Table I. Effects of Added Nucleophiles on Rate Constants for Cleavage of MTP[a]

nucleophile	concn,[b] M	pH	k_{av},[c] min^{-1}	k_{hydrol},[d] min^{-1}
$(HOCH_2)_3CNH_2$	0.10-0.30	9.87	0.055 ± 0.014	0.059 ± 0.021
	0.10-0.30	9.15	0.187 ± 0.008	0.25 ± 0.05
	0.74-1.5[e]	9.71	0.069 ± 0.009	0.082 ± 0.007
	2.0-3.0	9.90	0.055 ± 0.01	0.055 ± 0.006
H_2NNH_2	0.1-0.3	9.40	0.131 ± 0.005	0.155 ± 0.013
$HONH_2$	0.1-0.3	9.33	0.176 ± 0.007	0.178 ± 0.013

[a] The reaction mixtures included, at 25 °C, 10-30 mM K-CHES buffer and 63-76 μM Li_4MTP; the ionic strength was 0.5 maintained with KCl unless otherwise stated. The LiMTP was synthesized by a modification of the procedure described by Loewus and Eckstein.[6a,7] Cleavage of MTP to thiophosphate was monitored spectrophotometrically at 227 nm.[6b] [b] At each pH value, single rates were measured at each of three concentrations of nucleophile within the ranges indicated. [c] The values of k_{av} are the average of rate constants from three runs at different concentrations of added nucleophile within the ranges indicated in column 1, and the uncertainties listed are the mean deviations of individual rate constants from k_{av}. [d] The first-order rate constants for hydrolysis of MTP at the indicated pH were obtained by calculation from the pH-rate profile for hydrolysis.[6b] These are the rates at 25 °C and ionic strength 0.1 (KCl). [e] 0.1 M KCl.

Table II. Phosphoryl Group Capture by Tris and Water in the Cleavage of MTP[a]

[Tris], M	pH	% $(HOCH_2)_3$-$NHPO_3^{2-}$	% $H_2N(CH_2OH)$-$CH_2OPO_3^{2-}$	% PO_4^{3-}
1.7	9.8	16.5	14.5	69
3.0	9.9	28.8	23.3	48

[a] The partitioning of phosphoryl groups from MTP into P_i, *N*-phosphoryl-Tris, and *O*-phosphoryl-Tris was determined by ^{31}P NMR. The following values for chemical shifts were observed: PSO_3^{3-}, δ 35.2 ppm; PO_4^{3-}, δ 3.4 ppm; *N*-phosphoryl-Tris, δ 7.0 ppm; *O*-phosphoryl-Tris, δ 5.1 ppm (triplet, J = 5.8 Hz). The assignments of organic phosphates were based on the phosphorus-hydrogen coupling pattern for *O*-phosphoryl-Tris; this was confirmed by the observation that the species exhibiting the signal at δ = 7.0 ppm is labile in dilute acid, a property of phosphoramidates but not of phosphomonoesters.

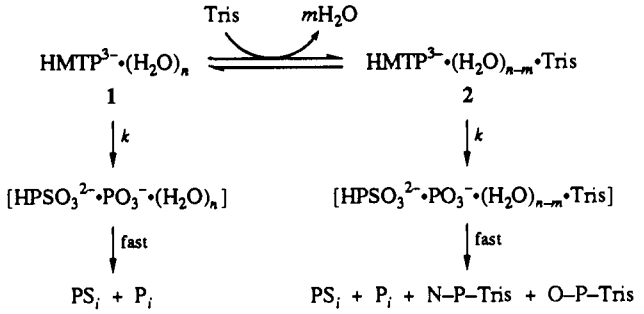
The rates at which phosphomonoesters and *N*-phosphopyridines are cleaved under hydrolytic conditions are enhanced by the presence of added nucleophiles such as carboxylates, bicarbonate, F^-, pyridines, and especially nucleophiles exhibiting the α-effect.[5] Enhanced cleavage rates result from the larger second-order rate constants for the added nucleophiles relative to water.

