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Perspectives in Biochemistry

Analysis of Biochemical Reactions with Molecular Specificity Using Fast Atom Bombardment Mass Spectrometry†

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Significant advances in the field of mass spectrometry over the past 7 years have afforded techniques that permit aqueous reaction systems to be directly sampled and analyzed. Intact ionic and polar molecules can be analyzed in complex mixtures of compounds such as those found in enzymic reactions and cell extracts without the need for time-consuming and labor-intensive purification, concentration, and derivatization steps. In addition, direct analysis of aqueous solutions can provide more accurate information since the molecular dynamics which exist in solution are not destroyed through extensive sample preparation steps.

Perhaps the most significant advances in mass spectrometry in regard to biochemical applications have taken place in the area of ionization techniques with the introduction of a number of desorption ionization methods, including fast atom bombardment mass spectrometry (FABMS), secondary ion mass spectrometry (SIMS), and Cf-252 plasma desorption mass spectrometry (PDMS), among others. This review will deal with the application of FABMS to biochemical analyses because it is a technique that has become widely used for a variety of biological applications. Although there are a number of reasons for its wide acceptance, a primary asset is its compatibility with liquid samples. A brief discussion of some basic aspects of FABMS is given below to provide some background for those who may not be familiar with the methodology. A number of excellent reviews and monographs have appeared over the past few years which provide detailed considerations of these desorption ionization techniques (DePauw, 1986; Lyon, 1985; Sundqvist & Macfarlane, 1985).

The applications of FABMS in biochemistry represent an enormous diversity in regard to the different types of molecules being analyzed as well as the structural information sought. Investigators have made extensive use of the technique in the analyses of proteins and peptides, glycoconjugates, oligosaccharides, oligonucleotides, porphyrins, drug metabolites, phospholipids, and steroids, as well as many other types of compounds. A survey of this field is beyond the scope of this review, and the reader is referred to other works for additional references and details (Gaskell, 1986; Burlingame & Castagnoli, 1985; McNeal, 1986).

The main focus of this review will be on a newly developing area in which FABMS is used in more dynamic applications for the time-dependent investigation of biochemical reactions at the molecular level. In these applications, mass spectrometry is used as the primary tool for following the rates of changes of specific molecules in relatively complex mixtures of substrates and enzymes. In some cases, these reactions are continuously analyzed using on-line monitoring techniques.

Fast Atom Bombardment Ionization

Although initial experiments involving the bombardment of samples with neutral atom beams were performed many years ago, a significant contribution was made in 1981 by two groups who refined the technique and for the first time made use of liquid samples (Barber et al., 1981; Surman & Vickerman, 1981). The term "fast atom bombardment" was coined by these investigators, and although the term is nonspecific in the context of current knowledge, its use has prevailed in the literature.

The procedure for obtaining a FAB mass spectrum is relatively simple. A sample solution of approximately 2–3 μL is placed on the tip of an insertion probe where, on entering the source of the mass spectrometer, it is bombarded with atoms having energies typically between 6 and 8 keV. Commonly, the bulk of the liquid is a viscous compound such as glycerol. The bombardment results in the sputtering of molecules in the surface layers of the liquid droplet, giving rise to both positively and negatively charged ion species as well as neutral molecules in the gas phase. Electrical fields in the

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Table I: FABMS Analysis of Glycan Processing Reactions[a]

reaction step	enzyme	% of total glycans in reaction mixture[b]							
		RM_6	RM_5	RM_4	RM_5Gn	RM_4Gn	RM_3Gn	RM_3Gn_2	RM_3Gn_3
$RM_6 \rightarrow RM_5$	α-mannosidase I	2	78	20					
$RM_5 \xrightarrow[SW]{UDP\text{-}Gn} RM_5Gn$	*N*-acetylglucosaminyltransferase I		5	3	76	5	5	3	3
$RM_5Gn \rightarrow RM_3Gn$	α-mannosidase II				1	1	98		
$RM_3Gn \xrightarrow{UDP\text{-}Gn} RM_3Gn_2$	*N*-acetylglucosaminyltransferase II						10	84	6

[a] Data from Shao et al. (1987). [b] Abbreviations: R = 6-(biotinamido)hexanoyl-Asn-Gn_2; M = mannose; Gn = *N*-acetylglucosamine; SW = swainsonine (mannosidase II inhibitor). The starting substrate (underlined) was 90–100% pure at zero time.

source of the mass spectrometer cause the ions to be transmitted into the analyzer region where their mass-to-charge ratios are measured. The ionization process itself appears to be complex and involves sputtering of preformed ions, gas-phase ion–molecule reactions, and perhaps other processes as well. Whatever the detailed mechanism, the bombardment results in the formation of positively charged species which are cation adducts, i.e., $(M + H)^+$, $(M + Na)^+$, $(M + K)^+$, etc., and also negatively charged species, mainly $(M - H)^-$. The mass spectra are characterized by these abundant molecular species with significantly less abundant fragment ions. Depending upon the sample and ionizing conditions, fragment ions may be of sufficient intensity to provide structural information.

A distinguishing feature of FAB in contrast to most other mass spectrometric ionization techniques is its compatibility with liquid samples. This gives rise to two major advantages: first, it makes use of a medium where polar and ionic compounds are soluble, thus allowing direct sampling of biological reactions, and second, it provides a sample surface which is fluid and is constantly refreshed with new molecular material. The liquid sample usually contains a high percentage (75–95%) of a viscous material such as glycerol, thioglycerol, triethanolamine, fomblin, or a similar compound. One of the primary purposes of this material is to maintain the liquid nature of the sample in the high-vacuum environment of the ion source of the mass spectrometer during the analysis. In addition, the solvent is believed to participate in the ionization process itself, although the precise mechanism is not well understood. Thus, under optimal conditions, ion signals can be recorded for up to about 15 min before the sample stage goes dry. A recent development, which will be described in some detail later, has employed a continuous-flow sample probe that allows the sample stage to be kept wet almost indefinitely (Caprioli et al., 1986).

One of the primary uses of FABMS in the analysis of biological samples has been for the measurement of the molecular weight of a compound. Modern magnetic instruments are able to scan beyond m/z 20 000 and quadrupole instruments up to about m/z 4000. However, a major limitation thus far has been the relatively low ion yields obtained from FAB and other desorption ionization techniques especially at higher masses. Thus, with present FAB sources, sufficient numbers of ions are produced for routine measurements up to about m/z 6000 and occasionally up to about m/z 12 000 (at low resolution). Although the sensitivity of these instruments can be in the femtomole range for some compounds under optimal conditions, practically, the sensitivity can be much less depending upon the molecular weight and chemical nature of the compound, the liquid support used, and instrumental parameters such as the scan range and the mass resolution required. For example, for peptides in the 2000-dalton molecular mass range, one can usually record signal-to-noise levels of better than 10:1 on the $(M + H)^+$ ion at unit mass resolution over a scan range of 1000 mass units or more using 5–10 pmol of the peptide dissolved in 2 μL of a 9:1 mixture of glycerol or thioglycerol and water. On the other hand, for underivatized oligosaccharides analyzed under these same conditions, the amount of compound needed could be as much as 1 nmol or more.

