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CHEMICAL MODIFICATION OF AMINOACYL LIGASES AND THE EFFECT ON FORMATION OF AMINOACYL-tRNA

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SUMMARY

Fluorodinitrobenzene (FDNB), Woodward's reagent K, and cyanate were reacted in separate experiments with a crude mixture of *Escherichia coli* aminoacyl-RNA ligases. Each of these reagents was found to induce selective inhibition of the capacity of certain of these enzymes to esterify ^{14}C-labeled amino acids to transfer RNA's. In particular, on treatment with FDNB, the arginyl-tRNA ligase activity was inhibited 88%, using conditions under which the lysyl-tRNA ligase activity was inhibited 20%. These ligases were amenable to purification, and this same preferential inhibition of the arginyl-tRNA ligase as compared to the lysyl-tRNA ligase was found to occur on treatment of these purified ligases with FDNB. The differential inhibitory effects on individual aminoacyl-RNA ligases varied from one reagent to another.

INTRODUCTION

The last few years have witnessed the elucidation of the triplet code which is responsible for the ordering of the primary structure of proteins. A fascinating, unknown recognition system, however, remains to be discovered between the transfer ribonucleic acid (tRNA) molecules within a single biological species and their respective aminoacyl ligases. Since it is at this point that an activated amino acid becomes attached to the correct tRNA molecule, to which it surrenders its identity in the later base-pairing step in polypeptide synthesis, the primary importance of this recognition scheme between the tRNA and the protein macromolecule is evident. Equally clear is our lack of information on the mechanism of the "recognition reaction". A plausible type of interaction between these two different kinds of macromolecule would appear to be hydrogen bonding of certain nucleic acid bases with side chains of particular amino acids in the protein. Besides hydrogen bonding, there is the possibility of π–π bond interactions and other hydrophobic forces.

For the above reasons we have begun to study the effect of chemical alteration

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of specific amino acid side-chains of the aminoacyl ligases on the aminoacyl-tRNA esterification process. Of the many possible protein "active-site" reagents among which to choose, a few were selected for preliminary exploration. The initial choice was guided by the degree of mildness of the reaction conditions (*i.e.* aqueous medium, pH near neutrality, temperature 37° or below, and a relatively short reaction time), and a known degree of specificity of the reagent in comparable situations. Our purpose was to conduct a preliminary screening test of active-site reagents for their ability to inhibit certain aminoacyl ligases preferentially, when reacted with a crude *Escherichia coli* enzyme mixture of all the ligases. The suggestion would then emerge, if a particular ligase were severely inhibited while others were spared, that the reagent was reacting with an amino acid side-chain related to one of the two reactive sites of that enzyme (*i.e.* the activation site or the recognition site). Such an inference would not necessarily be correct, but would serve as a guide for further studies pointed towards identification of the site involved, using a purified aminoacyl ligase of the type inhibited. We communicate herein some preliminary investigations which employ both a crude mixture of ligases and highly purified specimens of two of the aminoacyl ligases, with results which are comparable for both situations.

The pursuit of this initial goal in a mixed aminoacyl ligase system is a complex one, which requires testing the inhibitory effect on the initial catalytic rate of each enzyme, rather than obtaining a figure which might represent a final plateau value. It is necessary also to test a variety of concentrations of the site reagents and reaction times if one is to define conditions which will inhibit one aminoacyl ligase and spare another completely. After extensive trials of a number of reagents and of experimental circumstances, the results with three reagents: (1) fluorodinitrobenzene, (2) Woodward's reagent K, and (3) cyanate show some signs of promise, particularly for the first-mentioned.

