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PEROXIDASE-CATALYSED COVALENT BINDING OF THE ANTITUMOR DRUG 2N-METHYL-9-HYDROXY-ELLIPTICINE TO PROTEINS.

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2N-methyl-9-hydroxy-ellipticine (NMHE) (Fig.1) is a drug endowed with anticancer properties towards several experimental tumors (1) and actually used in the treatment of patients suffering from osteolytic breast cancer metastases (2). One hypothesis concerning the mechanism of action of NMHE takes into account its possible biotransformation to reactive and toxic metabolites. Such an activation has been found to be catalysed *in vitro* by peroxidases (3), enzymes widely distributed in human tissues. In the presence of hydrogen peroxide (H_2O_2) as electron acceptor, peroxidases catalyse the oxidation of NMHE to the quinone imine derivative 2N-methyl-9-oxo-ellipticine (NMOE) (Fig.1). NMOE is a strong electrophilic molecule which may undergo covalent binding with various N, O and S donors containing nucleophiles (4). These observations led us to consider the proteins as potential target for the drug if the occurence of covalent binding between the activated NMHE and proteins could be established. We have therefore investigated some characteristics of the peroxidase-catalysed binding of NMHE to bovine serum albumin (BSA) and human anti-B IgG *in vitro*.

MATERIALS AND METHODS

NMHE was a gift of SANOFI Co. (Sisteron, France). ^3H (2N-Me) labeled NMHE (0.5 mCi per mMole) was obtained from the CEA (France). BSA, horse radish peroxidase (HRP), glycine and aspartic acid were obtained from Sigma chemical Co. (St. Louis, USA). IgG anti-B was obtained from Ortho Diagnostic Inc. (USA). Pronase was obtained from Calbiochem (San Diego, USA). Glycine-NMHE (10Gly-2N-methyl-9-hydroxy-ellipticine) and aspartic acid-NMHE (10Asp-2N-methyl-9-hydroxy-ellipticine (Fig.1) were prepared as follows: 50 mg. NMHE and 250 mg. of the amino acid were dissolved together in distilled water. The solution was mixed (v/v) with 0.01 M

phosphate buffer (pH 7.40).0.02 mg/ml HRP and 40 mM H_2O_2 were then added to the mixture.After the end of the reaction,the solution was filtered and applied to a XAD-2 (Servachrom) column (2.5x30 cm.) previously equilibrated with 0.1 M ammonium acetate buffer (pH 7.40) containing 5% methanol.Various fractions were eluted from the column by a linear gradient of 0.1 M acetate buffer (pH 7.40) containing from 5% to 70% methanol.Amino acid-NMHE adducts were obtained in the last eluted fractions.The mixture containing the pure adduct was evaporated and the solid material was redissolved in distilled water and lyophilized.

Identification of the adducts: ^1H NMR (90 MHz) in D_2O : CD_3OD,50:50 (v/v).Gly-NMHE: 1.94 (s,3p,Ac);2.19 (s,3p,Me_5);2.82 (s,3p,Me_{11});4.21 (s,3p,N^+Me);7.00 (d, 1p,J=8.5Hz,H_7 or H_8);7.48 (d,1p,H_7 or H_8);7.56 (d,1p,J=7.2Hz,H_4);7.68 (d,1p,H_3); 8.34 (s,1p,C -H Gly) and 8.84.Mass spectrum,C.I. (NH_3) M cation: 348,observed peak at 302 ($-CO_2$,$-2H^+$).Asp-NMHE:Mass spectrum,C.I. (NH_3),M cation:406,observed peak at 316 ($-2CO_2$,$-2H^+$).

Binding of NMHE to BSA: Peroxidase-catalysed binding of ^3H NMHE (NMHE containing a tracer dose of ^3H NMHE) was performed as previously described (3) except that HRP was used instead of myeloperoxidase.

