Direct and Specific Photochemical Cross-linking of Adenosine 5′-Triphosphate to an Aminoacyl-tRNA Synthetase†

Vincent T. Yue and Paul R. Schimmel*

ABSTRACT: ATP has been photo-cross-linked to three different aminoacyl-tRNA synthetases from *Escherichia coli* B. Cross-linking was achieved by direct irradiation of the enzyme-ATP complexes with ultraviolet (254 nm) radiation. Detailed studies of the photoreaction were carried out with the complex of Ile-tRNA synthetase and ATP. These studies show that the entire nucleotide molecule, and not a fragment, is incorporated. Moreover, attachment occurs predominantly to the monomeric enzyme and not to aggregated species. Although irradiation of the enzyme alone leads to dose-dependent partial inactivation of catalytic activity, it is shown that inactivated molecules cannot cross-link to ATP. This and other controls strongly suggest that cross-linking under the conditions used requires active enzyme and only occurs at the ATP receptor site. Digestion with a single protease of [α-^{32}P]-ATP-Ile-tRNA synthetase complex gives rise to a single ^{32}P-labeled peptide. Using a combination of proteases, a labeled peptide small enough to sequence by manual methods was obtained. This peptide has the sequence Lys-Val-Ala-Gly-Asx-X, where X is a residue which has not yet been identified. (X does not correspond to any of the natural amino acids.) The site of attachment of ATP within this peptide has not been determined, although preliminary data make X a reasonable possibility. The sequence of the peptide from the ATP receptor site obtained here corresponds closely to a pentapeptide sequence in the partial sequence of the enzyme determined by another investigator.

Structural features of protein-nucleic acid complexes have recently been explored by means of photochemical cross-linking (Markovitz, 1972; Weintraub, 1973; Strniste and Smith, 1974; Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975; Anderson et al., 1975; Gorelic, 1975). In these studies, proteins are joined to nucleic acids under the direct action of ultraviolet light, without the introduction of extraneous reagent or of affinity labels on either of the reacting partners. This direct approach has been used to elucidate topological features of six different complexes of aminoacyl-tRNA synthetases with specific tRNAs (Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975). By determining the regions on the nucleic acid that are involved in the photo-cross-linking, it has been possible to construct a model for synthetase-tRNA complexes (Budzik et al., 1975; Schoemaker et al., 1975).

In the case of the synthetase-tRNA complexes, a variety of controls established that the cross-linking is specific; i.e., a firm complex must form in order to achieve good covalent linkage (Schoemaker and Schimmel, 1974; Budzik et al., 1975; Schoemaker et al., 1975). Although the regions on the tRNAs that are involved in cross-linking have been determined, the complexities of these macromolecule-macromolecule systems make it inherently much more difficult to determine segments of the polypeptide chain that are involved in photo-cross-linking. Because of this, certain important questions have not been approached. For example, it is important to determine whether polypeptide fragments involved in cross-linking are scattered throughout the protein structure or whether they are localized to a few specific regions. Demonstration of the latter would constitute further evidence that specific joining at the nucleic acid-protein interface had been achieved, and would also thereby locate in the primary structure of the protein the segments that bind nucleic acid.

In order to address these questions concerning the nature of the receptor sites on the proteins as identified by photochemical cross-linking, attention was directed at the much simpler synthetase-ATP complexes. Since this nucleotide is used to drive the aminoacylation reaction, all synthetases share the common feature of having an ATP receptor site. With this in mind, we attempted to photo-cross-link ATP to aminoacyl-tRNA synthetases and to examine in more detail some of the questions that were difficult to approach in the case of synthetase-tRNA complexes.

There is, however, additional motivation for investigating photo-cross-linking of ATP to synthetases. Since each specific synthetase carries out precisely the same chemical reaction, differing only in the particular amino acid and tRNA species that is used, it is plausible that certain structural features are preserved in each of the enzymes. For example, common structural elements are observed in other classes of enzymes such as the dehydrogenases and the serine proteases (Walsh and Neurath, 1964; Stroud, 1974; Rossmann et al., 1974; Rossmann and Liljas, 1974). Unfortunately, total amino acid sequences of two or more aminoacyl-tRNA synthetases are not yet available, so that such structural comparisons are not feasible. On the other hand, since the ATP receptor sites are common to all of the enzymes, this is the region most probable to contain analogous sequences of amino acids. Thus, a logical approach to the problem of structural similarities is to label and then isolate the peptides within the ATP binding domain. These peptides, from different enzymes, can then be sequenced and compared.

In the present article we present data demonstrating that ATP can be photo-joined to aminoacyl-tRNA synthetases under the direct action of ultraviolet light. Having established this point, a detailed study was then carried out with the cross-linked complex of isoleucyl-tRNA synthetase from *E. coli* B. These studies were done along lines described above so

† From the Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received April 1, 1977.* This work was supported by Grant No. GM 15539 from the National Institutes of Health.

as to answer specific questions concerning the photoreaction. In addition, the sequence of the peptide involved in the cross-linking reaction has been determined, thus laying the foundation for further structural comparisons with other synthetases.