MATERIALS AND METHODS

Amino acid acylation of tRNA (ref. 1)

(A) Amino acid + ATP + $E \rightleftharpoons$ aminoacyl-AMP·E + PP_i
(B) Aminoacyl-AMP·E + tRNA $\rightleftharpoons$ aminoacyl-tRNA + AMP + E

The reaction mixture (0.1 ml) contained: Tris–HCl, pH 7.5 (4 μmoles); $MgCl_2$ (0.5 μmole); KCl (4 μmoles); ATP (0.4 μmole); [^{14}C] amino acid (0.01 μmole), containing approx. 330 000 counts/min; *E. coli* tRNA (0.05 mg); and usually 5 μl enzyme, the latter being first diluted in bovine serum albumin (1 mg/ml). The assay measures the conversion of radioactive amino acid to aminoacyl-RNA. The reaction was started by the addition of enzyme, the concentration of which had been adjusted so as to be limiting, and the mixture was incubated at 37° for various times. With a given crude enzyme preparation, prior to definitive assay, determination of the limiting concentration of each of the aminoacyl synthetases to be tested later was first carried out. Thus the protein concentration of the crude enzyme preparation employed for assay of a particular aminoacylation varied, depending on the amino acid to be tested. Three time points within the first 15 min *plus* a zero time control were used for each rate determination. The reaction was stopped by the addition of cold 5% trichloroacetic acid (5 ml). The precipitate was collected on a standard type AA,

24-mm diameter Millipore filter, washed with 5% trichloroacetic acid (5 × 5 ml), dried for 10 min at 60°, and the radioactivity counted using a Nuclear-Chicago end-window gas-flow counter. Under these conditions the reaction was directly proportional to the enzyme concentration and to the time of incubation, during the first few minutes (10–15 min). One unit of activity is equivalent to the charging of tRNA with 1 mμmole of amino acid in 10 min at 37°.

ATP-pyrophosphate exchange reaction (this is the reverse of Reaction A above) (ref. 2)

The reaction mixture (0.1 ml) contained: Tris–HCl, pH 7.4 (10 μmoles); $MgCl_2$ (1.0 μmole); ATP (0.2 μmole); $Na_4{}^{32}P_2O_7$ (0.2 μmole, 10^4 counts/min per μmole); KF (1.0 μmole); L-amino acid (0.2 μmole); tRNA (0.05 mg); and enzyme. The enzyme was diluted (as described above) in bovine serum albumin (1 mg/ml).

The assay measures the incorporation of ^{32}P of inorganic pyrophosphate into ATP, which is adsorbed on charcoal. The reaction was started by the addition of enzyme, the concentration of which was adjusted so as to be limiting, and the mixture was incubated at 37° for various times. The reaction was stopped by addition of 7% $HClO_4$ in 0.2 M $Na_4P_2O_7$ (0.1 ml), followed by the addition of a suspension of Norit-A (0.5 ml) containing 30 mg/ml. The contents of the tube were collected on a Type-AA Millipore filter, washed with cold water (6 × 10 ml) and the radioactivity counted as described above. A 0.06% solution of polyvinylpyrrolidone (0.3 ml) was added to each filter after washing, so that the charcoal could adhere to the filter after drying. Under these conditions the reaction was directly proportional to the enzyme concentration and to the time of incubation during the first few minutes (10–15 min). A blank reaction without amino acid was run and its value subtracted from that of each sample. Enzyme activity is expressed as the number of mμmoles of pyrophosphate incorporated into ATP per mg of enzyme protein in 10 min at 37°.

Isolation of a crude mixture of ligases

All procedures were carried out at 0–5° and the tRNA acylation reaction was used for the enzyme assay system. *E. coli* strain B (late log-phase) cells from General Biochemicals were disintegrated in a Waring Blendor, in a medium consisting of 0.01 M Tris–HCl (pH 7.5); 0.01 M $MgCl_2$; 7 mM KCl (ref. 3). The supernatant solution was separated by centrifugation (30 000 × *g* for 40 min). Nucleic acids were removed from the crude extract by the addition of 50% streptomycin sulfate to the above medium to give a final concentration of 10 mg/ml of extract. The precipitate was removed by centrifugation (15 000 × *g* for 10 min) and discarded. To concentrate the solution, $(NH_4)_2SO_4$ (Merck reagent grade) was added to the streptomycin sulfate supernatant with stirring to give a 70% saturated solution at 0°. After 30 min the precipitate was collected by centrifugation (30 000 × *g* for 15 min). The precipitate was then dissolved in buffer and dialyzed extensively against the same solution. All dialyses were carried out overnight at 5°, with at least 3 changes of 2–4 l each of buffer as indicated. Protein concentrations were measured by the Lowry method[4].

