Evans, M. I., Silva, R., & Burch, J. B. E. (1988) *Genes Dev. 2*, 116–124.
Gerber-Huber, S., Nardelli, D., Haefliger, J.-A., Cooper, D. N., Givel, F., Germond, J.-E., Engel, J., Green, M., & Wahli, W. (1987) *Nucleic Acids Res. 15*, 4737–4760.
Germond, J.-E., Walker, P., ten Heggeler, B., Brown-Luedi, M., de Bony, E., & Wahli, W. (1984) *Nucleic Acids Res. 12*, 8595–8609.
Gruber, M., Bos, E. S., & AB, G. (1976) *Mol. Cell. Endocrinol. 5*, 41–50.
Gubler, U., & Hoffman, B. J. (1983) *Gene 25*, 263–269.
Huynh, T. V., Young, R. A., & Davis, R. W. (1984) in *DNA Cloning Techniques: A Practical Approach* (Glover, D., Ed.) pp 98–121, IRL Press, Oxford.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning, a Laboratory Manual*, pp 76–85, Cold Spring Harbor Laboratory, Cold Spring Habor, NY.
Marshall, R. D. (1972) *Annu. Rev. Biochem. 41*, 673–702.
McMaster, G. K., & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4835–4838.
Messing, J., & Vieira, J. (1982) *Gene 19*, 269–276.
Miller, M. S., Buss, E. G., & Clagett, C. O. (1981) *Biochim. Biophys. Acta 677*, 225–233.
Mount, S. M. (1982) *Nucleic Acids Res. 10*, 459–472.
Nardelli, D., van het Schip, F. D., Gerber-Huber, S., Haefliger, J.-A., Gruber, M., AB, G., & Wahli,W. (1987a) *J. Biol. Chem. 262*, 15377–15385.
Nardelli, D., Gerber-Huber, S., van het Schip, F. D., Gruber, M., AB, G., & Wahli, W. (1987b) *Biochemistry 26*, 6397–6402.
Neufeld, E. F., & Ashwell, G. (1980) in *The Biochemistry of Glycoproteins and Proteoglycans* (Lennarz, W. J., Ed.) pp 241–266, Plenum Press, New York.
Öfverstedt, L.-G., Hammarstrom, K., Balgobin, N., Hjerten, S., Pettersson, U., & Chattopadhyaya, J. (1984) *Biochim. Biophys. Acta 782*, 120–126.
Renugopalakrishnan, V., Horowtiz, P. M., & Glimcher, M. J. (1985) *J. Biol. Chem. 260*, 11406–11413.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schubiger, J.-L., & Wahli, W. (1986) *Nucleic Acids Res. 14*, 8723–8734.
Shainkin, R., & Perlmann, G. E. (1971) *J. Biol. Chem. 246*, 2278–2284.
Small, D., Chou, P. Y., & Fasman, G. D. (1977) *Biochem. Biophys. Res. Commun. 79*, 341–346.
Spieth, J., Denison, K., Zucker, E., & Blumenthal, T. (1985) *Nucleic Acids Res. 13*, 7129–7138.
Stifani, S., George, R., & Schneider, W. J. (1988) *Biochem. J. 250*, 467–475.
Taborsky, G. (1980) *J. Biol. Chem. 255*, 2976–2985.
van het Schip, F. D., Samallo, J., Broos, J., Ophuis, J., Mojet, M., Gruber, M., & AB, G. (1987) *J. Mol. Biol. 196*, 245–260.
Wahli, W., Germond, J.-E., ten Heggeler, B., & May, F. E. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6832–6836.
Wallace, R. A. (1985) in *Developmental Biology* (Browder, L. W., Ed.) Vol. I, pp 127–177, Plenum, New York.
Wallace, R. A., & Morgan, J. P. (1986) *Biochem. J. 240*, 871–878.
Wang, S.-Y., & Willams, D. L. (1980) *Biochemistry 19*, 1557–1563.
Wang, S.-Y., Smith, D. E., & Williams, D. L. (1983) *Biochemistry 22*, 6206–6212.
Wicks, R. J., & Clark, R. C. (1987) *Int. J. Biochem. 19*, 33–39.
Wiley, H. S., & Wallace, R. A. (1981) *J. Biol. Chem. 256*, 8626–8634.
Young, R. A., & Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1194–1198.
Yusko, S., Roth, T. F., & Smith, T. (1981) *Biochem. J. 200*, 43–50.

Evidence That the 3′ End of a tRNA Binds to a Site in the Adenylate Synthesis Domain of an Aminoacyl-tRNA Synthetase†

Kelvin Hill‡ and Paul Schimmel*
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
Received September 26, 1988; Revised Manuscript Received November 10, 1988

ABSTRACT: Aminoacylation requires that an enzyme-bound aminoacyladenylate is brought proximal to the 3′ end of a specific transfer RNA. In *Escherichia coli* alanyl-tRNA synthetase, the first 368 amino acids encode a domain for adenylate synthesis while sequences on the carboxyl-terminal side of this domain are required for much of the enzyme–$tRNA^{Ala}$ binding energy. The 3′ end of *E. coli* $tRNA^{Ala}$ has been cross-linked to the enzyme, and sequence analysis showed that Lys-73 is the major site of coupling. A mutant enzyme with a Lys-73 → Gln replacement has a 50-fold reduced k_{cat}/K_m (with respect to $tRNA^{Ala}$) for aminoacylation but has a relatively small alteration of its kinetic parameters for ATP and alanine in the adenylate synthesis reaction. The data provide evidence that the 3′ end of $tRNA^{Ala}$ binds to a site in the enzyme domain responsible for adenylate synthesis and that a residue (Lys-73) in this domain is important for a $tRNA^{Ala}$-dependent step that is subsequent to the synthesis of the aminoacyladenylate intermediate.

