AMINOACYL TRANSFER: CHEMICAL CONVERSION OF AN AMINOACYL ADENYLATE TO AN IMIDAZOLIDE

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SUMMARY:

N-Acetylglycyl adenylate anhydride has been shown to be readily converted in high yield to N-acetylglycyl imidazolide in the presence of excess imidazole at pH 7. The aminoacyl group can then be transferred from the imidazolide to become esters of mono- or polynucleotides. These observations suggest that histidine may be in the active site of the aminoacyl-tRNA synthetases, catalyzing the transfer of aminoacyl groups from the adenylate to tRNA.

INTRODUCTION:

The transfer of the acetyl group from the acetyladenylate anhydride to thiol groups using imidazole as a transfer agent was studied by Jencks and his co-workers (1,2). Their work showed that acetyl imidazolide was an activated intermediate in the transfer process. The formation of this compound was followed by observing its ultraviolet absorbance at 245 nm. An important implication of this work is that in the biological transfer of active acetyl groups, a histidine residue (i.e. imidazole side chain) may be at the active site of the enzyme involved.

We are interested in model peptide synthesizing systems that include the transfer of activated aminoacyl groups from adenylate anhydrides to polynucleotides and have explored the possibility that imidazole may

catalyze such transfers. Using two different methods, the work reported here shows that, under proper conditions, N-acetylglycyl adenylate anhydride can be converted in high yield to the N-acetylglycyl imidazolide. This work models the formation of activated intermediates of N-substituted amino acids from the adenylate anhydride, as in the initiation of protein synthesis (3). Subsequent papers will report the transfer of N-acetyl-aminoacyl groups to polyribonucleotides.

MATERIALS AND METHODS:

Preparation of the adenylate - N-acetylglycyl adenylate anhydride was prepared by the method of Berg (4) using dicyclohexycarbodiimide in aqueous pyridine. Using 2 millimoles each of N-acetylglycine (Sigma Lot 41C 3010) and 5'-adenylic acid (Sigma Lot 47B-7270), the product was dissolved in 20 ml of cold 0.1 N HCl and the pH adjusted to 3.5, filtered and then reprecipitated with 450 ml of acetone at -15° C. The final product was a white hygroscopic powder and was dissolved in 20 ml of cold distilled water. The resulting solution had a pH of 3.5 and was divided into 1.0 ml samples in screw cap test tubes and frozen at -70° C until use. The product was stable under these conditions.

The batch was assayed for active acyl using the hydroxamate assay of Lipmann and Tuttle (5) except that 10% $FeCl_3$-$6H_2O$ in 0.1 N HCl was used instead of 5%. The extinction coefficient used for the N-acetylglycyl hydroxamate was 57 as determined by Weber, et. al. (6), at 495 nm. The adenylic acid concentration was determined by measuring the absorbance of a diluted sample using $\varepsilon_{257} = 15.1 \times 10^3$.

Periodate assay - The spectrophotometric periodate assay method of Dixon and Lipkin (7) was used to estimate the amount of N-acetylglycyl adenylate ester present.

Stability of N-acetylglycyl imidazolide at various pHs - In the absence of excess imidazole, acetyl imidazolide is the most stable at about pH 7 and is extremely unstable at low and high pH (2). We carried

out similar experiments with N-acetylglycyl imidazolide by following the absorbance at 245 nm at 25° with the sample at various pHs in acetic acid buffer (pH 3.5 and 4.5) and phosphate buffer (pH 6 - 8.6). Buffer concentration was 10^{-2} M.

Conversion of N-acetylglycyl adenylate to the imidazolide - Two methods were used to study this conversion:

1) Absorbance at 245 nm. We had separately prepared N-acetylglycyl imidazolide by the method of Gottick et. al. (8) and found the product has an absorbance maximum at 245 nm with an extinction coefficient of approximately 1.7×10^3.

Using a Cary 15 spectrophotometer and cells with a 1 mm light path, 10λ of adenylate solution was added to the blank cell containing 1.0 ml distilled water and 10λ of adenylate solution was added to the sample cuvette containing 1.0 ml of imidazole solution of the desired concentration. Preliminary experiments were carried out to determine the pH of the imidazole solution required to give a final pH of 7.0 after addition of the adenylate. After adding the adenylate, the solution was shaken vigorously and the absorbance monitored at 245 nm until a constant reading was reached. Due to the high absorbance of the adenylic acid at 257 nm it was imperative that blank and sample contain the same amount of adenylate sample. The optimum adenylate concentration has about 1×10^{-3} M. These experiments were carried out at room temperature.

2) Acid decomposition of the imidazolides. Since the spectral method allows only low concentrations (0.001 M) of adenylate and we wished to eventually use higher concentrations (0.1 M), we had to develop a different method. This method was based on the fact that the imidazolide is very unstable (Fig. 1) at low pHs where the adenylate is stable. Consequently by running a hydroxamate assay for total active acyl (adenylate + imidazolide) and another on a sample (50λ) which had been dropped into 0.1 ml of 0.4 N HCl for two minutes we

could take the difference between the two values as the amount of imidazolide present. Correction was necessary for the dilution of the hydroxamate assay by the 0.1 ml of 0.4 N HCl.