Materials and Methods

Materials. Isoleucyl-tRNA synthetase was purified to ≥80% homogeneity from *E. coli* B by the method of Eldred and Schimmel (1972). Molar concentrations of active enzyme were determined by the ATP-PP_i isotope exchange assay using a procedure described elsewhere (Schimmel et al., 1972). Trypsin, protease type VI (Nomota et al., 1960), and pepsin were products of Calbiochem, Sigma, and Worthington, respectively. [α-^{32}P]ATP (10–20 Ci/mmol), [γ-^{32}P]ATP (10–20 Ci/mmol), [8-^{14}C]ATP (45 Ci/mol), [U-^{14}C]AMP (570 Ci/mol), and [U-^{14}C]ATP (800 Ci/mol) were purchased from New England Nuclear. Dansyl chloride was bought from BDH Chemicals (England). Cellulose thin-layer plates were a product of Brinkmann Instrument (Cellaplate-22, 0.1 mm, 20 × 20 cm). Polyamide thin-layer plates (15 × 15 cm, F 1700) were obtained from Schleicher and Schull. X-ray film was obtained from Kodak (RP/R-54). All other reagents were of best grades available.

UV Irradiation. In a typical experiment, the 100-μL reaction mixture contained 20–25 nmol of ATP, 1 nmol of Ile-tRNA synthetase, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8.0 at 4 °C. The mixture (not degassed) was placed as a droplet on Parafilm (on ice) and irradiated for varying times (30–40 min) at a distance of about 4 cm with a 15-W low-pressure mercury lamp (General Electric) equipped with a 2-mm thick Vycor filter. The lamp radiates predominantly at 253.7 nm while the filter eliminates far UV ($\lambda < 210$ nm) irradiation (Calvert and Pitts, 1967). Dosages were estimated with a uridine actinometer and corrected for the screening effect of the sample solution (Johns, 1968). The amount of cross-linking was determined from the amount of radioactive ATP precipitable by 5% trichloroacetic acid in the presence of the enzyme.

Protease Digestion. Irradiated complex (10–25 nmol) was separated from free ATP by passing through a Bio-Gel P-100 column (1 × 100 cm). It was then reduced with dithiothreitol in 6 M guanidium hydrochloride, followed by alkylation with iodoacetamide (Kula, 1973). After dialysis against 100 mM NH_4HCO_3, the protein was digested for 4 h at 37 °C with 2% trypsin in a 0.3–0.5-mL reaction mixture containing the dialysis buffer. Digestion was continued for 4 h with the addition of 2% protease type VI. The pH of the solution was adjusted to pH 3–4 with 6 M HCl. Then the sample was further digested with 2% pepsin for another 4 h.

Protease Type VI Digestion. The procedure for protease type VI digestion was identical with that of the other protease digestions, except that only protease type VI (2% weight of Ile-tRNA synthetase) was used. Digestion was done for 5 h at 37 °C.

Purification of ATP-Labeled Peptides. The completed digest was eluted through a Bio-Gel P-2 column (0.9 × 70 cm). The radioactive fractions were pooled and lyophilized. The residue was taken up with 5 μL of water. This material was then spotted on a cellulose thin-layer plate and chromatographed first in isobutyric acid–NH_4OH–H_2O (66:1:33) and then at a right angle in pyridine–methanol–H_2O (1:20:5). The separation was visualized by ninhydrin spray. The location of the radioactive spot on the plate was achieved by autoradiography. The ATP-labeled fragment was eluted from the plate with 100 mM NH_4HCO_3 and lyophilized.

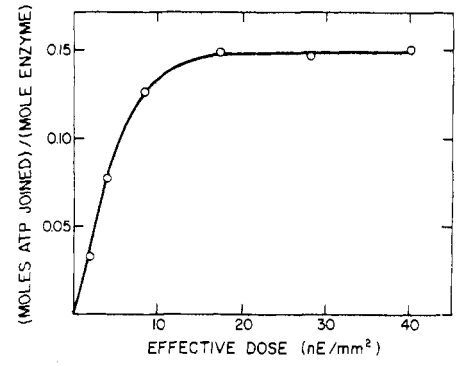

FIGURE 1: Moles of ATP joined per mole of enzyme vs. the effective irradiation dose in nEinsteins/mm^2. The 200-μL reaction mixture contained 0.25 mM ATP, 10 μM Ile-tRNA synthetase, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8.0. Irradiation was carried out as described in the text. Ten-microliter aliquots were withdrawn at different intervals of irradiation and assayed for Cl_3CCOOH precipitability.

