MASTER

POLYCYCLIC AROMATIC HYDROCARBON -PROTEIN INTERACTIONS

Progress Report
for Period September 1, 1977 - February 28, 1979

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October 1978

Prepared For

THE U. S. DEPARTMENT OF ENERGY
UNDER CONTRACT No. EE-77-S-02-4374.A000

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Abstract. In the interactions of bovine serum albumin (BSA) and benzo(a)pyrene (BaP), various BaP derivatives are formed. The BSA-BaP complex exhibits three types of fluorescences: visible fluorescence, modified by the formation of hydroxy-BaP derivatives, and two ultraviolet fluorescences (one probably from pyrene-like products and the other from unidentified products). When irradiated with UV(365nm), BaP products are photo-bound. The benzene extract, which removes non-covalently bound BaP and its products, shows a structural ESR signal in addition to 6-oxo-BaP radical. The lyophilized complex exhibits two broad ESR bands, one of which increases in lipid-free BSA. The effect of UV and mercaptoethanol suggests that one of the UV fluorescences originates from the radical species. The mixture of BaP and cysteine kept at room temperature produces both UV fluorescence and structural ESR spectrum, indicating the involvement of SH-groups in the interaction with BaP. When both are mixed at 65°C, no UV fluorescence and ESR signal are detected. Collagen binds BaP and bound BaP undergoes rapid photoreaction with oxygen and collagen. UV-irradiation of the complex causes photo-modification in collagen conformation, reducing the ability of forming native-type fibrils and lowering the denaturation temperature.

This research concerns carcinogenic polycyclic hydrocarbon-protein interactions that lead to the modification of both carcinogens and proteins. The interactions between benzo(a)-pyrene (BaP) and the proteins, bovine serum albumin (BSA) and collagen, the oxygenation of BaP in the presence of BSA, and the reactions of photo-activated BaP with BSA and collagen have been investigated. The interaction of BaP with cysteine has also been studied.

I. BSA - BaP Interactions

a) Fluorescence studies¹ When the mixture of aqueous solution containing 10% BSA and a small amount of BaP crystal was stirred in the dark for several days at 4°C and centrifuged to remove the remaining solid BaP, the resulting BSA-BaP complex exhibited the following three types of fluorescences:

1) Visible fluorescence: The fluorescence excited at 389nm showed three peaks at 406, 430 and 458nm with (a) higher intensities at 430 and 458nm as compared to those of BaP in benzene and (b) an increased shoulder around 415nm next to the highest peak at 406nm. The former change (a) can be accounted for by the formation of hydroxy-BaP derivatives, most of which fluoresce at about 430 and 460nm. This conclusion is supported by red-shifted excitation maxima at 396, 381 and 363nm for the fluorescence at 430nm of this complex (The excitation maxima of BaP in benzene are at 389, 369 and 351nm), and by a large red shift to about 520nm of the fluorescence maximum when excited at 420nm in alkaline solution (1N NaOH). The change (b) is due to BaP products strongly bound (probably covalently) to the protein. The fluorescence peak, red-shifted to about 416nm, was revealed after thorough benzene extraction of non-covalently bound BaP and its derivatives.

2) Ultraviolet fluorescence I: The excitation at 305nm of the BSA-BaP complex gave rise to a well-defined, vibrationally-structured UV fluorescence at 340, 357 and 378nm. The excitation spectrum for the fluorescence at 340nm showed a major maximum at 307nm with an additional band at 296nm which gradually developed. Upon addition of mercaptoethanol, this UV fluorescence decreased, while the excitation maximum shifted from 307nm to 296nm. This UV fluorescence was stable for days, provided that the complex was kept in the dark at 4°C.

3) **Ultraviolet fluorescence II:** The BSA-BaP complex also showed a weak, sharp fluorescence at 380nm, when excited at 330nm. The corresponding excitation spectrum exhibited maxima at 346 and 331nm. These spectra correspond to those of pyrene-like products, other oxidation products of BaP, and are in good agreement with the reported spectra of BaP-7,8-diol-9,10-epoxide (ultimate carcinogen of BaP), 7,8,9,10-tetrahydro-BaP or 7,8-dihydroxy-7,8,9,10-tetrahydro-BaP.

When a small amount of BaP crystal was added to aqueous solution containing 10% BSA and the fluorescence changes were followed as a function of time, the fluorescence at about 350nm of tryptophan residues, excited at 305nm, slowly decreased. As BaP incorporates into the hydrophobic region of the protein, the triple-peaked UV fluorescence I appeared, while the visible fluorescence of BaP gradually grew. The benzene extraction from the BSA-BaP complex removed most of the UV fluorescent BaP products, apparently regenerating the UV fluorescence of tryptophan residues.

