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A RAPID, SENSITIVE MASS SPECTROMETRIC METHOD FOR INVESTIGATING MICROSCALE CHEMICAL REACTIONS OF SURFACE ADSORBED PEPTIDES AND PROTEINS

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SUMMARY: A new mass spectrometric method for measuring the products of reactions of surface adsorbed peptides and proteins is described. The technique is rapid, convenient, and sensitive and provides detailed information concerning the molecular weights of the reaction products and the rate and extent of reaction. The properties of the technique are illustrated by an investigation of cleavage reactions of the disulfide bonds in bovine insulin, cyclic somatostatin, and conotoxin G1 utilizing the reducing agent dithiothreitol.

The detailed measurement of chemical reactions of small quantities of biologically important molecules bound to surfaces can facilitate the elucidation of both their structure and their complex chemical reactions. We have recently demonstrated (1) a new mass spectrometric method for investigating microscale chemical reactions of gas phase reagents with the surface of organic solids. In the present Communication we report for the first time an extension of this technique to the investigation of the chemical reactions of small quantities of surface bound peptides and proteins with condensed phase reagents. The technique involves essentially nondestructive mass spectrometric analysis (2) of monolayer to submonolayer amounts of noncovalently surface bound molecules (3-5) prior to and after chemical modification. The corresponding differences in the mass spectra yield information concerning the nature and the rate of the reaction under investigation.

We report here the results of an investigation of cleavage reactions of the disulfide bonds in bovine insulin, cyclic somatostatin and conotoxin G1 (Fig. 1) utilizing the reducing reagent 1,4-dithiothreitol (6). We

Abbreviations: NC: nitrocellulose; DTT: 1,4-dithiothreitol, m/z: mass to charge ratio.

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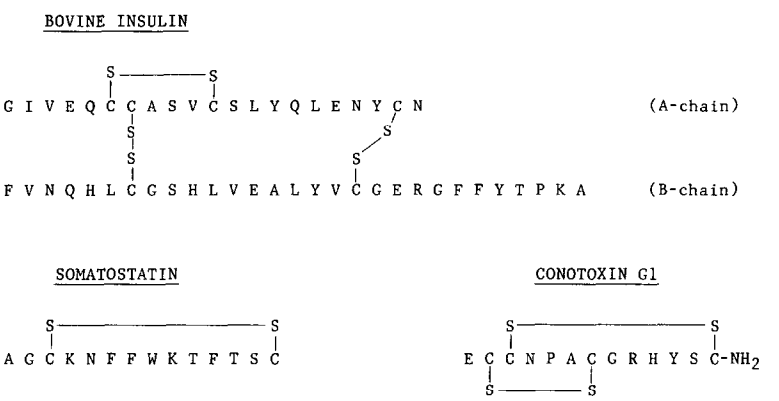

Fig. 1 The amino acid sequences and the positions of the S-S bonds in compounds used in the present study.

present this initial study concerning disulfide bond reactions as an example of what we think is a method of wide generality.

MATERIALS: Bovine insulin, synthetic cyclic somatostatin, conotoxin G1, DTT, and ammonium bicarbonate were all obtained from Sigma Chemical Co. (St. Louis, MO.) and used without further purification. Water and acetone were high purity, distilled in glass grade obtained from Burdick and Jackson Lab. Inc. (Muskegon, MI). Acetic acid was reagent grade purchased from Fisher Scientific. Nitrocellulose (NC) was obtained in the form of membranes (BA84-0.45μm pore size) from Schleicher and Schuell, Inc. (Keene, NH).

METHODS: General procedure: The general procedure for carrying out the new technique involves the following steps:

(a) The sample insertion probe of our ^{252}Cf fission fragment mass spectrometer (7,8) is coated with a thin film of NC. The NC is in turn coated with a solution containing a small quantity (10^{-9}-10^{-12} mol) of peptide or protein. The solvent is evaporated leaving a bare layer of the compound of interest bound to the NC. This sample preparation procedure follows closely that described by Sundqvist, Roepstorff, and co-workers (4), but with some differences which are described in detail below.

(b) The NC-sample covered sample insertion probe is inserted into the mass spectrometer and a spectrum of the compound is obtained, essentially nondestructively (1,9), as described previously (7,8).

(c) The probe is removed from the mass spectrometer and a small quantity of reagent is applied to the surface to effect the desired reaction with the sample under controlled conditions.

(d) The reagent is removed, and the probe with the chemically modified material is reinserted into the mass spectrometer and a new spectrum is obtained.

(e) Finally the spectra of the unmodified and the modified compounds are compared.