Although the FAB mass spectrum of a compound may appear simple, the interpretation of the data is not always straightforward. A number of interferences and artifacts can effect the formation and/or detection of ions from the liquid sample and markedly complicate the spectra, including high chemical background, interaction with salts, suppression of ion currents by other compounds, and ion cluster formation. However, some of these effects have been significantly reduced by the continuous-flow FAB (CF-FAB) technique, which makes use of liquid samples having 80–95% water and only 5–20% glycerol. The high water content of these samples is in contrast to that of the original or standard method (std-FAB) where very high glycerol concentrations are used. Both of these techniques will be illustrated in the applications which follow.

Applications Using Standard FAB Techniques

Oligosaccharide Processing. The use of FABMS for the indentification and quantitation of the products of enzymic reactions involving glycan processing in glycoprotein synthesis has been recently reported (Shao et al., 1987). The effect of the protein matrix on glycan processing by Golgi enzymes was studied by comparing the distributions of products formed by the processing of a free glycan and the same glycan linked to a protein. The glycan substrates had the general structure $R\text{–}Man_x$ [where R = 6-(biotinamido)hexanoyl-Asn-$GlcNAc_2$–]. By the use of different glycans as substrates and the presence or absence of substrate donors and enzyme inhibitors, it was possible to study individual steps of the processing of the glycan substrates. Sample preparation for mass spectrometry involved desalting the complex reaction mixture on a Bio-Gel P-6 column. Table I shows a portion of the data for the first four steps in the processing of the oligomannose glycan $R\text{–}Man_6$ under specific reaction conditions. For each reaction, the distribution of products formed after 8 h of incubation was measured. For example, Figure 1 shows the mass spectra obtained at $t = 0$ and 8 h for the reaction catalyzed by *N*-acetylglucosaminyltransferase I. The data obtained with FABMS is unique in providing the molecular specificity and sensitivity required to identify and quantitate the array of products formed in these reactions.

Many applications that make use of FABMS require the quantitation of compounds in mixtures or the comparison of the relative amounts of each of several compounds in a sample. Although this can be done with a high degree of precision and accuracy, there are a number of considerations and factors

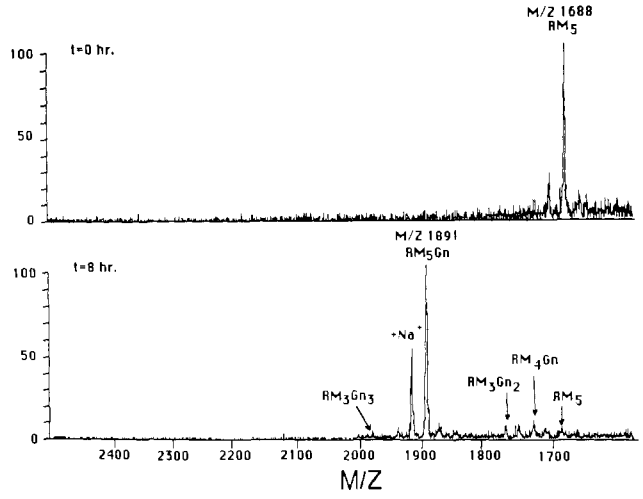


FIGURE 1: FAB mass spectra of the substrate RM_5 (top) and the products (bottom) for the reaction catalyzed by *N*-acetylglucosaminyltransferase I in the presence of UDP-Gn (Shao et al., 1987). Abbreviations and further details are given in Table I and the text.

that can affect such measurements. These include the chemical nature of the compounds of interest and their relative distribution in the surface layers of the sample droplet compared to their bulk concentrations, the presence of salts, the relative tendency of compounds to form ions, the stability of ions toward fragmentation, and the tendency of ions or molecules to form clusters, among others. For the most part, these factors can be collectively evaluated by the analysis of control samples having known concentrations. Such calibrations are of utmost importance in obtaining reliable quantitative measurements. Generally, it has been found that when compounds have the same chemical character, i.e., approximately the same mass, ionic and polar nature, hydrophobicity/hydrophilicity, etc., then quantitative measurements from mass intensities in a mixture are reliable. This is illustrated with the measurements of oligosaccharide mixtures described above where solutions of chemically similar oligosaccharides were determined to an accuracy of less than 5% (average deviation from the mean) by FABMS. This is also consistent with other work involving quantitative measurements of oligosaccharides (Townsend et al., 1984) where it was shown that, for a family of oligosaccharides differing only in the number of neutral sugars, quantitative comparisons of signal intensities were valid. However, when sialic acid residues were incorporated, positive ion intensities decreased significantly, although in this case correction constants could be derived for the effect of this sugar.

Reaction Monitoring. Mass spectrometry has been used in a time-course analysis to study the reaction catalyzed by enolase in which phosphoenolpyruvate (PEP) is reversibly hydrated to form 2-phosphoglycerate (2PG) (Caprioli, 1985). Starting with PEP and enolase, the reaction was allowed to proceed with samples removed at intervals, mixed with two parts of glycerol, and analyzed by FABMS. The molecular specificity of the analysis permitted the trianionic forms of the products, measured as $[M^{-3}Na^{+}_3 + H^+]$, to be measured directly. These data are shown in Figure 2. After about 60 min, the equilibrium ratio of the two compounds was measured and the concentrations of the products were obtained from a standard curve. The apparent equilibrium constant for the reaction calculated from this ratio was 5.1 for the dehydration reaction under the specific conditions of pH, metal ion concentration, and temperature used. This agrees well with the value of 5.4 reported in the literature for the same conditions by using chemical and spectroscopic techniques (Wold & Ballou, 1975). In the latter case, ionization constants of the products as well as the pH of the solution had to be accurately determined under the specific reaction conditions used in order to calculate the equilibrium constant.

