Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 10809–10813, September 1998
Immunology

Antigenicity of fullerenes: Antibodies specific for fullerenes and their characteristics

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Communicated by Seymour Lieberman, St. Luke's-Roosevelt Institute for Health Sciences, New York, NY, July 9, 1998 (received for review April 17, 1998)

ABSTRACT The recent interest in using Buckminsterfullerene (fullerene) derivatives in biological systems raises the possibility of their assay by immunological procedures. This, in turn, leads to the question of the ability of these unprecedented polygonal structures, made up solely of carbon atoms, to induce the production of specific antibodies. Immunization of mice with a C_{60} fullerene derivative conjugated to bovine thyroglobulin yielded a population of fullerene-specific antibodies of the IgG isotype, showing that the immune repertoire was diverse enough to recognize and process fullerenes as protein conjugates. The population of antibodies included a subpopulation that crossreacted with a C_{70} fullerene as determined by immune precipitation and ELISA procedures. These assays were made possible by the synthesis of water-soluble fullerene derivatives, including bovine and rabbit serum albumin conjugates and derivatives of trilysine and pentalysine, all of which were characterized as to the extent of substitution and their UV-Vis spectra. Possible interactions of fullerenes with the combining sites of IgG are discussed based on the physical chemistry of fullerenes and previously described protein-fullerene interactions. They remain to be confirmed by the isolation of mAbs for x-ray crystallographic studies.

Until 1985 there were only two known allotropic forms of carbon: graphite and diamond. In 1985, a novel allotrope was reported in which 60 carbon atoms were arranged as a truncated icosahedron, with 60 vertices and 32 faces, 12 of which were pentagonal and 20 hexagonal (1). It was dubbed Buckminsterfullerene (usually shortened to fullerene) because of its geodesic character, a name that has held through the present day.

Considerable activity followed this discovery particularly after procedures were developed to prepare fullerenes in workable quantities (2, 3). Various fullerene-based compounds have been prepared, and diverse uses were sought for them. Some were incorporated into photovoltaic cells (4) and nanotubes (5). Others were tested for biological activity (6), including antiviral (7, 8), antioxidant (9, 10), and chemotactic activities (11), and as neuroprotective agents in a mouse model of amyotrophic lateral sclerosis (12).

Practical application of fullerenes as biological or pharmacological agents requires that dosage and serum levels be capable of measurement, preferably by sensitive, simple immunological procedures. This, in turn, requires that specific antibodies to fullerenes be produced.

The clonal selection theory tells us that antigens elicit the production of antibodies by selecting for specific antibody-producing cells already present in the repertoire of immunized animals (13). Although there is debate about the size of the "available" repertoire (14, 15), immunologists usually work on the assumption that the repertoire is diverse enough to be counted on to produce antibodies to "any" molecule a researcher may choose. This is, of course, an unreliable assumption, as experimental failures rarely find their way into the literature. The question that arises, therefore, is whether the immune repertoire is "complete" enough (15) to recognize and respond to the unprecedented geodesic structure of the fullerenes or sufficient aspects of it—more particularly, whether the immune system can process a fullerene-protein conjugate and display the processed peptides for recognition by T cells to yield IgG antibodies. We report here that it does.

MATERIALS AND METHODS

The fullerene derivatives **1–4** relevant to this paper are shown in Fig. 1. Compounds **1** and **3** were prepared as described in ref. 16. For the synthesis of **2**, see ref. 17.

Preparation of the Bovine Thyroglobulin (TG) Conjugate of 1. Compound **1** (1.5 mg, 1.6 μmol) was dissolved in 0.25 ml of dry pyridine. *N*-Hydroxysuccinimide (Sigma) (8 mg, 70 μmol) was added and brought into solution with the fullerene compound. Dicyclohexylcarbodiimide (Fluka) (6 mg, 43 μmol) dissolved in 0.15 ml of dry pyridine was added, and the reaction was allowed to proceed at room temperature for 48 h. The reaction mixture then was added dropwise over a period of about 5 min to 10.4 mg (1.3 μmol) of TG dissolved in 1 ml of water and adjusted to pH 9.5. The pH was kept at 8.5 throughout the reaction by the addition of 1 M $NaHCO_3$. Some turbidity appeared during the reaction. The reaction was allowed to proceed for 4 h, and the reaction mixture then was dialyzed against PBS overnight at 4°C. The number of fullerene groups per molecule of TG was estimated, after clarification by centrifugation, to be *ca.* 20 by absorbance measurements at 320 nm (see below).

