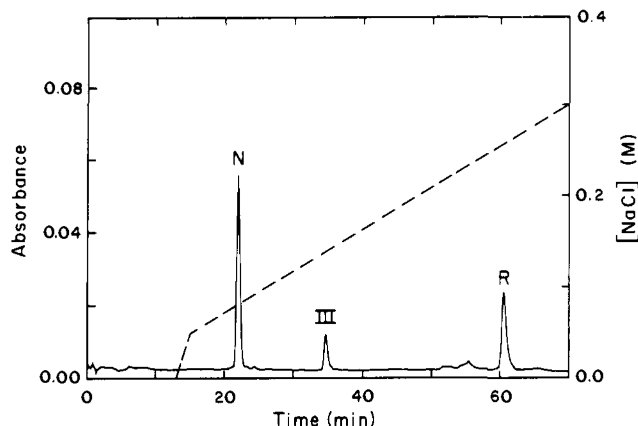

Figure 2. Chromatographic separation of disulfide intermediates in the reduction of RNase A. Native RNase A (73 μM) was reduced for 18 h at 25 °C and pH 8.0. The starting concentration of DTT^{red} was 40 mM. All other conditions are as described in Figure 1.

observed. The shortest $\tau_{1/2}$ that we have been able to observe is 128 min.[17] This compares favorably with the $\tau_{1/2}$ of 108 min obtained at the optimal ratio of oxidized and reduced glutathione under similar solution conditions.[5] Because DTT^{ox} regeneration is not limited by mixed disulfide formation, it is likely that higher ratios of DTT^{ox}/DTT^{red} will result in regeneration rates greater than those obtainable with glutathione.

The pathway for reduction of RNase A by DTT^{red} has been shown to have a native-like three-disulfide intermediate lacking the 65-72 disulfide.[18] This work has been reported by Creighton[8,10] to be nonreproducible. We have, however, been able to isolate this species in good yield, greater than 8% of the total protein, as shown in Figure 2. The primary difference between our result and previous results is the method used to block the free thiols in the protein. The blocking reagent that we used, 2-aminoethyl methanethiosulfonate (AEMTS),[19] is at least 5 orders of magnitude more reactive with thiols in solution than the reagent used in previous studies, iodoacetic acid.[20] It appears that the accessibility of the Cys-65 and Cys-72 thiols is somewhat restricted. We have found that they are not blocked by iodoacetic acid under the conditions normally employed.[3,8,10]

Conclusions[10,21] based on the previous inability of DTT^{ox} to regenerate native RNase A and the earlier failure to observe any stable intermediates upon reduction with DTT^{red} have been used to promote a specific regeneration model[3,8,22] and to attempt to discredit a more general model[5,23,24] for the regeneration pathways of RNase A. Clearly, given the data reported here, such contentions are unwarranted.

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Registry No. DTT^{ox}, 14193-38-5; DTT^{red}, 3483-12-3; RNase A, 9001-99-4.

(16) $\tau_{1/2}$ is determined from the average regeneration rate during the 2-h period corresponding to 1-3 h following the initiation of the oxidative process. The appearance of native protein as measured by chromatographic peak areas corresponds quantitatively to the regain of native activity.
(17) Conditions were 100 mM DTT^{ox}, 3 μM RNase A, pH 8.0, 25 °C.

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Detection of Noncovalent Receptor-Ligand Complexes by Mass Spectrometry

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Few methods are known for detecting and identifying enzyme-substrate, receptor-ligand, and antibody-antigen complexes, whose weak noncovalent interactions constitute the essential basis of molecular recognition in the biological world. With newer ionization techniques, mass spectrometry (MS) can be applied to problems of biological interest;[1] however, efforts to date have focused on sequencing carbohydrates,[2] oligonucleotides,[3] peptides, and proteins[4] and detecting other macromolecules.[5]

The newly developed technique of ion-spray (pneumatically assisted electrospray) MS forms gas-phase macromolecular ions directly from solution at atmospheric pressure via protonation and ion evaporation.[6,7] In contrast to electrospray, ion-spray MS can be performed in water without cosolvent, which is ideal for most biological systems. Multiple charging produces a family of molecular ions and dramatically reduces the mass-to-charge ratio, so that even quadrupole mass spectrometers having a typical mass range of 1000-2000 daltons (Da) can determine high MW species with unit mass resolution.

Since ionization of the targeted molecular species occurs under very mild conditions, fragmentation is usually not observed in ion-spray MS experiments, thus suggesting that noncovalent molecular association complexes might be detectable under conditions of real-time[8] reaction monitoring. Here we describe the first successful application of such a technique to a problem of considerable contemporary interest. The method should prove useful in probing a wide variety of macromolecular host-guest interactions.

