Laser Raman Spectroscopy as a Mechanistic Probe of the Phosphate Transfer from Adenosine Triphosphate in a Model System†

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ABSTRACT: Laser Raman spectroscopy has been used to study a phosphate transfer reaction from ATP to P_i or arsenate in dimethyl sulfoxide. The spectra support a mechanism involving Mg^{2+} binding to the α and β phosphates of ATP leaving the third phosphate free for the transfer reaction. The data also indicate the formation of a relatively stable intermediate which is facilitated by the presence of dimethyl sulfoxide and a dicarboxylic acid (maleate). The intermediate has a Raman spectrum with a band at 1090.5 cm^{-1} similar to the end product ADP, but is formed much more rapidly. Since the model reaction has many features in common (e.g., activation by maleate) with the transfer reactions catalyzed by coupling factors from spinach chloroplast, Raman spectroscopy may also prove to be a useful tool in the elucidation of biological energy transfer reactions.

A phosphate transfer reaction in 70% dimethyl sulfoxide from ATP to either P_i or arsenate has been described (Nelson and Racker, 1973) which resembles in some respects energy transfer reactions catalyzed by coupling factor 1 from chloroplasts (Nelson et al., 1972). Earlier studies by Lord and Thomas (1967a,b), Rimai et al. (1969, 1970), and others (see Koenig, 1972; Lewis and Spoonhower, 1974) suggested that laser Raman spectroscopy might yield information on the mechanism of this reaction.

The transfer reactions in 70% dimethyl sulfoxide required Mg^{2+} or another divalent cation (Ca^{2+} or Mn^{2+}) and were markedly stimulated by dicarboxylic acids, particularly maleate or malonate. The hydrolysis of ATP depended on the presence of arsenate (approximately 5 m*M*) suggesting a common feature with the well-known effect of arsenate in biological energy transfer reactions.

Experimental Section

The experiments were performed in a final volume of 1.5 ml containing 20 m*M* ATP, 20 m*M* $MgCl_2$, 40 m*M* Tricine-maleate (pH 8), 5 m*M* arsenate, and 1 ml of dimethyl sulfoxide. Several controls were run to test the dependence of the spectra on various components of the above solution. Samples were made with triply distilled water obtained from the Cornell Crystal Growing facility and introduction of dust particles was avoided. The solutions were passed through 0.4-μ Millipore filters into capillaries which were sealed. These procedures successfully minimize the background and thus facilitate the observation of the Raman spectra in solutions at relatively low concentrations. To obtain information on reaction times a syringe attached to the cuvet was used to inject 5 m*M* arsenate into a solution already containing the other required components. A particular vibrational frequency was monitored and recordings within 40 sec were made.

Spectra were obtained with a Spex 1401 double monochromator and were detected with an EMI 6256S photomultiplier. Photons were counted at each step of the monochromator and were stored in the memory of a Nuclear Data Model 1100 multichannel analyzer. The spectra were then averaged and plotted out with a PDP/11 mini computer and a Houston plotter. Laser powers of up to 1.2 W were obtained with a Model 52G-A Coherent Radiation argon ion laser.

Results

The Raman spectra seen in Figure 1 were obtained immediately after dimethyl sulfoxide was introduced into a solution containing ATP, Tricine-maleate, Mg^{2+}, and arsenate in the concentrations outlined in the Experimental Section of this paper. The frequency range covered in these spectra include the P=O bond stretching motion of the phosphate moiety (Rimai et al., 1969). The spectra were not dependent on the nature of the anion of the magnesium salt (Figure 1a and b is a comparison of the Raman spectrum of the above solution with $MgCl_2$(a) and $MgSO_4$(b)) or on the presence of Tricine. However, if dimethyl sulfoxide was added to a solution free of Tricine-maleate or to a solution without arsenate or to a solution without maleate, the spectra in Figure 2a-c, were obtained. In all these spectra the main peak at 1123 cm^{-1} has a shoulder around 1116 cm^{-1} and the ratio of the 1123-cm^{-1} band to the 1116-cm^{-1} band is approximately 2:1. The spectra of solutions in which dimethyl sulfoxide is left out but in which Mg^{2+} ions are present is seen in Figure 3a-e. Except for the solution used to obtain the spectrum in Figure 3b which had half the required concentration of dimethyl sulfoxide, a broadening of the 1123-cm^{-1} band is observed in these spectra and the shoulder seen in the spectra in Figure 2 disappears. If, however, Mg^{2+} ions are omitted the spectra obtained give a single band in this region at 1117 cm^{-1} which is essentially identical with the spectrum obtained from a solution of simply ATP. These spectra are compared in Figure 4a-e. Finally, it is interesting to compare all of the above spectra to

