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USE OF PHOTOACOUSTIC FOURIER-TRANSFORM INFRARED SPECTROSCOPY TO STUDY PHOSPHATES IN PROTEINS

Donald J. Graves[1] and Siquan Luo

Department of Biochemistry and Biophysics,
Iowa State University, Ames, Iowa 50011

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SUMMARY: Photoacoustic Fourier-transform infrared spectroscopy was used to study phosphoamino acids and phosphoproteins. Using this method, we have found that the spectral properties of phosphate esters depend on the nature of the linkage, pH, and binding of metal ion. At high pH values, dianionic symmetric stretching of phosphotyrosine and phosphoserine occurs at 984 and 974 reciprocal centimeters, respectively. Analysis of the IR bands of bound phosphate in phosvitin at different pH values gives the pKa value for the esterified phosphates. Addition of aluminum ions to phosvitin at different pH values causes a shift in the phosphate band consistent with a direct binding of aluminum ions to the esterified phosphate. The phosphate signal for 40 μg of Pepsin (1 P/mole) is detectable by this method.

Enzymatic phosphorylation-dephosphorylation of seryl, threonyl, and tyrosyl residues in proteins is associated with the regulation of numerous biological processes of living systems. The incorporation of phosphate by changing the charge of a region can change interactions in the protein and interactions with ligands including other proteins. Much information about phosphoproteins and their binding interactions has been provided by X-ray crystallography and NMR spectroscopy (1), but there is a need for other methods that don't require crystalline materials and large amounts of protein.

[1] To whom correspondence should be addressed. Fax: 515-294-8961.

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Partial acid hydrolysis and phosphoamino acid analysis is used to determine whether a protein contains seryl, threonyl, and/or tyrosyl phosphate (2). This procedure is commonly used with proteins labeled with ^{32}P to identify reaction products of protein kinases. A drawback is, however, that this procedure will not give any information about endogenous nonradioactive phosphates or phosphate esters like aspartyl, histidyl, cysteinyl, or arginyl phosphate because of their acid lablility (3). Specific antibodies have found much use in the identification of phosphotyrosine in proteins (4). Fourier-transform transmission infrared spectroscopy can be used to identify phosphates in proteins and evaluate some of their characteristics, including pKa values, but unfortunately the method requires considerable sample (5).

We showed earlier that photoacoustic Fourier-transform infrared spectroscopy can give information on the secondary structure of proteins using less than 10 μg of protein (6). Here we report the use of photoacoustic Fourier-transform infrared spectroscopy to study phosphates in amino acids and proteins. The method can give information about the ionization state of phosphate esters of amino acids and proteins and binding of aluminum ions, a neurotoxic substance.

EXPERIMENTAL METHODS

Materials-Phosphoserine, phosphotyrosine, pepsin, and phosvitin were obtained from Sigma Chemical Co. Aluminum phosphate was purchased from Aldrich Co. All other chemicals were reagent grade.

Methods-Samples for infrared spectroscopy were prepared as a thin layer by spreading 10μl of protein solution (1-4 mg/ml.) or phosphate esters on a 7mm diameter polyethylene membrane disk made from the window of 3M disposable IR cards (product KC-0061). The membranes were treated first with methanol for a few seconds, washed with distilled water, and then allowed to dry at room temperature before application of the sample. Photoacoustic spectra were measured directly on these membrane-supported samples by using a Bio-Rad Model FTS-60A FT-IR spectrometer and MTEC Photoacoustic Model 200 photoacoustic detector (7). Two hundred fifty six cycles were accumulated at a frequency of 2.5KHz at 4 cm^{-1} resolution. Sample spectra were divided by a spectrum of carbon black to normalize for spectral variations due to the infrared source and spectrometer.

RESULTS AND DISCUSSION

A schematic for the photoacoustic Fourier-transform infrared spectrometer is shown in Fig. 1. By using an IR beam with intensity modulation, intensity can be made to vary in the acoustic frequency range. The light absorbed is detected by a slight heating of the solid sample. The heat oscillation in the sample can cause corresponding changes in the pressure of a gas which can be detected as sound by a microphone in the photoacoustic cell. Amplification of the signal and Fourier transformation yields the IR spectrum (6,7).