Bovine and Rabbit Serum Albumin (RSA) Conjugates. Similar procedures were used for the BSA and RSA conjugates. The UV-Vis spectrum of the RSA conjugate is shown in Fig. 2. It has a peak at 254 nm and a shoulder at about 320 nm. Others have seen these fullerene characteristics, albeit with slight shifts in wavelength (11, 16–19). The rise after 254 nm is characteristic of polypeptides, as shown by the spectrum of an equal concentration of RSA in Fig. 2. In both cases the proteins were substituted with about 10 molecules of the fullerene derivatives per molecule of protein, as determined by UV-Vis spectral analysis at 320 nm and by titration of the unsubstituted amino groups by trinitrobenzenesulfonic acid (20).

Conjugation of 1 to Lys-Lys-Lys·3HCl (3L). *N*-Hydroxysuccinimide (0.5 mg, 4.3 μmol) was dissolved in 0.125 ml of dry pyridine. The solution was added to 0.5 mg (0.54 μmol) of **1**,

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Abbreviations: TG, thyroglobulin; RSA, rabbit serum albumin.

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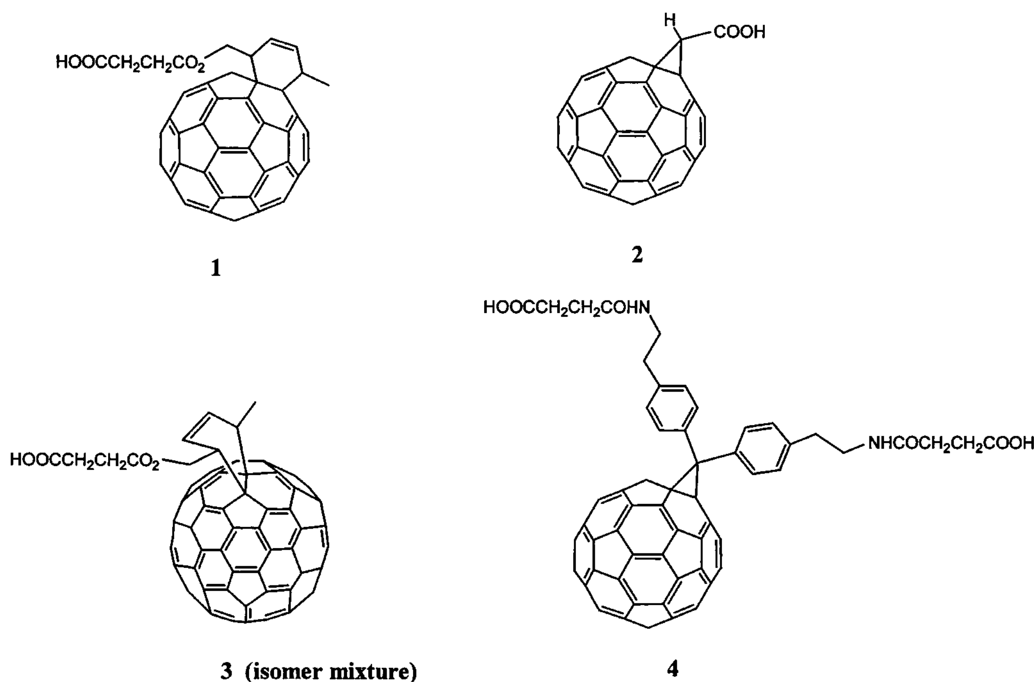

FIG. 1. Fullerene derivatives used in this study.

which then was allowed to dissolve. Dicyclohexlycarbodiimide (2.5 mg, 18 μmol) was dissolved in 0.1 ml of dry pyridine, and the solution was transferred to the solution containing **1** and *N*-hydroxysuccinimide. The total reaction mixture was allowed to stand at room temperature for 48 h. It then was added dropwise to 0.1 mg (0.2 μmol) of trilysine trihydrochloride (21) in 0.9 ml of 0.1 M $NaHCO_3$, with stirring. The pH was kept at 8.5 by addition of the bicarbonate solution. The reaction was allowed to proceed for 4 h, and the resulting solution was taken to dryness over P_2O_5 *in vacuo*. The resulting residue was taken up in 250 μl of distilled water and clarified by centrifugation. Its UV-Vis spectrum is shown in Fig. 3. Like **1**-RSA, it has a shoulder and a peak, albeit somewhat shifted. The shoulder is at 335 nm and the peak is at 260 nm. From both its spectrum and reaction of its free amino groups with trinitrobenzenesulfonic acid (20) it was found to have an average of 1.8 mol of **1** per trilysine molecule.