The complementary use of chemical modification and site-directed mutagenesis has been applied recently in a number of cases to probe structure–activity relationships in enzymes [Huynh et al., 1988; Kato et al., 1988; Nickbarg et al., 1988; reviewed in Profy and Schimmel (1988)]. This approach identifies regions of functional interest in a protein by chemical modification techniques yet overcomes many inherent limitations of interpretation through the introduction of unam-

† This work was supported by Grant GM15539 from the National Institutes of Health.
* Correspondence should be addressed to this author.
‡ Recipient of a postdoctoral fellowship from Loma Linda University.

0006-2960/89/0428-2577$01.50/0

biguous amino acid substitutions at specific locations in the protein structure. A comparison of the properties of the mutant enzyme with those of the wild-type enzyme then provides an assessment of the functional importance of the amino acid residue(s) under study. We have employed both a chemical modification technique and site-directed mutagenesis to study the specific interaction between the amino acid acceptor end of a transfer RNA and an aminoacyl-tRNA synthetase.

Aminoacyl-tRNA synthetases are a class of enzymes responsible for the esterification of amino acids to a hydroxyl group at the 3′ end of their cognate tRNAs, prior to the coded incorporation of these amino acids into a growing polypeptide chain during protein synthesis [for reviews, see Schimmel and Soll (1979) and Schimmel (1987)]. The esterification process is accomplished via two steps: first the condensation of the amino acid with ATP to form an aminoacyladenylate intermediate and then reaction of this enzyme-bound intermediate with the cognate tRNA. In spite of the analagous reactions catalyzed by each aminoacyl-tRNA synthetase, few characterized similarities exist among their structural features (Schimmel, 1987). Among the bacterial enzymes alone, quaternary structures range from a single subunit (α) (Hoben et al., 1982; Webster et al., 1984; Breton et al., 1986) to multiple different subunits ($\alpha_2\beta_2$) (Webster et al., 1983; Mechulam et al., 1985), and polypeptide sizes range from 303 (Webster et al., 1983) to 939 (Webster et al., 1984) amino acids. With respect to similarities among the synthetases, there appears to be a common arrangement of functional domains along each polypeptide chain. That is, in the cases examined to date, sequence units important for the synthesis of aminoacyladenylate are located in the amino-terminal halves of the enzymes; sequences responsible for RNA recognition include these and other units located on the carboxyl-terminal side (Jasin et al., 1983; Waye et al., 1983; Bedouelle & Winter, 1986; Regan et al., 1987; Schimmel, 1987).

Studies on the nature of the tRNA binding site of aminoacyl-tRNA synthetases have included the generation of covalent enzyme–substrate complexes with cross-linking techniques such as ultraviolet irradiation (Schoemaker & Schimmel, 1974) or reagents such as dithiobis(succinimidyl propionate) (Valenzuela & Schulman, 1986; Leon & Schulman, 1987a,b), *N*-succinimidyl [(bromoacetyl)amino]benzoate (Leon & Schulman, 1987c), and periodate-oxidized tRNA (Renaud et al., 1982; Hountondji et al., 1985, 1986b, 1987). The latter reagent is an affinity label specific for lysine residues inside the CCA binding site of tRNA on the enzyme, the use of which has led to the identification of peptide sequences inside this pocket on several synthetases. This reagent is used here as an affinity label to identify the CCA binding site of $tRNA^{Ala}$ on alanyl-tRNA synthetase from *Escherichia coli*.

E. coli alanyl-tRNA synthetase is a tetramer comprised of identical 875 amino acid subunits (Putney et al., 1981b,c). From analysis of the activities of truncated proteins generated from in vitro deletion mutagenesis of its cloned gene (Putney et al., 1981a), regions of this enzyme essential for aminoacyladenylate formation, charging of $tRNA^{Ala}$, and oligomerization have been identified (Jasin et al., 1983). Specifically, with regard to $tRNA^{Ala}$ interactions, a protein with the amino-terminal 461 amino acids (461N) can charge $tRNA^{Ala}$, whereas proteins with 76 or 93 less amino acids (385N and 368N, respectively) can still activate alanine to the aminoacyladenylate intermediate but cannot charge $tRNA^{Ala}$. The active site for adenylate synthesis is thus contained in fragment 368N, in which computer modeling studies suggest the presence of sequence elements responsible for the formation of a nucleotide binding fold (Webster et al., 1987). In vitro tRNA filter binding assays showed that 461N and 385N bind $tRNA^{Ala}$ (with dissociation constants 20-fold and 220-fold higher than for wild-type enzyme, respectively) but no $tRNA^{Ala}$ association with the slightly shorter 368N has been detected (Regan et al., 1987).

Although polypeptide sequences on the carboxyl-terminal side of Arg-368 in alanyl-tRNA synthetase are required for the in vitro binding of $tRNA^{Ala}$, no peptide sequences have been identified on the amino-terminal side that are responsible for the functional integration of adenylate synthesis with $tRNA^{Ala}$ binding and aminoacylation. The affinity labeling studies described in this work provide evidence that the 3′ end of $tRNA^{Ala}$ binds to a site in the enzyme domain responsible for adenylate synthesis. In this domain, Lys-73 has been shown by site-directed mutagenesis to be important for a $tRNA^{Ala}$-dependent aminoacylation step that is subsequent to the synthesis of the aminoacyladenylate intermediate.

Materials and Methods

Proteins. The plasmid-encoded *E. coli* protein alanyl-tRNA synthetase (875N), the truncated fragment 461 (Jasin et al., 1983), and a mutant 875N in which Lys-73 has been changed to glutamine [875N(KQ73)] were prepared from *E. coli* strain W3110 *laqI*^{q} *recA* Δ1Kan^r *alaS*Δ2 (Regan, 1986). In this strain, the gene (*alaS*) that encodes 875N is deleted from the chromosome so that there is no contamination of plasmid-encoded 875N with wild-type species. Typically, 0.4 L of late log phase cells containing the desired plasmid was harvested by centrifugation, washed with cold 0.85% saline, and either ground with alumina or sonicated in a minimal volume of 0.1 M NaCl, 50 mM potassium phosphate, pH 7.5, 50 mM β-mercaptoethanol, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF).[1] The resulting extracts were stored in 40–50% glycerol at −20 °C.