RESULTS:

Production and analysis of adenylate - The N-acetylglycyl adenylate produced was obtained in approximately 50% of theory in several batches produced. The ratio of active acyl to adenylic acid was found to be approximately 1.0 in all batches of N-acetylglycyl adenylate. Based on periodate assay of the adenylate it was estimated to be 80% or more anhydride.

Stability of N-acetylglycyl imidazolide - The hydrolytic stability of the N-acetylglycyl imidazolide was found to vary considerably with pH as shown in Fig. 1 in which the rate constants for hydrolysis are

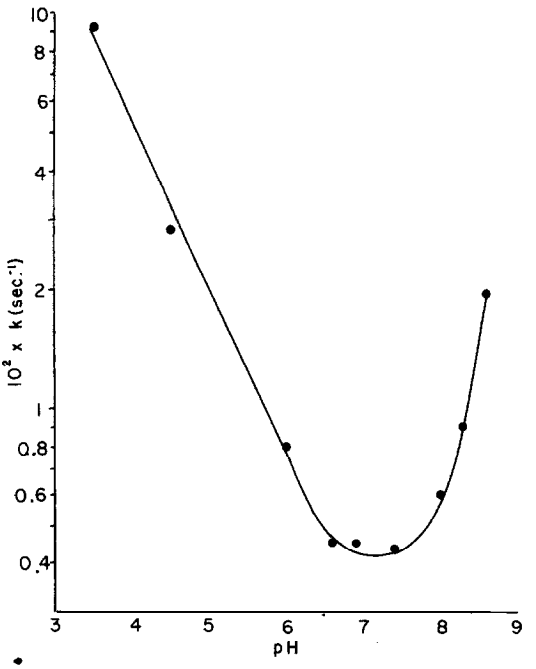

Fig. 1 Logarithmic plot of the rate constant (assuming first order rate law) for hydrolysis of N-acetylglycyl imidazolide as a function of pH at room temperature (25° C) and 0.01 M buffer concentration. The pH 3.5 and 4.5 samples were with acetic acid buffer and the rest were with phosphate. No attempt was made to maintain constant ionic strength.

plotted as a function of pH. The most stable region is obviously at about pH 7.0, the pKa of imidazole.

Conversion of N-acetylglycyl adenylate to N-acetylglycyl imidazolide - 1) Followed by spectral method at low concentrations (0.001 M). The absorbance at 245 nm (Fig. 2) due to the imidazolide being formed and then hydrolyzed showed very rapid increases and quite rapid decreases, presumably due to hydrolysis. Both the rate of appearance and the rate of disappearance were directly proportional to the concentration of imidazole

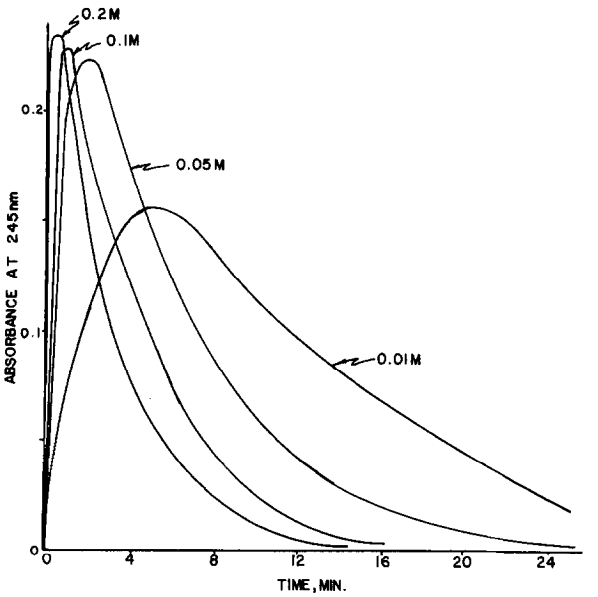

Fig. 2 Formation and disappearance of N-acetylglycyl imidazolide from N-acetyl glycyl adenylate (0.0014 M starting concentration) and imidazole (at various concentrations shown), pH 7.0 and room temperature (25° C). Reaction was followed by monitoring the absorbance at 245 nm. At imidazole concentrations of 0.05 M and greater conversions to imidazolide are near 100%. A Cary 15 spectrophotometer was used with 1 mm path length cells. The blank contained adenylate but no imidazole.

in reaction mixture. Based on the amount of active acyl initially present, the maximum conversion to imidazolide is approximately 94%. This high conversion is true at high ratios of imidazole to adenylate; however 0.01 M imidazole (i.e. a ratio of 10/1 imidazole to adenylate) showed the maximum amount present at the peak to be considerably less.