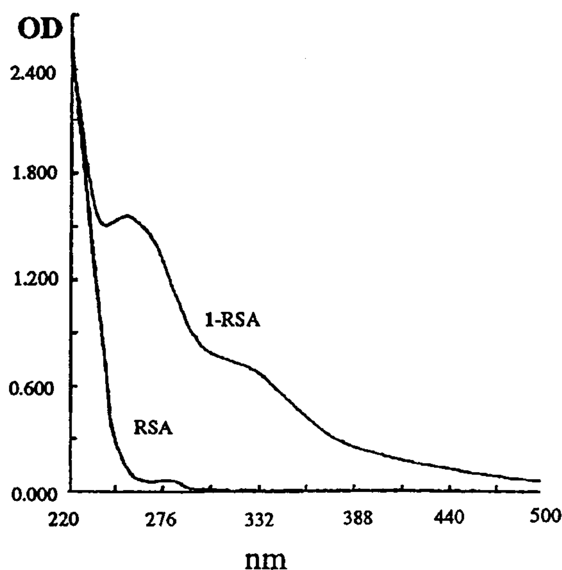

FIG. 2. UV-Vis spectrum of **1**-RSA and RSA, both at concentrations of 100 μg/ml in PBS.

Conjugation of 1 to Penta-L-Lysine (Sigma). This reaction was carried out in a similar fashion as the trilysine conjugation. The final product was substituted to the extent of 2.7 mol of **1** per pentalysine.

Immunization Procedure and Detection of Antibodies. BALB/c mice were immunized i.p. with **1**-TG in complete Freund's adjuvant for the primary immunization and incomplete adjuvant for subsequent immunizations.

After a total of three immunizations at 3-week intervals, confirmation of an immune response was determined by direct ELISA in which polystyrene plates (Corning) were coated with the **1**-RSA conjugate (0.5 mg/ml in 0.1 M $NaHCO_3$, pH 9), and binding of preimmune and immune sera were determined by standard procedures. Development was with horseradish peroxidase-labeled goat anti-mouse IgG (Sigma), which tested negative for crossreaction with an IgM preparation. The substrate used was *o*-phenylenediamine.

Specificity of the response was determined by two procedures: double diffusion in agar (22) and competitive inhibition, as determined by ELISA using RSA and BSA conjugates of the fullerenes, as well as the oligo lysine derivatives. The fullerenes themselves were not soluble enough in aqueous solutions.

There were two controls for the ELISA experiments: (*i*) RSA, to show that the protein moiety did not participate in the inhibition, and (*ii*) adenosine-6-hexanoyl RSA (23), to show that the linkage group did not participate. The adenosine conjugate had been synthesized using the same strategy used

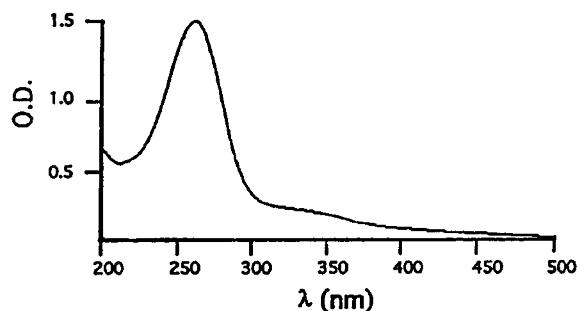

FIG. 3. UV-Vis spectrum of **1**-trilysine in water (80 μg/ml).

for the fullerenes, i.e., linkage to the epsilon amino groups of the lysines via an *N*-hydroxysuccinimide ester derivative.

RESULTS

Immune Response to 1-TG. The immune response of mice immunized with **1**-TG first was determined by ELISA. Because of the extreme hydrophobicity of fullerenes, it was important to show that nonspecific binding to serum components did not occur in the preimmune serum. The results are shown in Fig. 4 for sera taken from a BALB/c mouse immunized i.p. with one primary and two booster injections 3 weeks apart. The result was a high titer of specific antibody, as measured with **1**-RSA. No antibody or nonspecific binding was seen with components of the preimmune serum. Development was with a peroxidase-labeled anti-mouse antibody specific for mouse IgG.