Fragment 461N was purified from crude extracts as described by Regan (1986). Partial purification of proteins 875N and 875N(KQ73) was conducted by fast protein liquid chromatography. Aliquots of the crude extracts were diluted 3-fold in 25 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1.0 mM β-mercaptoethanol, and 0.5 mM PMSF and loaded onto a Mono-Q HR 5/5 column (Pharmacia) equilibrated in the same buffer. Proteins were eluted with a 14-mL NaCl gradient (0.10–0.18 M over 4 mL; 0.18–0.22 M over 6 mL; 0.22–0.40 M over 4 mL) at a flow rate of 0.5 mL min^{-1}. One-milliliter fractions were collected and assayed for alanyl-tRNA synthetase activity via the aminoacylation assay. Both the wild-type and the mutant proteins eluted with 0.2 M NaCl. Fractions containing activity were pooled, concentrated in a Centricon-30 microconcentrator (Amicon), and stored in 40–50% glycerol at −20 °C. The protein composition of these pools was shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) to be approximately 50% 875N or 875N(KQ73).

When further purification was desired, the pooled fractions containing alanine-dependent aminoacylation activity were concentrated in a Centricon-30 microconcentrator and applied to an S-300 (Pharmacia) column (1 × 47 cm) equilibrated in 10 mM sodium phosphate, pH 7.5, 1.0 mM β-mercaptoethanol, and 0.5 mM PMSF at 4 °C. The column was washed with the same buffer at a flow rate of 0.3 mL min^{-1}. One-

[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; PP_i, inorganic pyrophosphate; RNase, ribonuclease; SDS, sodium dodecyl sulfate.

milliliter fractions were collected and assayed for aminoacylation activity. Those containing activity (fractions 17–23) were examined for protein purity by SDS–PAGE (Laemmli, 1970), pooled appropriately (fractions 17–19), concentrated in a Centricon-30 microconcentrator, and stored in 40–50% glycerol at −20 °C. At this point, the synthetases were judged to be >98% pure.

All restriction enzymes and DNA and RNA modifying enzymes were purchased from New England Biolabs or Boehringer Mannheim and used according to manufacturers' instructions. Trypsin was purchased from Cooper Biomedical in the L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated form and further purified by passage over an SP-Sephadex C-50 column equilibrated in 5 mM sodium citrate, pH 3.0, and 0.3 M NaCl.

Nucleic Acid Material. Purified *E. coli* $tRNA^{Ala}$ was purchased from Subriden RNA, the concentration of which was determined from its total aminoacylation capacity. All buffers employed in the storage and incubation of tRNA material were pretreated with diethyl pyrocarbonate (Sigma). Ethanol precipitation of RNA and separation via PAGE were performed essentially as described by Reilly and RajBhandary (1986). Elution of RNA material from polyacrylamide gels was conducted by modification of published procedures (Maxam & Gilbert, 1977). The gel slices were diced and placed in a 2× volume of 0.5 M ammonium acetate, 0.1% SDS, and 0.1 mM EDTA. Following incubation overnight at 37 °C, the suspension was subjected to centrifugation, the supernate was filtered through a Millex-GV filter unit (Millipore), and the RNA was recovered by ethanol precipitation. ATP was purchased from Boehringer Mannheim; deoxynucleotides and dideoxynucleotides were purchased from Pharmacia.

Enzyme Assays. The concentration of active alanyl-tRNA synthetase in protein preparations was determined by the adenylate burst assay as described by Fersht et al. (1975), with the exception that each incubation contained 3 mM alanine. The concentration of active sites obtained from each burst was divided by four to obtain the concentration of tetrameric synthetase.

The ATP–inorganic pyrophosphate (ATP–PP_i) exchange assay was adapted from Calendar and Berg (1966). Assay mixtures contained the following in 1.0 mL: 100 mM Tris-HCl, pH 8.0, 0.01–2.0 mM ATP, 2.0 mM $NaPP_i$ (0.2 mCi $mmol^{-1}$, New England Nuclear), 10 mM KF, 0.05–2.0 mM alanine, 10 mM β-mercaptoethanol, and 5.0 mM $MgCl_2$. Reactions were carried out with 4 nM enzyme for 15 min at 37 °C.

Aminoacylation of $tRNA^{Ala}$ was performed according to Schreier and Schimmel (1972) and Jasin et al. (1985) at 37 °C. The assays were performed over a range of $tRNA^{Ala}$ concentrations (0.2–72 μM) and at fixed concentrations of ATP (4.0 mM) and [^{3}H]alanine (22.4 μM, 5 mCi μmol^{-1}, ICN Biomedicals). It should be noted that the aminoacylation assay is performed at subsaturating alanine concentrations; to raise [^{3}H]alanine to saturating concentrations requires impractical amounts of radioactive substrate. Previous data suggested that, under our assay conditions, the K_m for $tRNA^{Ala}$ is not strongly dependent on the alanine concentration (Jasin et al., 1985). The rate of formation of alanine–$tRNA^{Ala}$ was determined from five time points during the initial 2.5 min of each reaction, using 3 nM enzyme.

Radiolabeling and Oxidation of $tRNA^{Ala}$. Transfer RNA^{Ala} was 5′-^{32}P labeled by modification of the phosphatase and polynucleotide kinase treatment described by Silberklang et al. (1977). The incubation for the first reaction contained 20 mM Tris-HCl, pH 8.0, 1–60 μg of $tRNA^{Ala}$, and 20–24 units of calf intestinal alkaline phosphatase (molecular biology grade) in a final volume of 50 μL. Dephosphorylation was carried out for 30 min at 55 °C and then terminated by phenol extraction and ethanol precipitation. The incubation for the second reaction mixture (20 μL) contained the above dephosphorylated $tRNA^{Ala}$ and 20 units of T4 polynucleotide kinase in 50 mM Tris-HCl, pH 8.0, 15 mM β-mercaptoethanol, 10 mM $MgCl_2$, and 0.8 μM [γ-^{32}P]ATP (4 mCi $nmol^{-1}$, New England Nuclear). Incubation was at 37 °C for 30 min, after which time the mixture was lyophilized and subjected to PAGE.