Sequence Determination of the Labeled Peptide. The details of the microdansyl-Edman procedure used in our sequence determination, N-terminal analysis, and the determination of amino acid composition were as described elsewhere (Fleischman, 1973). Basically, in each cycle of the procedure the N terminus was chemically removed from the peptide. Then in an aliquot of the sample solution, the newly exposed N terminus was labeled with a fluorescent group. This was subsequently hydrolyzed in 6 M HCl. The fluorescent amino acid derivative was identified chromatographically as the corresponding amino acid residue. The N-terminal analysis is equivalent to the first cycle of the procedure in which the peptide is directly labeled with the fluorescent group without prior removal of the N terminus. In the composition determination, the peptide was first hydrolyzed, then derivatized with the fluorescent label, and finally analyzed chromatographically.

Results

Characteristics of the Cross-linking Reaction. Figure 1 shows the dependence of the formation of Cl_3CCOOH-precipitable [8-^{14}C]ATP upon the dosage of irradiation at 254 nm. Control experiments established that none of the ATP becomes Cl_3CCOOH precipitable in the absence of irradiation or in the absence of Ile-tRNA synthetase. The figure shows that the amount of Cl_3CCOOH precipitable ATP increases in a monotonic fashion until a plateau of about 0.15 mol of ATP per mol of synthetase is reached.

Similar experiments were also carried out with Val-tRNA synthetase and Tyr-tRNA synthetase from *E. coli* B. In these cases it was also found that Cl_3CCOOH-precipitable ATP is formed as a result of irradiation in the presence of the respective synthetases. Further characterization of the cross-linking reaction was carried out with the Ile-tRNA synthetase system. This enzyme is a single polypeptide chain of mol wt 112 000 (Baldwin and Berg, 1966); it is believed to contain one principal site for ATP (see Schimmel, 1973).

One may ask whether in the course of the photoreaction ATP is attached to the enzyme as a whole molecule or as a fragment containing ^{14}C. Experiments were carried out to check on this point. Table I gives a summary of the plateau photo-cross-linking yields obtained with differently labeled ATP, i.e., [U-^{14}C], [α-^{32}P], and [γ-^{32}P]ATP. As shown in the table, the maximal yield of cross-linking is the same for each labeled ATP. These data indicate that the intact ATP molecule is incorporated during photochemical joining.

TABLE I: Photo-cross-linking Yields for Ile-tRNA Synthetase Labeled ATP Mixtures.[a]

Compound	Moles of ATP joined/mol of Ile-tRNA synthetase
[U-^{14}C]ATP	0.142
[α-^{32}P]ATP	0.163
[γ-^{32}P]ATP	0.148

[a] [U-^{14}C]ATP/[α-^{32}P]ATP/[γ-^{32}P]ATP = 0.96:1.10:1.00. The solutions contained in 100 μL: 50 mM Tris-HCl (pH 8), 10 μM Ile-tRNA synthetase, 0.25 mM ATP, and 5 mM $MgCl_2$. Effective irradiation dose was 31 nEinsteins/mm^2 over a period of 40 min at 0–4 °C.

TABLE II: Photo-induced Joining of ATP.[a]

Protein	Nucleotide	Cl_3CCOOH-precipitable cpm	Moles joined per mole of enzyme
Ile-tRNA synthetase	[8-^{14}C]ATP	1148	0.15
Ile-tRNA synthetase	[U-^{14}C]AMP	59	0.0
Serum albumin	[8-^{14}C]ATP	37	0.0
(Ile-tRNA synthetase	[8-^{14}C]ATP)[b]	20	0.0

[a] All irradiations were carried out to give an effective dosage of about 30 nEinsteins/mm^2 (over a period of 30–40 min), until maximal yields were obtained. Reaction mixtures contained (in 100 μL) about 10 μM protein, 200 μM mononucleotide, 5 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.0). [b] No irradiation.

Two further controls were done to investigate other aspects of the photoreaction. [8-^{14}C]ATP was isolated from an irradiated Ile-tRNA synthetase–[8-^{14}C]ATP mixture and subjected to paper chromatographic analysis. Approximately 75% of the isolated radioactive material was intact ATP, while the remainder was ADP and AMP. This indicates that some hydrolysis occurs during the irradiation procedure.

It was considered possible that irradiation might induce some covalent aggregation of enzyme and that ATP cross-links to an aggregated form. This issue was explored by preparing an [α-^{32}P]ATP–Ile-tRNA synthetase complex which was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by autoradiography. This experiment showed that none of the radioactivity occurs in the region one would expect to find aggregates.

Specificity of Cross-linking. Several experiments were carried out to determine whether cross-linking requires specific complex formation or whether it results from random collisions between the nucleotide and the protein. One set of experiments aimed at this question is summarized in Table II. These experiments sought to determine whether a nonspecific protein such as serum albumin, or whether a closely similar nucleotide such as AMP, can photoreact in the same way as the synthetase–ATP complex. Under conditions where good cross-linkage between Ile-tRNA synthetase and ATP is achieved, no joining occurs between the synthetase and AMP nor between serum albumin and ATP. The small amount of joining between serum albumin and ATP is quite plausibly due to weak binding of this nucleotide to the enzyme. Collectively the data in Table II suggest that the photoreaction between ATP and the synthetase is due to specific complex formation.