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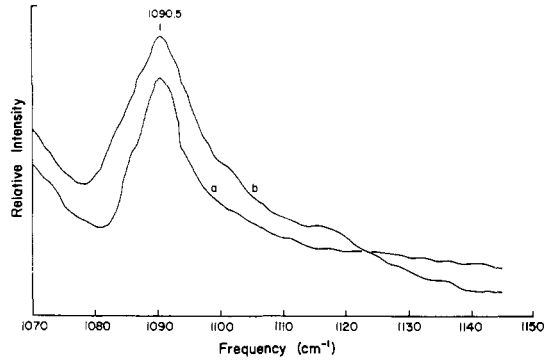

FIGURE 1: (a) The Raman spectrum of the vibration associated with the triphosphate moiety in a solution containing 20 mM ATP, 20 mM $MgSO_4$, 40 mM Tricine-maleate (pH 8), 5 mM arsenate, and 1 ml of dimethyl sulfoxide. (b) Same as a except $MgSO_4$ was replaced by 20 mM $MgCl_2$.

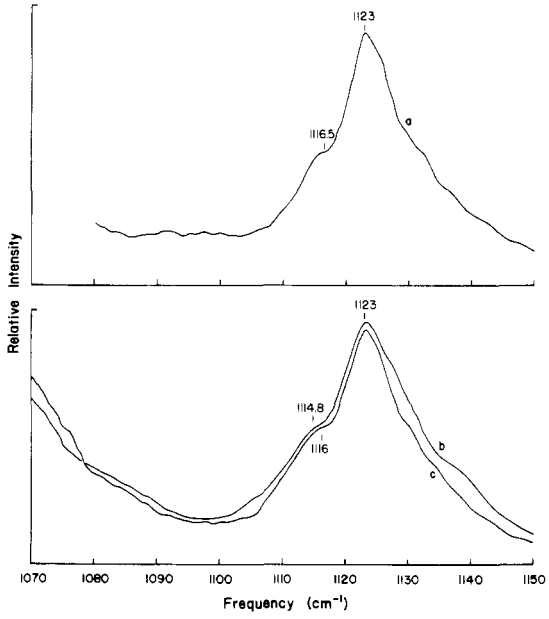

FIGURE 2: (a) The Raman spectrum of the vibration associated with the triphosphate moiety in a solution containing 20 mM ATP, 5 mM arsenate, 20 mM $MgCl_2$, and 1 ml of dimethyl sulfoxide. (b) Same as a except 40 mM Tricine-maleate (pH 8) has been added and arsenate has been deleted. (c) Same as a except 40 mM Tricine-chloride (adjusted to pH 8) has been added.