The dialdehyde derivative of $tRNA^{Ala}$ was obtained according to Buonocore and Schlesinger (1972) with the exception that smaller quantities were used. Typically, 0.6 mg of $tRNA^{Ala}$ was treated in 0.3 mL of 4 mM KIO_4.

Preparation and Purification of tRNA-Labeled Tryptic Peptides of Alanyl-tRNA Synthetase. The 875N–$tRNA^{Ala}$ and 461N–$tRNA^{Ala}$ covalent complexes were obtained by modifications of procedures described previously (Hountondji et al., 1985, 1986b, 1987). Reaction mixtures contained 20 mM imidazole hydrochloride, pH 8.0, 25% glycerol, 10 mM $MgCl_2$, and 3.0 mM sodium cyanoborohydride and were conducted at 37 °C. When conducted on an analytical scale, a typical reaction contained 4.3 μM 875N and 8.0 μM oxidized [^{32}P]$tRNA^{Ala}$ in a volume of 10 μL. Following an 80-min incubation, the reduction reaction was completed by the addition of 1 μL of 50 mM sodium borohydride (to 5 mM) and a further 5-min incubation. When desired, exhaustive trypsin digests were performed by the addition of 5 μL of 10 M urea (to 3 M), 0.5 μL of 5 mM $CaCl_2$ (to 0.15 mM), and 0.15 μg of RNase-free trypsin and a further 4.5-h incubation at 37 °C. In preparation for PAGE, each sample was dried down to approximately 5 μL in a Speed Vac concentrator (Savant) and then dissolved in 10 μL of deionized formamide loading buffer. The samples were then subjected to electrophoresis through a 6.5% polyacrylamide gel (0.4 mm thick). When larger amounts of peptide–$tRNA^{Ala}$ complex were required for peptide sequence analysis, the above analytical procedure was conducted on a larger, preparative scale. A typical reaction contained 87 μM 875N and 28 μM [^{32}P]$tRNA^{Ala}$ in a volume of 600 μL, and all other reagents were scaled up accordingly. Electrophoresis was conducted with 0.1 mM sodium thioglycolate (Sigma) in the cathode tank buffer and a 6.5% polyacrylamide gel (2.5 mm thick) containing deionized urea (Hunkapiller et al., 1983).

Oligonucleotide-Directed Mutagenesis. The KQ73 substitution was introduced into protein 875N by site-directed mutagenesis with a synthetic oligonucleotide. We followed the procedure of Zoller and Smith (1983) as described by Toth and Schimmel (1986). DNA manipulations were according to Maniatis et al. (1982). The entire *alaS* gene was cloned into the *Eco*RI and *Sma*I sites of the single-stranded DNA phage M13mp8 as an *Eco*RI/*Bal*I fragment derived from pMJ301 (Jasin et al., 1984). Mutagenesis of *alaS* was directed by the permuted 21′-mer TGCGGGTGGTCAGCACAACGA synthesized on a Systec Microsyn 1450A automated DNA synthesizer. Except for the permuted positions (underlined), this oligonucleotide is complementary to the coding strand of *alaS* that includes the codons for amino acid residues 70–75. The permuted triplet encodes glutamine at position 73.

Mutant mp8 plaques were detected at a frequency of 4% by screening for hybridization with the 5′-^{32}P-labeled permuted

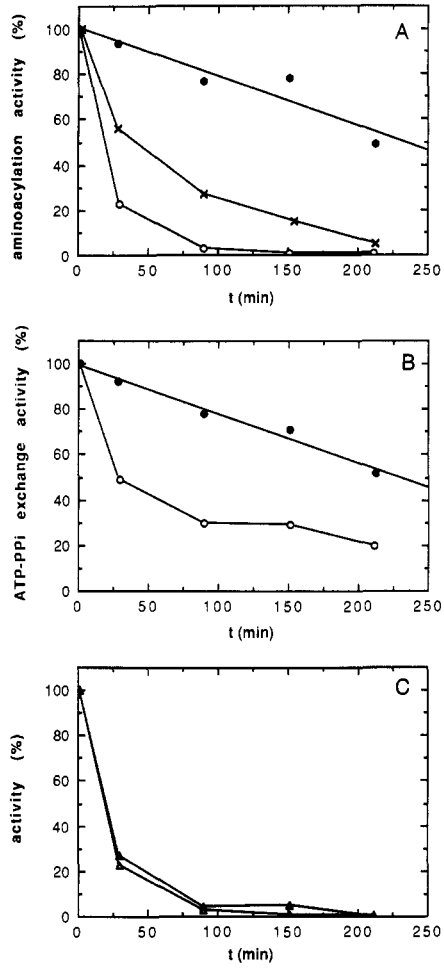

FIGURE 1: Inhibition of 875N catalytic activity by oxidized $tRNA^{Ala}$. Protein 875N (4.1 μM) was incubated as described under Materials and Methods in the presence of 52 μM oxidized $tRNA^{Ala}$ (○), 52 μM native $tRNA^{Ala}$ (●), or 52 μM oxidized $tRNA^{Ala}$ and 282 μM native $tRNA^{Ala}$ (×). At the times indicated, aliquots were withdrawn and assayed for aminoacylation (A) and ATP–PP_i exchange (B) activity. The ATP–PP_i exchange data obtained in the presence of oxidized $tRNA^{Ala}$ were normalized (▲) by subtraction of the activity remaining upon termination of the incubation and are plotted for comparison with the corresponding aminoacylation inhibition data (△) (C).

oligonucleotide (Carter et al., 1984). Single-stranded DNA was prepared from these plaques, and the presence of the new glutamine codon confirmed by dideoxy sequencing (Sanger et al., 1977; Biggin et al., 1983) with an oligonucleotide that primes 56 base pairs 5′ to codon 73. An approximate 600 base pair *Eco*RV/*Bam*HI fragment was cut from the mutant mp8 double-stranded DNA and subcloned into the *Eco*RV/*Bam*HI sites of pMJ301. The result is a plasmid containing a mutant *alaS* that encodes 875N(KQ73), as confirmed by sequence analysis of the double-stranded plasmid DNA.

