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High-performance liquid chromatography in enzymatic analysis

Opening lecture

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ABSTRACT

During the previous two decades, high-performance liquid chromatography (HPLC) has proven to be an extremely useful technique with which to study the activity of enzymes and this paper will explore some of these uses. The success of the method can be seen not only from the increase in the number of papers utilizing this technique but also from the insights gained from its use on cellular phenomena. Given this success, it is no wonder that HPLC has become the technique of choice for many biologists seeking a more quantitative understanding of biological processes. Based on past experience, there is every reason to expect that the application of HPLC to the assaying of enzymatic activities will usher in another era of fundamental discoveries in the biological sciences. HPLC is particularly well suited to the assay of one activity in the presence of other activities obviating the need for extensive and tedious purification of biological samples. This advantage makes this technique particularly well suited to those who wish to use enzymes as markers for cellular processes, as indicators of metabolic activity and as evidence of gene function. To date, well over 100 activities have been assayed by this method. The method is particularly suited to problem-solving especially in such cases as when the presence of competing reactions prevents the recovery of the expected reaction products. Of the many applications, examples will be given on the use of HPLC for (1) monitoring the activity of an enzyme in a cell-free system, (2) monitoring the flow of metabolites through a multienzyme system and (3) the detection and study of new enzymatic activities. Some generalizations about the use of HPLC methods for the analysis of enzymatic activities will be presented.

INTRODUCTION

Among the various products of genes, enzymes are special in that they act as catalysts for those processes by which we define living systems. Given this central role, it is no wonder that an advance in the technology for measuring enzymatic activities usually paved the way for an advance in our understanding of cellular phenomena. Based on this past experience, we have every reason to expect that the application of high-performance liquid chromatography (HPLC) to enzymes will usher in another era of fundamental discoveries in the biological sciences.

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By 1987 nearly 100 activities had been measured by this method. And now, just three years later, this number has doubled and with each issue of the *Journal of Chromatography*, the number increases. The list of enzymes measured by HPLC includes those for the metabolism of carbohydrates, steroids, purines, porphyrins, amino acids, peptides and proteins and now DNA. Clearly, the method is very versatile. Some examples of this versatility are summarized as follows: HPLC is useful for (1) monitoring the activity of an enzyme in a cell-free system, (2) monitoring the flow of metabolites through a multienzyme system and (3) the detection and study of new enzymatic activities. In this paper some illustrations of these three points will be presented.

EVALUATING THE REGULATORY ROLE OF SECONDARY REACTIONS ON PRIMARY REACTIONS

Illustrative of the first point are the results of early studies which showed that it was possible to measure the activity of one enzyme in the presence of others. The reaction catalyzed by the enzyme under study has been referred to as the primary reaction and others as secondary reactions [1]. At that time secondary reactions were problems to be dealt with. With time, however, and the accumulation of data, a role for secondary reactions in regulating the primary reaction was suspected.

As an example, consider the following activities for the activation of sulfate. These are shown in Fig. 1 and include two primary reactions, catalyzed by ATP

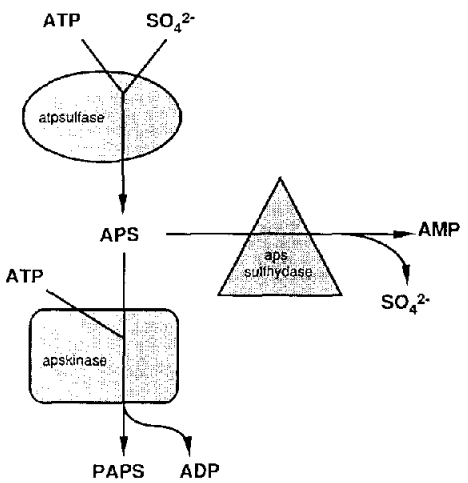


Fig. 1. Pathways for the evaluation of the role of secondary enzymatic reaction in the regulation of a primary reaction pathway. In the diagrammatic representation of the enzymatic reactions for activation of sulfate, two primary reactions are shown. The first catalyzed by ATP-sulfurylase (atpsulfase) and the second by APS kinase (apskinase). The secondary reaction catalyzed by sulfohydrolase (apssulfhydase), is also shown.