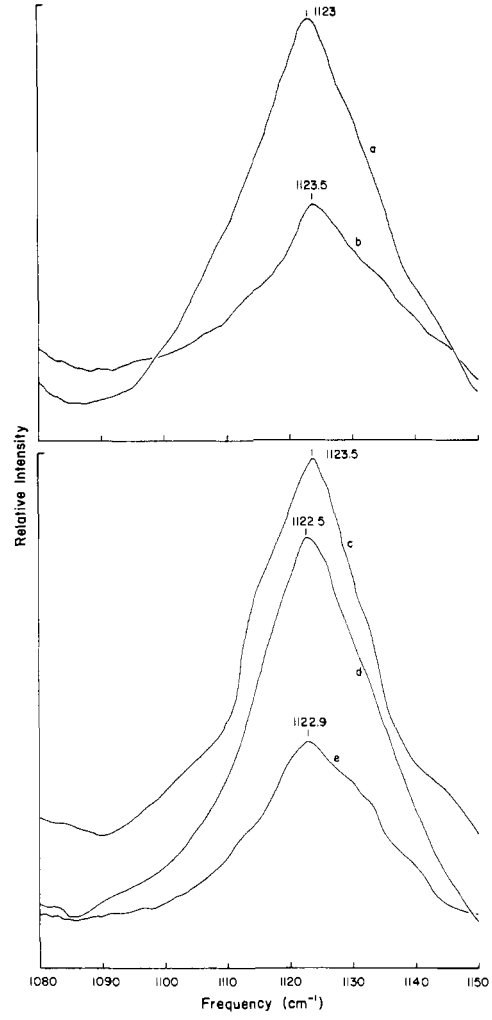

FIGURE 3: (a) The Raman spectrum of the vibration associated with the triphosphate moiety in a solution containing 20 mM ATP, 40 mM Tricine-maleate (pH 8), 5 mM arsenate, and 20 mM $MgCl_2$. (b) Same as Figure 3a except 0.5 ml of water and 0.5 ml of dimethyl sulfoxide have been added. (c) Same as a except arsenate was deleted. (d) Same as a except Tricine-maleate was deleted. (e) Same as a except 1 ml of H_2O was added.

a solution of AMP with Tricine-maleate, arsenate, $MgCl_2$, and dimethyl sulfoxide. The only scattering we observe is a band at 1123 cm^{-1} (see Figure 5). It is significant to note that although all spectra seen in the previous four figures had bands that were polarized, the band of AMP in Figure 5 was depolarized, thus indicating a fundamental difference in the character of the vibrational mode distinct from that of ATP.

The spectrum obtained in the presence of ATP, Tricine-maleate, arsenate, $MgCl_2$, and dimethyl sulfoxide (Figure 1) is very similar to that of ADP when examined under the same experimental conditions (Figure 6, upper curve).

Discussion

A clue to the molecular nature of thehkractions present in these solutions is obtained from Figure 2. It is obvious from these spectra that the symmetric stretch of the phosphate group which occurs at 1117 cm^{-1} in an ATP solution free of Mg^{2+} ions (see Figure 4) has shifted and split into two vibrations. The principal band now occurs at 1123 cm^{-1} while there is a definite shoulder at approximately 1116 cm^{-1} in all the spectra in Figure 2. It seems clear to us that this suggests that the Mg^{2+} ions are interacting with at least some of the phosphate groups. The observation of a shoulder at 1116 cm^{-1} which is about half as intense as the band at 1123 cm^{-1} makes it quite plausible to assume that of the three phosphate groups two are complexed to the Mg^{2+} ions while one remains free. This explanation is certainly supported by the frequencies of the principal band and the shoulder. The vibration at 1123 cm^{-1} is higher in frequency than the band at 1117 cm^{-1} obtained for ATP solutions without Mg^{2+} ions. Such a frequency shift has been observed previously for ATP solutions with Mg^{2+} and with Ca^{2+} by Rimai et al. (1970). The shoulder at 1116 cm^{-1}, on the other hand, would correspond to a free phosphate and thus the stretching frequency is quite similar to the 1117-cm^{-1} band for ATP without Mg^{2+} ions.

All of the solutions used to obtain the spectra seen in Figure 2 contained dimethyl sulfoxide. However, the solutions from which we obtained the spectra seen in Figure 3 lacked