RESULTS

Cross-Linking of Alanyl-tRNA Synthetase with Oxidized $tRNA^{Ala}$. Observed changes in the catalytic activity of 875N upon incubation with $tRNA^{Ala}$, in the presence of sodium cyanoborohydride, are presented in Figure 1. When the aminoacylation activity of 875N was assayed as a function of incubation time (Figure 1A), the presence of oxidized $tRNA^{Ala}$ was seen to cause irreversible inactivation of the synthetase in an apparent first-order process, leading to essentially complete (97%) inactivation within 90 min under the conditions employed. Similar inactivation was obtained when fragment 461N was utilized (data not shown). These data are in contrast to a control experiment that employed native $tRNA^{Ala}$, in which an apparent zero-order inactivtion occurred at a much slower rate (20% inactivation at 90 min). (There was no more than a 6% loss of activity after a 210-min incubation with native $tRNA^{Ala}$ in the absence of sodium cyanoborohydride.) Other data in Figure 1A show that the inactivation of aminoacylation activity conferred by the dialdehyde derivative of $tRNA^{Ala}$ was partially blocked by native $tRNA^{Ala}$. Specifically, the presence of a 5.4-fold higher concentration of native $tRNA^{Ala}$ led to a 2.7-fold reduction in the rate of inactivation by the oxidized species.

The observed inhibitory effect of oxidized $tRNA^{Ala}$ on 875N catalytic activity was not unique to the aminoacylation assay. Data presented in Figure 1B establish that synthesis of the aminoacyladenylate intermediate, as assayed by the rate of ATP–PP_i exchange, was also inhibited by prior incubation of the enzyme with the dialdehyde derivative of $tRNA^{Ala}$. In this case, however, no more than 80% inhibition of ATP–PP_i exchange activity was obtained, even after an incubation period of over 8 h (data not shown). For a direct comparison of the inhibition of ATP–PP_i exchange activity with that of aminoacylation activity, the ATP–PP_i exchange rate data were normalized by subtraction of the approximate 20% residual activity and are plotted with the aminoacylation data in Figure 1C. The data from the two different assays now coincide on the inactivation plot.

Inactivation similar to that reported in this work has been observed for other aminoacyl-tRNA synthetases upon incubation, under reducing conditions, with the dialdehyde derivatives of their cognate tRNAs (Baltzinger et al., 1979; Hountondji et al., 1985, 1986b, 1987). In these cases, the inactivation resulted from covalent cross-linking, via a Schiff base intermediate (Fayet et al., 1979), between one of the 3′-aldehydes on the oxidized cognate tRNA and the ϵ-amino group of a lysine in the synthetase polypeptide chain. In order to establish that a stable complex is formed between 875N and oxidized $tRNA^{Ala}$, we incubated these materials together, in the presence of sodium cyanoborohydride, and subjected the resulting protein–nucleic acid mixture to PAGE. When SDS–PAGE and Coomassie blue staining were employed, we detected the formation of one major and several minor species that migrate with R_f values less than that of 875N (data not shown). The rate of formation of the major species resembled closely the rate of inactivation observed in the same experiment (Figure 1C). Furthermore, upon digestion with RNase, all of these higher molecular weight species were reduced to a molecule that comigrated with 875N. No such higher molecular weight species were observed when native $tRNA^{Ala}$ was employed.

The results of similar incubations with 5′-radiolabeled $tRNA^{Ala}$, following electrophoresis through a denaturing polyacrylamide gel, are presented in Figure 2. Autoradiography confirmed that no protein was complexed with native $tRNA^{Ala}$ (lane 1), but apart from material that remained in the well, one major putative 875N–$tRNA^{Ala}$ complex was detected when the dialdehyde–$tRNA^{Ala}$ derivative was employed (lane 2). Exhaustive trypsin digestion of the protein material in the incubation mixture, prior to electrophoresis, yielded one major and several minor peptide–$tRNA^{Ala}$ species (lane 3) that migrated through the gel with R_f values slightly less than that of free $tRNA^{Ala}$.

Because 461N is an active truncated fragment containing only the amino-terminal portion of alanyl-tRNA synthetase, we anticipated that the use of this protein may aid in the determination of the region of 875N to which oxidized

Table I: Sequence of the $tRNA^{Ala}$-Labeled Peptide[a]

cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
461N[b]	A	G	G	x	x	N										
875N[b,c]	x	x	x	x	x	N	D	L	E	N	V	x	Y	x	x	x
	x	x	x	v	H	N	y	p	E	N	V	G	Y	x	A	x
known sequence[d]	A	G	G	K	H	N	D	L	E	N	V	G	Y	T	A	R
residue	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85

[a] Peptide sequences were determined at the Whitehead Institute for Biomedical Research on an Applied Biosystems, Inc., Gas-phase protein sequencer. Amino acid residues listed for each cycle correspond to chromatograph peaks showing the strongest increase over the previous cycle. Cycles that produce either no amino acid signal or no signal increase over the previous cycle are designated as x. Upper and lower case letters designate, respectively, amino acid residues that match or do not match the known sequence that is shown. [b] Peptide–$tRNA^{Ala}$ complexes were purified from polyacrylamide gels and sequenced either with the $tRNA^{Ala}$ moiety intact or following digestion with RNase A. [c] Results of two independent experiments are shown. [d] Peptide Ala-70–Arg-85 from 875N; the sequence is from Putney et al. (1981b).

