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Structural Analysis of Human and Bovine α-Fetoprotein by Electron Microscopy, Image Processing, and Circular Dichroism†

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ABSTRACT: The images of human and bovine α-fetoprotein molecules have been enhanced by combining dark-field electron microscopy with a laser-assisted optical system. This system filters out random background noise while permitting true averaged signal reconstruction of the molecule. A single averaged molecular image was digitized into a matrix, each pixel being assigned a gray scale level to produce a relative mass map for each molecule. These maps were interpreted from the α-helix, β-form, and random coil of the purified proteins as determined by circular dichroism. Results showed that both molecules are "U shaped", apparently monomeric, with outside dimensions of approximately 80 Å. Both molecules have asymmetrical structural features, notably three mass dense regions at both extremities and at the vertex of the molecules. Circular dichroism data suggest a high degree of similar stabilized α-helix and extensive β-form in these regions. Mass map analysis of hAFP correlates with the subdomains organized by disulfide bridges.

Various methods have been used to elucidate the conformation of biological molecules among which are X-ray diffraction analysis (Kendrew, 1963; Blundell, 1976), optical rotary dispersion and circular dichroism (Crabbé, 1972), and electron microscopy (EM)[1] using various staining procedures combined with computer averaging (Frank et al., 1978). The structure of naturally occurring crystalline specimens has also been accomplished by EM (Unwin & Henderson, 1975).

More recently a relatively rapid technique has been developed for the direct visualization of sulfur atoms and small biological molecules in the noncrystalline state such as vasopressin (1056 daltons) and myokinase (21 000 daltons). The procedure combines the features of high contrast dark-field EM, low specimen damage through minimal beam exposure, and image enhancement by means of a laser-assisted optical filter system (Ottensmeyer et al., 1973, 1975, 1977). This technique has been termed "filtering of arrays of images in reciprocal space" (FAIRS) which filters out random background noise while permitting true averaged signal reconstruction of the molecule. EM/FAIRS was first employed on small molecules of known structure, and it was demonstrated that molecular images obtained by this technique were in good agreement with images obtained by X-ray crystallography (Ottensmeyer et al., 1977). Conformations of low molecular weight proteins of unknown structure have also been

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[1] Abbreviations: hAFP, human α-fetoprotein; bAFP, bovine α-fetoprotein; EM, electron microscopy.

0006-2960/83/0422-5978$01.50/0

determined by EM/FAIRS (Ottensmeyer et al., 1978) and by dark-field EM on a small protein containing heavy metal (Fiskin et al., 1977).

The major objective of the present investigation was to apply the EM/FAIRS technique to the conformational imaging of two larger biological molecules, human α-fetoprotein (hAFP) and bovine α-fetoprotein (bAFP), both fetus-specific serum glycoproteins consisting of single polypeptide chains with molecular weights of 64 600 and 68 000, respectively (Nishi, 1970; Lai et al., 1978). Another objective was to compare possible secondary conformational features of these two molecules by mass analysis and circular dichroism.

Materials and Methods

Specimen Preparation. hAFP was isolated from fetal cord serum by a two-step procedure, which we have previously employed, involving immunoaffinity chromatography (Forrester et al., 1975) and preparative gel electrophoresis (Lai et al., 1976). bAFP was prepared from fetal calf serum by concanavalin A affinity chromatography, gel filtration, and preparative gel electrophoresis (Lai et al., 1978). Both proteins were purified to electrophoretic and immunochemical homogeneity and standardized. Proteins were diluted to a concentration of 1 μg/mL in quartz D·H_2O and 1 drop of each solution was applied to an electron microscope grid allowing molecules to adsorb onto an ultrathin carbon film (Johansen, 1974). After 4–5 min the grids were washed with 20 drops of quartz D·H_2O to remove excess AFP and air-dried at room temperature. No staining procedure was used in preparation of the specimens.

Dark-Field Electron Microscopy. Dark-field transmission EM (Dupouy, 1967) was performed by using a Philips EM-300 in beam-tilt mode at a calibrated nominal magnification of 51300×. In order to achieve maximum illumination, a 500 μM second condensor aperture was installed. Astigmatism was corrected by focusing on the carbon film. To reduce radiation damage to molecules, the minimal beam exposure techniques of Williams & Fisher (1970) were employed by using Kodak electron image plates. Exposures were less than 4 s at 80 KeV. A suitable developed plate was determined by an in-focus exposure with no specimen drift or astigmatism (Ottensmeyer et al., 1975).

