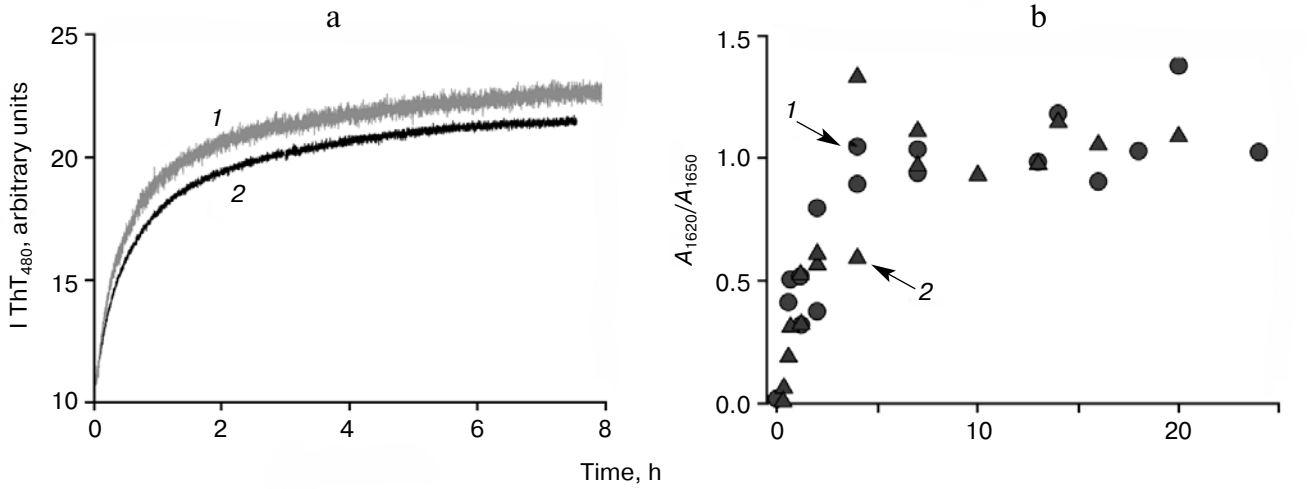

Fig. 4. Kinetics of amyloid formation by apoMb mutants as shown by fluorescence and IR spectroscopy. a) Time dependence of ThT fluorescence upon protein binding; b) the change of 1620 cm^{-1}/1650 cm^{-1} (A_{1620}/A_{1650}) absorption ratio calculated from IR spectra (normalized). *1*) Val10Phe; *2*) Val10Ala.