Purification of L-*arginine-tRNA ligase (EC class 6.1.1) (arginyl synthetase)*

Initial procedure. The starting material for both the arginyl and lysyl enzymes was a partially purified extract from *E. coli* strain B cells (late log-phase) (5 lbs) kindly supplied by our colleague, Dr. C. M. JANEWAY. The procedure of STERN *et al.*[3,5]

had been followed for the initial extraction and purification, and is therefore not given below in complete detail. In general, the initial enzyme purification procedure is also similar to the earlier one of BERGMANN, BERG AND DIECKMANN[6], and to the contemporary purification of lysyl-tRNA synthetase by KALOUSEK AND RYCHLIK[7].

The cell extract had been treated with streptomycin sulfate to remove nucleic acid, followed by adsorption of the lysyl synthetase activity on calcium phosphate gel. Approx. 30% of the arginyl synthetase activity* was also adsorbed onto the gel in this step (designated for convenience as "step C"), and it was this portion of the arginyl activity that was purified as a byproduct of the lysyl enzyme purification. The purification of the arginyl activity which remained in the supernatant after treatment with calcium phosphate gel was carried out separately and is not reported in detail here. It was in brief found that all of the arginyl synthetase activity could be obtained from the original nucleic acid-free extract by taking a $(NH_4)_2SO_4$ cut (55–68% saturation), followed by adsorption onto calcium phosphate gel (gel:protein ratio of 0.58)*.

The calcium phosphate gel in step C was washed with water, and the enzymes were eluted with 0.05 M potassium phosphate (pH 7.5). The arginyl- and lysyl-synthetase activities in an aliquot of this eluate were separated by chromatography on DEAE-Sephadex A-50, using a linear gradient from 0.15 M potassium phosphate buffer (pH 7.0) to 0.35 M potassium phosphate buffer (pH 6.5)[5]. The arginyl synthetase activity emerged with the bulk of the protein at the beginning of the gradient, while the lysyl synthetase activity emerged later. The portion of the DEAE-Sephadex column eluate containing the arginyl synthetase activity was used as the starting material for further purification.

This arginyl enzyme fraction (900 ml containing 0.35 mg protein per ml) was brought to 70% saturation with $(NH_4)_2SO_4$ (430 g) and after standing for 1 h the precipitate was collected by centrifugation (10 000 × *g* for 20 min). The precipitate was dissolved in 50 ml of 0.05 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA and was dialyzed against the same buffer. The material (53 ml; 227 mg of protein) had a specific activity, in the aminoacyl esterification assay, of 550 units of arginyl enzyme per mg protein.

Chromatography on DEAE-Sephadex. The dialyzed concentrate was adsorbed on a column of DEAE-Sephadex A-50 (87 cm × 1.6 cm) previously equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA. The column was washed with the same buffer (120 ml) using a flow rate of 2 ml/min. A linear gradient from 0.1 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA to 0.25 M sodium phosphate buffer (pH 6.5) and 1 mM EDTA in 3 l was run at 1.6 ml/min. 16-ml fractions were collected, and the bulk of the arginyl activity was found in fractions 76–115. These fractions (636 ml) were combined and dialyzed against water, then concentrated about 2-fold by reverse sucrose dialysis. The material (255 ml containing 0.18 mg protein per ml) had a specific activity of 2400 units/mg (step yield 88%).

Adsorption and elution from alumina. A suspension of alumina Cγ gel (total dry weight 724 mg) was added to this material (245 ml, 44 mg protein). After 15 min

* M. L. STEPHENSON, personal communication.

the gel was removed by centrifugation (2000 × *g* for 10 min), washed with water (2 × 40 ml) and the enzyme was eluted with 0.1 M sodium phosphate buffer (pH 7.5) and 1 mM EDTA (3 × 40 ml). The eluates were combined and concentrated by reverse sucrose dialysis and dialyzed against 0.02 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA. The concentrated protein (16.8 ml containing 0.80 mg protein per ml) had a specific activity of 5800 units/mg (step yield 74%). Analytical polyacrylamide-gel electrophoresis, kindly carried out by our colleague Dr. L. SHUSTER[8], showed a main band and 5 minor components.