Photographic Array Construction. EM plates were scanned by a stereomicroscope (15×) for repeating molecular images of AFP corresponding to an approximate size of 15–2300 Å based on a globular or linearly arranged protein estimated to contain 600 amino acid residues (in the case of hAFP), the distance between residues being 3.8 Å. The location of individual AFP molecules were marked, and an intermediate photographic enlargement (23×) was made on a microscope with flat-field optics. A sufficient number of human and bovine AFP molecules were individually photographed and arranged into a small 3 × 3 photoarray which was then repeated 9 times to enhance uniform averaging for a total of 81 images. The periodicity of the array was one. A photonegative of this composite array was reduced onto a 50 × 50 mm Kodak (medium grade emulsion) slide plate such that the image of each individual AFP molecule covered an area of approximately 1 mm^2.

Laser-Assisted Optical Filtering. The photonegative slide plate was subjected to a laser beam, and the composite diffraction pattern of the image array was transmitted through an optical filter to remove random noise signals while allowing repeated signal information to be averaged, reconstructed optically, and photographed. A 6-mW helium–neon laser beam, made uniform by a spatial filter, was transmitted through a beam expander (Spectra Physics, Mountain View, CA), and the beam diameter was adjusted by an iris diagragm. All components were mounted on an optical bench with adjustable carriages. The converging beam was transmitted through the photonegative slide plate to produce a diffraction pattern which, at the beam's focus, was optically filtered by a hole reciprocal lattice arrangement which matched the composite array. The filtered diffraction pattern was reconstructed by a lens to produce an array of averaged molecular images. This process significantly increased the image's signal to noise ratio and attained a molecular image resolution of better than 8 Å. Reconstructed images were photographed by using a standard 35 mm camera and Kodak Technical Pan film 2415.

Optical Filter Design. The optical filter was constructed by using the Interactive Graphics Package (Tektronix, Beaverton, OR) with a Calcomp plotter to produce a 41 × 41 matrix of small blackened circles on a sheet of plotter paper measuring 90 × 90 cm. The plot was reduced onto a photonegative so that each hole diameter was 0.22 mm repeating at 1.27-mm intervals. Copper foil (1-mil thickness) was attached to a fiberglass board with rubber cement and photoetched by using the reduced matrix plot photonegative. The copper foil containing holes was removed from the fiberglass by immersion of the board in toluene for 12 h. The optical filter was mounted in a rigid fiberglass frame.

Image Processing. Photographic prints of averaged images of hAFP and bAFP molecules were digitized by using a video camera and Grinnell image processor (Grinnell Systems Corp., San Jose, CA) into a 512 × 512 array of pixels. The data were reduced to a 64 × 64 matrix by a DEC PDP 11/23 minicomputer with UNIX (Bell Laboratories Murray Hill, NJ) attached on line to the Grinnell image processor. Each pixel had associated with it a gray scale integer from 0 to 255 depending on the averaged intensity of a matrix position on the photonegative. The 64 × 64 matrix images were subsequently photographed on the Videoprint system (Image Resource Corp., West Lake Valley, CA). To obtain a mass contour map of the molecule based on the density of scattered electrons (Dubochet, 1973) and hence the image's gray scales, the image data were transferred via magnetic tape to a Honeywell DPS Level 2 computer with Multics (Honeywell Information Systems, Waltham, MA) to produce the image's contour map by the DISSPLA plotting package (Integrated Software Systems Corp., San Diego, CA). We were unable to calibrate the nonlinear response of the EM plates exposed to scattered electrons.

Circular Dichroism. Circular dichroism spectra were recorded on a Jasco J 41 A spectropolarimeter (Jasco Inc., Easton, MD). Protein samples were dissolved in D·H_2O at a concentration of 50 μg/mL as determined by Waddell (1956). Spectra were recorded in a 205–250 nM range with each 1-nM interval ellipticity converted to mean residue ellipticity (Reed et al., 1975). The proportions of α-helix, β-form, and random coil in the two molecules were determined by fitting the data to a standard curve by using the parameters of Chen (Fasman, 1976).