The macrolides FK506 **1** (MW 804 Da, Figure 1) and rapamycin **2** (RM, MW 913 Da) are promising new immunosuppressive agents with approximately 100-fold better activity than the widely used immunosuppressant cyclosporin A.[9] Both **1** and **2** inhibit T-cell activation in a complex series of events triggered by binding of the drugs to their naturally occurring cytoplasmic receptor FKBP, a member of the immunophilin family of im-

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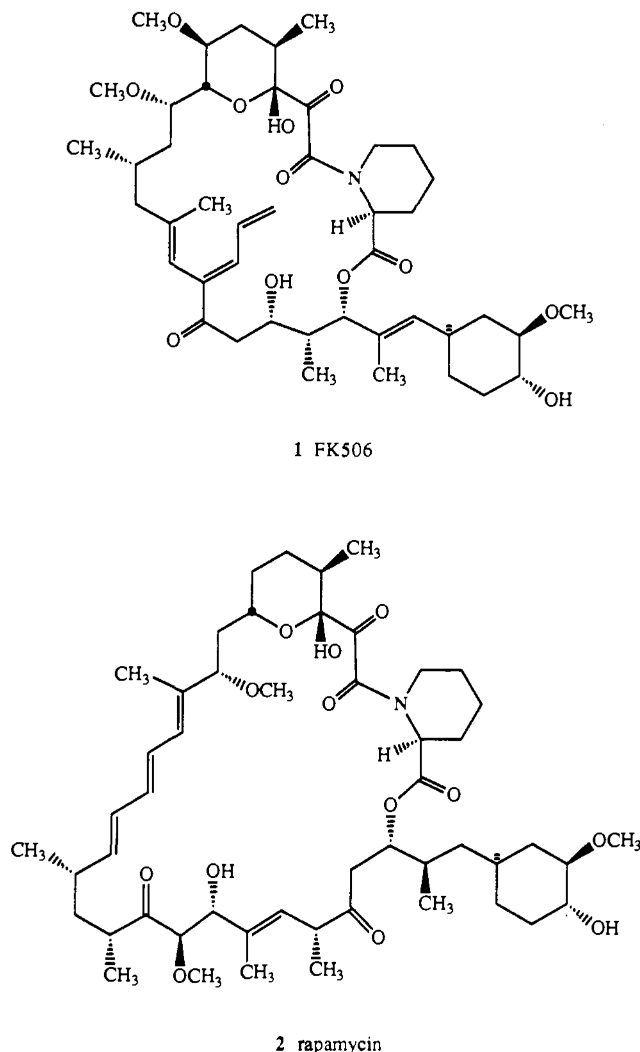

Figure 1.

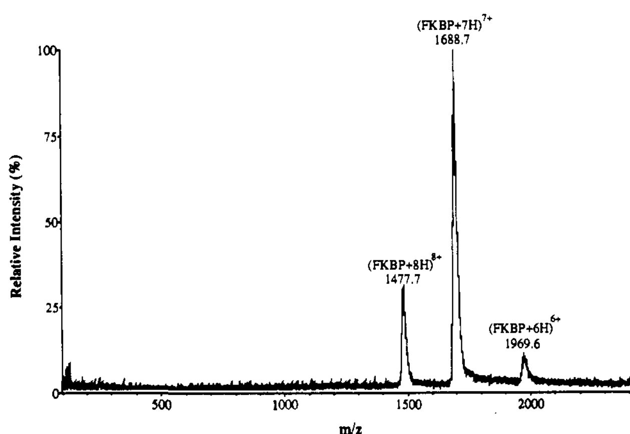

Figure 2. Ion-spray mass spectrum of FKBP at pH 7.5.

munosuppressive binding proteins.[10] Human FKBP is a small, hydrophilic protein (exact MW 11 812 Da) which has been cloned and overexpressed in *E. coli*.[11] The immunophilin binds both FK506 (K_d = 0.4 nM, pH 7.8) and rapamycin (K_d = 0.2 nM) with high affinity.