Chromatography on Sephadex. A portion of this material was concentrated (5.3 ml, containing 9.7 mg of protein and $5.6 \cdot 10^4$ enzyme units) and was applied to a column of Sephadex G-100 (90 cm × 4 cm) previously equilibrated with 0.02 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA. The protein was eluted, using the same buffer, at a rate of 20 ml/h; and after the void volume of 210 ml was reached, fractions of 5 ml were collected. Arginyl synthetase activity was found in a symmetrical peak in fractions 20–33. These were combined, concentrated by reverse sucrose dialysis, and dialyzed against 0.02 M sodium phosphate buffer (pH 7.0) and 1 mM EDTA. The final solution (15 ml containing 0.17 mg protein per ml) had a specific activity of 11 400 units/mg (step yield 52%) and gave a single band on polyacrylamide-gel electrophoresis.

This solution was used as the purified arginyl synthetase in the chemical modification experiments described later. The enzyme was frozen in liquid nitrogen and kept at −70°. It retained its activity over a period of several months and was stable to thawing and refreezing.

Preparative polyacrylamide-gel electrophoresis was also used by Dr. SHUSTER[8] to purify the protein to a single band.

Purification of L-*lysine-tRNA ligase (EC 6.1.1.6) (lysyl synthetase)*

The starting material for the purification consisted of a portion of the 0.05 M potassium phosphate buffer (pH 7.5) eluate from the calcium phosphate gel treatment of the original *E. coli* extract, following step C. The purification procedure was a modification of that described in detail by STERN AND MEHLER[3,5], and was also similar to that of KALOUSEK AND RYCHLIK[7]. The steps consisted of chromatography on DEAE-Sephadex A-50, chromatography on Sephadex G-100, alumina Cγ gel adsorption–elution, and preparative polyacrylamide-gel electrophoresis. Following the last-mentioned procedure, kindly carried out by Dr. L. SHUSTER[8], the lysyl synthetase had a specific activity of 540 units/mg (considerably less than that of later preparations of this purified enzyme), and traveled as a single band on analytical polyacrylamide-gel electrophoresis.

The material from the preparative polyacrylamide-gel electrophoresis was used as the purified lysyl synthetase in most chemical modification experiments. This enzyme preparation was frozen in liquid nitrogen and kept at −70°. It retained its activity over a period of several months and was stable to thawing and refreezing.

EXPERIMENTAL

Effect of FDNB on aminoacyl ligases

Experiments on crude mixed ligases. The crude mixed ligase (2.5 ml containing

15 mg protein per ml) in 0.1 M potassium phosphate buffer (pH 7.0) was treated with 5 μl of FDNB (40 μmoles) at 25° for 15 and 60 min. After removal of the excess reagent by extensive dialysis against buffer (0.01 M Tris, pH 7.0, 0.01 M $MgCl_2$, 0.007 M KCl), the enzymatic activity of the various ligases was measured. As a control, a solution of enzyme (15 mg protein per ml) in 0.1 M potassium phosphate buffer (pH 7.0) was kept at 25° for 15 min, then dialyzed against buffer.

When the mixed ligase system was treated with FDNB at pH 7.0 for 15 min, the arginyl, aspartyl, histidyl and leucyl enzymes lost 88–99% of their activities, while the lysyl enzyme retained 80% of its activity (see Table I). It may be seen that the reaction with FDNB for 60 min resulted in extensive loss of activity for all enzymes tested.