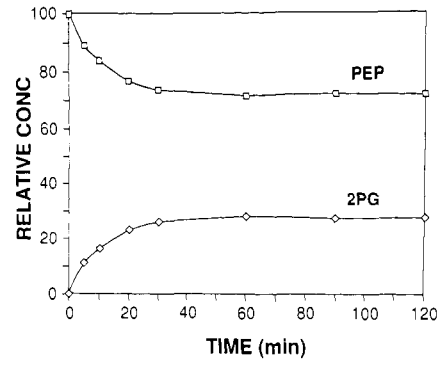


FIGURE 2: Time course for the hydration of phosphoenolpyruvate (PEP) catalyzed by enolase to give 2-phosphoglycerate (2PG). The data were obtained from direct analysis of the reaction solution using FABMS after being mixed 1:1 with glycerol. The $(M + H)^+$ ion of the trisodium salt was measured for both compounds.

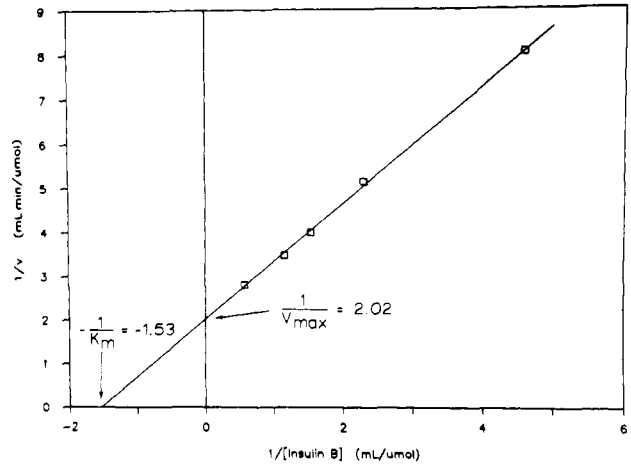


FIGURE 3: Double-reciprocal plot of rate data obtained for the hydrolysis of oxidized bovine insulin B chain by trypsin. Rate measurements were made by using FABMS to determine the rate of disappearance of the molecular species at m/z 3495 and the appearance of the peptide product at m/z 2583.

Kinetic Measurements. The effectiveness of FABMS for the determination of kinetic parameters for the tryptic cleavage of polypeptide substrates in the molecular mass range 500–3500 daltons was recently shown (Caprioli, 1987). Kinetic measurements were made by removing aliquots of the reaction mixture with time, adding 2 volumes of glycerol containing 0.3% trifluoroacetic acid (TFA), and measuring the decrease in the intensity of the molecular species at different substrate concentrations. Reaction rates were plotted as a double-reciprocal (Lineweaver–Burk) plot, and K_m and V_{max} were calculated from the appropriate intercepts. For the peptide M-R-F-A, for example, V_{max} was measured to be 0.31 μmol mL^{-1} min^{-1} and K_m was 1.90 mM. On calculating k_{cat}, the reaction constant for the formation of product from the enzyme–substrate complex, the ratio k_{cat}/K_m had a value of 3622 M^{-1} s^{-1}. These measurements were also obtained for the reaction for the tryptic hydrolysis of oxidized bovine insulin B chain in order to test the procedure using larger substrates. Although two clips are possible, cleavage of the peptide at Arg_{22} is considerably faster than that at Lys_{29}. Thus, for the Arg–Gly bond, K_m = 0.65 mM, V_{max} = 0.495 μmol mL^{-1} min^{-1}, and $k_{cat}/K_m = 5.75 \times 10^4$ M^{-1} s^{-1}. The double-reciprocal plot of these data is shown in Figure 3. The molecular