Figure 2 shows the ion-spray mass spectrum of FKBP at pH 7.5 recorded on a Sciex TAGA 6000E triple quadrupole mass spectrometer. The spectrum was recorded by infusion of FKBP

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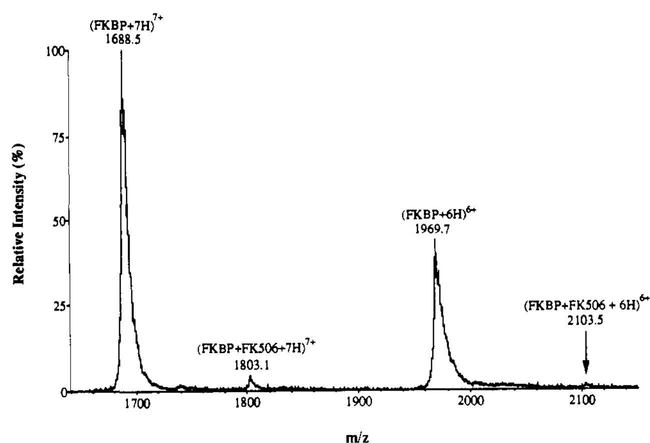

Figure 3. Ion-spray mass spectrum of FKBP + FK506 at pH 7.5.

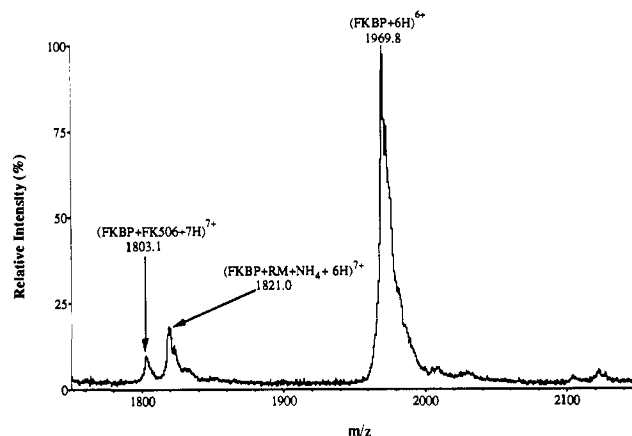

Figure 4. Competitive binding of FKBP with FK506 and rapamycin (RM) at pH 7.5.

(10 ng/μL in 10 mM NH_4OAc buffer) at 2 μL/min through the ion spray interface. Gas-phase ions were formed at atmospheric pressure and sampled into the high vacuum region of the mass spectrometer, which was calibrated with the multiply charged ions of FKBP. The spectrum is an averaged sum of 32 scans from m/z 100 to 2400 at a scan rate of 2 s/scan. The envelope of multiply protonated, multiply charged ions ranges from the (M + 6 H)$^{6+}$ to the (M + 8 H)$^{8+}$ charge states of FKBP. The mass-to-charge regions between the three charge states are of interest for detecting noncovalent complexes.

When FKBP was mixed with a slight excess of FK506 at pH 7.5 (receptor/ligand 1:1.6), a new signal appeared at m/z 1803.1, corresponding to the FKBP–FK506 complex in the 7^+ charge state (Figure 3). A much weaker signal for the same complex in the 6^+ charge state was also evident at m/z 2103.5. Increasing concentrations of FK506 had no effect on the relative intensity of these signals, indicating saturation of the protein with its high-affinity ligand (K_d = 0.4 nM). Nevertheless, a substantial amount of free FKBP was detected in the mass spectrum, suggesting that some dissociation of the noncovalent complex occurred during vaporization. Two important controls established that no adduct was observed either (i) when FK506 was combined with denatured FKBP or (ii) when FK506 was replaced by cyclosporin A (MW 1202 Da), which binds to FKBP several orders of magnitude more weakly than does **1**.[10]

Binding of FKBP with RM (1:1.6 ratio, pH 7.5) was also readily detected, giving rise to signals at m/z 1821.3 and 2124.4 for the (FKBP + RM + NH_4 + 6 H)$^{7+}$ and (FKBP + RM + NH_4 + 5 H)$^{6+}$ charge states, respectively, of the receptor–ligand complex (spectrum not shown). Moreover a competition experiment between FKBP and equimolar amounts of **1** and **2** clearly showed both receptor–ligand complexes at m/z 1803.1 and 1821.0 (Figure 4). Peak integration revealed that the RM complex was twice as abundant as the FK506 complex, in agreement with the

above-mentioned K_d values.[11] This result indicates that noncovalently bound species can be detected directly in a complex mixture without chromatographic separation.

Other macromolecular complexes may be detectable under conditions which are compatible with ion-spray MS.[12] In the negative-ion mode, this technique might be appropriate for highly acidic proteins or oligonucleotides. The use of MS to detect and identify complexes of charged macromolecules with their specific molecular ligands (or vice versa) may also find application in exploring signal transduction,[13] cellular adhesiveness,[14] and other multicellular processes.[15] Antibody-antigen recognition and aggregation phenomena may likewise be amenable to study with antibody Fab fragments,[16] single chain V_L/V_H and V_H antigen-binding proteins[17,18] as well as other, lower MW immunoglobulins.[19] Ongoing research in our laboratories will address these issues.