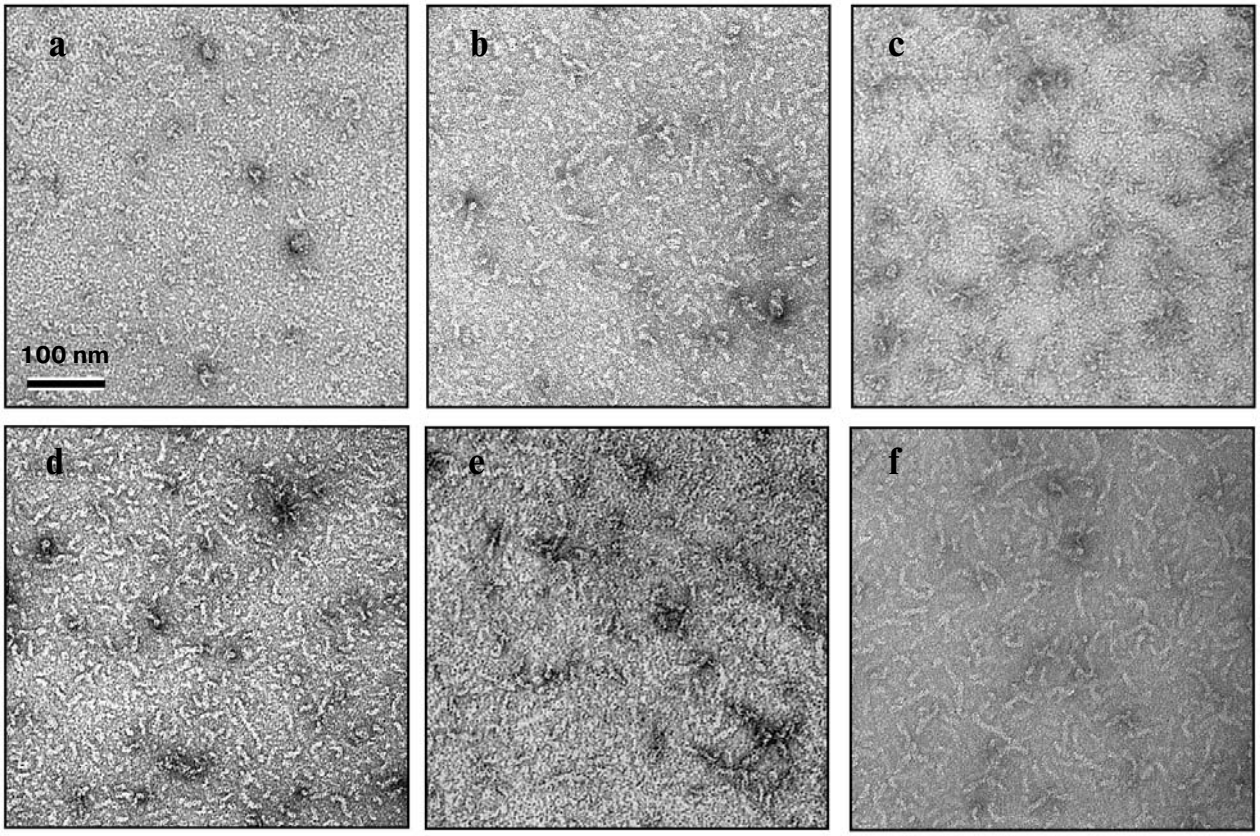

Fig. 5. EM images of aggregates formed by the mutant Val10Phe after: a) 20 min; b) 40 min; c) 1 h; d) 2 h; e) 6 h; f) 11 h.

structures that bound ThT and possessed cross-β-structure (Fig. 3).

It has been shown that an early stage of amyloid formation mostly yields globular aggregates that already contain, though in small proportion, the β-structure (Fig. 3). Later in the process, there appear fibril-like structures (Fig. 5) with increased proportion of cross-β-structure (Fig. 4), although globular oligomers are still present. As