μ-Monothiopyrophosphate (MTP) is an analogue of pyrophosphate that undergoes hydrolysis millions of times faster than pyrophosphate, owing to the dissociative character of the reaction and the weakness of the P-S bond relative to P-O.[6b] The cleavage rate for MTP is not increased by the addition of any of a variety of nucleophiles, including α-effect nucleophiles. For example, as shown in Table I, Tris at concentrations from 0.01 to 3.2 M does not alter the rate at which MTP is cleaved to thiophosphate at pH values ranging from 9.15 to 9.90.

Although Tris does not affect the rate under hydrolytic conditions, it participates in the cleavage of MTP, as shown by the data in Table II, which shows that Tris at high concentrations captures a phosphoryl group from MTP to form both *N*-phosphoryl-Tris and *O*-phosphoryl-Tris. The phosphoryl groups captured by Tris are partitioned as 54 ± 1% *O*-phosphoryl-Tris and 46 ± 1% *N*-phosphoryl-Tris. Inasmuch as an amino group should be orders of magnitude more reactive than a hydroxyl group when the value of β_{nuc} is in a normal range, the observed capturing ratio is consistent with little or no nucleophilic participation in the transition state (i.e., $\beta_{nuc} = 0$).

Tables I and II show that Tris captures a phosphoryl group from MTP in competition with water, producing both *N*-phosphoryl-Tris and *O*-phosphoryl-Tris in comparable amounts, but it has no significant effect on the rate at which MTP is cleaved to thiophosphate. The simplest interpretation of these facts is that the reaction proceeds by a preassociation, stepwise mechanism such as that in Scheme I for the trianionic form of MTP ($HMTP^{3-}$), the dominant and most reactive form under the conditions of Tables I and II.[6b] Tris reversibly enters the solvation sphere, presumably by displacing water, in a preassociation step. Phosphoryl transfer proceeds *at the same rate* within both solvated complexes **1** and **2**. That phosphoryl transfer to nitrogen and oxygen of Tris is almost random within complex **2** suggests the production of a common, highly reactive intermediate, such as PO_3^-, in the rate-limiting step. Cleavage of MTP in complexes **1** and **2** to PO_3^- (and PS_i) with the same rate constant, k in Scheme I, followed by fast, essentially random capture of PO_3^- by water and Tris in the solvation sphere, accounts for our results.

Scheme I



Earlier studies of phosphomonoesters and phosphoramidates showed that there is nucleophilic participation in phosphoryl group transfer; that is, b_{nuc} is positive. These reactions required cleavage of P-O and P-N bonds, which are much stronger than the P-S bond. The present work shows that phosphoryl group transfer from μ-monothiopyrophosphate in water is insensitive to the nucleophilic reactivity of the acceptor, that is, $\beta_{nuc} = 0$. This is positive evidence that monomeric metaphosphate is a discrete intermediate that is randomly captured by nucleophiles in the solvation sphere.

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Observation of an Imine Intermediate on Dehydroquinase by Electrospray Mass Spectrometry

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We report the direct observation of an imine intermediate (Schiff base) in an enzyme-catalyzed reaction using electrospray mass spectrometry. Dehydroquinase (E.C. 4.2.1.10) catalyzes the third step on the shikimate pathway, the conversion 3-dehydroquinate **1** to 3-dehydroshikimate **6**.[1] The reaction involves loss of the less acidic *pro-R* hydrogen[2] and proceeds via a multistep

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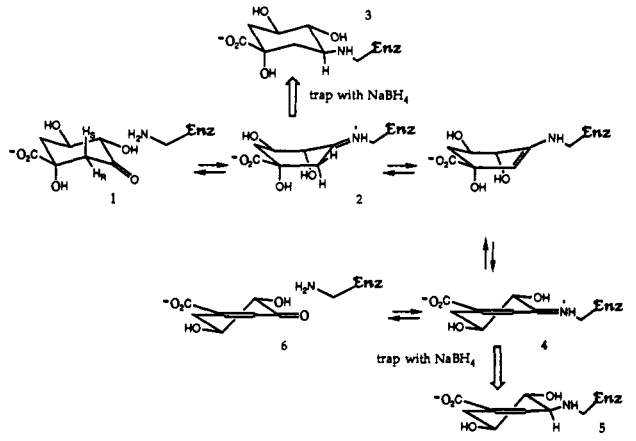
Scheme I. Proposed Reaction Mechanism for the Conversion of 3-Dehydroquinate **1** to 3-Dehydroshikimate **6** Catalyzed by Dehydroquinase[a]



[a]The large arrows signify the trapping of imine intermediates with sodium borohydride.

mechanism involving an imine intermediate (Scheme I).