However, further experiments demonstrated that in the absence of the nucleotide the enzyme is progressively inactivated with respect to the ATP–PP_i isotope exchange reaction (Calendar and Berg, 1966) with increasing doses of irradiation.

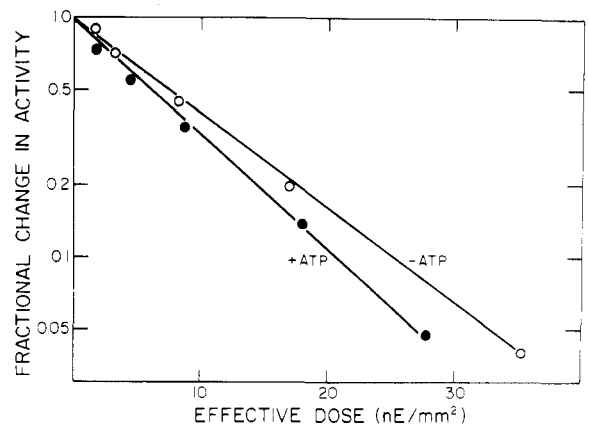

FIGURE 2: Semilogarithmic plot of fractional enzyme activity vs. effective dose of irradiation in nEinsteins/mm^2. The reaction mixtures contained in 200 μL: 10 μM Ile-tRNA synthetase, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 8), and in one case (see figure) 0.25 mM [U-^{14}C]ATP. Ten-microliter aliquots were withdrawn at periodic intervals to assay for enzyme activity and for cross-linking (see Figure 3). The lines through the points were calculated according to the method of least squares, and requiring them to intersect at the ordinate at 1.0.

The same inactivation occurs in the presence of ATP to an extent considerably greater than the amount of ATP that is cross-linked to the enzyme. Thus, in spite of the encouraging results of Table II, there arose a serious question as to whether the nucleotide actually joins to the active site on the active enzyme or whether labeling of one or more sites on an inactive species occurs. Further experiments were designed to answer this question.

The inactivation in the presence or absence of ATP is first-order in the dose of irradiation. That is, the inactivation can be described by an equation of the form

$$d(E) = -\sigma_I(E)dL$$

or

$$\ln(E)/(E_0) = -\sigma_I L \qquad (1)$$

where (E) is the concentration of active enzyme, (E_0) is the initial concentration before irradiation, σ_I is a phenomenological photochemical reaction cross section, and L is the fluence (or dose) of irradiation (corrected for effects of absorbance (see Johns, 1968)). In actual practice prolonged irradiation does not produce completely inactivated enzyme but instead yields some residual activity. Thus, eq 1 may be modified to account for the final residual activity

$$\ln[((E) - (E_r))/((E_0) - (E_r))] = -\sigma_I L \qquad (2)$$

where (E_r) is the residual activity. In the absence of ATP, (E_r) $\cong 0.14(E_0)$ while in the presence of ATP, (E_r) $\cong 0.04(E_0)$. Although the molecular basis for the residual activity is not currently known, several possibilities exist.

Figure 2 gives plots of $\ln[((E) - (E_r))/((E_0) - (E_r))]$ vs. L in the presence and absence of ATP. It is clear that the inactivation conforms closely to a first-order photochemical process, with the inactivation being somewhat greater in the presence than in the absence of ATP. Values for the phenomenological coefficient are $\sigma_I = 0.092$ mm^2/nEinstein (−ATP) and $\sigma_I = 0.104$ mm^2/nEinstein(+ATP).

From the data in Figure 2 it appears that during photo-cross-linking of ATP there are competing processes giving photoinactivation. The question we sought to answer was whether in such a complex situation it could be determined if ATP specifically joins to active enzyme. A simple experiment

TABLE III: Effect of Preirradiation on Cross-linking Yields.[a]

Effective preirradiation dose (nEinsteins/mm^2)	% act.	% of maximal cross-linking
0	100	100
1.7	91	89
26	21	23
51[b]	15	15
51[b]	14	≤14
102	5	≤14

[a] Solutions initially contained in 50 μL: 50 mM Tris-HCl (pH 8), 10 μM Ile-tRNA synthetase, and 5 mM $MgCl_2$. After irradiation with varying dosages, lyophilized [U-^{14}C]ATP was added to give a final concentration of 0.25 mM. An irradiation dose of 35 nEinsteins/mm^2 over a period of 40 min was then given. [b] Separate experiments.

was designed to answer this question. Enzyme alone was irradiated with varying dosages to give different amounts of inactivation. At this point ATP was then added and irradiation was continued until maximal cross-linking yields were obtained. The idea is to determine if the amount of cross-linking correlates with the amount of active enzyme remaining after the initial preinactivation irradiation.

Table III summarizes results of these experiments. The table compares the percent activity remaining before addition of ATP with the percent yield of cross-linking (normalized to 100% for enzyme that was not preinactivated). It is seen that there is a close correlation between the amount of active enzyme and the amount of cross-linking. That is, a 10% decrease in activity gives approximately 10% less cross-linking, and an 80% decrease in activity gives an approximately equal decrease in cross-linking. These experiments suggest strongly that cross-linking occurs only to active, and not to preinactivated, enzyme.