sulfurylase and APS kinase, and one secondary reaction, a sulfohydrolase. To study these reactions required the separation of the nucleotides AMP, ADP, ATP and the sulfate compounds adenosine 5′-phosphosulfate (APS) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) which was accomplished using an anion-exchange HPLC method and isocratic elution with a phosphate-buffered mobile phase containing sodium bicarbonate [2]. With such a chromatographic system it was possible to monitor the APS formed. An analysis of the reaction mixture at three time points, 30 s, 15 min and 45 min, is shown in Fig. 2 with the elution position of APS indicated by the arrow. The increase of APS is graphed on the inset panel. Because it was also possible to follow AMP during the incubation, it was demonstrated that the enzyme for the secondary reaction was active and, when tissues from several sources were compared, it was found that the level of activity depended on the source of the tissue [2]. These findings illustrate the point that with HPLC we can monitor single enzymes in a cell-free system and evaluate the importance of a secondary reaction to the overall regulation of a complex pathway.

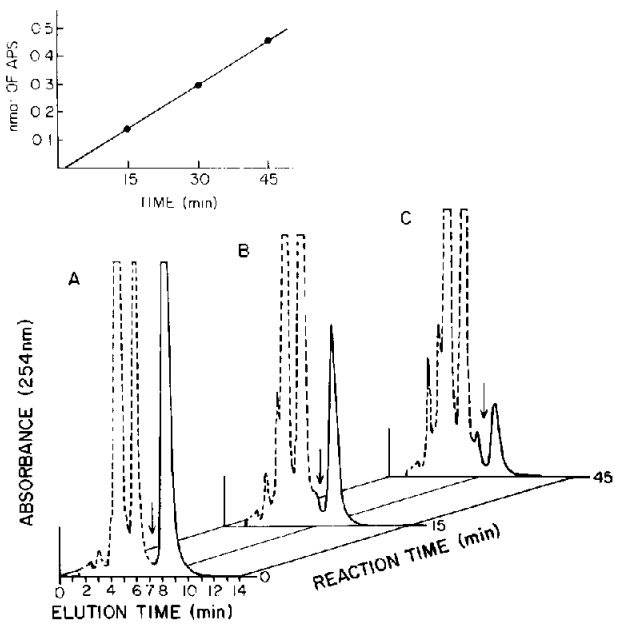


Fig. 2. Analysis of reaction mixture for the activities involved in the activation of sulfate. The separations were performed on an anion-exchange column eluted isocratically with a phosphate-buffered mobile phase containing sodium bicarbonate. Analyses of the reaction mixture at three time points, 30 s, 15 min and 45 min, are shown. The peak containing APS is indicated by the arrow. The increase in APS with time is shown graphically in inset panel. The elution time for AMP, the secondary reaction product which also increases with time, is about 4 min. (From ref. 2.)

MONITORING THE FORMATION OF MULTIPLE PRODUCTS DURING A REACTION

The second point mentioned above was that with HPLC we can monitor the flow of metabolites through a multienzyme system. In studies on metabolite flow HPLC is useful for several reasons. Firstly, HPLC can simultaneously monitor changes in the concentration of several compounds in the reaction mixture. Thus, several enzymes in a cell-free system can be followed. Secondly, we can use analogues in place of the natural substrates. This allows a specific metabolite to be followed through a multienzyme system. And thirdly, we can use HPLC to isolate the product of a reaction for characterization.

As an example of points 1 and 2, the processing of adenosine (Ado) through a multienzyme system will be discussed. Replacing Ado with fluorescent and radiochemical analogues allows for the study of the specific pathway by which Ado was metabolized.

One enzyme for Ado metabolism is adenosine kinase which catalyzes the phosphorylation of Ado according to the reaction shown in Fig. 3. ATP is one substrate, the phosphate donor, and Ado the second substrate, the phosphate acceptor. ADP and AMP are the reaction products. However, when working in cell-free preparations, other enzymes are able to form ADP and AMP including an ATPase and an apyrase. While it is an easy matter to separate these metabolites by HPLC, clearly the loss of ATP and the formation of AMP will not be sufficient to prove that the adenosine kinase is active.