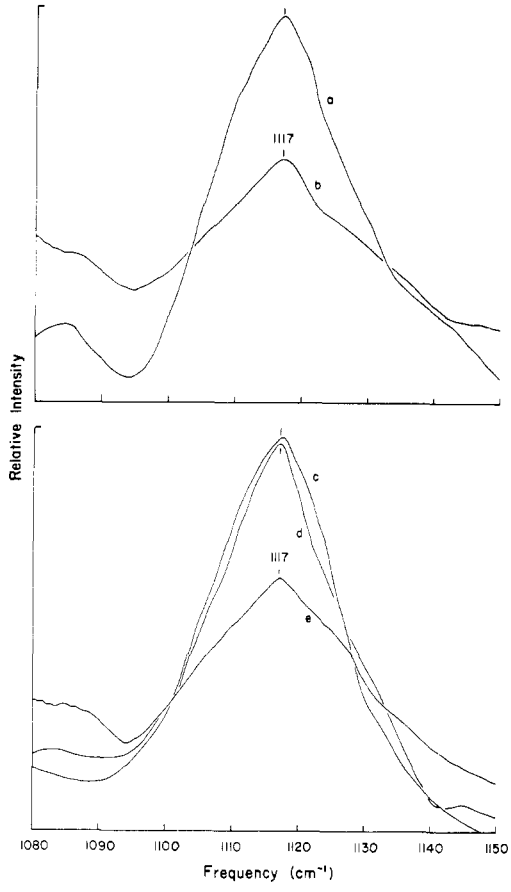

FIGURE 4: (a) The Raman spectrum of the vibration associated with the triphosphate moiety in a solution containing 20 m*M* ATP, 40 m*M* Tricine-maleate (pH 8), and 5 m*M* arsenate. (b) Same as a except 20 m*M* NaCl and 1 ml of dimethyl sulfoxide were added. (c) Same as a except Tricine-maleate and arsenate were deleted. (d) Same as a except Tricine-maleate was deleted. (e) Same as a except 1 ml of dimethyl sulfoxide was added.

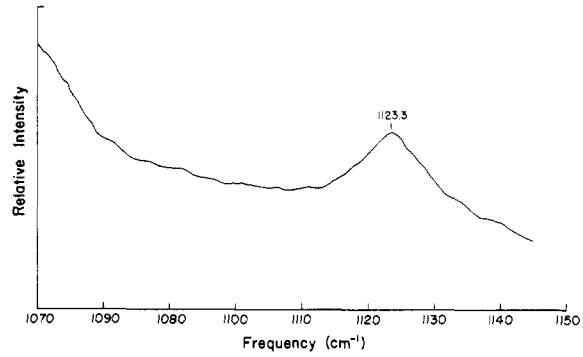

FIGURE 5: The Raman spectrum of the vibration associated with the phosphate moiety in a solution containing 20 m*M* AMP, 40 m*M* Tricine-maleate (pH 8), 5 m*M* arsenate, 20 m*M* $MgCl_2$, and 1 ml of dimethyl sulfoxide.

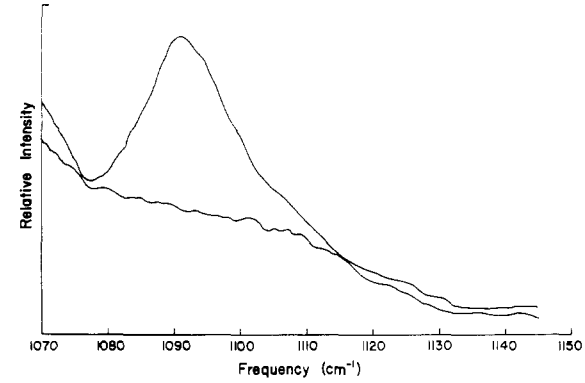

FIGURE 6: The Raman spectrum of the vibration associated with the diphosphate moiety in a solution similar to the one used to obtain the spectra in Figure 1 except that instead of 20 m*M* ATP, 20 m*M* ADP (upper curve) or 2 m*M* ADP (lower curve) was used.

dimethyl sulfoxide and the resulting spectra seem quite diffuse. In fact, although the peak of the band is still observed at 1123 cm^{-1} the shoulder at 1116 cm^{-1} cannot be detected. This indicates that the dimethyl sulfoxide aids the Mg^{2+} ions in forming a tight complex with the phosphate groups. Solutions of AMP show a similar band at 1123 cm^{-1} (Figure 5) which is definitely depolarized. In contrast the vibration of ATP solutions is polarized. Thus the vibration of AMP solutions appear to have an origin which is different from the similar vibration seen in ATP solutions. This explanation is supported by assignments made by Rimai et al. (1969) on the vibrational spectra of AMP, ADP, and ATP.

If dimethyl sulfoxide is added to a solution of ATP, Tricine-maleate, arsenate, and $MgCl_2$ (in essence if all the key ingredients are present for the phosphate transfer reaction) the spectrum seen in Figure 1 is observed. The spectrum looks very similar to that of ADP, the end product of the ATP transfer reaction (Figure 6, upper curve). However, it can be readily deduced from the rate of ADP formation (Nelson et al., 1972) that during the short time period required to take the spectrum (ca. 10 min) less than 5% of the ATP could have been converted to ADP. This amount of ADP could not be detected under the experimental conditions. When instead of ATP an amount of ADP corresponding to 10% of the ATP was added, no detectable vibrations at 1091 cm^{-1} were observed (Figure 6, lower curve). Moreover, when the scattering at 1091 cm^{-1} was monitored within 40 sec after addition of arsenate the counts per second have risen to their maximum value. At this time period only negligible amounts of ADP could have formed. Thus the intermediate formed with a band at 1090.5 cm^{-1} has spectral properties similar to ADP suggesting a complex formed between Mg^{2+} and the α and β phosphate of ATP with the γ phosphate available for transfer. This is also suggested by the splitting of the symmetric phosphate stretch (Figure 2) into two vibrations with intensity ratio of 2:1. Dimethyl sulfoxide appears to enhance the splitting in the vibrational frequencies of the free and bound phosphate group.