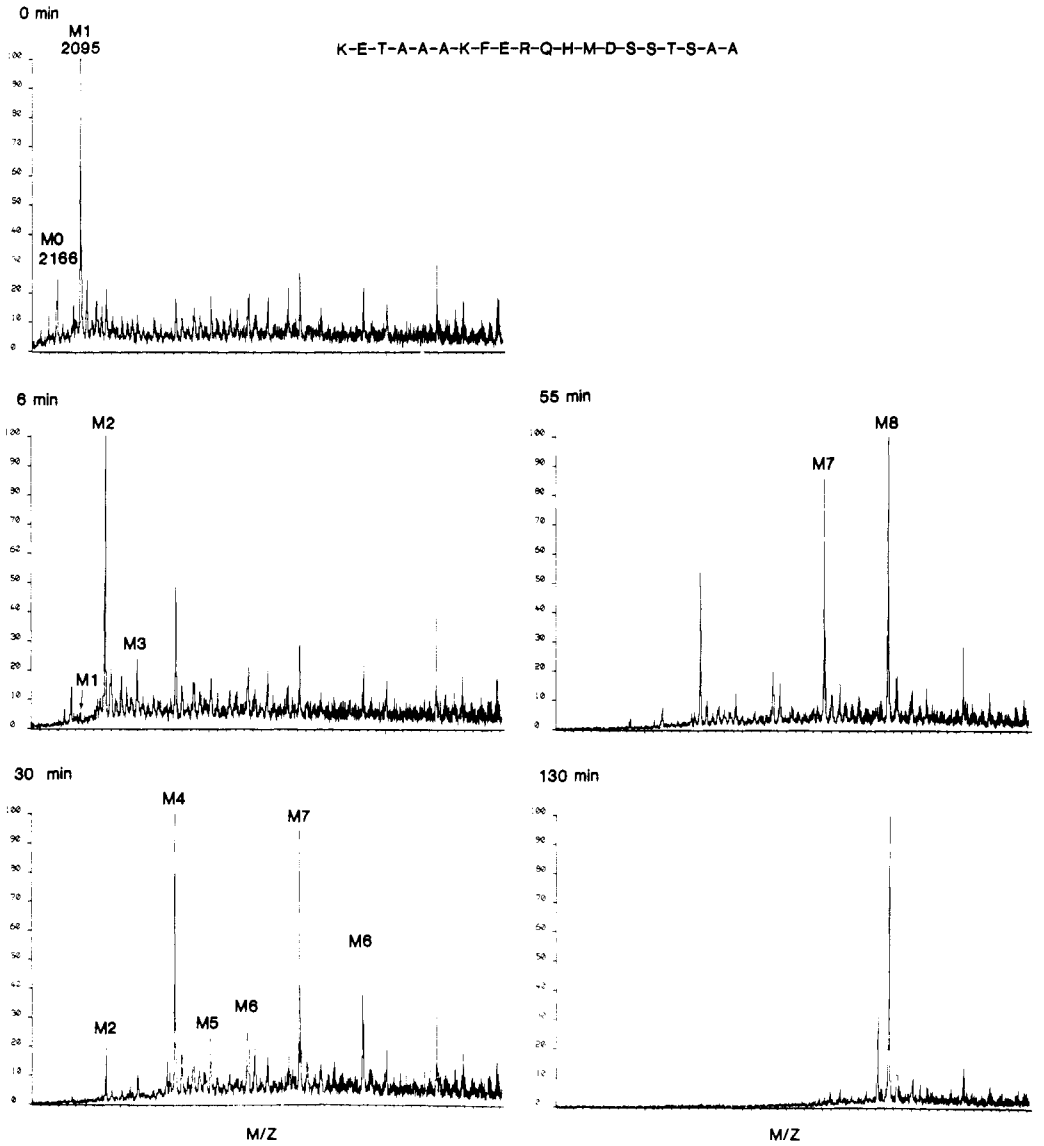


FIGURE 4: FAB mass spectra of aliquots taken from a reaction mixture of the hydrolysis of ribonuclease S-peptide with carboxypeptidase Y. M0 denotes the $(M + H)^+$ ion for the substrate at m/z 2166; M1 that for the peptide with one C-terminal residue removed at m/z 2095; M2 for two C-terminal residues removed at m/z 2024; M3, m/z 1937; M4, m/z 1836; M5, m/z 1749; M6, m/z 1662; M7, m/z 1547; and M8, m/z 1416 (Cprioli & Fan, 1986a).

specificity that mass spectrometry brings to these measurements is essential for the determination of such kinetic parameters especially on natural substrates of high mass.

Peptide Sequencing. One of the common applications of FABMS in biochemistry involves the structural analysis of peptides, including both primary sequence analysis and the identification of modified residues. Several investigators have demonstrated the utility of following the time-course digestion of a peptide substrate to obtain sequence information (Caprioli & Fan, 1986a; Self & Parente, 1982). We have described a general technique based on the use of FABMS which employs a mixture of several carboxypeptidases to give C-terminal sequence data by following the production of the series of truncated molecular species produced by the enzymic hydrolysis. The mass difference between two such molecular species identifies the residue lost in that step of the reaction. In this way, the extreme variation in the rates of hydrolysis seen with these enzymes does not cause difficulty. The primary sequence information becomes the $(M + H)^+$ value of the polypeptide and not the relative rate and identification of the terminal residue released. For this reason, repeating residues in a sequence are easily identified. Figure 4 shows several mass spectra of samples taken at various times from a reaction solution in which ribonuclease S-peptide was being digested with carboxypeptidase Y. A series of $(M + H)^+$ ions corresponding to the truncated polypeptide chains can be seen, and under the conditions used, eight C-terminal residues were released after about 30 min.

Although the emphasis of this review is on the use of FABMS in time-course analyses, a few brief comments concerning the sequencing of peptides by FAB/tandem mass spectrometry are in order. A number of investigators have demonstrated the successful application of MS/MS as a peptide sequencing tool, including those who have used a high-resolution double-focusing instrument (Biemann & Martin, 1987) and a tandem quadrupole instrument (Hunt et al., 1986). Generally, for a peptide up to about 2000 daltons, one could expect to obtain much of the sequence directly by mass spectrometry (Mathews et al., 1987). The overall procedure involves digestion of the protein with a protease such as trypsin and fractionation of the resulting peptides by HPLC. Although the technique can be used with mixtures of peptides, it is best to limit the sample to simple mixtures to avoid interferences. The peptides are then subjected to FABMS/MS analysis where the sequence is deduced from the fragment ion patterns. Peptide sequence overlaps

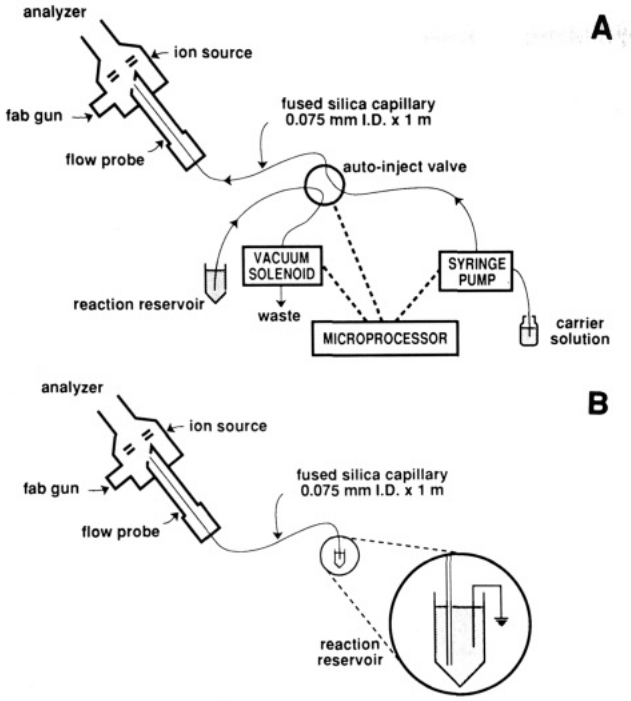


FIGURE 5: Instrumental setup for use of the continuous-flow FAB interface for on-line monitoring of enzymic reactions. (A) Flow-injection mode for repetitive analysis of 1.0-μL samples from a reaction reservoir. (B) Constant-flow mode for continuous monitoring. Further details are given in the text.

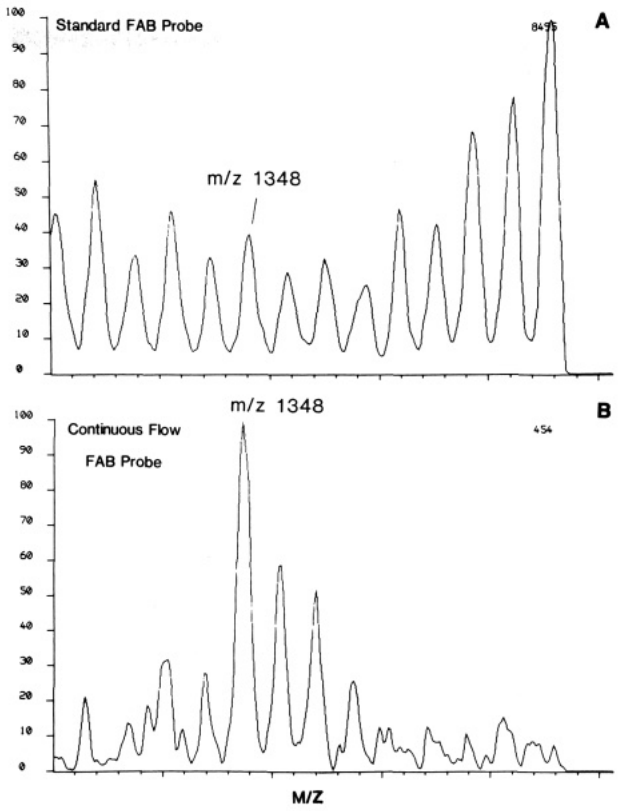


FIGURE 6: Sensitivity of FAB measurements using 100 fmol (135 pg) of substance P [$(M + H)^+ = m/z$ 1348]. (A) Standard FAB method where sample contains 95% glycerol and 5% water and (B) continuous-flow FAB analysis where sample contains 20% glycerol and 80% water (Caprioli & Fan, 1986b).

are obtained from similar analyses using a different protease. It is clear that FABMS plays an integral role in the strategy for sequencing a protein, together with the established methods of amino acid analyses and chemical and enzymic hydrolyses of the protein, to provide specific molecular weight and structural information on peptide fragments.