To test for adenosine kinase activity in such a situation, Ado was replaced in the reaction mixture with the fluorescent analogue formycin (FoA) [3]. As illustrated in Fig. 4, in the presence of an adenosine kinase, the AMP analogue,

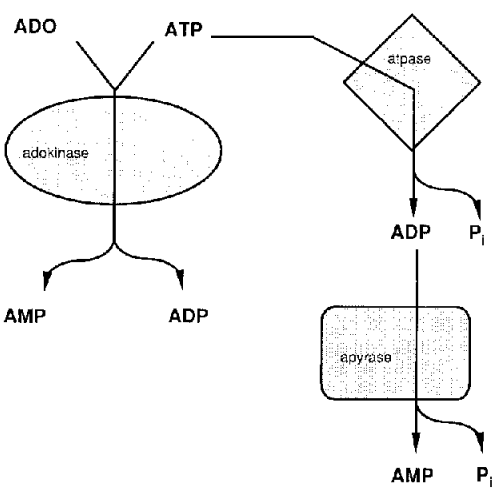


Fig. 3. Schematic used for monitoring the flow of adenosine (ADO) through several pathways. In the diagrammatic representation, three activities are shown. ADO kinase (adokinase), ATPase (atpase) and ADPase (apyrase).

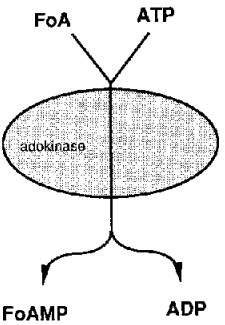


Fig. 4. Pathway for evaluation of metabolism of a substrate analog to monitor metabolite flow. The replacement of Ado with its analogue, formycin (FoA), results in the synthesis of the formycin analogue of AMP (FoAMP) as the reaction product of ADO kinase (adokinase).

formycin AMP (FoAMP), would be formed. The results of this experiment, shown in Fig. 5, in which the column effluent was monitored with a fluorescence monitor, illustrate the decline in FoA and the increase in FoAMP proving that the adenosine kinase was active [3].

But what of the fate of the AMP or in this case the FoAMP? A more complete analysis of the reaction mixture, shown in Fig. 6, indicated that the formycin-containing compound FoATP was present [4]. The presence of this compound suggested that FoAMP was processed by an AMP kinase in the reaction sequence shown in the right hand panel of Fig. 6.

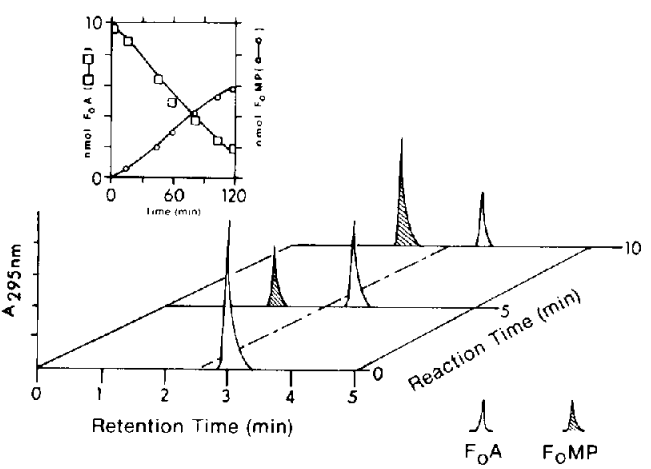


Fig. 5. Analysis of a reaction mixture for adokinase activity with FoA as the substrate at three times after the initiation of the reaction, 30 s, 5 and 10 min. The time-dependent decline in the substrate, FoA, and increase in the product, FoAMP (FoMP), are shown in the chromatogram and in graphical form on the inset. (From ref. 3.)