The proposal of an imine intermediate is founded on a well-precedented series of experiments. Treatment of a mixture of enzyme and substrate with sodium borohydride irreversibly inactivates the enzyme, presumably by reduction of the iminium group (as in **2** or **4**) to form a secondary amine (**3** or **5**).[3] The biophysical properties of this inactivated enzyme have been compared with those of the native, underivatized enzyme.[4] Using sodium [^{3}H]borohydride a radiolabel was introduced into the enzyme–ligand adduct. Proteolytic degradation and sequencing of the labeled enzyme identified the modified residue as lysine-170 in the *Escherichia coli* enzyme.[5] This residue is strongly conserved in all type I dehydroquinases.[6] Such evidence is considered diagnostic of an imine intermediate in a catalytic mechanism despite the fact that it is indirect. Direct spectroscopic evidence of an imine intermediate is limited to specific examples, e.g. pyridoxal chemistry.[7]

The development of electrospray mass spectrometry to determine accurately the molecular weight of proteins[8] prompted us to attempt to observe the imine intermediate on the enzyme directly. In electrospray mass spectrometry, a series of multiply charged gas-phase protein ions, $(M + nH)^{n+}$, are formed. Each ion gives rise to a signal in the spectrum, and from the series of signals the molecular weight of the protein is deduced very precisely. The physical state of the highly charged protein species in the spectrometer is uncertain, although it is likely to be extensively unfolded and dissociated from non-covalently bound ligands. Electrospray mass spectroscopy has been used to detect covalently bound enzyme–substrate[9] and enzyme–inhibitor complexes.[10]

Dehydroquinase is a homodimer of calculated subunit molecular weight 27466.8.[5,11] Using an homogeneous sample of dehydroquinase[12] the molecular weight was determined by electrospray mass spectrometry to be 27467.3 (four determinations, standard deviation 5.6).[13,14]

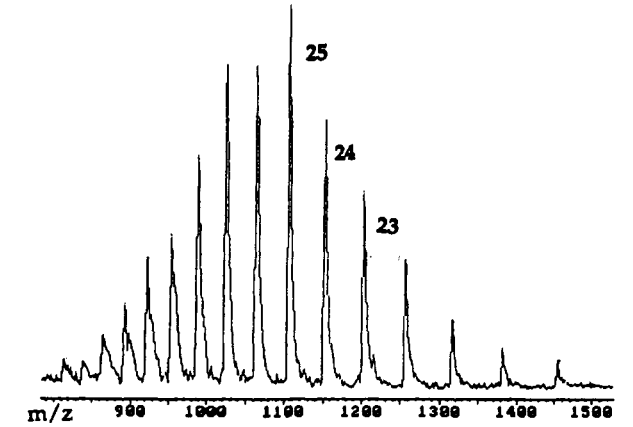


Figure 1. The electrospray mass spectrum of the adduct isolated after three sequential treatments of dehydroquinase with sodium borohydride in the presence of an equilibrium mixture of 3-dehydroquinate and 3-dehydroshikimate. The number beside a peak is n in the formula $(M + nH)^{n+}$.