To gain further insight into the relationship between the cross-linking and the inactivation process, a different kind of analysis was carried out. Although the various elementary photochemical events that occur during the cross-linking reaction may be complex, the simple results of Table III suggest that the relationship between the differential amount of cross-linking dC accompanying an increment in radiation dL might phenomenologically be described as

$$dC = \sigma_C(E)dL \tag{3}$$

where σ_C is a phenomenological irradiation cross section for the cross-linking reaction. This equation simply says that the differential amount of cross-linking for dose dL is directly dependent upon the concentration of active enzyme. Although eq 3 cannot be analytically integrated, it is possible to treat data by a simple numerical integration given by

$$\Sigma\Delta C_i/(\tilde{E}_i) = \sigma_C\Sigma\Delta L_i = \sigma_C L \tag{4}$$

where ΔC_i is the incremental amount of cross-linking resulting from the ith incremental amount of irradiation ΔL_i and $(\tilde{E}_i)$ is the average concentration of active enzyme during the ith interval of irradiation. An experiment was carried out to test the validity of eq 4. A synthetase–ATP mixture was subjected to varying dosages of irradiation. At periodic intervals an aliquot was removed and assayed for the extent of remaining activity[1] and the degree of cross-linking. The results were then plotted according to eq 4.

[1] It was assumed that enzyme molecules were either completely active or completely inactive. Strictly speaking the highly irradiated enzyme still has low (~4%) activity.

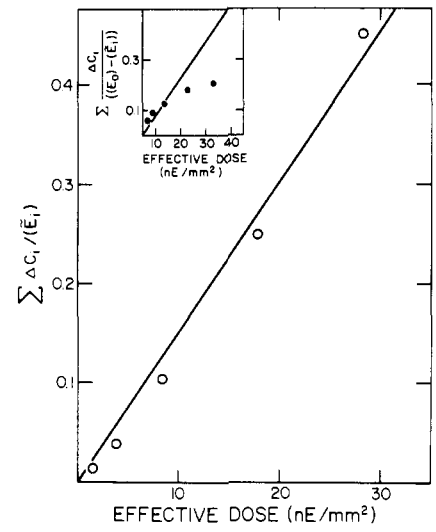

FIGURE 3: $\Sigma_i\Delta C_i/(\tilde{E}_i)$ vs. the effective dose of irradiation. The line is drawn according to the method of least squares, but requiring it to pass through the origin. In the inset, $(E_0) - (\tilde{E}_i)$ replaces $(\tilde{E}_i)$. Reaction conditions are identical with those described in Figure 2.

Figure 3 gives a plot of $\Sigma\Delta C_i/(\tilde{E}_i)$ vs. L. The plot covers approximately 90% of the total inactivation and cross-linking that occurs during the experiment. Beyond this range the experimental data are not sufficiently accurate to warrant including the data points. It is clear that an excellent linear relationship is obtained with $\sigma_C = 0.015$ mm^2/nEinstein.

To be certain that the close correlation between amount of active enzyme and cross-linking yield is not due to an insensitivity of the numerical integration, $(\tilde{E}_i)$ in eq 4 was replaced by $(E_0) - (\tilde{E}_i)$, where $(E_0) - (\tilde{E}_i)$ is simply the average amount of inactive enzyme that is present during the ith irradiation interval. The corresponding plot of the data is shown in the inset to Figure 3. It is clear that the degree of cross-linking does not correlate with the amount of inactive enzyme. Thus, the cross-linking reaction appears to follow closely eq 3, indicating that the active enzyme species is involved in the cross-linking reaction.

Both inactivation and cross-linking are described by simple first-order phenomenological equations (eq 1 and 3, respectively). A straightforward way for this to arise is if both events independently occur, with each requiring as a primary process the absorption of a photon by the enzyme. In the presence of ATP there are two reactions

$$E \xrightarrow{h\nu} E_I \tag{5a}$$

$$E \xrightarrow{h\nu} C \tag{5b}$$

where E_I is inactivated, un-cross-linked enzyme and C is the cross-linked species (presumed to be inactive in the ATP–PP_i exchange reaction). The total differential decrease in active enzyme accompanying a dose dL is

$$-d(E) = \sigma_i(E)dL + \sigma_C(E)dL = \sigma_I(E)dL$$

where σ_i is the inactivation cross section for eq 5a and $\sigma_I = \sigma_i + \sigma_C$. From values given above for σ_I in the presence of ATP and for σ_C, we calculate $\sigma_i = 0.089$ mm^2/nEinstein.

It is of interest to note that $\sigma_C \cong 0.15\sigma_I$. That is, the cross section for cross-linking is about 15% of that for overall inactivation. This fits well with the observation that the maximal cross-linking yield at the plateau is about 15% (see Figure 1).