Results

Figure 1 shows the original composite photoarrays made from its EM images of hAFP (A) and bAFP (B) molecules with insets of the filtered molecular images and respective enlargements of single averaged molecules (C and D). Both molecules are "U shaped" possessing three domains. Asymmetrical structural features are evident as mass dense regions at both ends and at the vertex of the two molecules. The

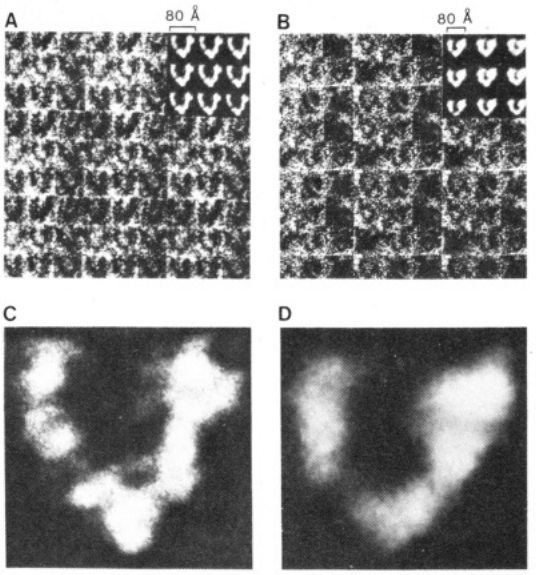

FIGURE 1: Composite photoarrays for human α-fetoprotein (A) and bovine α-fetoprotein (B) with insets (upper right) of the filtered molecular images and respective enlargements of single averaged molecules (C and D).

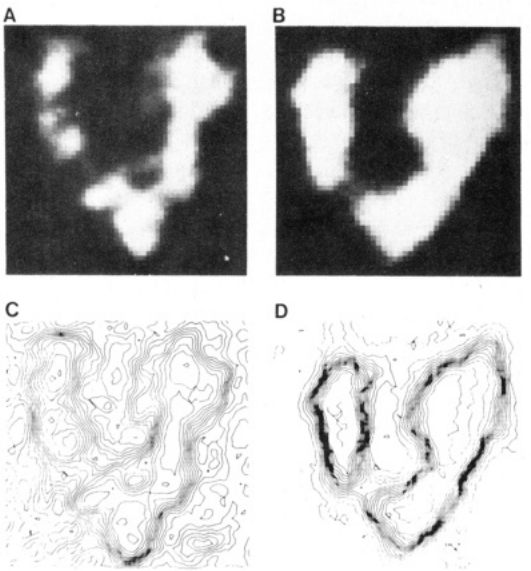

FIGURE 2: Digitized matrices of mass-scattered electrons from single averaged molecular images of human α-fetoprotein (A) and bovine α-fetoprotein (B) with respective contoured mass maps (C and D).

distance across the top of each molecule is approximately 80 Å while the "arm" widths range from 25 to 50 Å.

Figure 2 illustrates the digitization into a matrix of gray scales for single molecular images of hAFP (A) and bAFP (B) with their respective contouring into mass maps (C and D). bAFP exhibits a lower background contribution due to higher contrast in the original array. The mass maps accentuate the locations of the three domains on each molecule.

Figure 3 presents the circular dichroism spectra for hAFP and bAFP, with the α-helix, β-form, and random coil composition for these two molecules. The secondary structural conformations of hAFP and bAFP show compositions for α-helix of 49% and 55%, β-form of 17% and 17%, and random coil of 34% and 28%, respectively.

Discussion

In this paper we have shown that the EM/FAIRS technique and image processing combined with circular dichroism studies could rapidly elucidate the general molecular conformation

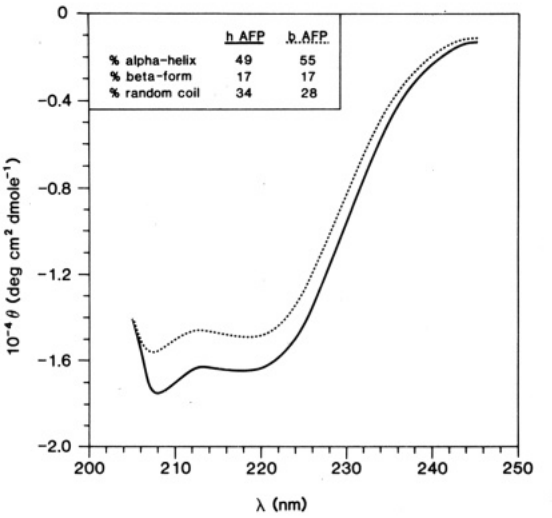

FIGURE 3: Circular dichroic spectra of human α-fetoprotein (hAFP) and bovine α-fetoprotein (bAFP). θ is the mean residue ellipticity.

of hAFP and bAFP in the noncrystalline state. Our results showed a high degree of conformational homology in these molecules.