Specificity of the Immune Response. Specificity of the immune response first was determined by double diffusion in agar. The results are shown in Fig. 5.

Confluent lines of precipitation were seen associated with wells 1–5 with a spur between wells 2 and 3, pointing toward 3, i.e., toward **1**-BSA. This finding is evidence of a population of antibodies reactive with both **1** and **2**, with an additional population specific for the carrier protein of the immunogen, **1**-TG. The identity of precipitation with **1** and **2** is evidence for the lack of participation of the linker group, which is missing from **2**. A visible, albeit smaller, precipitate was seen with **3**-RSA (well 5), the C_{70} fullerene. Well 6, containing unsubstituted TG, showed no visible precipitate. However, when the TG solution was diluted 5-fold, a line of precipitation was seen (not shown), indicating a low titer of antibody specific for unsubstituted TG, i.e., in the original experiment (Fig. 5) TG was in antigen excess. Anti-TG also could be detected by ELISA (below).

Specificity also was determined by ELISA inhibition studies (Fig. 6). Binding to **1**-RSA was inhibited by **1**-RSA, **1**-BSA, **1**-TG, **3**-pentalysine, **2**-pentalysine, and **1**-pentalysine. Inhibition by **2**-pentalysine is additional evidence for nonparticipation of the linker group, i.e., specificity for the C_{60} moiety. Although not shown in Fig. 6 no inhibition was seen by TG, RSA, or adenosyl-6-hexanoyl-RSA, which has the same linker joining the hapten to the protein carrier (23).

In an additional control for specificity, the fullerene derivatives **1**-pentalysine, **1**-RSA, and **1**-BSA were tested by ELISA for nonspecific inhibition of an antibody to a retinoic acid derivative (unpublished work) with its hapten-RSA conjugate. No inhibition by the fullerene derivatives was seen, in contrast to the inhibition seen with the retinoic acid derivative (data not shown).

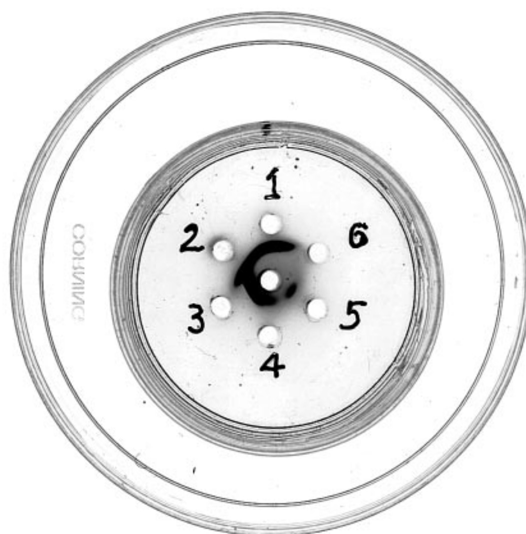

FIG. 5. Results of double diffusion in agar. Well 1, **1**-TG (the immunogen); 2, **2**-TG; 3, **1**-BSA; 4, **1**-RSA; 5, **3**-RSA; and 6, TG.

Taken together, the results show that there was an IgG response directed at the fullerenes, and not at the functional groups linking the fullerenes to the carriers. Moreover, a subpopulation of the antibodies raised to a C_{60} fullerene crossreacted with the C_{70} fullerene.

DISCUSSION

Immunization of mice with a C_{60} fullerene-TG conjugate produced a polyclonal response comprised of antibodies specific for C_{60} fullerenes and a subpopulation that crossreacted with a C_{70} fullerene derivative (Figs. 5 and 6). Detection was possible by ELISA using an IgG-specific second antibody showing that the antibodies raised were of the IgG isotype (Figs. 4 and 6). It follows from this that derivatization of TG by a fullerene molecule did not prevent intracellular processing and subsequent peptide display to T cells presumably by the process of linked recognition (24).