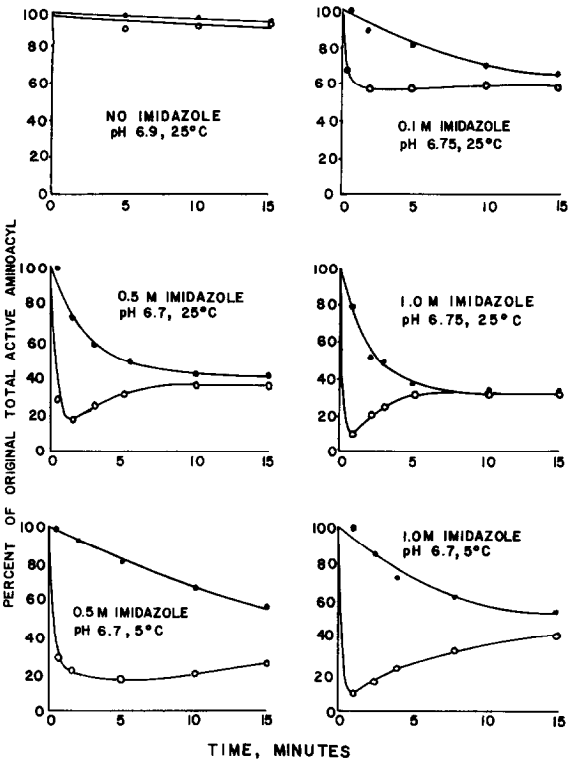

Fig. 3 Formation of N-acetylglycyl imidazolide from N-acetylglycyl adenylate and imidazole at various imidazole concentrations. The upper curve in each case is total active acyl (adenylate plus imidazolide). The lower curve is active acyl remaining after treatment at low pH to decompose the imidazolide. The initial N-acetylglycyl adenylate concentration was 0.1 M. The area between the two curves represents imidazolide. The active acyl was determined by hydroxamate method of Lipmann and Tuttle (5).

2) Assayed by acid decomposition of the imidazolide. At higher adenylate concentrations (0.1 M), higher concentrations of imidazole are required for good conversion to the imidazolide. The data in Fig. 3 show the conversion to be dependent on both the imidazole concentration and the temperature. The best conditions for maintaining high levels of imidazolide over long periods using 0.1 M adenylate was with 0.5 M imidazole and at 5° C.

DISCUSSION:

The transfer of N-blocked aminoacyl and free aminoacyl groups from

their adenylate anhydrides to the 3' terminal adenosine residue on tRNAs is an important aspect of protein biosynthesis. This transfer is catalyzed by the aminoacyl synthetases. We would like to understand the mechanism by which such transfers take place. Since Jencks, et. al. (2) had shown that imidazole could catalyze the transfer of acetyl groups from the adenylate anhydride to thiol groups, we felt the same might be true of N-acetylaminoacyl and aminoacyl groups. The data presented here show quite conclusively that N-acetylglycyl adenylate can be converted to N-acetylglycyl imidazolide. The yield is a function of the ratio of imidazole to adenylate and is also concentration dependent as shown by the fact that at 0.1 M adenylate a five fold excess of imidazole gives about 90% conversion to the imidazolide but at 0.001 M adenylate a tenfold excess of imidazole gives conversions in the range of 70%. The spontaneous formation of the imidazolide from the adenylate anhydride, makes it seem likely that the aminoacyl synthetases contain a histidine in the active site.

Jencks (1) had observed the formation of acetyladenylate ester from the anhydride. The data in Fig. 3 show that this is also true with N-acetylglycyl adenylate. The lower curves in the figure represent acid stable active acyl. At high levels of imidazole, the acid stable active acyl drops to a low level. With time the acid stable active acyl rises and in most cases becomes the same as the total active acyl (upper curves). These data show that the acid unstable imidazolide is being converted in time to an acid stable active acyl compound. The most logical candidate for the final product is the aminoacyl adenylate ester. Periodate assay does show decreases in the amount of periodate positive material although we have not yet explored this aspect quantitatively. The ester is periodate negative.

If the active acyl plateaus, reached in the presence of imidazole, are due to the adenylate ester, the data imply that the imidazole will

not catalyze the decomposition of the ester. This in turn indicates that the imidazolide does not form from the ester. Consequently, it would appear that peptidyl transfer in protein synthesis is not mediated by an imidazolide intermediate. That is, the activity of peptidyl transferase may not depend on a histidine residue in the active site. However, if the peptidyl group is reactivated to an anhydride form of GTP or ATP, then peptidyl transfer could be mediated by histidine. Most workers in the field of protein synthesis do not feel that the peptidyl group is reactivated before peptide bond formation (9).

The data presented here are consistent with histidine mediated transfer of N-acetylaminoacyl groups from adenylate anhydrides to the -OH groups of adenosine via an imidazolide intermediate, but do not indicate further imidazole catalyzed transfer from the adenylate ester unless reactivation takes place. The amino acid sequence of the active site of aminoacyl tRNA synthetases has not been determined, however, Bruton and Hartley (10) have isolated a histidine containing octapeptide from methionyl tRNA synthetase. This peptide is possibly contained in the active site but absolute evidence is not at hand to support such a conclusion.

We are presently studying the transfer of N-acetylamino acids from the adenylate to polynucleotides as catalyzed by imidazole.

ACKNOWLEDGEMENT

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