It can be seen from Figure 1 that both AFP molecules have a planar distance across the top of the molecule of approximately 80 Å. This suggests that the molecule has a compact conformation since the distance of adjacent α-carbons in a protein is 3.8 Å which, if multiplied by 590 residues for hAFP (Morinaga et al., 1983), would result in a linear length of 2240 Å. The compact nature of AFP further suggests a high degree of ordered secondary structures such as α-helices and β-form. This interpretation is supported by the circular dichroism data which reveals that both AFPs have a relatively large amount of α-helix and a typical amount of β-form structure. Moreover, these secondary compositions of both proteins are remarkably similar, possibly demonstrating an interspecies relatedness which may explain the partial antigenic identity that we have observed between hAFP and bAFP (Lai et al., 1978).

Three mass dense regions projecting in either direction from the image plane seen in Figure 2 indicate the relative size and asymmetry of the three domains. Since hAFP and bAFP have carbohydrate contents of 3–6% (Nishi, 1970; Marti et al., 1976), glycosidic side chains or other appendages may comprise portions of these mass dense regions. The low degree of scattered electrons on the lower left "arm" of both molecular images (Figure 1) may indicate a polypeptide link between domains. This putative link may serve as a "hinge segment" to allow flexible movement of domains and facilitate binding of potential ligands. The high α-helix composition also suggests the presence of tertiary structures in these mass dense regions since the compact nature of the AFP molecule would not accommodate a single stretch of α-helix. It is possible, to judge from the width of the domains on either molecule, that three or more α-helices could lie adjacent to each other.

Since we could not ascertain whether molecular dimerization might have occurred during isolation or when AFP was dissolved we first scanned for monomers (assuming the U shapes were dimers) and then for dimers (assuming the U shapes were monomers). We did not find structures that were half the size of the U shape or structures that were twice the size of the U shape. Our preparation, therefore, must be of a uniform population of AFP molecules that are exclusively in a monomer form and not mixed with other proteins.

During image selection it would be expected that AFP molecules would orientate themselves on the carbon film in

a variety of energy stable positions. A noncritcal selection of molecules from electron micrographs clearly revealed the general U shape of both molecules, while a more rigorous selection and alignment of individual molecules produced the images with asymmetrical structural features shown in Figure 1.

The EM/FAIRS technique employed in the present investigation had a maximum resolution of 4 Å. Calculations were derived from the formulas of Ottensmeyer et al. (1977). Limitations of the EM/FAIRS system have been discussed (Misell, 1978; Fujiyoshi et al., 1980) which suggest that future improvements in the EM/FAIRS technique might include liquid helium cooling of the specimen combined with scanning transmission EM to reduce radiation damage, modifications of the thin carbon film to further reduce background noise, and densitometric acquisition of image data with computer alignment by cross-correlation and rotation of the unfiltered array components. Other potential applications of the EM/FAIRS system (Ottensmeyer, 1982) to molecular biology and chemistry remain to be explored, including attempts to demonstrate the association between molecules such as AFP and their ligands or antibodies.

Morinaga et al. (1983) have now elucidated the complete amino acid sequence of hAFP which has three domains similar to those as defined for human serum albumin (Brown et al., 1978). Morinaga et al. (1983) have shown a notable absence of disulfide bridges in hAFP from residues 295–396, which would enhance flexibility between domains 2 and 3. Furthermore, their study indicates that the N-terminus region (domain 1), containing residues 1–79, is also characterized by flexibility of the polypeptide backbone. These observations lead us to assign the right arm of our molecular image (Figure 1C) to domain 1 of hAFP, since the "hinge" region adjacent to domain 3 is likely represented in the Morinaga et al. (1983) sequence by the relatively flexible and uncompact region between domains 2 and 3. Furthermore, the flexibility in domain 1 correlates strongly with many of our other electron micrographs (unpublished observations) showing structural heterogeneities in this region. With the domains for hAFP assigned in this way, an approximate mass integration of the three prominent areas (Figure 2A) gives comparable values to the amino acid masses of each domain proposed by Morinaga et al. (1983). The densest regions of the mass map for hAFP (Figure 2C) correlate strongly with the subdomains organized by the disulfide bridges assigned by Morinaga et al. (1983) to the protein, an interesting finding since it is known that with optical filtration sulfur atoms, as found in disulfide bridges, diffract electrons significantly more than do other biological prevalent elements (Ottensmeyer et al., 1973). We speculate that the hinge segment of AFP may delineate domains which are products of gene duplication as has been postulated for the murine AFP gene (Eiferman et al., 1981).

Acknowledgments

We thank E. Morris (Lion's Sight Centre), W. F. Conn, and D. Orford for their technical assistance with the design of the optical bench and computer software for image processing. We also thank D. Andrews for his technical advice and K. Naka for his comments on the manuscript.

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