Phosphoamino acids and proteins were applied in the liquid state to the disk used in the photoacoustic cell and analyzed after the solvent had evaporated. Using samples containing 30μg of phosphotyrosine or phosphoserine, two phosphoamino acids found in proteins, strong signals were seen at 979 and 984 cm^{-1}, respectively, for these two amino acids obtained from solutions at pH 8.5 (Fig. 2A and 2B). A signal at about these frequencies has been previously attributed to symmetric stretching of the dianionic form (5). Phosphoserine and phosphotyrosine at pH 4.4 do not have these peaks but do have peaks at 1082 and 1085 cm^{-1} (Fig. 2A and 2B), respectively, which are likely due to symmetric stretching of the monoanion (5). Peaks around 1190 cm^{-1} at pH 4.4 for the two phosphoamino acids could be due to

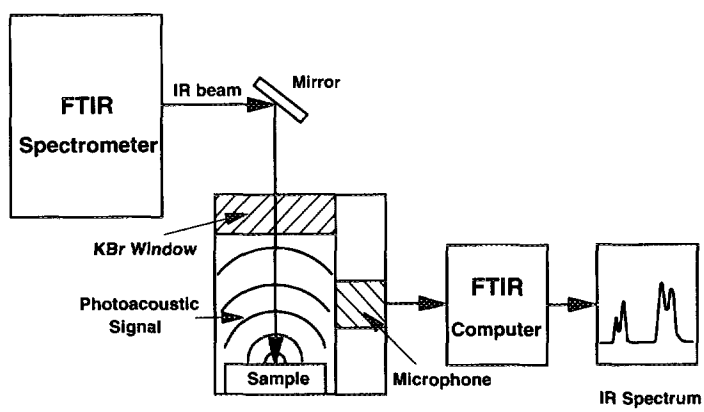


Fig.1. Schematic for Photoacoustic Fourier-transform infrared spectroscopy.

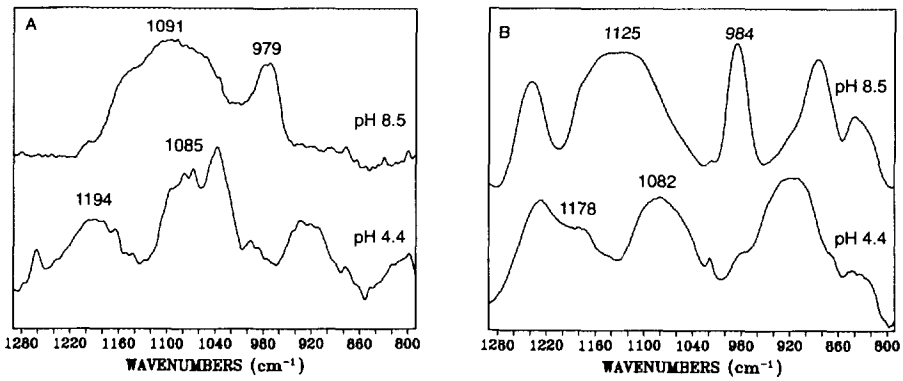


Fig.2. IR spectra for phosphoamino acids at pH 8.5 and 4.4. A, phosphotyrosine; B, phosphoserine.

antisymmetric stretching of the monanion (5). Other signals detected were not identified. Thus, its seems this method, like transmission IR spectroscopy in solution, distinguishes ionic states of the phosphate group and can be used to differentiate between seryl and tyrosyl phosphate.

The protein phosvitin (4mg/ml), which contains mutliply phosphorylated seryl residues, has been previously analyzed at different pH values by Fourier-transform transmission infrared spectroscopy (5). With the photoacoustic method and only 40 μg of sample, strong signals were obtained for phosvitin at high and low pH values (Fig. 3). A signal at 1002 cm^{-1} is seen for the dianionic form of the covalently

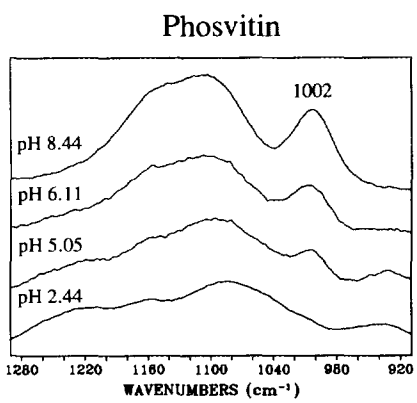
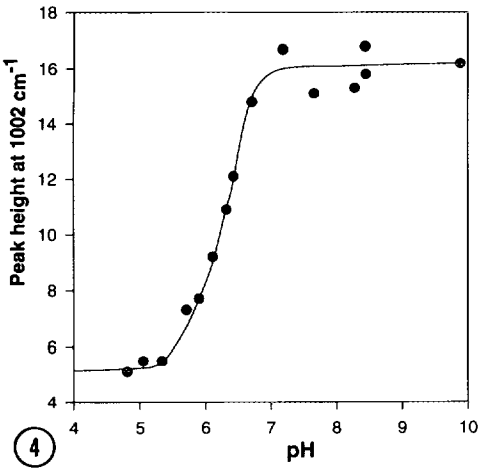


Fig.3. IR spectra of phosphate in phosvitin. A, Spectra at different pH values; phosvitin (4mg/ml.) in 50mM KCl at different pH values. 10μl of sample was applied to the disk.