Effect of FDNB on purified arginyl ligase. A solution of purified L-arginine-tRNA ligase (10 μl containing 1.67 μg protein) was treated with 40 μl of a saturated solution of FDNB, 0.08 μmole in 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA at 25° for 15 min. A saturated solution of FDNB at 25° is approx. 2 mM[9]. Bovine serum albumin (20 μl of a 1 mg/ml solution) was added and the mixture placed

TABLE I

INHIBITION OF AMINOACYL LIGASE ACTIVITY BY FLUORODINITROBENZENE

Amino acid	*Rate of labeling of RNA (mμmole amino acid/min/mg protein)*			*% retention of activity a/c*
	(c) Control reaction	*(a) Reaction with FDNB (15 min)*	*(b) Reaction with FDNB (60 min)*	
Alanine	0.98	0.57	0.04	58
Arginine	2.6	0.30	0.04	12
Aspartic acid	0.58	0.02	0.02	3
Histidine	0.99	0.05	0.04	5
Leucine	7.1	0.04	0.02	1
Lysine	1.5	1.2	0.10	80
Serine	4.9	2.6	0.05	53
Valine	2.8	1.4	0.40	50

on a column of Sephadex G-25 (3 × 0.5 cm) previously equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA. The enzyme was eluted with a solution of albumin (1 mg/ml) in 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA and the first 1.0 ml collected. Under these conditions the free FDNB still remained on the column.

A control solution of L-arginine-tRNA ligase was treated similarly. Quantitative recovery of enzyme without loss of activity was obtained off the column. The eluates of the FDNB-treated and control enzymes from the Sephadex columns were assayed for their ability both to catalyze the acylation of tRNA with [^{14}C]-arginine, and the incorporation of ^{32}P of inorganic pyrophosphate into ATP.

In the acylation assay, the control had a specific activity of 15 600 units/mg enzyme protein while the FDNB-treated enzyme had a specific activity of 425 units/mg, indicating a 97% loss of activity when assayed for the acylation reaction. In

the pyrophosphate exchange reaction the control enzyme had a specific activity of $3.6 \cdot 10^5$ units/mg, while the FDNB-treated material had a specific activity of $1.6 \cdot 10^3$ units/mg resulting in a 99.5% loss of activity.

Effect of FDNB on purified lysyl ligase. A solution of the purified L-lysine-tRNA ligase (5 μl containing 1.89 μg protein) was treated with 40 μl of a saturated solution of FDNB, 0.08 μmole in 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA at 25° for 15 min. Bovine serum albumin (20 μl of a 1 mg/ml solution) was added and the mixture placed on a column of Sephadex G-25 (3 cm × 0.5 cm) as described above. The column was eluted with a solution of albumin in 0.1 M potassium phosphate buffer (pH 7.0) and 0.001 M EDTA and the first 1.0 ml collected.

As a control a solution of L-lysine-tRNA ligase was similarly treated, and was quantitatively recovered from the Sephadex column. The eluates were assayed for their ability to catalyze both the acylation of tRNA with [^{14}C]lysine, and the incorporation of ^{32}P of inorganic pyrophosphate into ATP.

When assayed for the acylation reaction, the control had a specific activity of 1450 units/mg enzyme protein, while the FDNB-treated material had a specific activity of 754 units/mg, showing a 48% loss of activity. For the pyrophosphate exchange reaction, the control enzyme had a specific activity of $9.4 \cdot 10^4$ units/mg, while the FDNB-treated material had a specific activity of $6.6 \cdot 10^4$ units/mg, indicating a 30% loss of activity. The inhibition of the lysyl and arginyl ligases is summarized in Table II.

Gel electrophoresis of FDNB-treated purified enzymes. A solution of the purified arginyl enzyme (250 μl containing 33 μg protein) in 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA was treated with 50 μl of a saturated solution of FDNB (0.1 μmole) in buffer at 25° for 15 min. As a control, a similar aliquot of

TABLE II

INHIBITION OF AMINOACYL LIGASES BY FDNB

Enzyme	*% of control aminoacylation rate*		*% of control PP exchange*
	Crude enzyme	*Purified enzyme*	*Purified enzyme*
Lysyl ligase	80	52	70
Arginyl ligase	12	3	1

arginyl enzyme (33 μg protein in 250 μl buffer) was treated with water (50 μl) at 25° for 15 min. After the incubation, polyacrylamide-gel electrophoresis was performed[8]. On destaining the gels after electrophoresis, no difference between the control and the FDNB-treated material was observed. Each ran as a single band.