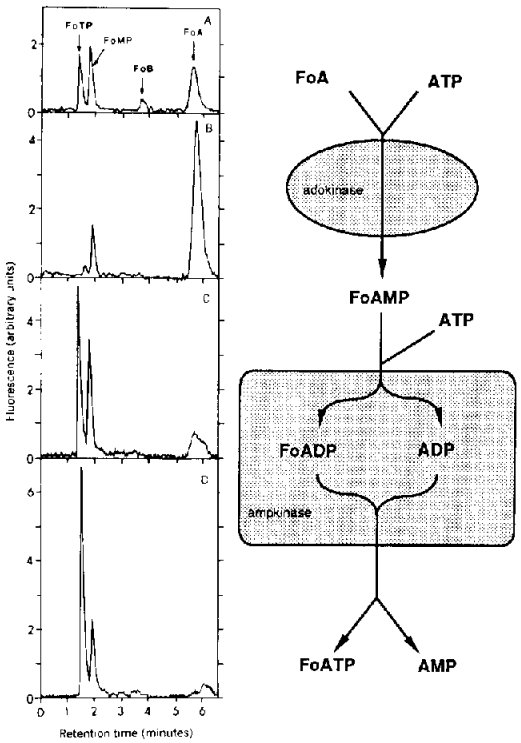


Fig. 6. Metabolic fate of the Ado analogue, FoA, following incubation with cell-free extract. Left panel: (A) chromatographic separation of several standards including FoA, the inosine analogue, FoB, FoMP and the ATP analogue, FoTP; (B–D) chromatograms of the reaction mixture at several times after the initiation of the reaction. Several compounds can be identified including FoMP and, with time, the formation of FoTP. (From ref. 4.) The right panel represents the enzymatic reactions implied to be present in the cell-free extract, adenosine kinase (adokinase) and AMP kinase (ampkinase).

However, as illustrated in Fig. 7, cell-free systems of this kind often contain an ATPase which can be an additional source of ADP obscuring the measurements of an AMP kinase activity. In order to evaluate the importance of any ATPase activity to the AMP kinase pathway, radiolabeled AMP was used as a substrate (indicated in Fig. 7 by the asterisk). After processing by AMP kinase, the formation of radiolabeled ADP and ATP would be expected. As illustrated in Fig. 8, unlabeled compounds are shown as open peaks and radioactive compounds as cross-hatched peaks. The figure shows an analysis of the reaction mixture at three times after the start of the reaction, 30 s, 5 min and 10 min. The compounds shown include AMP, ADP and ATP. Note the disappearance of radiolabeled AMP while radiolabeled ATP appears. There was very little change in the amount of unlabeled ATP. Note also the absence of radiolabeled ADP contrasting with the formation of unlabeled ADP. These findings were consistent with the

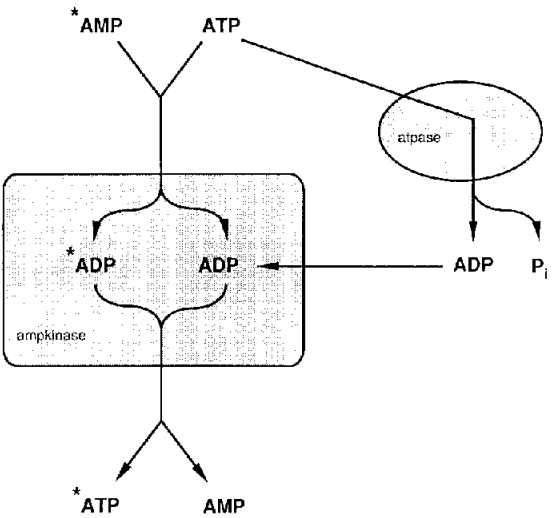


Fig. 7. Use of a radiolabeled analogue to evaluate secondary reactions. A schematic representation of the metabolic fate of radiolabeled AMP (*AMP) and unlabeled ATP in a cell-free exract. Phosphorylation of AMP by ampkinase and the formation of radiolabeled ATP (*ATP) is shown. Formation of additional unlabeled ADP by ATPase is also shown.

presence of those pathways shown in Fig. 7, where AMP kinase activity would account for the decline in labeled AMP and the formation of labeled ATP, while the ATPase activity would account for the formation of the unlabeled ADP [5]. Thus, using the HPLC method and analogues it is possible to monitor the flow of adenosine and ATP through a multienzyme system consisting of adenosine kinase, which forms AMP, AMP kinase, which forms ATP, and ATPase which forms ADP.

SYNTHETIC SUBSTRATES FOR DETECTION OF NEW ENZYMATIC ACTIVITIES

Turning now to one of the more interesting aspects of biological research, an interesting discovery is made in your laboratory and an enzyme is postulated as being responsible. The first problem in proving this postulate will be identifying a substrate which may not have been proviously isolated.