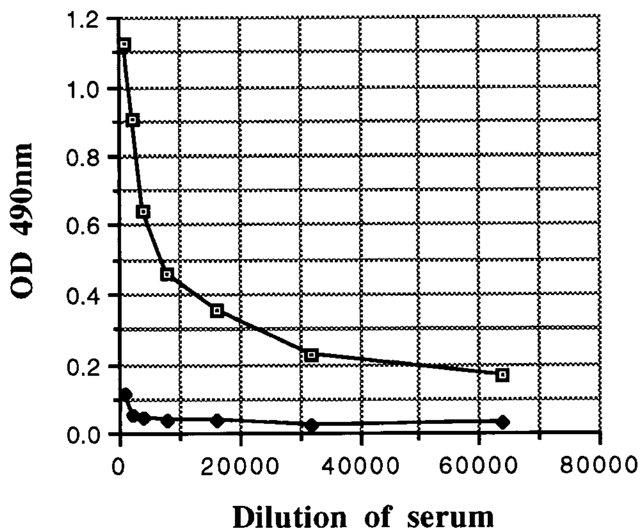

FIG. 4. ELISA study of antibody response to **1**-RSA. ⊡, immune serum. ◆, preimmune serum.

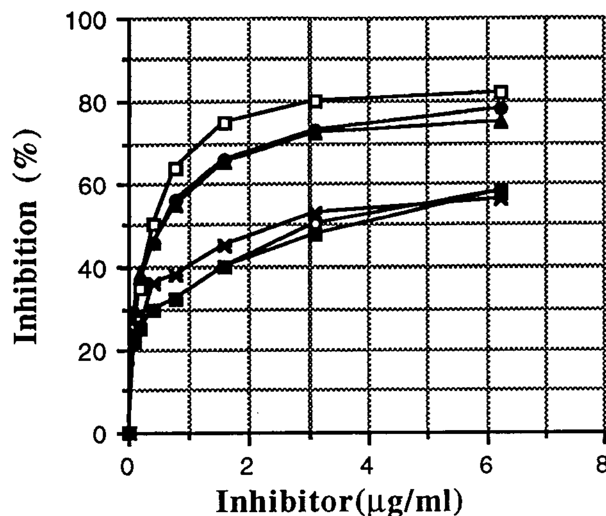

FIG. 6. ELISA inhibition experiments: ▲, **1**-BSA; ○, **1**-(Lys)$_5$; ●, **1**-RSA; □, **1**-TG; ■, **3**-(Lys)$_5$; ×, **2**-(Lys)$_5$.

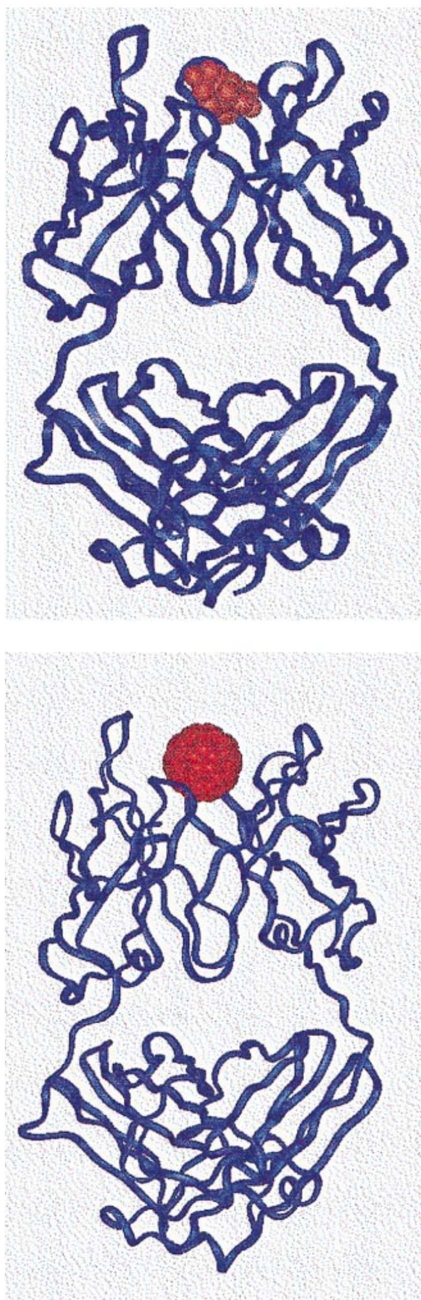

FIG. 7. (*Upper*) Binding of the progesterone analog, 5-α-pregnane-20-one-3-β-ol hemisuccinate to the Fab′ fragment of a mAb specific for progesterone. This computer model was displayed in INSIGHT II from the x-ray crystal structure coordinates reported in ref. 26. Steroid is in red. (*Lower*) The molecular docking of fullerene-C_{60} by deletion of the steroid and manual docking using INSIGHT II. Fullerene is in red.