CONTINUOUS-FLOW FAB

The continuous-flow technique for FABMS provides a means of introducing aqueous samples directly from reaction mixtures into the mass spectrometer, in either on-line or off-line applications (Caprioli et al., 1986). This eliminates the need for extensive sample handling and the addition of high concentrations of glycerol or other organic liquids that must be used for std-FAB. More importantly, use of this introduction system provides several performance benefits, described later, which are particularly worthwhile for many biological applications.

The basic principle of operation involves the balance of the flow of liquid to, and evaporation from, the sample stage of the insertion probe in the source of a FAB mass spectrometer. A narrow bore (75 μm) fused silica capillary, chosen because of its electrical insulating property, is used to deliver the liquid to the probe tip. For many commercial instruments, source pumps can handle flow rates of 5–10 μL/min. For reaction monitoring, the CF–FAB probe may be operated in the flow-injection mode or in the constant-flow mode. Each method has particular advantages depending on the nature of the process being monitored and also the type of information desired. Basically, the flow-injection mode shown schematically in Figure 5A is best for following enzymic reactions where continuous monitoring is not essential and where reaction conditions are not conducive to ion production in the source. For example, many peptides are detected with highest sensitivity at a pH <2 on the probe tip, although protease digestions with few exceptions are carried out in the pH 5–9 range. Thus, for flow-injection operation, aliquots of approximately 1 μL of the reaction solution are injected into a carrier flow, delivered by a microflow pump, which contains sufficient acid to maintain the pH at <2. A second method of operation of the CF–FAB probe is the constant-flow mode shown schematically in Figure 5B where the reaction being monitored is introduced directly into the source. This case is especially useful if the reaction time is very short or rapid changes in concentrations are expected. For this mode the capillary is immersed into the reaction solution and atmospheric pressure used to deliver the solution to the ion source.

Performance Benefits. Several benefits have been noted when the CF-FAB probe is used for sample introduction as compared to the std-FAB probe. Mass spectra show a significantly lower background, primarily as a result of the higher aqueous content of the solutions being analyzed (Caprioli & Fan, 1986b). Thus, more detail can be seen in the FAB mass spectrum, and a lower limit of detection is achieved. For example, in the case of the analysis of femtomole quantities of the peptide substance P (M_r 1347), an approximately 150-fold increase in signal-to-noise was achieved relative to that obtained by the std-FAB probe, as shown in Figure 6.

A second benefit of the CF-FAB probe is a decreased ion "suppression" effect (Caprioli et al., 1987b), i.e., the suppression of the signal of one compound in a mixture by another that preferentially occupies the surface of the sample droplet. Thus, although two compounds may be present at equimolar concentrations in the bulk liquid, only one compound is seen in the FAB spectrum. Figure 7 shows a portion of the mass spectra obtained for the analysis of the tryptic digest of glucagon. Although two ions, m/z 1352 and 1357, should be seen in this region, m/z 1357 is completely suppressed when the std-FAB method for analysis is used. This suppression effect has been rationalized on the basis of the hydrophobicity/hy-

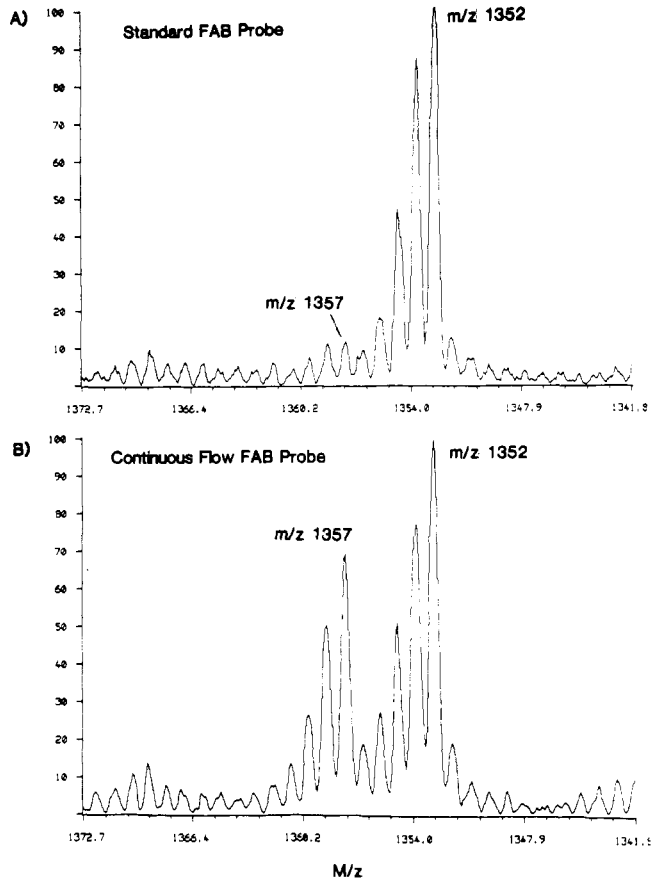


FIGURE 7: Comparison of the ion suppression effect observed with FAB using (A) standard FABMS and (B) continuous-flow FABMS. The sample represents 100 pmol of the tryptic digest of glucagon. Two peptides are present in this mass region and have $(M + H)^+$ values at m/z 1357 and 1352 (Caprioli et al., 1987b).