That was the situation in a study from my laboratory on chemotaxis when the existence of a chemoattractant containing an acid-labile phosphoamide bond was proposed [6]. As chemotaxis requires hydrolysis of the chemoattractant, we hypothesized that these cells would contain a phosphoamidase. Clearly to find this activity a substrate was needed and, because we had not yet isolated the true substrate, we synthesized one. The compound we synthesized is shown in Fig. 9. It is composed of two parts, lysine and AMP, with the nitrogen from the epsilon amino group and the phosphorous from the 5′-phosphate of the AMP forming

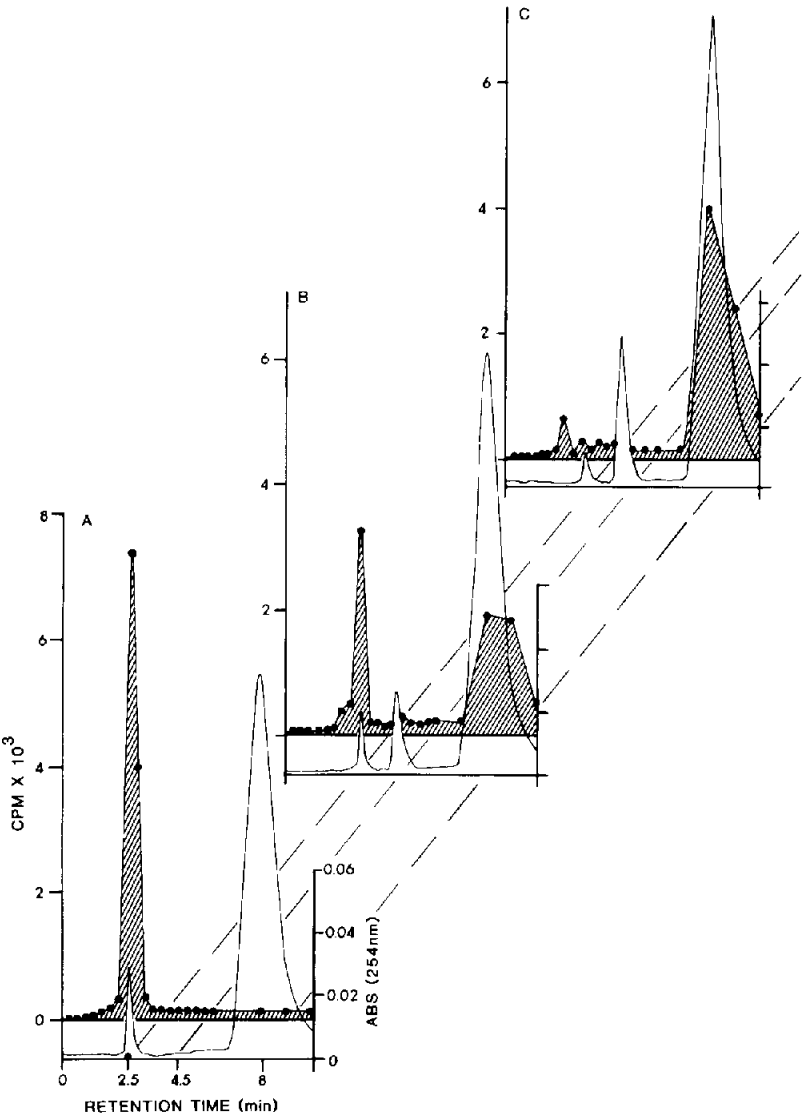
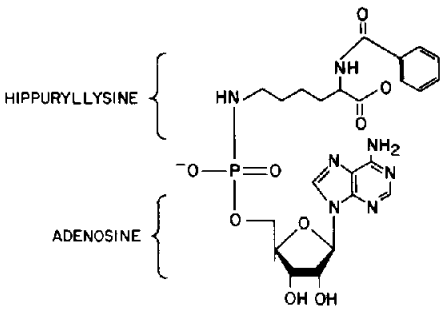


Fig. 8. Experimental results obtained with radiolabeled AMP (*AMP) and unlabeled ATP in cell-free extract. Representative chromatograms of the incubation mixture are shown at three time points after the initiation of the reaction, 30 s, (A), 5 min (B) and 10 min (C). The compounds, identified in the order of elution, are AMP, ADP and ATP. Unlabeled compounds are shown as open peaks and radiolabeled compounds are shown as cross-hatched peaks. (From ref. 5.)

the phosphoamide bond. Thus, phosphoamidase activity would generate lysine and AMP. As the formation of AMP could be monitored with an absorbance detector, we used hippuryllysine (HL) in order to monitor the formation of the other with the same detector. This compound, HLAMP as we called it, was added



HIPPURYLLYSYL(N-ε-5'-PHOSPHO)ADENOSINE

Fig. 9. Structure of the synthetic phosphoamidase substrate, hippuryllysyl (N-epsilon-5'-phospho) adenosine, HLAMP. The phosphoamide bond is formed by the condensation of the 5'-phosphate of the AMP and the nitrogen of epsilon amino group of the amino acid hippuryllysine in a carbodiimide mediated reaction.

to an incubation mixture containing a cell extract, and the results were monitored by HPLC. A representative profile of the reaction mixture at several times after the addition of the extract is shown in Fig. 10. Note the elution position of the substrate and, with time, the formation of the two reaction products hippuryllysine and AMP. These results indicated that a phosphoamidase was present in these cells.