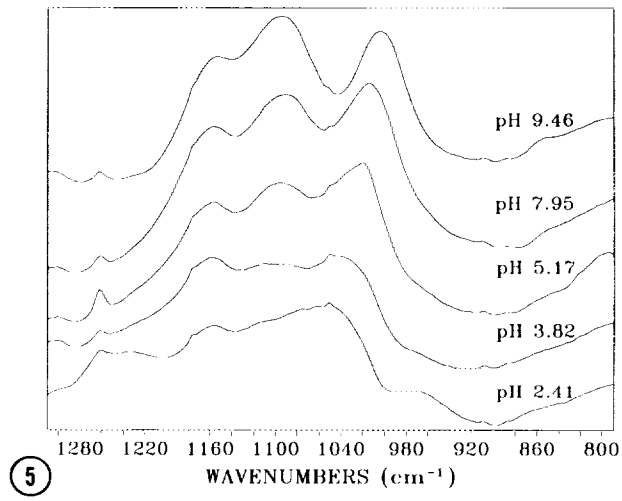


Fig.4. Titration curve of phosphate in phosvitin; conditions as in Fig.3.

Fig.5. Effect of aluminum ions on the IR spectral characteristics of phosvitin. 30 µl of phosvitin (0.4 mg/ml.) at different pH values in 50mM KCl and aluminum chloride (10^{-3} M) was applied to the disk.

bound phosphate in phosvitin. This interpretation is based upon the reduction of this signal with decreasing pH from 8.44 to 2.44. In Fig. 4, a titration curve was generated by measuring changes of the dianionic form as a function of pH. A pKa value of 6.3 was obtained by this method similar to a previously reported value by transmission IR spectroscopy (5). Therefore, we conclude, that photoacoustic IR spectroscopy can be used effectively to gain information about the ionization characteristics of phosphate esters in a protein.

Phosphate groups in proteins can interact with metal ions. To learn if the photoacoustic method could be used to examine such interactions, we studied a protein, phosvitin, which is known to interact strongly with metal ions through its phosphoryl groups (8). Aluminum ions interact strongly with phosphate, and its neurotoxicity may be connected with abnormal protein phosphorylation in the brain (9). Recently, phosvitin was used as a model to study effects of aluminum on a mutiphosphorylated protein (10). It was shown that aluminum ions influence the structure of phosvitin and inhibit dephosphorylation by acid phosphatase, but no proof

was provided that the aluminum ions interact with the phosphate groups. Fig. 5 shows the IR spectrum of phosvitin with aluminum ions. Note that the band at 1002 cm^{-1}, characteristic of the dianion (Fig.3), is present only at high pH. As the pH is lowered the phosphate band shifts to higher wave numbers. This is in contrast to the IR spectra without aluminum ions (Fig. 3) which show a progressive loss of this signal with decreasing pH. The band at 1002 cm^{-1} is also missing in solid samples of aluminum phosphate but is present in potassium phosphate (results not illustrated). We interpret these results to mean that aluminum ions interact directly with the phosphate groups in phosvitin.

Last, photoacoustic IR spectroscopy was applied to the study of pepsin, a protein containing a single phosphorylated seryl residue. The infrared spectra obtained with 40μg of sample showed a signal for the dianion at 987cm^{-1} although the signal was weak in comparison to phosvitin (results not illustrated).

In conclusion, photoacoustic IR spectroscopy can be used to distinguish serine phosphate from tyrosine phosphate. With phosvitin, a phosphoseryl-containing protein, it was used to determine pKa value and binding of aluminum ions. Although the method can detect small amounts of phosphate in protein as found with pepsin, photoacoustic IR spectroscopy may find better application in the study of multiphosphorylated proteins, e.g., Tau and its hyperphosphorylated forms in Alzheimer's disease (11). It is not known yet whether this methodology can differentiate between the various phosphate esters found in proteins.

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