$tRNA^{Ala}$ specifically cross-links. We first showed that the aminoacylation activity of 461N is inhibited by oxidized $tRNA^{Ala}$ (unpublished data) and then conducted cross-linking experiments that utilized this fragment in parallel to the experiments that employed 875N, the results of which are also presented in Figure 2. One major putative 461N–$tRNA^{Ala}$ complex was observed upon incubation with the dialdehyde derivative of $tRNA^{Ala}$ (lane 5). As expected, due to the smaller protein employed, this complex migrated further into the gel than the larger 875N–$tRNA^{Ala}$ complex (lane 2). Following exhaustive digestion with RNase-free trypsin, autoradiography revealed a peptide–$tRNA^{Ala}$ complex that comigrated with the corresponding material originating from 875N. This suggested that the major site of cross-linking with oxidized $tRNA^{Ala}$ is located in the amino-terminal half of 875N.

Isolation and Sequencing of Labeled Peptide. In order to determine the precise location of the primary alanyl-tRNA synthetase amino acid residue(s) that participate(s) in the oxidized $tRNA^{Ala}$/sodium cyanoborohydride modification reaction, we analyzed the amino acid sequence of the peptide–$tRNA^{Ala}$ material obtained upon exhaustive tryptic digestion of the 875N–$tRNA^{Ala}$ and 461N–$tRNA^{Ala}$ complexes. The required quantity of peptide–$tRNA^{Ala}$ complex was prepared by scaling up the analytical procedure and was purified partially by denaturing PAGE. Due to the inherent presence of numerous nonlabeled peptides (48 are possible from 461N and 93 from 875N) in each of the trypsin digest mixtures, the major peptide–$tRNA^{Ala}$ complex was eluted from the polyacrylamide and purified further by a series of ethanol precipitations. This procedure was sufficient to remove peptides that comigrate with the peptide–$tRNA^{Ala}$ complex. [Comigrating non-cross-linked peptides were observed when the peptide–$tRNA^{Ala}$ complex band was electroblotted from the polyacrylamide onto a poly(vinylidine difluoride) membrane (Millipore) and sequenced directly as described by Matsudaira (1987).]

The results of sequence analysis of the major $tRNA^{Ala}$-labeled peptide, derived from 461N and 875N, are shown in Table I. Sequencing reactions were carried out for 6 cycles with the limited material available from the 461N complex and for 16 cycles with the peptide from 875N. Strong signals for alanine, glycine, and glycine were always detected in cycles one, two, and three, respectively, but were not always increased over the previous cycle, and thus are designated as x in some cases. The combined results of one experiment with 461N and two experiments with 875N provide the amino acid sequence AGGvHN(Dy)(Lp)ENVGYXAX (Table I). Upon comparison of this sequence with the published amino acid sequence of 875N (Putney et al., 1981b), the only close similarity was found in the peptide Ala-70–Arg-85 (AGGKHNDLENVGYTAR), where there is a match of 13/16 residues. Assuming that this is the peptide of interest, the lack of an unambiguous signal in cycle 14 is not surprising due to the ready degradation of tryptophan, and no signal is expected in cycle 16 because of the inherent low yield of the carboxyl-terminal residue. Furthermore, the lack of a lysine signal in cycle four could reflect chemical modifications at this position. Because residues 69 and 85 of 875N are arginine, it is reasonable to expect a tryptic digest peptide with the amino-terminal sequence $A^{70}GG$ and the carboxyl-terminal sequence TAR^{85}. By the same token, one would expect tryptic cleavage to occur at Lys-73, a cleavage site that is apparently blocked in the $tRNA^{Ala}$-labeled synthetase. Because incubations of the nature described in this work result in covalent cross-linking between one of the 3′-aldehydes on the oxidized cognate tRNA

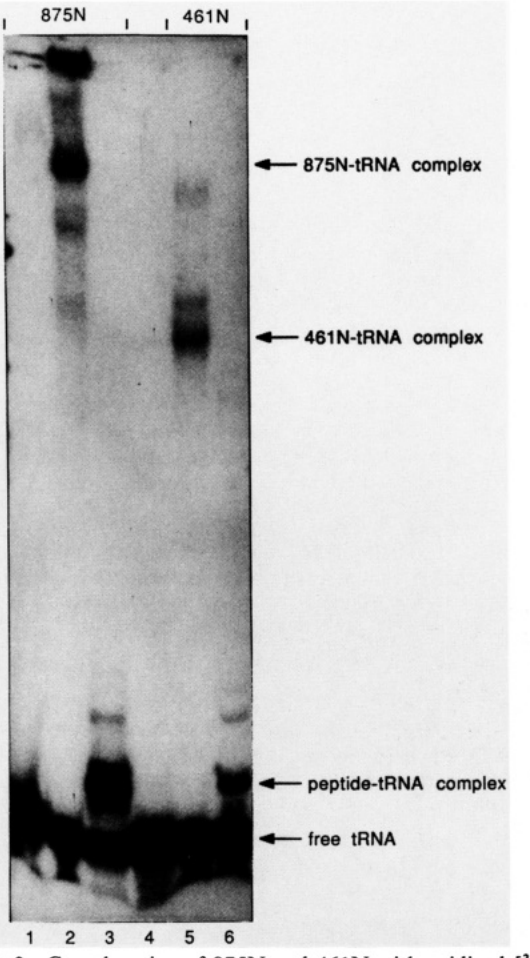

FIGURE 2: Complexation of 875N and 461N with oxidized [^{32}P]-$tRNA^{Ala}$. Shown is an autoradiograph of a denaturing polyacrylamide gel through which radioactively labeled products of various incubations were subjected to electrophoresis. Proteins 875N (25 μM, lanes 1–3) or 461N (22 μM, lanes 5 and 6) were incubated as described under Materials and Methods in the presence of native [^{32}P]$tRNA^{Ala}$ (14 μM, lane 1) or oxidized [^{32}P]$tRNA^{Ala}$ (13 μM, lanes 2, 3, 5, and 6). Prior to electrophoresis, the protein material in lanes 3 and 6 was digested exhaustively with RNase-free trypsin. The sample in lane 4 contains oxidized [^{32}P]$tRNA^{Ala}$ (13 μM) in the absence of protein.