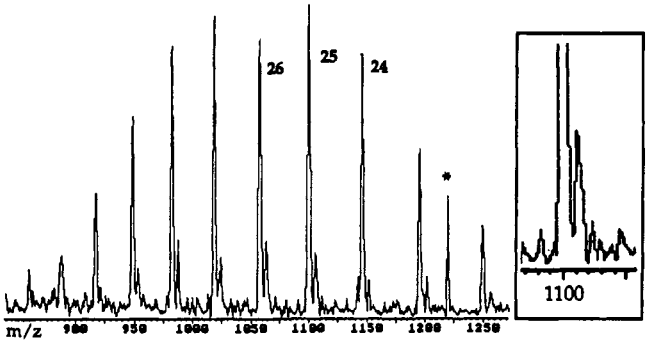


Figure 2. The electrospray mass spectrum of dehydroquinase mixed with 3-dehydroquinate immediately before injection into the mass spectrometer (inset: expanded detail from the 25^+ signal). The marked peak (*) is an artefact.

An identical sample of dehydroquinase was 95% inactivated by three sequential additions of sodium borohydride in the presence of an equilibrium mixture of 3-dehydroquinate and 3-dehydroshikimate (total concentration 2 mM).[5] The electrospray mass spectrum of this sample reveals a single protein species (Figure 1). This shows that all the active sites are modified and confirms that the dimeric enzyme does not exhibit half-sites reactivity. The molecular weight of the modified protein is 27623.3 (three determinations, standard deviation 5.3). This corresponds to the reduced enzyme–product adduct **5** (calculated molecular weight 27622.9) rather than the reduced enzyme–substrate adduct **3** (calculated molecular weight 27640.9). The detection of **5** may reflect the relative concentrations of the enzyme-bound species, but alternatively could arise due to enhanced reactivity or accessibility of the intermediate **4** to borohydride.

The spectrum of a mixture of native and modified dehydroquinase showed that signals for the two species were resolvable and suggested that the imine adduct would be detected if it were

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(13) Typically, dehydroquinase (100 pmol) in 5 mM TrisHCl, pH 7.2 (10 μL), was injected into the carrier solvent stream (50:50 mixture of water: methanol containing 1% acetic acid, flow rate of 4 μL/min) of a VG BIO Q quadrupole mass spectrometer. The spectrometer scanned over a mass range of 700–1500 Da and was calibrated with myoglobin.
(14) The spectrum for native dehydroquinase is identical with that in Figure 1 except that the single series of peaks occur at slightly lower m/z values.

stable in the mass spectrometer. Catalytically active dehydroquinase was mixed with 3-dehydroquinate immediately prior to injection into the mass spectrometer. By using a large excess of dehydroquinate (10000:1 substrate:enzyme) it was possible to observe a second series of signals of lower intensity (Figure 2). These correspond to a species of molecular weight 27618 ± 7.4, which is that expected for **4**, the imine adduct between the enzyme and the product (calculated molecular weight 27621.9).

The low intensity of the imine adduct observed in Figure 2 is probably a consequence of the extreme conditions the liganded enzyme encounters upon injection into the spectrometer.[13,15] The observation of the imine adduct with the product rather than the substrate is consistent with the result from the borohydride trapping experiment and suggests that **4** is the major covalently bound intermediate on the enzyme. This is reasonable as the equilibrium constant for the dehydration is 15.[16]

In summary, the use of electrospray mass spectrometry has allowed direct observation of an imine intermediate in an enzyme reaction. The molecular weight of both the imine intermediate and the adduct formed after reduction with sodium borohydride suggests that the major covalent enzyme adduct is the imine with the product 3-dehydroshikimate. The inactivation experiment also showed the enzyme does not exhibit half-sites reactivity. These experiments provide a powerful demonstration of the use of electrospray mass spectrometry to study enzyme mechanisms.

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(15) The acidity of the carrier stream in the mass spectrometer (pH 3)[13] will protonate the substrate (increasing K_m) and the active site lysine on the enzyme inhibiting imine formation. Furthermore acetate is a competitive inhibitor of dehydroquinase.[12]

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Externally Bound Metal Ion Complexes of Buckminsterfullerene, MC_{60}^+, in the Gas Phase