Sample preparation: NC membranes were autoclaved at 120°C (10) for 30 min and then dissolved in acetone to produce a solution containing 1.0 mg/μl. This solution was electrosprayed (11) onto a 2 μm thick aluminized polyester foil (diameter = 1.2 cm) to form a film with a weight of ~50μg. The peptide or protein sample was dissolved in 10% acetic acid in water to give concentrations ranging from 10^{-9} to 10^{-12} mol/μl, and 1 μl of this solution was deposited in a drop on the center of the NC film. The sample solution was spread over the NC coated foil by placing a microscope coverslip over the drop, which spread the liquid. The solution was

maintained between the coverslip and the NC layer for 5.0 min to allow adsorption of the peptide or protein. The solvent was then evaporated by insertion of the sample foil into the vacuum lock of the mass spectrometer leaving a bare layer of molecules bound to the surface of the NC.

Reduction reaction: The reaction solution contained 0.06M ammonium bicarbonate buffer and 0.08M DTT (PH 8.6). N_2 was passed through the solution for 30 min just prior to use. 1 µl of this solution was dropped onto the peptide or protein covered NC surface, spread with a coverslip, and allowed to react for 5.0 min at 24°C. With the fixed amount of DTT, the molar excess of DTT ranged between 10^2 and 10^4 depending on the surface concentration of peptide or protein. After reaction, the coverslip was removed and the reacted surface was promptly inserted into the mass spectrometer where the volatile reagents evaporated and the spectrum obtained.

Mass spectrometry: The ^{252}Cf time-of-flight mass spectrometer has been described in detail previously (7,8). In the present configuration the ion flux through the sample foil is 3400 fission fragments/sec. Sample ions are accelerated by a 9.95kV potential and are postaccelerated just prior to detection by a further 8.75kV. Spectra were accumulated for periods ranging between 3 and 30 min.

RESULTS AND DISCUSSION: The mass spectrometric response (number of protonated molecules detected/unit time) of the reactant materials was measured as a function of the amount of material applied to the NC layer (Fig.2). The response increases linearly between 10^{-12} and 5 x 10^{-11} mol with yield saturation occurring between 10^{-10} and 10^{-9} mol. Since we observe the microtopography of the NC surface to be granular, the actual surface area available for binding peptide or protein is in considerable excess of the geometric surface area of 1.1 cm^2. We calculate (based on an estimate of the available surface area and the area occupied by an insulin molecule (14)) that yield saturation occurs when approximately a single monolayer of material on the NC has been deposited.

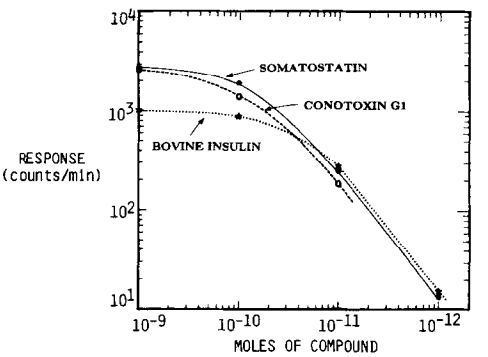

Fig. 2 The mass spectrometric response (number of protonated molecules detected/min) versus the amount of material applied to the NC layer for bovine insulin, somatostatin, and conotoxin G1.

DTT reductive cleavage reactions:

Bovine insulin: The high mass portion of the ^{252}Cf ionization mass spectrum obtained from 5 x 10^{-10} mol of bovine insulin deposited on NC, prior to chemical treatment, is shown in Fig.3(A). The two dominant peaks correspond respectively to the singly and doubly protonated molecular ions of insulin (12). Fig 3(B) shows the mass spectrum obtained from the same sample foil after DTT treatment for 5.0 min. The disappearance of the protonated molecular ion peaks and the appearance of a series of lower mass product peaks indicates that the reduction reaction has progressed almost to completion. The measured m/z ratios (13) of the peaks labeled $(B+H)^+$ and $(B+2H)^{2+}$ in Fig.3 (m/z 3401.2±0.5 and 1701.5±0.5) agree with the values calculated (13) for the protonated and doubly protonated reduced B-chain