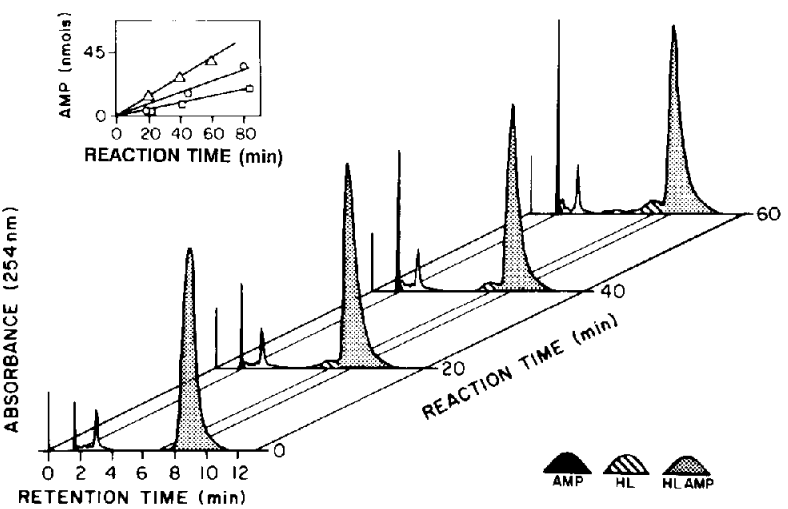


Fig. 10. Use of the synthetic substrate to detect a new enzyme. The chromatographic analyses of a reaction mixture following incubation of HLAMP with a cell-free preparation for 30 s, 20, 40 and 60 min are shown. Several compounds can be identified on chromatograms including HLAMP, AMP and hippuryllysine. The time-dependent loss of HLAMP is shown along with the increase in hippuryllysine and AMP. The rate of formation of AMP at three protein concentrations is shown in the inset. (From ref. 6.)

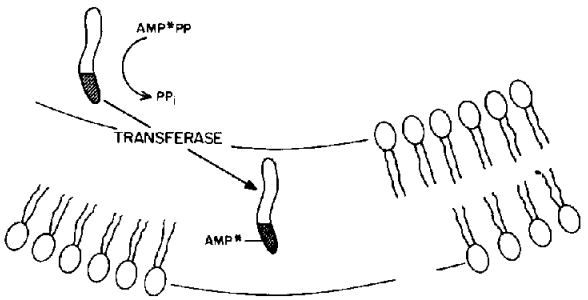


Fig. 11. Development of a model enzymatic pathway. A schematic representation of a membrane-bound reaction catalyzed by an ATP transferase in which the radiolabeled adenylyl moiety (AMP) of ATP, shown as AMP*PP, is transferred to a membrane-bound acceptor protein to form a radiolabeled (adenylylated) protein, the proposed natural substrate.