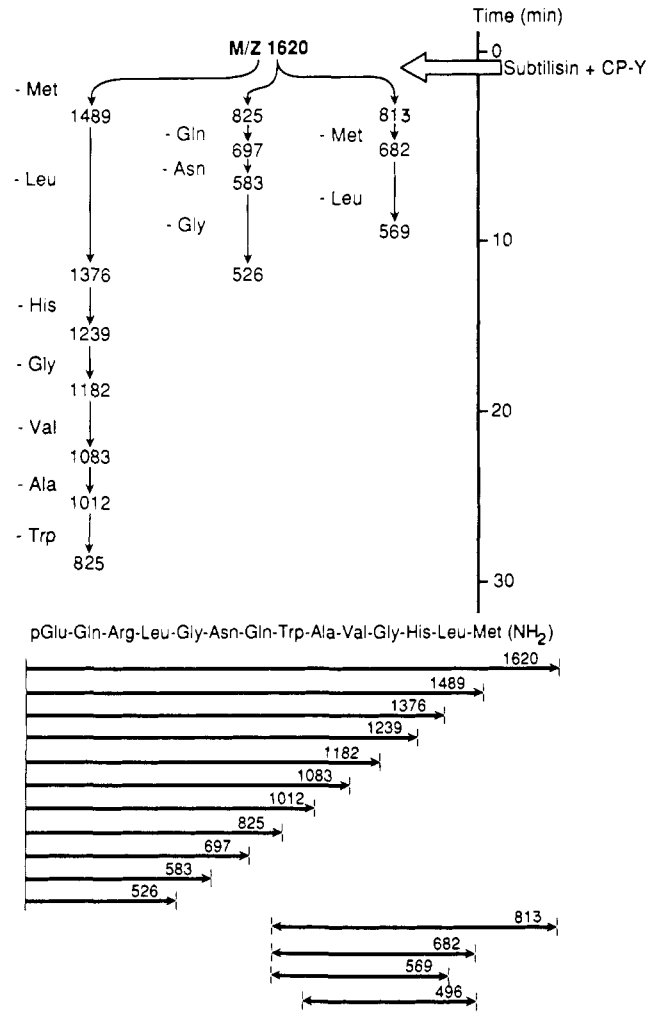


FIGURE 8: Time-course production of peptides identified by FABMS from the on-line hydrolysis of bombesin with a 10:1 mixture of carboxypeptidase Y:subtilisin Carlsberg. The arrangement of $(M + H)^+$ ions, which are related by the mass difference of an amino acid residue, is shown with the sequence of the substrate.

drophilicity index of peptides (Naylor et al., 1986). However, when the same reaction solution was measured by CF-FAB, a significant ion intensity was observed at both m/z 1357 and 1352. Further studies with a number of tryptic digests showed that use of CF-FAB significantly reduced this suppression effect, although not always eliminating it entirely. It is believed that the constant refreshment and mixing of the surface layers in a more vigorous manner than can be achieved in a static droplet are responsible for this observed decrease in the ion suppression effect.

Peptide Mapping. Separation techniques such as paper chromatography, electrophoresis, and more recently, high-performance liquid chromatography (HPLC) have been employed as analytical tools to separate and visualize peptide fragments produced in enzymic and chemical hydrolyses of proteins. "FAB mapping" is a technique that utilizes FABMS to identify and mass measure these peptides (Morris et al., 1983).

We have used CF-FAB for the time-course analysis of the appearance of peptides produced from proteolytic digestion of polypeptides, producing a time-ordered peptide map. Both the identification of the mass of the peptides liberated and also the relative times of appearance and/or disappearance of peptides can be obtained. In the simplest case where a single endopeptidase is used, the time-course data provide a means of linking some of the peptide fragments to each other, although not necessarily providing their complete arrangement in the parent fragment. However, if an exopeptidase such as carboxypeptidase or aminopeptidase is used together with an endopeptidase, then the identification of only a few terminal residues of each fragment is sufficient to order the fragments in the parent peptide or protein. For example, Figure 8 shows the results of the hydrolysis of bombesin (M_r 1619) by a mixture of subtilisin and carboxypeptidase Y. Within the first few minutes of the reaction, the first C-terminal residue was found to have been cleaved from the original $(M + H)^+$ molecular ion at m/z 1620 to give m/z 1489, corresponding to the loss of the mass of a methionyl residue. At the same time, a portion of the original peptide was hydrolyzed by subtilisin to give two fragments, having $(M + H)^+$ values of 825 and 813. These two ions appear and maximize in intensity in the same time (scan) period, and their residue masses add up to that corresponding to the original peptide. In later scans, C-terminal residues are observed to be lost from these peptides as well. Since the C-terminal residues lost from the peptide fragment at m/z 813 match those lost from the intact peptide, this fragment is derived from the C-terminus of the original peptide and that of m/z 825 from the N-terminus. In all, the sequences of 10 C-terminal residues were readily deduced from these data.

Automated On-Line Kinetic Measurements. The molecular specificity that FABMS brings to analytical biochemistry is of immense importance to kinetic studies because it permits individual cleavages to be followed on large natural substrates. To facilitate such measurements, we have developed an autosampling device for use with CF-FAB for the determination of enzyme kinetics as shown in Figure 5A. This device consists

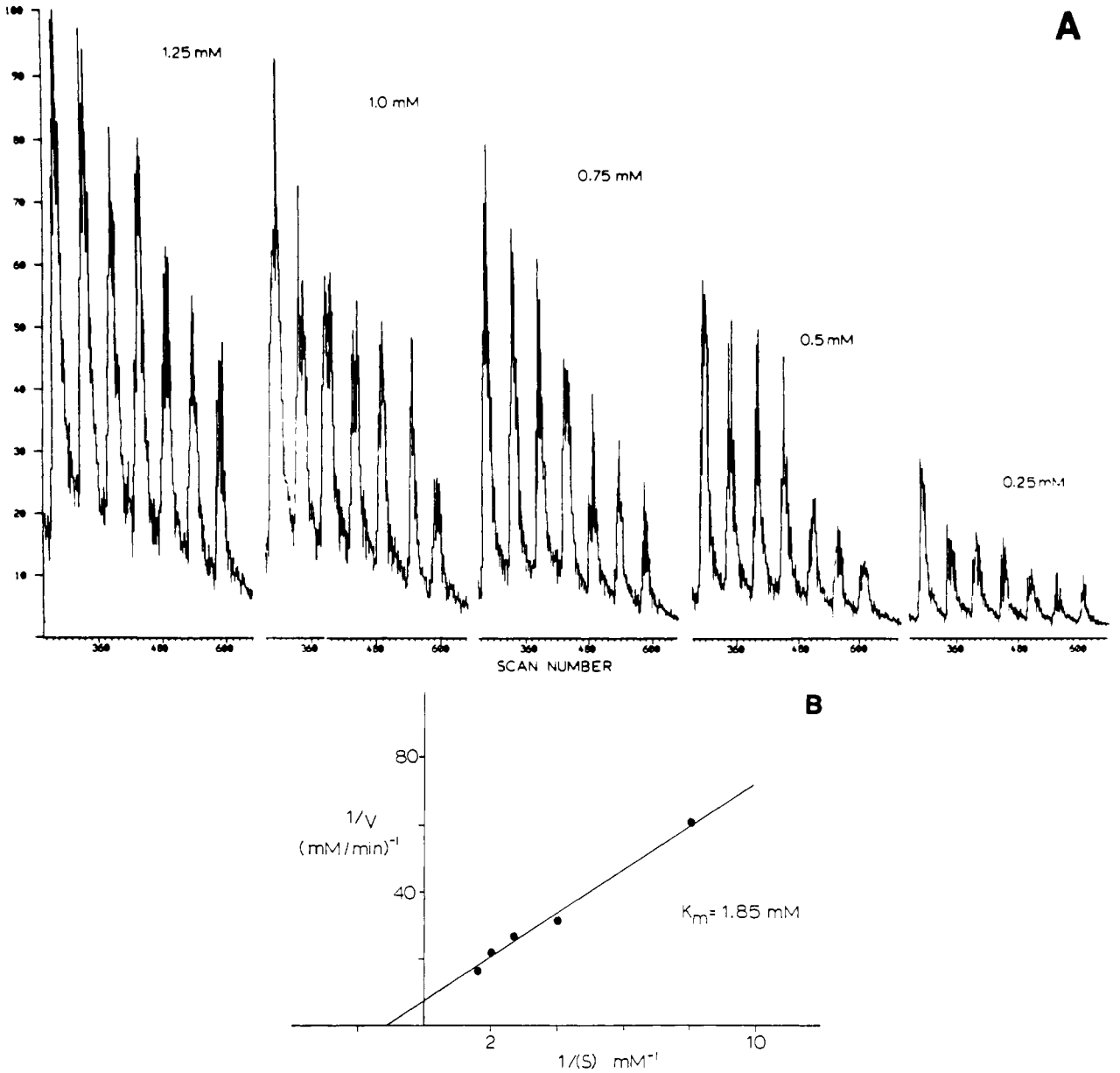
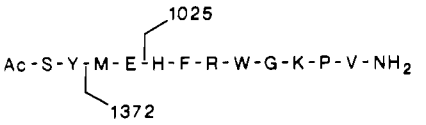