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Polyacrylamide Gels Copolymerized with Active Esters. A New Medium for Affinity Systems†

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ABSTRACT: A new and versatile method for linking biologically active ligands to a polyacrylamide matrix is reported. Active esters of acrylic acid (*N*-succinimidyl acrylate and *N*-phthalimidyl acrylate) were synthesized, then copolymerized with acrylamide and *N,N'*-methylenebisacrylamide. Displacement of the active ester in the gel thus formed by various ligands containing aliphatic amino groups resulted in the formation of stable amide bonds between the ligands and the polyacrylamide gel. The affinity gel thus prepared has the following advantages: (i) resistance to chemical and microbiological degradation, (ii) ease of control of ligand level and higher levels of ligand possible, (iii) ease of control of porosity, and (iv) total displacement of the active ester under suitable conditions. Efficacy of this system was tested by preparation of 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside derivative of polyacrylamide gel by the described method. It was found to be more effective for purification of wheat germ agglutinin than the previously published affinity chromatography systems and the wheat germ hemagglutinin was obtained in crystalline form. In addition, partial resolution of isolectins was obtained by elution from the affinity gel with a pH gradient.

Immobilization of biologically active components has become an important tool in biological research, especially in its application to affinity chromatography for purification of biological molecules. Some of the variables for such immobilization are the insoluble support used and the type of bonding of biologically active compounds to that support. The use and comparative advantages of agarose, cellulose, dextran, glass, and polyacrylamide as insoluble carriers have been reviewed (Silman and Katchalski, 1966; Cuatrecasas and Anfinsen, 1971; Scouten, 1974). The most popular among the systems currently in use are the polysaccharides (especially agarose beads) activated by cyanogen halide treatment to accept ligands with amino terminals. However, this system has the disadvantages that the polysaccharide is labile to attack by chemicals as well as microbes, and the bonds linking the polysaccharides and the biologically active compounds are alkali labile.

Some of these disadvantages were absent in the system developed by Inman and Dintzis (1969), in which commercially available preformed polyacrylamide beads (Bio-Gel) were modified to provide acyl azide and other active functional groups suitable for coupling ligands. Although this method led to high capacity of the carrier beads for the ligands, porosity was prohibitively reduced, greatly decreasing the effectiveness of the gel in macromolecule purification (Cuatrecasas, 1970; Steers et al., 1971).

In a preliminary investigation, we have successfully formed polyacrylamide gels containing desired ligands by first coupling acrylic acid to the amino terminal of the ligands, and then copolymerizing them with acrylamide and cross-linking reagent (Lee, 1973). This approach has an obvious advantage of allowing easy control of porosity as well as level of ligand incorporation. In addition, the total level of ligand that can be incorporated can be made considerably higher than by the use of CNBr-activated polysaccharides. In a similar approach that has been reported independently (Hořejší and Kocourek, 1974), allyl glycosides were copolymerized with acrylamide to form an affinity gel system useful for purification of plant hemagglutinins. Although these methods overcame most of the disadvantages discussed above, the necessity of synthesizing individual ligands containing a double bond discourages general application of this method.

The objectionable features of the previous methods were resolved in the present studies. The acrylic acid esters of *N*-hydroxysuccinimide and *N*-hydroxyphthalimide were synthesized and copolymerized with acrylamide and *N,N'*-methylenebisacrylamide. The resulting "active" gels reacted readily with ligands containing primary amino groups (Scheme I). Thus, the present method provides a new and versatile system of wider applicability than the earlier systems. The applicability of this system to affinity chromatography is demonstrated by efficient purification of wheat germ hemagglutinin on polyacrylamide gel in which 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside was incorporated by the new method.

Experimental Section

Materials. Acryloyl chloride and *N*-hydroxyphthalimide were purchased from Aldrich Chemical Co. and used with-

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