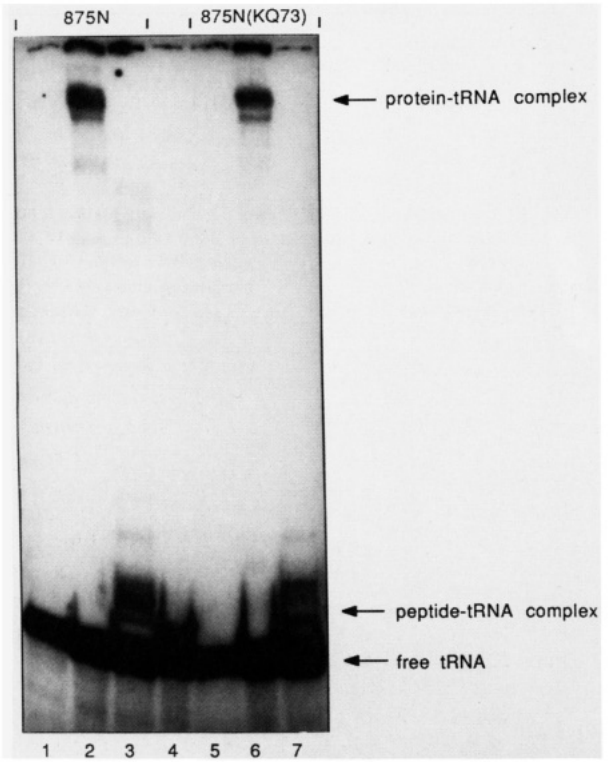

FIGURE 3: Complexation of 875N and 875N(KQ73) with oxidized [^{32}P]tRNA^{Ala}. Shown is an autoradiograph of a denaturing polyacrylamide gel through which radioactively labeled products of various incubations were subjected to electrophoresis. Proteins 875N (4.3 μM, lanes 1–3) or 875N(KQ73) (4.2 μM, lanes 5–7) were incubated as described under Materials and Methods in the presence of native [^{32}P]tRNA^{Ala} (8.0 μM, lanes 1 and 5) or oxidized [^{32}P]tRNA^{Ala} (7.2 μM, lanes 2, 3, 6, and 7). Prior to electrophoresis, the protein material in lanes 3 and 7 was digested exhaustively with RNase-free trypsin. The sample in lane 4 contains oxidized [^{32}P]tRNA^{Ala} (7.2 μM) in the absence of protein.

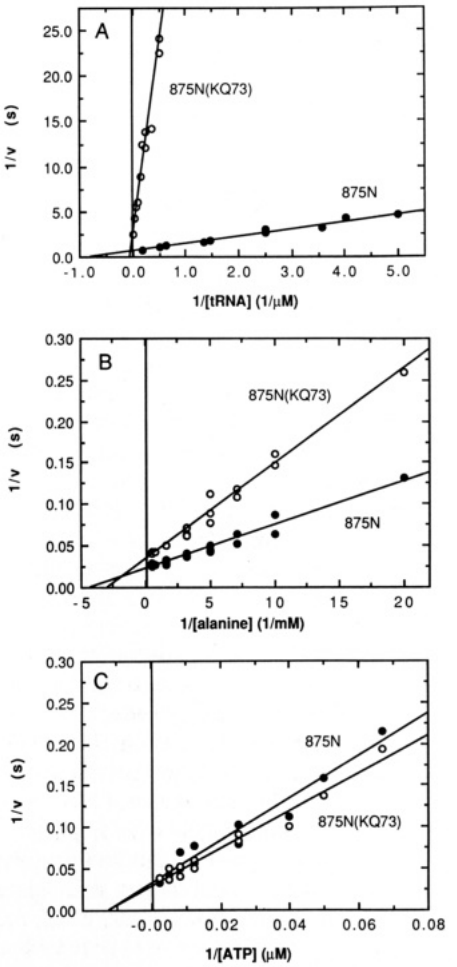

FIGURE 4: Comparison of 875N and 875N(KQ73) catalytic activities. Kinetic data obtained with 875N and 875N(KQ73) are presented in Lineweaver–Burk plots of initial rates versus initial substrate concentrations. The data were obtained via the aminoacylation (panel A, [alanine] = 22 μM; [ATP] = 4.0 mM) and ATP–PP$_i$ exchange (panel B, [ATP] = 2.0 mM and panel C, [alanine] = 5.0 mM) assays. Rates are presented as picomoles of alanyl-tRNA^{Ala} formed per second per picomole of enzyme (A) or picomoles of [γ-^{32}P]ATP formed per second per picomole of enzyme (B and C).

and the ϵ-amine of a lysine residue in the synthetase polypeptide chain (Fayat et al., 1979), these data provide evidence that the side chain of Lys-73 is the major site of cross-linking between alanyl-tRNA synthetase and the 3′ terminus of tRNA^{Ala}.

Modification of Alanyl-tRNA Synthetase by Oligonucleotide-Directed Mutagenesis. Our peptide sequencing data suggest the probability that Lys-73 of alanyl-tRNA synthetase is proximal to the 3′ end of its bound cognate tRNA. We decided to test this hypothesis through the introduction of a single amino acid change at position 73 via oligonucleotide-directed mutagenesis. The substitute amino acid of choice was glutamine, in order to eliminate the positive charge yet maintain closely the steric and hydrophilic properties of lysine.

The KQ73 substitution was introduced into 875N as described under Materials and Methods, and the phenotype of this mutation was tested in the *E. coli alaS* deletion derivative of strain W3110 (Regan, 1986). When encoded on a multicopy plasmid, the mutant protein was found to be functional by its ability to complement the growth-defective phenotype of this deletion strain. It is not known whether there is sufficient activity for complementation by a single copy of the gene.