FIGURE 9: Flow-injection FABMS analysis of the hydrolysis of the peptide M-F-R-A by trypsin. (A) Selected ion recording of the molecular species at various substrate concentrations. (B) Double-reciprocal plot of rate data obtained from measurements of areas under peaks in panel A.

of a Brownlee microgradient pump, an autoinjector valve (Rheodyne Model 7410 with a 0.5-μL loop), and a sample loop loader. Timed event markers from the pump are used to operate the automatic valves. Samples may be injected as rapidly as every 12 s if necessary, although it was found that the optimal rate is approximately once every 2 min over a 10–15-min period for most enzymic reactions. For comparison with data obtained earlier, the kinetics of the tryptic hydrolysis of the peptide M-R-F-A were obtained in this manner. Figure 9A shows the injection profiles obtained for five substrate concentrations, and Figure 9B, the double-reciprocal plot obtained. The calculated kinetic value for K_m was 1.85 mM, in close agreement with the value of 1.90 mM obtained earlier.

On-Line Continuous Reaction Monitoring. In certain cases, it is advantageous to monitor a reaction on a continuous basis. If the reaction conditions are compatible with FAB ionization, and low concentrations of glycerol are acceptable in the reaction mixture, then the solution may be admitted directly into the mass spectrometer. For example, Figure 10 shows the selected ion chromatograms for the hydrolysis of the peptide α-MSH (M_r 1664) by pepsin to give fragments at m/z 1372 and 1025.



The reaction solution, containing 20% glycerol and adjusted to pH 1.0, was allowed to flow into the FAB source at a rate of 0.8 μL/min. The enzyme was added at t = 0. The initial 2-min delay corresponds to the time the reaction solution takes to reach the source at this flow rate. The rate of increase of the two product peptides as well as the decrease of substrate was found to be approximately the same over the first 10 min of the reaction.

HPLC/MS of Proteolytic Digests. The combination of mass spectrometry and high-performance liquid chromatography represents a powerful tool for the characterization of complex reaction mixtures. Conceptually, this application is analogous to reaction monitoring in that one is measuring a

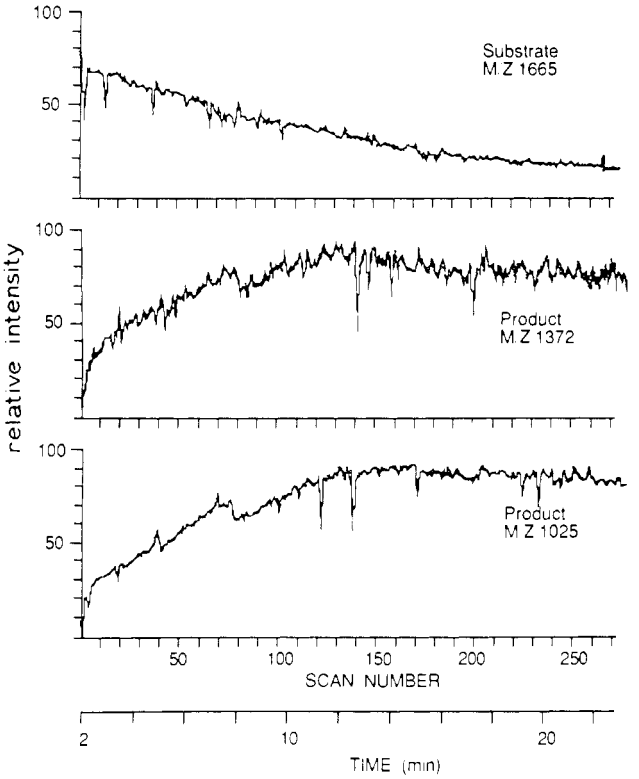


FIGURE 10: Constant-flow FABMS analysis of the hydrolysis of α-MSH [$(M + H)^+ = m/z$ 1665] by pepsin. The selected ion chromatograms of the substrate and two products are shown. Enzyme was added at $t = 0$ and the reaction monitored starting at $t = 2$ min.

change of molecular species over a specified time period, i.e., the duration of the chromatograph gradient. Use of the CF-FAB probe provides an effective interface for chromatographic separations performed at the low flow rates of microbore HPLC (Caprioli et al., 1987a,b). A number of tryptic digests, including myoglobin, cytochrome *c*, glucagon, and ribonuclease A, were analyzed by this HPLC/FABMS procedure. For example, Table II shows the peptides identified from the analysis of 100 pmol of the tryptic digest of sperm whale myoglobin using a Brownlee RP-300 (1 × 250 mm) microbore column with gradient elution (solvent A, 5% glycerol in water containing 0.1% TFA, and solvent B, 60% acetonitrile, 5% glycerol, and 0.1% TFA) at a flow rate of 5 μL/min. In all, 19 peptides are expected for the tryptic hydrolysis of this 17 000-dalton protein, and 16 peptides were found, ranging in mass from m/z 2000 to 300. Two dipeptides and one tripeptide expected in the mixture were not identified and were believed to be masked by the background present at very low mass.