A solution of purified lysyl enzyme (150 μl containing 38 μg protein) in buffer was treated with 50 μl of a saturated solution of FDNB (0.1 μmole) in buffer at 25° for 15 min. As a control an aliquot of lysyl enzyme (38 μg of protein in 150 μl of buffer) was treated with water (50 μl) at 25° for 15 min. After the reaction polyacrylamide-gel electrophoresis was carried out. On destaining of the gels after electro-

phoresis, no difference between the control and FDNB-treated material was observed. Each ran as a single band. There was thus no evidence of breakdown of arginyl- and lysyl-tRNA ligases into subunits as a result of treatment with FDNB.

Treatment of the purified synthetases with [^{14}C]FDNB. A solution of purified arginyl enzyme (210 μl containing 33 μg of protein) in 0.1 M potassium phosphate buffer (pH 7.0) and 0.001 M EDTA was treated with 100 μl of [^{14}C]FDNB (Nuclear-Chicago) in 50% ethanol (0.57 μmole, $2.82 \cdot 10^6$ counts/min per μmole), at 25° for 15 min. A solution of the lysyl enzyme (110 μl containing 38 μg of protein) in buffer was treated with 100 μl of the same [^{14}C]FDNB reagent at 25° for 15 min.

The protein in each case was precipitated with cold 5% trichloroacetic acid (5 ml) and filtered on Type-EM Millipore filters (24 mm diameter). The filters were washed with cold 5% trichloroacetic acid (4 × 5 ml) followed by cold ethanol (6 × 5 ml) until no radioactivity was observed in the washings. The filters were air dried and counted. As a blank, the buffer (200 μl) was treated with the same [^{14}C]-FDNB reagent as that described above (100 μl) and also filtered in a similar manner on the EM Millipore filter. Assuming a molecular weight of 75 000 as determined by our colleague Dr. R. D. MARSHALL for arginyl synthetase*, for both of the ligases, the molar ratio of protein-bound FDNB to protein was found to be 4.2 for the lysyl enzyme and 3.6 for the arginyl synthetase.

FDNB has long been used as a reagent for N-terminal group determination in polypeptides[10]. It reacts with free amino groups to give a dinitrophenyl derivative. At pH 8.0–8.8 the sites of attack on a polypeptide chain are the α-amino group of the N-terminal amino acid, the ε-amino group of lysine, and to some extent the hydroxyl group of serine, and imidazole group of histidine. HIRS, HALMANN AND KYCIA[11] have shown that FDNB reacts at pH 8.0 with the ε-amino group of a lysine in a special position in bovine pancreatic ribonuclease A. The bifunctional reagent 1,5-difluoro-2,4-dinitrobenzene reacts with ribonuclease at pH 8.4 to form a bridge across the ε-amino groups of lysine in positions 7 and 41 (refs. 12, 13). At pH 9.3 RONCA, IPATA AND BAUER[14] found evidence for small amounts of *O*-tyrosine and *S*-cysteine derivatives, in addition to lysine derivatives from dinitrophenylation of adenosine deaminase.

The present studies with FDNB were carried out at pH 7.0, under which condition one might expect dinitrophenylation of a particularly reactive amino group but not of the usual ε-amino group of lysine ($\gamma K_a = 10.7$); possibly, however, an α-amino terminal group ($\gamma K_a \sim 8$) might react. Reaction with tyrosine remains an open question. In line with the above reasoning, a greater selectivity in the inhibitory effect of FDNB on mixed amino-acyl ligases was found at pH 7.0 than at pH 8.0–8.5, and thus the lower pH was chosen.