Of interest is the manner of recognition of fullerenes by the immune system. Until we are in the position to examine the immune complex by direct means, e.g., x-ray crystallographic analysis, we can speculate based on characteristics of fullerenes that could provide potential for recognition.

Hydrophobicity. Fullerenes, being made up solely of carbon atoms, are very hydrophobic. It would be expected, therefore, that antibodies that recognize fullerenes will have hydrophobic amino acids in their binding sites. Such has been reported for the combining site of an Fab′ fragment of a mAb specific for progesterone (25, 26), a molecule highly apolar in character (see below). Contact with amino acids in the combining site of the heavy chain included three tryptophans, one tyrosine, and a phenylalanine in a buried combining site of 254 Å^2 in surface area (26).

Curvature. Theoretical studies of various fullerenes show that curvature, expressed as the pyrimidalization angle P, significantly influences fullerene properties and reactivity (27). Although the completely flat graphite molecule has a P angle of 0°, the most curved fullerene, C_{60} has angles uniformly bent at 11.6°. The angles of the C_{70} molecule vary from P = 8.8° to almost 12° (see Fig. 1 for its shape). Curvature of a normally planar aromatic ring induces local charge differences.

pi stacking. The pi system of fullerenes would be expected to interact with molecules in a combining site of an antibody via pi-stacking interactions. Experimental data from the x-ray structure of a C_{60}/benzene solvate clearly show this kind of interaction (28). Three benzenes are associated with each C_{60} at distances of 3.27, 3.24, and 3.31 Å and are localized over the electron-rich interpentagon bonds. The C_{60} molecule is not rapidly "jumping" from one orientation to another as observed in the unsolvated C_{60} by neutron diffraction structure (29).

Uneven charge distribution. Although the charge distribution of C_{60} is necessarily uniform, the charge distribution of a mono-substituted C_{60} derivative such as **1** (Fig. 1) can show perturbations in its electron distribution (30). Moreover, it has been observed that C_{60} and other fullerenes interact with donor $-NH_2$ and $-SH$ groups (31, 32).

Combining site fit. We referred earlier to the interaction between progesterone and specific mAbs. The dimensions of C_{60} and progesterone are very similar. Progesterone is longer and somewhat narrower (5.8 Å by 13 Å) (26); C_{60} is a uniform sphere, 7.2 Å in diameter. The overall surface area, however, is very similar. There is no question about the "fit" of a fullerene in the combining site of an antibody.

Fig. 7 (*Upper*) shows the x-ray structure of an Fab′ fragment of a monoclonal progesterone-specific antibody bound to 5α-pregnane-20-one-3β-ol-hemisuccinate (26). The protein (Brookhaven PDB code 2DBL) is displayed as ribbons and the steroid as a space-filling model by using INSIGHT II (Molecular Simulations, San Diego, CA). The binding site is a large hydrophobic cavity lined with Trp, Phe, and Tyr groups. Using INSIGHT II, we replaced the steroid with C_{60} to provide the model shown in Fig. 7 (*Lower*). As we did not relax the geometry using molecular dynamics, the fit is tight, but a slight side-chain adjustment would provide a very good fit.

Solvent displacement. The free energy released on removing a hydrophobic surface from contact with water has been shown to correlate well with binding constants (33). Molecular modeling studies of **4** (Fig. 1), a competitive inhibitor of an HIV protease, removed 298 Å^2 of solvent exposure (33). This stabilization reaction was mainly caused by carbon-to-carbon contacts with hydrophobic residues at the enzyme's active site: Leu, Ileu, Tyr, Trp, Pro, Gly, and Ala.

We intend to answer the question of the binding of C_{60} and C_{70} fullerenes to Fab′ or Fv fragments of monoclonal anti-fullerene antibodies by x-ray crystallographic studies.

Finally, as part of this investigation we have prepared three fullerene peptide derivatives that are highly water soluble and have presented the UV-Vis spectra of two of them.

B.F.E. acknowledges the support of the National Institutes of Health (HL47377-03) and the American Cancer Society (RPG-93-006-06 CDD).

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