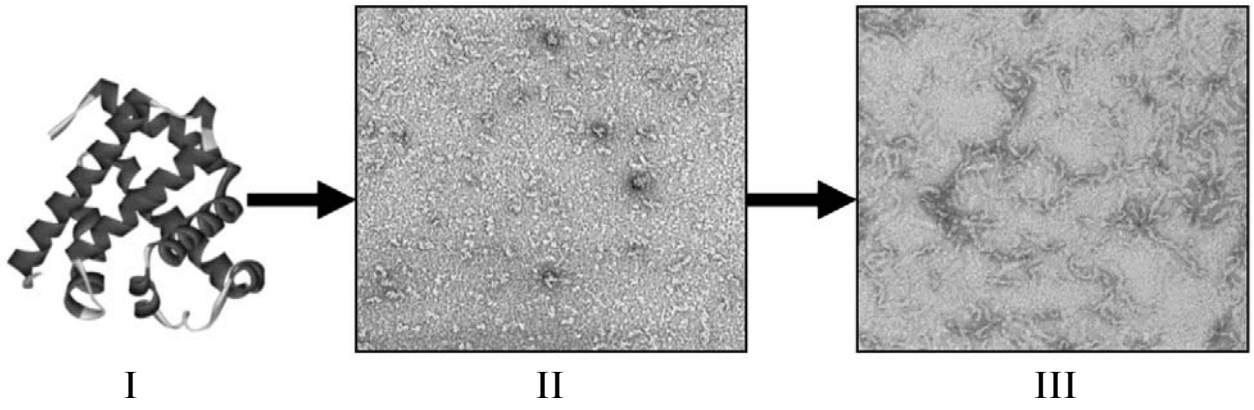

Fig. 6. A pilot scheme of apoMb amyloid formation. I) The original state (folded mutant at 40°C). II) Formation of globular aggregates comprising 20-40 monomers and a small number of elongated aggregates, as well as appearance of cross-β-structure. III) Appearance of protofibrils with a developed cross-β-structure.

a possible explanation of the protofibril growth, their presence suggests protofibril elongation by joining one of them. Experiments on heat denaturation of the monomers and protofibrils showed that the latter had no absorption peak typical of the native apoMb and its mutants, but on the other hand, they could be unfolded by addition of urea (data not shown).

Totally, the data obtained for Val10Ala and Val10Phe by a variety of techniques allow us to present a very simple pilot scheme (Fig. 6) that describes amyloid protofibril formation by apoMb in the course of its long incubation at permissive temperature.

It follows from numerous experiments on amyloid formation by unfolded proteins that hydrophobicity of amino acid residues belonging to the amyloidogenic regions is a key factor for protein aggregation [13, 14]. However, the results reported here indicate that the changed hydrophobicity (caused by Ala and Phe substitution for Val10) has no effect on the rate of the β-structure growth or aggregate properties when the amyloids are formed by folded apoMb. This may suggest that the studied substitutions have been made at a position uninvolved in the intermolecular contacts that arise during apoMb amyloid formation. Although the important role of the apoMb N-terminus has been emphasized previously [23-26], it should be noted that the double mutation reported there resulted in a dramatic protein destabilization.

The fact that hydrophobicity produces no effect on amyloid formation by folded apoMb may have still another explanation. The process of protein aggregating into amyloids in native-like conditions is accompanied by both conformational rearrangements within the protein molecules and formation of intermolecular contacts. It may be that the slower of the two processes determines the rate of aggregating. Under conditions close to native ones, stability of the protein native state is high enough, so the factor governing (restricting) the aggregation rate is likely to be the protein conformational rearrangements. This is why the fluctuation-promoting destabilization of the protein structure predominates over hydrophobicity of the substituting residues in the course of amyloid formation under the native conditions.

In contrast, under the unfolded state conditions, the rate of intermolecular contact formation may determine the aggregation rate. Since hydrophobicity of amino acid residues promotes formation of intermolecular contacts, its increase under strongly destabilizing conditions may possibly be a major accelerating factor of the protein aggregation. The Phe-for-Val10 substitution was meant to increase the number of contacts at this position, which probably explains why we observed no intensification of the aggregation process under the chosen conditions. To have a direct answer to this question, experiments on replacement of residues from other apoMb regions are needed.

Although the reported results on amyloid formation in virtually native conditions are not numerous, they do not fail to indicate the difference between aggregation of folded and unfolded proteins. Specifically, as shown, some algorithms of aggregation rate prediction for acyl phosphatase from *Sulfolobus solfataricus* that are well applicable for the unfolded state of the protein cannot be used to predict the protein aggregation rate in the native-like conditions [36].

Thus, the results presented here show that in predicting the possibility of amyloid formation and in determining the aggregation-governing factors we have to take proper account of the original conformation of the protein molecule in question.

The reported experimental data allow the unequivocal conclusion that a slight destabilization of the apoMb molecule (fluctuations, but not denaturation, caused by a point mutation and incubation at permissive temperature) results in formation of amyloids. This indicates that

altered cellular conditions like disease-caused fewer, though not beyond permissive limits, or local pH decrease (acidification) accompanying infarct or a stroke [37, 38] can cause appearance, short as it may be, of amyloid-like aggregates, the growth and role of which can hardly be predicted.

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