Gel electrophoresis of protein-NMHE adducts: Lyophilized protein-NMHE was either dissolved in 0.1 M phosphate buffer (pH 8.60) or in 1.2% SDS containing 6 M urea and 1.7% β-mercaptoethanol.In the latter case,the mixture was heated in a boiling water bath for 5 min.Samples were subjected to electrophoresis on polyacrylamide gel (10%) (with or without SDS) at pH 8.60.Protein-NMHE bands were detected by fluorography upon excitation at 366 nm.The protein-NMHE band was cut into strips, dried and assayed for radioactivity by combustion followed by liquid scintillation counting.

HPLC experiments: HPLC was performed on a Water apparatus using a C_{18} micro-bondapak column.Mobile phase:methanol/water 50/30 containing 0.1 g.% heptane sulfonate and 0.1% acetic acid.Flow rate was 1.2 ml/min.

Isolation of an amino acid-NMHE adduct (Asp-NMHE) from BSA-NMHE: 1 g. of BSA-NMHE was dissolved in 50 ml of Krebs phosphate buffer (pH 7.40) and incubated for 24 hr at 37°C with 50 mg. of pronase.After centrifugation,the supernatant was lyophilized and then extracted with 10 ml methanol.After filtration,the methanol extract was subjected to HPLC.A major radioactive and fluorescent peak was eluted at 25 min. corresponding to the retention time of the authentic Asp-NMHE adduct. This peak was collected and the material was subjected to mass spectrometry.As observed for the authentic Asp-NMHE,a peak was obtained at 316.

RESULTS AND DISCUSSION

HRP-H_2O_2 catalyses the two-electron oxidation of NMHE to the quinone imine NMOE (Fig.1).

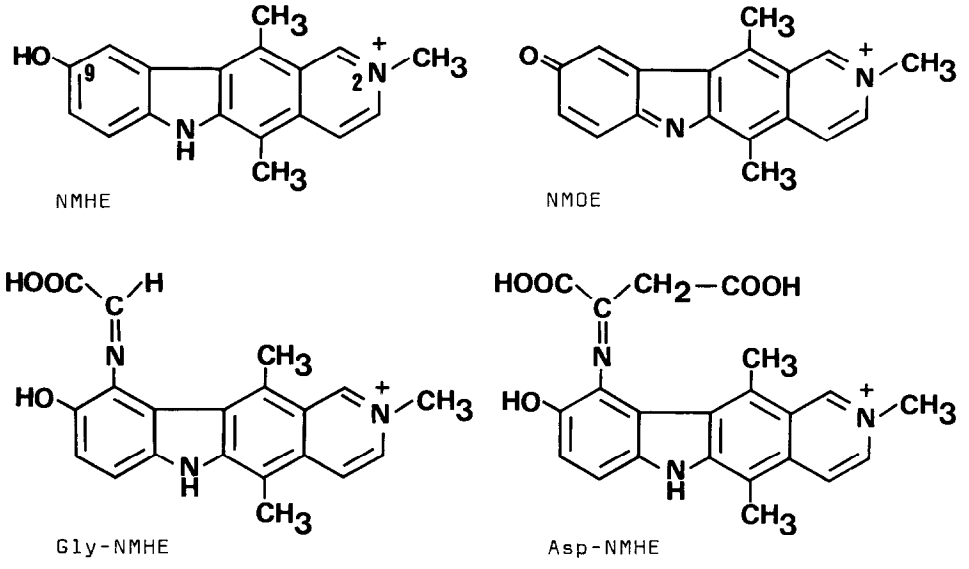

Figure 1. Structure of ellipticinium and amino acid ellipticinium derivatives.

The NMHE oxidation occurs with a catalytic constant of 1,120 and with an apparent Km for NMHE of 5.5 µM at pH 7.40 and at 37°C.In the presence of BSA, the HRP-catalysed oxidation of NMHE results in an irreversible binding of the drug to the protein.Fig. 2A shows the time-course of NMHE binding to BSA in the presence of various concentrations of HRP.The amount of NMHE incorporated increases linearly with time and enzyme concentration.When incorporated to BSA, NMHE exhibits a fluorescence (Ex:315,375,402,440.Em:525) in aqueous media.The fluorescence intensity is a direct function of the amount of NMHE bound (Fig.2B). The covalent nature of the NMHE binding was obvious first of all from polyacrylamide gel electrophoresis (PAGE) of SDS-denaturated BSA-NMHE (gel 1. in fig. 2C). BSA-NMHE can be detected by both radioactivity (see methods) and by fluorescence (Fig. 2C).Similar results were obtained with various proteins including human immunoglobulins.Gel 2 in fig. 2C shows the fluorogram of PAGE of the native anti-B IgG-NMHE.It must be pointed out that HRP-catalysed oxidation of NMHE can be used to prepare NMHE containing fluorescent immunotoxins.