The BSA-BaP complex undergoes UV(365nm)-induced decrease of both the UV fluorescence I and the visible fluorescence. The fluorescence excited at 305nm of the UV-irradiated complex showed two unique changes: (a) a definite peak at 411nm and a relatively high peak at 430nm in the visible fluorescence and (b) the higher intensities of the UV fluorescence peaks at 358 and 378nm than that at 340nm. After benzene extraction, red-shifted, intense fluorescence peaks of photo-bound BaP products appeared at 415 and 437nm [responsible for the change (a)], whereas the UV fluorescence mainly consisted of a single maximum at 371nm, probably due to photo-oxidized products of tryptophan residues. This tryptophan-related 350 to 370nm shift, as well as the possible increase of pyrene-type products (UV fluorescence II), could be responsible for the fluorescence change (b).

The interesting aspects of this study are that the protein BSA can induce the oxygenation (hydroxylation) of BaP and that some of these products can covalently bind to the protein, particularly when BaP is photo-activated.

b) **Electron spin resonance (ESR) studies**² In the interaction of BaP with serum albumins (bovine and human, native and lipid-free), a new kind of free radical has been found in addition to the known 6-oxo-BaP radical. The lyophilized BSA-BaP complex exhibited at least two broad ESR bands on either side of the 6-oxo-BaP radical ($g = 2.004$, band width: ~ 12 gauss). When the lyophilized sample was dissolved in minimal volume of distilled water, the ESR signal was converted to a six-line spectrum plus the single absorption due to the 6-oxo-BaP radical. Upon re-lyophilization of this aqueous solution of the BSA-BaP complex, one of the broad ESR bands, at lower g -value ($g < 2.00$), disappeared. On the other hand, when lipid-free BSA was used, the intensity of the lower g -valued band was much higher than that in the native BSA-BaP complex. (Similar results were obtained with native and lipid-free human serum albumin). Re-lyophilization of the lipid-free BSA-BaP with added water did not reduce that signal. However, upon addition of distilled water with a small amount of mercaptoethanol, followed by re-lyophilization, the whole broad signal was replaced by a narrow signal at g -value slightly higher than 2.00. Furthermore, a greater enhancement of the lower g -valued band occurred in the lyophilized sample of the stirred mixture of BSA in 0.01N NaOH and BaP. The ESR signal of 6-oxo-BaP considerably decreased in this alkaline sample. From these results, it appears that removal of lipids enhances the low g -valued signal, while it reduces the formation of 6-oxo-BaP. It should be noted that when BSA was treated with the detergent sodium dodecyl sulfate (0.1%) and subsequently with BaP, the lyophilized mixture showed no detectable ESR signal.

The new radical was stable in its complex with BSA, and although it was bound strongly to BSA, it could be transferred to a benzene matrix where it was very stable. The benzene extract of the lyophilized material from BSA (0.1%) - BaP aqueous solution exhibited a sharp ESR signal of 6-oxo-BaP radical superimposed on a weak, broad, structural background signal. This broad, structural ESR signal due to the new radical became pronounced in the condensed benzene extract from concentrated BSA (10%) - BaP aqueous solution and was similar to that of the BSA-BaP complex dissolved in the minimal volume of distilled water. When this benzene solution was degassed under vacuum, the well-resolved ESR spectrum was obtained under low modulation amplitude. The separation between each signal was fairly large with $\Delta g \approx 0.05$.

UV(365nm)-irradiation of the BSA-BaP complex in aqueous solution increased the concentration of 6-oxo-BaP radical as in BaP alone in benzene. While UV irradiation of the benzene extract from the BSA-BaP complex increased the signal intensity of 6-oxo-BaP, it reduced signal intensities of the new radical species. The signal intensities of this radical also diminished unevenly upon addition of mercaptoethanol. The intensities decreased rapidly in a few minutes and then gradually in hours. The decrease was fastest in the signal with the highest g-value and slowest in the two signals with the lowest g-values. The whole set of signals disappeared and was replaced by a weak signal, apparently similar to that of a lyophilized mixture of the BSA-BaP complex which was treated with mercaptoethanol. Thus, the effects of UV irradiation and mercaptoethanol upon the new radical appear similar to those on the UV fluorescence I. Although the nature of the new radical and the mechanism of its formation are not known, these findings suggest that the UV fluorescence I originates from the radical species. This implication, as well as the possible involvement of SH-groups in producing new BaP products, may be supported by the interaction of cysteine with BaP, described below.