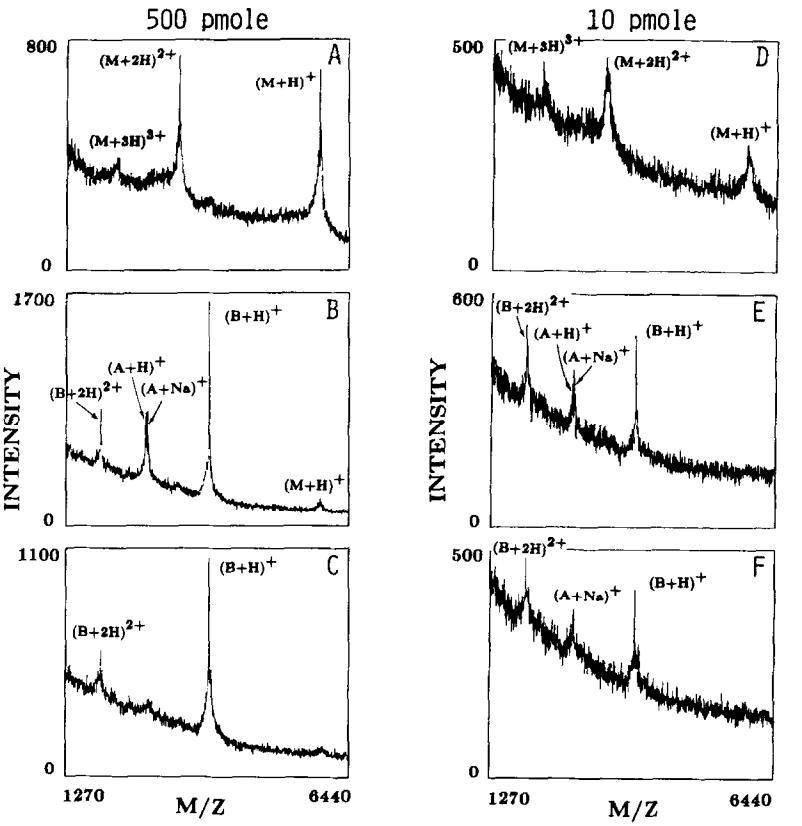

Fig. 3 Reduction of disulfide bonds in surface adsorbed bovine insulin. ^{252}Cf time-of-flight mass spectra of: (A) 500 pmol sample prior to reaction; (B) after 5.0 min DTT reaction; (C) after H_2O rinse of reacted sample; (D) 10 pmol sample prior to reaction; (E) after 5.0 min DTT reaction; (F) after H_2O rinse of reacted sample. Spectra accumulation times: (A-C)=15 min, (D-F)=30 min.

of insulin (m/z 3400.9 and 1701.0). The measured m/z value (2339.3±0.5) for the protonated A-chain product [$(A+H)^+$ in Fig.3(B)] is, however, slightly lower than the value calculated (m/z = 2340.7) on the assumption that all S-S bonds are fully reduced. Close inspection of the protonated A-chain product peak (not visible in the compressed form shown) reveals that it is composed of two incompletely resolved components separated by ~ 2 Da indicating incomplete reduction of the intrachain A6-A11 disulfide. This finding is consistent with previous observations (14) that the intrachain disulfide is buried and is relatively unreactive in the native molecule. Fig.3(C) shows the spectrum from the same sample foil after the surface was thoroughly washed with water. The peaks corresponding to the A-chain ions have disappeared indicating that the A-chain products are not strongly bound to the NC and are removed by the washing. Similar experiments performed with smaller amounts of insulin showed that the reduction reaction can be successfully effected as the surface concentration is lowered (Fig.3(D-F)).

All of these results demonstrate that the reductive cleavage reaction occurs rapidly on surface bound insulin and that the signal to noise ratio is still adequately high at the 10^{-11} mol level to allow ready identification of the reaction products.

Somatostatin:

The DTT reduction reaction was carried out on somatostatin (Fig.1). Space limitations prevent the inclusion here of the mass spectra for this system, but they show that the cyclic reactant was converted into a linear peptide with molecular weight 2 Da higher than that of the reactant. The reaction proceeded to completion rapidly ($\lesssim$5 min) and provided clear information regarding the progress of the reduction reaction even when the amount of reactant was as small as 10^{-11} mol. The relative intensity of the protonated reaction product was only about 60% that of the protonated reactant. This intensity decrease is probably the result of an increased

mass spectrometric fragmentation of the linear protonated product in comparison with that of the cyclic protonated reactant.

Conotoxin G1: Complete reduction of Conotoxin G1(15) (Fig.1), which contains two disulfide linkages, should yield a linear product with molecular weight 4 Da higher than that of the starting material. After 5 min of reaction with DTT the mass spectra demonstrated that the starting material disappeared and the reduced product was indeed formed, although the observed intensity of the protonated product was only 10% that of the starting material.

From our initial study we can summarize the general properties of the new mass spectrometric reaction probe as follows: Sensitivity is high (10^{-11} moles of peptide or protein required) and further increase in sensitivity by reducing the area of the sample probe (presently 1.1 cm^2) is feasible. Low yields of product (<10%) can be detected reliably. The mass spectrometric analyses are essentially nondestructive (9). The analyses are rapid and convenient since the chemical operations are easily performed and since the mass spectra described here required a measurement time of only 3-30 min. Sequential reactions can be conveniently carried out and monitored, e.g., we have methylated the sulfhydryl groups of reduced somatostatin utilizing p-nitrobenzenesulfonic acid methyl ester.

We do not here make comparison of the present technique with existing methods of investigating disulfide bonds (16). We conclude, however, that the unique attributes and detailed information available with the present technique make it at least an interesting complementary addition to existing techniques. Beyond that, we believe that it will be a valuable general tool for studying biomolecules bound to surfaces.

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