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Irreversible Inhibition of 3-Dehydroquinate Synthase

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An analogue[1] (**1**, Scheme I) of a reactive intermediate (A, Scheme I) formed during 3-dehydroquinate synthase (DHQ synthase) catalyzed[2] conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) to 3-dehydroquinate (DHQ) has been discovered to be an irreversible inhibitor. Ketocarbaphosphonate

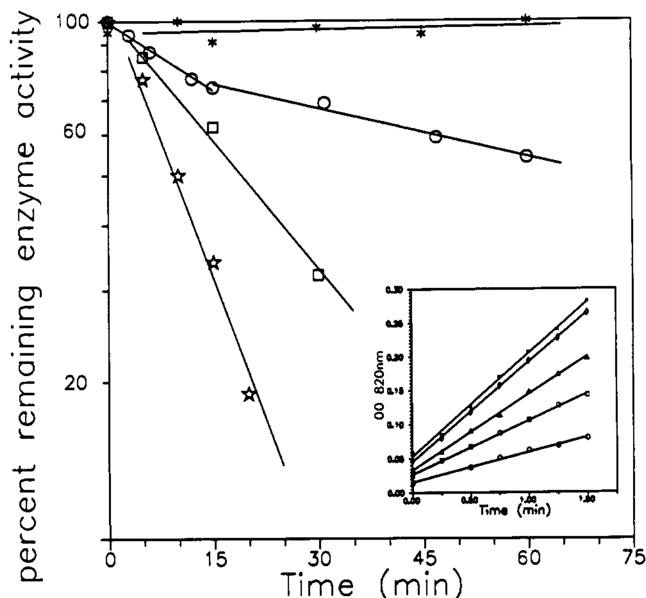
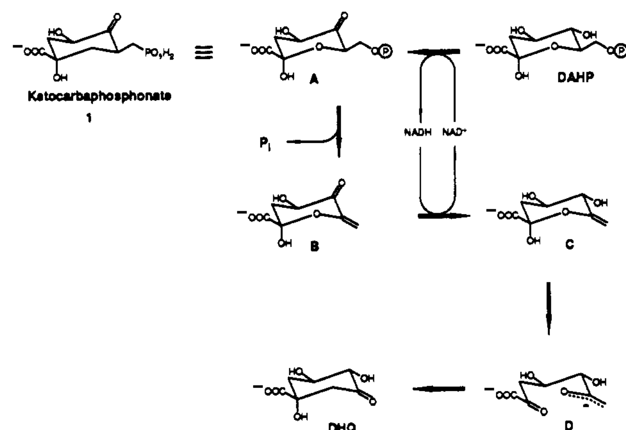


Figure 1. Time-dependent inhibition of DHQ synthase by ketocarbaphosphonate **1**. Enzyme (0.2 μM) was incubated at 15 °C in MOPS buffer (50 mM, pH 7.5) containing NAD (0.25 mM), $CoCl_2$ (0.25 mM), and one of the following: (*) 800 μM DAHP and 0 μM **1**; (O) 0 μM DAHP and 8 μM **1**; (□) 800 μM DAHP and 8 μM **1**; (★) 800 μM DAHP and 160 μM **1**. Aliquots were removed at timed intervals and diluted. Enzyme activity was then determined by colorimetric quantitation (OD_{820}) of product inorganic phosphate.[10] Grade V-C NAD (Sigma) was used for all experiments. All lines are based on linear regression analysis of each set of data points. Insert: Steadily increasing concentrations of DAHP (50, 100, 200, 500, and 800 μM) restore enzyme activity when DHQ synthase (0.025 μM) is incubated for a relatively short time (1.5 min) with a reduced concentration (2 μM) of ketocarbaphosphonate **1**.

Scheme I



1 is the first reported irreversible inhibitor of DHQ synthase and one of the few examples[3] of irreversible inhibition of an enzyme in the common pathway of aromatic amino acid biosynthesis.[4] The unique enzymology associated with ketocarbaphosphonate's inhibition of DHQ synthase also suggests a general strategy for irreversibly inhibiting enzymes that exploit nicotinamide adenine dinucleotide (NAD) as a catalyst rather than a cosubstrate.[5]

Ketocarbaphosphonate **1** was synthesized (Scheme II) from quinic acid in 12 steps with an 8% overall yield.[6] Incubation of

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