Effect of KCN on crude mixed ligases

2 ml of enzyme solution (15 mg/ml) were treated with 2 ml of 1 M KCN in 0.01 M $MgCl_2$ at pH 6.5 for 1 h at 25°. The pH was maintained at 6.5 by occasional addition of dilute HCl in a pH stat. Excess cyanate was removed by dialysis against buffer (0.01 M Tris–HCl, pH 7.2, 0.01 M $MgCl_2$, 7 mM KCl) and the enzymatic activities of the various ligases measured.

* R. D. Marshall, personal communication.

TABLE III

INHIBITION OF AMINOACYL LIGASE ACTIVITY BY CYANATE

Amino acid	*Rate of labeling of RNA (mμmole amino acid/min/mg protein)*		*% retention of activity a/c*
	(c) Control reaction	*(a) Reaction with cyanate*	
Arginine	3.0	0.62	21
Aspartic acid	0.51	0.03	6
Leucine	0.12	0.02	17
Lysine	1.8	0.52	29
Proline	0.31	0.07	23
Serine	2.5	0.90	36
Valine	3.1	0.37	12

As a control, a solution of protein (7.5 mg/ml) in 0.01 M $MgCl_2$ (pH 6.5) was kept at 25° for 1 h. The solution was dialyzed against buffer and the various enzymatic activities assayed.

Table III shows the effect of cyanate on the mixed ligase system at pH 6.5 and 25°. Considerable loss of activity for each of the ligases tested was observed. When the mixture of ligases (7.5 mg protein per ml) was treated with 0.5 M $K^{14}CN$ for 1 h at pH 6.5, an average of 1 protein molecule reacted with 17 molecules of cyanate.

At pH 8 cyanate reacts with α-amino terminal residues and the ε-amino group of lysine in polypeptides[15], probably by the mechanism shown in Fig. 1. However,

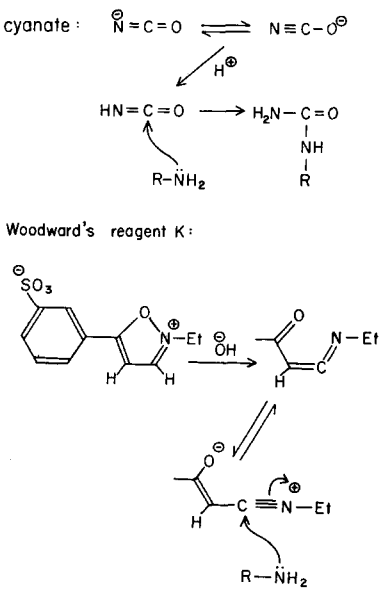

Fig. 1. Possible mechanisms of reaction of cyanate and Woodward's reagent K.

at a pH below 7.0, cyanate would react more selectively with terminal α-amino groups[16,17]. Serine at the active site of chymotrypsin is also attacked by cyanate[18]. Under pH conditions of 6.5, attack of cysteine would probably be reversible[19].

Effect on aminoacyl ligases of N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K)

Experiments on mixed aminoacyl ligases. 2 ml of enzyme solution (15 mg of protein per ml) in 0.01 M $MgCl_2$ were treated with Woodward's reagent K (100 μmoles in 2 ml of 0.01 M $MgCl_2$) at pH 7.5 for 1 h at 25°. The pH was maintained at 7.5 on a pH stat by addition of dilute alkali. Excess reagent was removed by extensive dialysis against buffer (0.01 M Tris–HCl, pH 7.5, 0.01 M $MgCl_2$, 7 mM KCl). The ultraviolet absorption spectrum of the protein in 0.14 M NaCl, 0.014 M sodium citrate (pH 7) showed an additional peak at 340 mμ. The enzymatic activity of various ligases was measured.

As a control, a solution of protein (7.5 mg/ml) in 0.01 M $MgCl_2$ was kept at pH 7.5 for 1 h at 25°, then extensively dialyzed against buffer.

Table IV shows the results of the action of Woodward's reagent K at pH 7.5 on the mixed ligase system. Serine retained only 7% while alanine retained more than 50% of its activity. Intermediate values were found for other ligases.