Isolation of Photo-cross-linked Peptide. Having reasonable

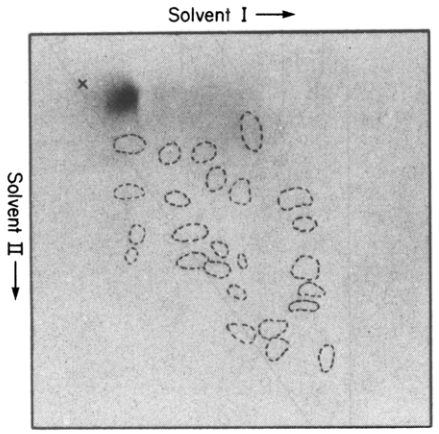

FIGURE 4: Two-dimensional chromatogram of protease type VI digest of cross-linked [α-^{32}P]ATP-Ile-tRNA synthetase. The origin is marked by an X. The areas of ninhydrin-positive material are indicated by dotted outlines. The dark spot corresponds to that obtained by autoradiography of the chromatogram. Solvent compositions are given in the text.

assurance that the irradiation specifically attaches ATP to its receptor site on the active protein, the question of the nature of and location of the cross-linking site(s) can be addressed. For this purpose, a cross-linked [α-^{32}P]ATP-Ile-tRNA synthetase complex was reduced, alkylated, and subjected to digestion with protease type VI. The peptide fragments released were separated by two-dimensional chromatography and visualized by ninhydrin. The location of [α-^{32}P]ATP peptides was determined by autoradiography.

Figure 4 gives the results of the chromatographic separation. The mark X indicates the origin and the dotted outlines enclose ninhydrin-positive material; a dark smudge results from the autoradiogram. It is clear that, although the ninhydrin positive material is spread over a large area, the radioactivity is concentrated in a single spot. Moreover, this labeled spot migrates distinctively away from AMP, ADP, and ATP. These results indicate that cross-linking has occurred to a single specific peptide.

The radioactive material was eluted from the chromatogram and chromatographed on a Bio-Gel P4 molecular sieve column. A single major peak of radioactivity was obtained; from the elution position a molecular weight of about 2000, or 15–20 amino acid residues, is estimated. (Starting from the cross-linked ATP-protein complex in the irradiation mixture, this peptide was obtained in about 40% yield.) An N-terminal analysis indicated the presence of a single amino acid residue—alanine. These results give further confirmation that cross-linkage has occurred at a single specific site on the protein.

Sequence of Amino Acids around the Photo-cross-linking Site. The labeled peptide isolated as described above is rather large for microsequencing work. Therefore, a different kind of digestion was carried out using a combination of proteases. The reduced, alkylated cross-linked protein was digested with trypsin, protease type VI, and pepsin. Two-dimensional chromatography (see Materials and Methods) and autoradiography were used to locate the labeled fragment. This procedure gave rise to a small peptide that could be easily handled by the micro-dansyl-Edman procedure.[2]

[2] One major and a closely spaced minor spot were obtained. Each was separately eluted and determined to have the same amino acid composition and the same residues in the first two positions of the sequence. We suspect the minor spot is the identical peptide as the major one, but that one of them may have undergone some hydrolysis of one of the phosphate linkages so as to migrate somewhat differently.

The amino acid composition of the peptide was determined by the standard procedure of acid hydrolysis followed by dansylation and chromatography. This showed that the peptide has a composition Ala, Asx, Gly, Lys, Val, and X. The residue X does not correspond to any of the natural amino acids. Using the micro-dansyl-Edman procedure described by Fleischman (1973), the sequence of the peptide was determined as

Lys-Val-Ala-Gly-Asx-X

The residues determined by the sequencing procedure correspond exactly to those determined independently by the amino acid composition analysis.[3]

Control experiments established that the acid hydrolysis procedures used in the dansyl-Edman method are sufficiently harsh to rupture the linkage between the attached nucleotide and the peptide. Therefore, we cannot be certain as to which residue is linked to ATP. However, a reasonable possibility is that the unknown residue X represents a modified form of a standard amino acid which was originally attached to ATP. A preliminary check of this possibility was carried out by subjecting the labeled peptide to digestion with a mixture of carboxypeptidases A and B. Chromatographic separations were done at different times to determine whether a labeled adduct was among the first products released by the enzyme. It was in fact found that a labeled, dansyl chloride positive product is quickly released by the carboxypeptidases. Although further verification is required, this suggests that X may be the site of attachment of ATP.

At this point lack of sufficient materials makes it unfeasible to characterize further the cross-linked adduct. For example, with a 15% cross-linking yield and a 40% yield of labeled peptide from a preparation of cross-linked complex, only about 6 nmol of peptide can be isolated from 10 mg ($\approx$100 nmol) of irradiated enzyme (0.15 $\times$ 0.40 $\times$ 100 nmol). Hopefully a characterization can be done when larger amounts of enzyme are available and, additionally or alternatively, better cross-linking yields are attainable.