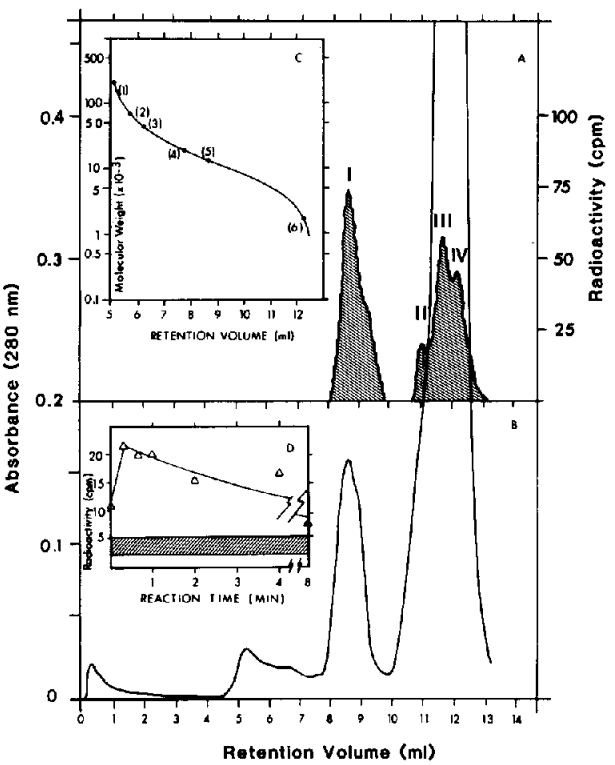


Fig. 12. Testing the model. Chromatographic analysis of reaction mixture containing [α-^{32}P]ATP and a membrane preparation. After incubation, the reaction mixture was fractionated by gel-permeation HPLC and the effluent analyzed for radioactivity (A) and absorbance at 280 nm (B). Peak I, the 13-kDa protein, peak II, the 1-kDa peptide, peaks III and IV contain nucleoside phosphates. (C) (inset on panel A) retention volume for standards used for calibration of column. Standards included (1) IgG, (2) bovine serum albumin, (3) ovalbumin, (4) myoglobin, (5) avidin monomer and (6) cyanocobalamin. (D) (inset on panel B) kinetics of formation of protein-AMP conjugate isolated in peak I. (From ref. 7.)

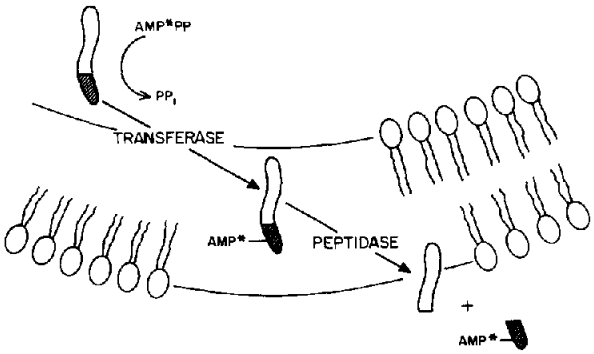


Fig. 13. Revising the model. A schematic representation of the reactions predicted by the results shown in Fig. 12. These include the ATP transferase and a protease to cleave the original reaction product to form a small, labeled peptide.

Given this success, our attention turned to those enzymes involved in the synthesis of the true chemoattractant. A membrane-bound ATP transferase was suspected which would catalyze the reaction shown in Fig. 11, where one substrate, ATP, would transfer its adenylyl group to an acceptor, a lysine residue in a protein. Clearly, what was needed to prove such a reaction was ATP, radiolabeled in the AMP moiety as shown by the asterisk on the AMP, and a method for following the formation of protein-bound radioactivity, that is to separate the unreacted ATP from any protein containing covalently linked radiolabeled AMP. Gel-permeation HPLC was used for this separation [7]. And, as shown in Fig. 12, by monitoring both absorbance at 280 nm and radioactivity, the location of protein, protein-bound AMP and any free nucleotides could be determined. In the first peak (peak I), the compound predicted was found, the AMP was covalently linked to a protein of about 13 kDa. Free nucleotides, including ATP, were found in peaks III and IV. However, there was also an additional, unexpected peak, peak II, whose further characterization showed AMP covalently bound to protein of about 1 kDa. The presence of this peak II necessitated a revision of the reaction scheme shown in Fig. 11 to that illustrated in Fig. 13. It was proposed that the second product, peak II, resulted from proteolytic cleavage of the first, peak I, to produce a smaller unlabeled protein and the smaller 1-kDa peptide containing the radiolabled AMP. Thus the use of the HPLC allowed for the detection not only of the originally hypothesized ATP transferase, but also to detect a second product and to predict a second activity.

CONCLUSION

HPLC is a method of great versatility for studies of enzyme chemistry. With this method it is possible to monitor single enzymatic reactions and to evaluate their relative importance and to study the regulatory role of a secondary reaction

in a cell-free system. The method also allows for the substitution of analogues for the natural substrates and the monitoring of the flow of metabolites through a multienzyme system. And finally, the HPLC method is useful in studies looking for new enzymes by providing a complete analysis of a reaction mixture which can lead to the discovery of new and unexpected reaction products.

ACKNOWLEDGEMENTS

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