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Shortly after their discovery of the special stability of the fullerenes,[1] Smalley and co-workers demonstrated that metal-fullerene species could be generated in the gas phase by growing them in a supersonic expansion source following laser desorption from a graphite target impregnated with various metals, M = La, K, and Cs.[2,3] Cox and co-workers made similar observations, but did not consider these to be fullerene derivatives.[4,5] As with the fullerenes themselves, the metallated species were detected mass spectrometrically and their structures probed by multiphoton photodissociation. These complexes, and in particular MC_{60} and its singly charged counterpart, MC_{60}^+, were found to be highly stable, requiring significant laser fluence to cause fragmentation by sequential C_2 loss.[3] Further loss of C_2 ceased at some critical even number of carbon atoms depending on the metal, such as $C_{44}La^+$ (possibly $C_{42}La^+$), $C_{44}K^+$, and $C_{48}Cs^+$. These results led Smalley and co-workers to predict metal-included structures in which the central metal is enclosed in an inert carbon cage. If such species could be synthesized in macroscopic amounts, as demonstrated for C_{60} and C_{70},[6] they could exhibit important chemical and physical properties. Most notably to date is the observation that preformed C_{60} doped with potassium exhibits a superconducting transition,[7] although an externally bound metal-fullerene association has been demonstrated.[8] In this work, we extend our initial study[9] on FeC_{60}^+ to several other metal ions and demonstrate unequivocally that metals added to preformed C_{60} generate externally bound complexes. In addition, externally bound LaC_{60}^+ is observed to exhibit dramatically different properties than its metal-included isomer.

MC_{60}^+ (M = Fe, Co, Ni, Cu, Rh, La, and VO) species were formed in a Nicolet FTMS-2000 Fourier transform mass spectrometer via a multistep sequence[10] initiated by laser desorption to generate M^+ from the pure metal targets.[11] The next step depended on the metal ion, but the majority of the ions including Co^+, Ni^+, Cu^+, Rh^+, and La^+ were then permitted to react directly with a background pressure of C_{60} heated off a solids probe at 350 °C. Using a system temperature of 250 °C, charge transfer to background potassium was found to be a problem, although K^+ was not observed to form KC_{60}^+ under these conditions. Reducing the temperature to 150 °C, however, removed the potassium background and permitted longer trapping periods to make up for the reduced C_{60} pressure. Under these conditions, the metal ions formed MC_{60}^+ by direct attachment together with varying amounts of C_{60}^+ by charge transfer. V^+ and La^+ form very strong bonds with oxygen[12] and rapidly reacted with the background gas ($\sim 2 \times 10^{-7}$ Torr using the solids probe) to form VO^+ and LaO^+. Only VO^+ was observed to directly attach to C_{60} to form the fullerene complex, but conditions probably exist favorable to the formation of $LaOC_{60}^+$. In order to generate LaC_{60}^+, it was necessary to suppress LaO^+ formation. This was accomplished by encasing the external elements of the solids probe with a flexible plastic sheath through which Ar flowed. Finally, Fe^+ reacts with C_{60} predominantly by charge transfer and, thus, a background of *n*-pentane at about 4×10^{-8} Torr was added which yields $Fe(C_nH_{2n})^+$ (n = 2-5) species.[13] These ions undergo ligand exchange reactions with C_{60} to form FeC_{60}^+. A similar procedure could be used on selected metal ions to enhance MC_{60}^+ intensities over that observed by direct attachment.

After a total reaction period, which varied from about 0.3 to 10 s depending upon the system and the conditions, the MC_{60}^+ was isolated by double resonance ejection pulses[14] and subjected to low-energy collision-induced dissociation (CID)[15] using Ar as the target gas at about 2×10^{-6} Torr. The collision energies ranged from 30 to 130 eV, laboratory frame. CID on FeC_{60}^+, CoC_{60}^+, NiC_{60}^+, and CuC_{60}^+ yields C_{60}^+, while LaC_{60}^+ and VOC_{60}^+ yield La^+ and VO^+, respectively, and RhC_{60}^+ yields a mixture of C_{60}^+ and Rh^+, with the Rh^+ predominating. Figure 1 for LaC_{60}^+ illustrates the type of data obtained.

Given the ionization potentials[16] of IP(Fe) = 7.87 eV, IP(Co) = 7.86 eV, IP(Cu) = 7.726 eV, IP(Ni) = 7.635 eV, IP(Rh) =

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