Discussion

Several lines of evidence indicate that ATP cross-links specifically to its receptor site on Ile-tRNA synthetase. First, under conditions where good cross-linkage of ATP to synthetase is achieved, AMP does not significantly cross-link to synthetase nor does ATP cross-link to serum albumin (Table II). Second, ATP does not cross-link to catalytically inactivated enzyme (Table III). Third, the extent of cross-linking is quantitatively correlated with the amount of active enzyme present (Figure 3). Finally, the isolation of a single ATP-cross-linked peptide further substantiates that a specific receptor has been affinity labeled, and rules out the possibility of random surface labeling.

Another way to gain information on the specificity of cross-linking is to test whether the extent of cross-linking at a given dose of irradiation is a saturatable function of the nu-

[3] The question arises as to why lysine occurs in the first position of the peptide since this residue is one that should be cleaved by trypsin. It should first be mentioned that the sequence determination was done on two independent isolations of the peptide. The two independent determinations gave exact agreement. In addition, a third determination was done of the N terminus which further verified the presence of lysine in this position. As stated in Materials and Methods, digestion was performed first with trypsin, followed by protease type VI, and finally by pepsin. We found that trypsin alone did not give a radioactive peptide that was small enough to sequence by manual methods. This suggests that not all potentially trypsin sensitive sites are cleaved. The subsequent addition of protease type VI and pepsin may inactivate residual trypsin activity and thus prevent cleavage at the N-terminal lysine of the peptide that is finally isolated.

cleotide concentration; if so, a "K_m" for the cross-linking reaction may be obtained and compared with that from the usual steady-state kinetic analysis of the enzymatic activity (cf. Cooperman et al., 1975). However, this procedure, while perhaps valid in certain cases, can in others be misleading. It is based on the premise that for a given dose of irradiation the amount of cross-linking is a strictly linear function of the amount of complex formation. But a priori there is no reason why this should be the case. In particular, the amount of cross-linking for a fixed dose and its relation to concentration will in general depend on the details of the kinetic pathways (and lifetimes of photoactive species) that lead to cross-linking. As a result, the midpoint ("K_m") of a concentration profile may itself depend on the amount of the fixed irradiation dose. The situation may be even more complex when competing photoinactivation reactions are also present, as is often the case. Thus, a determination of the "K_m" for cross-linking at a single fixed dose is not in general subject to unambiguous interpretation. Moreover, for substrates with K_m's on the order of 10^{-4} M (such as ATP with Ile-tRNA synthetase (Cole and Schimmel, 1970)), saturation with ligand is not achieved until 10^{-3} to 10^{-2} M. With ligands having high extinction coefficients at the exciting wavelength (as is the case here), such high concentrations seriously attenuate the incident light. In the face of these ambiguities and difficulties, the approaches used here seem to be a more satisfying and more practical test of the question of specificity of labeling.

The data in Table I indicate that the entire ATP molecule is incorporated as a result of photochemical cross-linking. This leaves open the posibility that the 8-position on the intact adenine ring is the site of attachment. Studies of Steinmaus et al. (1969) indicate that this position is a prime site for photoreaction. This issue and the related one concerning the mechanism of photojoining will be better understood after determination of the molecular structure of the cross-linked adduct. An obvious start will be to examine the fate of ^3H in the cross-linking of [8-^{3}H]ATP.

As mentioned earlier, at this time there is no complete sequence published on any aminoacyl-tRNA synthetase. However, Kula (1973) has determined the sequence of several cysteine containing peptides of Ile-tRNA synthetase from *E. coli* MRE 600. One of these peptides has the sequence

Cys-Val-Ser-*Asx-Val-Ala-Gly-Asx*-Gly-Glx-Lys

The italicized portion has a sequence identical with the one determined by us except that the first residue is Asx in Kula's peptide whereas Lys is first in ours. It is of interest to observe that Asn and Lys are related by a single base difference in their triplet codons. The difference in amino acid at this position may be due to the fact that the enzyme in Kula's case is isolated from a different strain of *E. coli*.

The peptide isolated by Kula has Gly in the position corresponding to X in the peptide isolated by us. In this regard it is interesting to note that Toth and Dose (1976) have recently reported that glycine is the residue which participates with the highest frequency in photo-cross-linking reactions between DNA and two different proteins that bind to it. Thus, it is possible that the residue X is glycine and that this residue is attached to ATP.

The results obtained here are perhaps of more general interest, particularly since there has been so much recent activity in the area of photoaffinity labeling. For example, several nucleotide affinity analogues that make use of pendant diazo or azido groups have been described (Brunswick and Cooperman, 1971, 1973; Haley and Hoffman, 1974). These analogues depend upon the formation of nitrene or carbene intermediates upon irradiation of the bound nucleotide analogue. Notwithstanding the attractive features of these compounds, they suffer the disadvantage that an extraneous group has been introduced that may distort or otherwise perturb the complex under investigation. This difficulty is avoided by using the natural, unmodified material, as is the extra labor of synthesis and isolation of reactive analogues.