II. Cysteine - BaP Interactions³

The lyophilized mixture from BaP and cysteine-HCl (0.1%) in aqueous solution, stirred at room temperature for one day in the dark, gave a widely separated six-line ESR spectrum centered around $g \approx 2.00$, in addition to the 6-oxo-BaP radical. This ESR spectrum was similar to that obtained from the concentrated aqueous solution of the BSA-BaP complex. When the concentration of cysteine was increased to 4%, the amount of the radical with the six-line spectrum also increased. The radical decayed slowly in days in the lyophilized samples. On the other hand, the same mixture, when stirred at 65°C for 5-8 hrs, gave no detectable ESR signal for the low concentration of cysteine (0.1%). A very weak signal consisting of the above six-line spectrum was obtained for the high concentration of cysteine (4%) kept at 65°C with BaP.

The aqueous solution of cysteine (4%) and BaP mixed at room temperature showed, in addition to the known visible fluorescence of BaP, a structureless broad fluorescence spectrum in the UV region. The maximum occurred at 330nm. The corresponding excitation spectrum of the UV fluorescence had a maximum at 280nm. This UV fluorescence attained its maximum intensity within 24 hr at room temperature and the intensity fell off to a low level in about one week. Neither the cysteine nor the BaP in aqueous media have any fluorescence in this UV region. At the low concentration of cysteine (0.1%) at room temperature or at the high concentration (4%) kept at 65°C, no UV fluorescence was observed. In all cases, however, a weak fluorescence with a maximum at 380nm was observed when excited at 340nm. This corresponds to the UV fluorescence II, and the fluorescence at 330nm may correspond to the UV fluorescence I, in the BSA-BaP complex.

These results show that the production of both the BaP radical and the UV fluorescence depends on the concentration of cysteine and the temperature which control the formation of the reaction intermediate between BaP and cysteine in the presence of air, suggesting the significance of SH-groups in the reaction with BaP. Our preliminary studies indicate the possible involvement of oxygen in this reaction.

III. Collagen - BaP Interactions⁴

The fluorescence intensity of BaP in buffer was very low. In the presence of collagen type I isolated from rat tail tendon, the fluorescence was enhanced and the intensity at 406nm was proportional to the concentration of BaP up to 3×10^{-7} M BaP for 0.2% collagen in 0.05M acetate buffer (pH 4.0). At higher concentrations of BaP, a plateau was observed and accompanied by the appearance of a fluorescence peak at 490nm which is due to a microcrystalline state of BaP. The fluorescence maxima of BaP bound to the collagen molecule, when excited at 387nm, were at 406, 430 and 456nm and essentially the same as those in benzene solution.

In the presence of air, UV(365nm)-induced decrease of the fluorescence in the collagen-BaP complex was very rapid compared to the decrease of BaP fluorescence in benzene. In degassed solution of the collagen-BaP complex, the decrease was slowed down except during the initial period of UV irradiation probably due to residual oxygen present in solution. Since the fluorescence of bound BaP was not quenched by oxygen, the participation of bound BaP in its triplet state and oxygen is suggested in the photochemical reaction of BaP in the complex. The fluorescence at 305nm of tyrosine residues present only in the non-helical terminal peptides, observed when excited at 275nm, was not affected by the photochemical reaction of BaP.

Although, judging from the intrinsic viscosity of native collagen, BaP-collagen, and UV-irradiated BaP-collagen, the dimensions of a majority of collagen molecules were not altered, a small degree of photo-fragmentation was observed in SDS-acrylamide gel-electrophoresis experiments. In the formation of native-type fibrils, both the lag period and the growth phase were delayed, and the amount of fibrils formed was lowered, by the presence of BaP. UV irradiation of the collagen-BaP complex further extended the lag period and lowered the rate in the growth process. The circular dichroism spectrum of native collagen has a positive band at 222nm which changes its sign upon thermal denaturation occurring at 38-39°C. In the circular dichroism changes as a function of temperature, the collagen-BaP complex showed a broadening in the temperature range of thermal denaturation. After UV-irradiation, further broadening lowered the denaturation temperature to 35-36°C. These results indicate that BaP bound to the collagen molecule affects the conformation of the protein and that UV(365nm)-irradiation of the collagen-BaP complex causes further conformational changes in collagen molecules.

- 1) Srinivasan, B. N. and Fujimori, E. "Serum albumin-Benzo(a)pyrene Interactions. 1. Fluorescence Studies", in preparation.
- 2) Srinivasan, B. N. and Fujimori, E. "Serum albumin-Benzo(a)pyrene Interactions. 2. Electron Spin Resonance Studies", in preparation.
- 3) Srinivasan, B. N. and Fujimori, E. "Cysteine-Benzo(a)pyrene Interactions", in preparation.
- 4) Fujimori, E. "Collagen-Benzo(a)pyrene Interactions", in preparation.