In order to assess the high-mass capability of the technique, the separation and analysis of 400 pmol each of bovine, ovine, porcine, and equine insulins were investigated. The compounds were nearly completely separated and the $(M + H)^+$ values measured with the same instrument conditions as above. The average mass assignment was accurate to ±0.4 mass unit.

Conclusions

The integration of methods that employ enzymes for specific molecular reactions and the analytical technique of mass spectrometry for mass-specific detection of these compounds provide a powerful investigative technique. A crucial link between the use of FABMS in the analysis of biochemical processes in a routine and facile manner is the capability for direct analysis of aqueous solutions. The studies cited above help establish the viability of this combination and point to the advantages that can be obtained as this methodology and technology are further improved and developed.

Table II: Identification of Peptides in the Tryptic Digest of Myoglobin Using Combined HPLC/FABMS

sperm whale myoglobin sequences	$(M + H)^+$ calcd	$(M + H)^+$ found (HPLC/MS)
VLSEGEWQLVLHVWAK	1894	1894
VEADVAGHGQDILIR	1593	1593
LFK	407	407
SHPETLEK	940	940
FDR	437	437
FK	294	?
HLK	397	?
TEAEMK	708	708
ASEDLK	662	662
HGVTVLTALGAILK	1392	1393
GHHEAELK-PLAQSHATK	1855	1856
HK	284	284
IPIK	470	470
YLEFISEAIIHVLHSR	1927	1928
HPGNFGADAQGAMNK	1518	1519
ALELFR	748	748
DIAAK	517	517
YK	310	?
ELGYQG	665	665

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Accelerated Publications

Hybrid Hexanucleotide Duplex Containing Cyclonucleotides and Deoxynucleotides: The d(TA) Segment Can Adopt a High Anti Left-Handed Double-Helical Structure†

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ABSTRACT: It is known that oligonucleotides containing cyclonucleosides with a high anti (intermediate between anti and syn) glycosidic conformation adopt left-handed, single- and double-helical structures [Uesugi, S., Yano, J., Yano, E., & Ikehara, M. (1977) *J. Am. Chem. Soc. 99*, 2313–2323]. In order to see whether DNA can adopt the high anti left-handed double-helical structure or not, a self-complementary hexanucleotide containing 6,2′-*O*-cyclocytidine (C°), 8,2′-*O*-cycloguanosine (G°), thymidine, and deoxyadenosine, C°G°dTdAC°G°, was synthesized. Imino proton NMR spectra and the results of nuclear Overhauser effect experiments strongly suggest that C°G°dTdAC°G° adopts a left-handed double-helical structure where the deoxynucleoside residues are involved in hydrogen bonding and take a high anti glycosidic conformation. A conformational model of the left-handed duplex was obtained by calculation with energy minimization. Thus it appears that DNA can form a high anti, left-handed double helix under some constrained conditions, which is quite different from that of Z-DNA.

8,2′-*S*-Cycloadenosine (A^s),[1] where the adenine C8 and sugar C2′ are bridged with a sulfur atom, has a fixed glycosidic torsion angle in a high anti region (Prusiner et al., 1973; Tanaka et al., 1979). In the course of studying oligonucleotide derivatives of purine cyclonucleosides, we found that a dinucleoside monophosphate of 8,2′-*S*-cycloadenosine, A^{s}pA^s, takes a stable stacking conformation with a left-handed screw axis (Ikehara et al., 1970; Uesugi et al., 1972). Homooligonucleotides containing A^s adopt a left-handed helical structure (Ikehara & Uesugi, 1972) and form left-handed duplexes with homooligonucleotides containing 6,2′-*O*-cyclouridine (U°), which has the same glycosidic conformation as that of A^s (Uesugi et al., 1976). Studies on ApA analogues containing cyclonucleosides with different torsion angles in an anti range and adenosine reveal that the high anti conformation (χ = 110–120° according to Sundaralingam's definition; Sundaralingam, 1969) is required for most stable left-handed stacks (Uesugi et al., 1977). Studies on pairs of dimer sequence isomers containing different base species (adenine, uracil, and hypoxanthine) confirmed the left-handedness of stacking (Ikehara et al., 1980; Uesugi et al., 1980a,b). These studies also revealed that the order of sequence-dependent stability of stacking (U°pA^s > A^{s}pU°) and the order of sequence-dependent stability of the ethidium complex (A^{s}pU° > U°pA^s) for A^{s}pU° and U°pA^s are reversed with respect to those for ApU and UpA. These phenomena are a reflection of the difference in the modes of stacking in left-handed and right-handed stacks. Energy calculations on the A^{s}pA^s conformation also support the view that the left-handed stack is the most stable one (Fujii & Tomita, 1976). Extension of the dimer structure to a polymer structure gives a regular left-handed helix with no unusual conformational parameters, including those for the phosphodiester bonds (Fujii & Tomita, 1976; Yathindra & Sundaralingam, 1976).

Discovery of the Z-DNA structure (Wang et al., 1979), which adopts quite different left-handed double-helical structure from the high anti one, and subsequent studies on DNA structures in crystals, fibers, and solution revealed that DNA is conformationally very flexible. On the basis of these advances, it was of great interest to see whether DNA can adopt the high anti left-handed double-helical structure or not. For this purpose, we synthesized a self-complementary hexanucleotide containing 6,2′-*O*-cyclocytidine (C°), 8,2′-*O*-cycloguanosine (G°), thymidine, and deoxyadenosine, C°G°dTdAC°G°, in which a d(TA) segment is sandwiched between two C°G° segments (Figure 1). It was expected that the flanking C°G° segments might force the d(TA) segment

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[1] Abbreviations: A^s, 8,2′-anhydro-9-β-D-arabinofuranosyl-8-mercaptoadenine; U°, 6,2′-anhydro-1-β-D-arabinofuranosyl-6-hydroxyuracil; C°, 6,2′-anhydro-1-β-D-arabinofuranosyl-6-hydroxycytosine; G°, 8,2′-anhydro-9-β-D-arabinofuranosyl-8-hydroxyguanine; NOE, nuclear Overhauser effect; DSS, sodium 1-(trimethylsilyl)propane-3-sulfonate; EDTA, ethylenediaminetetraacetic acid.

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