The present results give more confidence in using direct irradiation of natural, unmodified materials as a means for achieving covalent affinity labeling. Not long ago Antonoff and Ferguson (1974) gave data that indicated that direct irradiation of cAMP mixed with a crude extract from testes and from adrenal cortex gave specific attachment to cAMP receptor proteins. Although a number of the experiments that were carried out here were not done in that case, the data indicate that specific attachment was achieved. In another vein, Cooperman et al. (1975) have cross-linked puromycin to ribosomes using direct irradiation; some indication of a specific affinity labeling was obtained. In a more recent study, Sperling and Havron (1976) have used acetone as a sensitizer and wavelengths longer than 300 nm to attach cytidine nucleotide inhibitors to ribonuclease A. This study is an important benchmark since it involves coupling to an enzyme of known three-dimensional structure. They found, like us, that attachment occurs to a single peptide; they also found that this peptide is derived from the known nucleotide binding site of the enzyme. Sperling (1976) has also used the same procedure to cross-link ATP to histone H4. From these examples it appears clear that labeling by light activation can be achieved in a variety of situations without the introduction of synthetic substrate analogues. And as demonstrated in the present study, even with competing photoinactivation processes it is possible to achieve specific active site labeling.

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Molecular Weights of Particles and RNAs of Alfalfa Mosaic Virus. Number of Subunits in Protein Capsids†

R. A. Heijtink,‡ Corrie J. Houwing, and E. M. J. Jaspars*

ABSTRACT: The molecular weights of the four main RNAs of alfalfa mosaic virus, viz., the three genomic RNAs 1, 2, and 3 and the subgenomic RNA 4, which carries the coat protein cistron, were determined by two different methods. First, the weights of purified particles in which these RNAs occur were obtained by sedimentation equilibrium measurements. Since the particles are composed of RNA and a single species of protein subunits for which nucleotide and amino acid compositions are known, the weights of the RNA contents could be calculated from the particle weights and the phosphorus and nitrogen contents of the particles. They turned out to be 1.13, 0.80, 0.66, and 0.56×10^6, respectively. Second, sedimentation equilibrium distributions of the isolated and purified RNA species yielded molecular weights of the polyanions of 0.95, 0.66, 0.58, and 0.22×10^6, respectively. As a mean of these results, values of 1.04, 0.73, and 0.62×10^6 were accepted for RNAs 1, 2, and 3, respectively. It was concluded that two molecules of RNA 4 are encapsidated together. Thus a mean molecular weight of 0.25×10^6 was calculated for RNA 4. The molecular weights are lower than most values reported in the literature, which in many cases had been obtained from sedimentation coefficients and electrophoretic mobilities using empirical relationships. From the above values we calculated the alfalfa mosaic virus RNAs to consist of 3250, 2250, 1950, and 800 nucleotide residues, respectively. This leaves 240 and 140 extracistronic residues in the case of RNAs 3 and 4, respectively. With the aid of the particle weights, the RNA weight fractions, and the molecular weight of the coat protein (24 280), the numbers of protein subunits in the capsids were calculated. It is proposed that the RNAs are in capsids of 60 + ($n \times 18$) subunits, n being 10, 7, 5, and 4, respectively.

Alfalfa mosaic virus is an interesting virus from a biological and from a structural point of view. The genome of the virus consists of three RNA molecules called RNA 1, RNA 2, and RNA 3 in order of decreasing length. The genome parts are found in separate particles, thus giving rise to three different size classes, which in order of decreasing sedimentation velocity are referred to as components B, M, and Tb, respectively. Virus preparations contain also subgenomic RNAs. In general these occur in minor quantities, with the exception of a small RNA which encodes for the sole coat protein of the virus and is referred to as RNA 4. Most of this RNA is found in component Ta, which sediments slightly slower than component Tb (Van Vloten-Doting and Jaspars, 1977).

Host plants can be infected by a mixture of the three nucleoprotein components B, M, and Tb, but not by a mixture of their RNAs. The mixture of the three genomic RNAs becomes infectious after addition of a small amount of coat protein or RNA 4 (Bol et al., 1971).

In recent years the tripartite genome has been found to be a characteristic of several groups of plant viruses (for reviews, see: Jaspars, 1974; Van Vloten-Doting and Jaspars, 1977). All these viruses appear to have four major RNAs not differing very much in length from those of alfalfa mosaic virus. In some of these viruses the coat protein or its messenger is necessary for infectivity; with others it is not.

AMV[1] differs from other viruses with tripartite genomes by its particles being bacilliform instead of spherical. This is

†From the Department of Biochemistry, University of Leiden, Leiden, The Netherlands. *Received March 7, 1977; revised manuscript received June 29, 1977.* This work was sponsored in part by the Netherlands Foundation for Chemical Research (S.O.N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

‡Present address: Department of Virology, Medical Faculty, Erasmus University, Rotterdam, The Netherlands.

[1]Abbreviations used: AMV, alfalfa mosaic virus; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.