Experiments on partially purified lysyl ligase. When a partially purified sample

TABLE IV

INHIBITION OF AMINOACYL LIGASE ACTIVITY BY WOODWARD'S REAGENT K

Amino acid	*Rate of labeling of RNA (mμmole amino acid/min/mg protein)*		*% retention of activity a/c*
	(c) Control reaction	*(a) Reaction with Woodward's reagent K*	
Alanine	0.66	0.36	55
Arginine	2.7	1.3	50
Leucine	5.3	1.7	32
Lysine	1.5	0.28	19
Serine	2.1	0.14	7
Valine	4.5	1.6	36

of the lysyl enzyme (0.1 mg protein per ml) was treated with 0.01 M ^{35}S-labeled Woodward's reagent K at pH 7.5 at 25° for 1 h, an average of one chain in 5 reacted with a molecule of reagent. Under similar conditions one molecule of ^{35}S-labeled reagent reacted with one bovine serum albumin molecule. Since the lysyl enzyme is considerably inhibited (80%) under the above conditions, the data suggest, according to the reasoning below, that in the reaction with the lysyl enzyme some cross-linking may have taken place.

At pH 7.2 *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) loses a proton to give the α-ketoketimine, which readily undergoes nucleophilic

attack (see Fig. 1). At pH 7.5 it would presumably react with free amino groups. Reaction with a carboxylate anion would be reversible unless a cross-linkage occurred. MARFEY, GILL AND KUNZ[20] have shown the formation of cross-linkages in synthetic peptides reacted with Woodward's reagent at pH 7.2. There is also evidence for reaction with ε-amino groups of lysine in ribonuclease*.

Treatment of the mixed ligase system with 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent)

KOSHLAND, KARKHANIS AND LATHAM[21] report that this reagent reacts specifically with tryptophan at pH 3. We were unable to use this pH, however, without producing considerable loss in enzymatic activities in the absence of reagent. The enzyme (1.5 ml containing 11 mg protein per ml) was therefore treated at pH 8 with 1.5 ml of a saturated solution of Koshland's reagent (0.02 M) in buffer (0.01 M Tris–HCl, pH 8.0, 0.01 M $MgCl_2$, 7 mM KCl) at 25° for 30 min. The excess reagent was removed by dialysis against buffer. The ultraviolet absorption spectrum of an aliquot of the reacted protein was measured in the presence of 0.14 M NaCl, 0.014 M sodium citrate. It showed an additional peak at 410 mμ, which corresponds to the ε_{max} of 2-hydroxy-5-nitrobenzyl bromide[21]. A control enzyme (5.5 mg protein per ml) in buffer at pH 8.0 was kept at 25° for 30 min, then dialyzed against buffer.

The enzymatic activities of various ligases were measured and no catalytic loss of activity compared with the control was found with the following ligases: alanine, arginine, aspartic acid, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. It must be emphasized that no degradative work was carried out to identify possible sites of reaction of the reagent.

DISCUSSION

The present experiments were designed as a screening procedure to search for the active site reagents with selective inhibitory power toward a few aminoacyl ligases, as compared with other members of the same family of enzymes. A similar quest was initiated recently by STERN *et al.*[5]. One may presume that this family of enzymes would have certain common structural characteristics. Each enzyme should, however, bear two distinctive features: its amino acid-activating site, and its tRNA-recognition site. A reagent providing a sharp inhibitory specificity toward a few aminoacyl ligases might be further tested by comparing the degree of inhibition of aminoacyl esterification and of pyrophosphate exchange. A reagent particularly inhibitory to the esterification reaction but not to pyrophosphate exchange (or hydroxamate formation) would offer promise of relationship to the recognition reaction of the aminoacyl-RNA ligase. Such inhibition might be due to involvement in the recognition site itself, or might be more remotely involved by way of interference with tertiary structural requirements necessary for development of the recognition site. The present results appear to justify further efforts to improve the reaction conditions, and to locate the site of attachment of FDNB to the purified arginyl and lysyl ligases.

* P. S. MARFEY, personal communication.

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