In proteins,among amino acids concerned by covalent binding with electrophilic molecules are the basic amino acids lysine and arginine as well as the end amino acid having a free amino group.Models of such adducts involving N-C bond

between NMHE and the primary amine of amino acids can be obtained using free amino acids.We have therefore prepared various amino acid-NMHE adducts using the procedure described in methods.The structure of these adducts has been

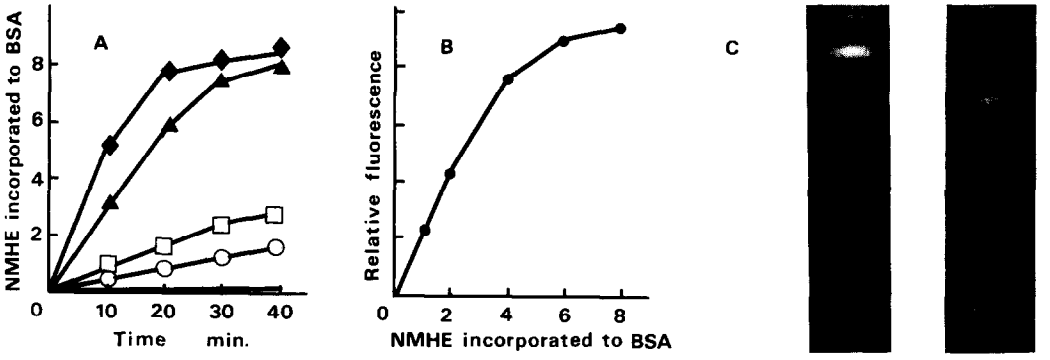

Figure 2. HRP-catalysed binding of NMHE to proteins. A) Experiments were performed in mixtures composed of 0.05M phosphate buffer pH 7.40, 10^{-4}M H_2O_2, $5x10^{-5}$M NMHE and $5x10^{-6}$M BSA. ◆—◆ 10^{-10}M HRP, ▲—▲ $5x10^{-11}$M, □—□ $2x10^{-11}$M, ○—○ 10^{-11}M.Temp. was 37°C.NMHE incorporated was expressed in terms of binding ratio drug/protein. B) The fluorescence (Ex:315 nm.,Em:525 nm.) intensity of BSA-NMHE was measured in 0.05M phosphate buffer pH 7.40 on aliquots of the solutions used for the radioactivity counting. C) SDS-PAGE of BSA-NMHE (gel 1) and PAGE of anti-B IgG-NMHE (gel 2).Both BSA-NMHE and IgG-NMHE were prepared in mixtures composed as described above,using 10^{-10}M HRP and 60 min. incubation time. Protein-NMHE bands were revealed by fluorescence upon excitation at 366 nm.

determined by ^{1}H NMR and mass spectrometry.The structure of glycine-NMHE adduct is given as exemple in fig. 1.Fluorescence spectra of these adducts are similar to those obtained with BSA-NMHE.The adduct resulting from the covalent binding between the N-terminus amino acid of BSA aspartic acid and NMHE should exhibit the structure of asp-NMHE adduct indicated in fig.1 after it was released by proteolysis.Accordingly,the enzymatic hydrolysis of BSA-NMHE liberates a fluorescent adduct identified as asp-NMHE by HPLC and mass spectrometry (see methods).In conclusion,the results indicated in the present paper show that the HRP-catalysed oxidation of NMHE results in covalent binding of the drug to proteins in vitro.The binding reaction may involve the nucleophilic addition of primary amines to NMOE yielding fluorescent adducts.These will be of major interest for further studies on the covalent binding of NMHE in vivo.

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