In order to substantiate the finding that Lys-73 is the major site of cross-linking between 875N and oxidized tRNA^{Ala}, we attempted to complex this dialdehyde derivative with the KQ73 mutant protein. The results of these experiments, and parallel experiments with the wild-type protein, are presented in Figure 3. Autoradiography revealed that when 875N or 875N(KQ73) was incubated with oxidized tRNA^{Ala}, in the presence of sodium cyanoborohydride, protein–tRNA^{Ala} complexes were generated (lanes 2 and 6). These complexes did not form when native tRNA^{Ala} was employed (lanes 1 and 5). Upon exhaustive trypsin digestion, it was observed that the major peptide–tRNA^{Ala} complex formed with 875N (lane 3) was not generated when 875N(KQ73) was employed (lane 7). The minor sites observed with the wild-type enzyme were the only sites of cross-linking between oxidized tRNA^{Ala} and 875N(KQ73). These results confirmed the peptide sequencing data and established Lys-73 to be the major site of cross-linking between 875N and oxidized tRNA^{Ala}.

Kinetic Evaluation of Mutant Alanyl-tRNA Synthetase. Although the mutant alanyl-tRNA synthetase was functional in vivo, an evaluation of its kinetic parameters provided a more sensitive indication of subtle functional changes caused by the KQ73 substitution. The functional importance of Lys-73 in alanyl-tRNA synthetase was assessed by comparing the steady-state kinetic parameters of 875N and 875N(KQ73) for both the ATP–PP$_i$ exchange and aminoacylation reactions. The data from these experiments are presented in Figure 4, and the apparent kinetic parameters obtained upon extrapolation of the plots are summarized in Table II.

The initial rates of aminoacylation of tRNA^{Ala} by 875N and 875N(KQ73) are plotted, in Lineweaver–Burk form, as a

Table II: Apparent Kinetic Parameters for Enzymes 875N and 875N(KQ73)

	aminoacylation assay[a]			ATP–pyrophosphate exchange assay			
protein	K_m(tRNA^{Ala})[b] (μM)	k_{cat}[c] (s^{-1})	k_{cat}/K_m(tRNA^{Ala})[d]	K_m(alanine)[e] (μM)	K_m(ATP)[f] (μM)	k_{cat}[g] (s^{-1})	k_{cat}[h] (s^{-1})
875N	1.4 ± 0.3	1.7 ± 0.3	1.0	240 ± 50	83 ± 17	46 ± 9	33 ± 7
875N(KQ73)	17 ± 3	0.42 ± 0.08	0.02	450 ± 90	79 ± 16	30 ± 6	35 ± 7

[a] Aminoacylation assays were conducted at subsaturating concentrations of alanine; see comment under Materials and Methods. [b] Determined from the negative reciprocals of the abscissa intercepts of Figure 4A. [c] Determined from the reciprocals of the ordinate intercepts of Figure 4A. [d] Relative values are shown. Actual values are 1.2 ± 0.2 μM^{-1} s^{-1} for 875N and 0.025 ± 0.005 μM^{-1} s^{-1} for 875N(KQ73). [e] Determined from the negative reciprocals of the abscissa intercepts of Figure 4B. [f] Determined from the negative reciprocals of the abscissa intercepts of Figure 4C. [g] Determined from the reciprocals of the ordinate intercepts of Figure 4B. [h] Determined from the reciprocals of the ordinate intercepts of Figure 4C.

function of tRNA^{Ala} concentration in Figure 4A. Upon inspection of this graph, it is immediately obvious that the lysine to glutamine substitution at position 73 caused a significant change in the apparent kinetic parameters of alanyl-tRNA synthetase. Specifically, the point mutation resulted in a 12-fold increase in the apparent K_m for tRNA^{Ala} and a 4-fold decrease in the apparent k_{cat} (Table II). This corresponds to a 50-fold (98%) decrease in the efficiency of this synthetase to aminoacylate its cognate tRNA under the conditions of the assay.

In addition to the above evaluation of k_{cat} and K_m with respect to tRNA^{Ala}, we compared the apparent K_m's for alanine and ATP via the ATP–PP$_i$ exchange reaction. The initial rates obtained in these assays are plotted, in Lineweaver–Burk form, as functions of alanine and ATP concentrations in Figure 4B and Figure 4C, respectively. The apparent kinetic parameters obtained from the data in these plots show small or no changes resulting from the lysine to glutamine mutation at position 73. Specifically, the mutation led to an approximate 2-fold increase in the apparent K_m for alanine with no significant alteration in the apparent K_m for ATP or the apparent k_{cat} for this reaction (Table II).

Discussion

Much effort has been directed in recent years at developing a deeper understanding of the factors responsible for the efficient and functional protein–RNA interactions that occur between aminoacyl-tRNA synthetases and their cognate tRNAs (Schimmel, 1987). Although high-resolution X-ray diffraction data have been obtained for two members of this class of enzymes [*E. coli* methionyl-tRNA synthetase (Zelwer et al., 1982) and *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (Bhat et al., 1982)], rigorous structural information on a synthetase–tRNA complex is not yet available (Lorber et al., 1983; Frederick et al., 1988; Perona et al., 1988). Studies on the protein–RNA interaction have thus been limited to the use of other physical and chemical techniques, with the addition of the more recent developments in molecular biology. With regard to the specific interaction between aminoacyl-tRNA synthetases and the amino acid acceptor arm of their cognate tRNAs, the periodate-oxidized tRNA affinity label has proven to be a valuable tool in the identification of peptide sequences that may be proximal to the 3′ end of the bound tRNA molecule (Renaud et al., 1982; Hountondji et al., 1985, 1986b, 1987). When combined with information generated by site-directed mutagenesis, the importance of such amino acid residues can be addressed directly.

Affinity labeling of *E. coli* alanyl-tRNA synthetase with periodate-oxidized tRNA^{Ala} caused inactivation of this enzyme in a time-dependent, irreversible manner. The inactivation was observed when assayed by either the two-step aminoacylation reaction or the one-step ATP–PP$_i$ exchange reaction, and comparison of the two processes (Figure 1C) suggests that inactivation was caused in each case via the same phenomenon. However, inactivation of the adenylate synthesis reaction was only partial, with a residual activity of 20% after long incubation with the oxidized tRNA^{Ala}. In other work, essentially total inactivation of both aminoacylation and ATP–PP$_i$ exchange activities was obtained in cross-linking experiments with *E. coli* methionyl-tRNA synthetase (Hountondji et al., 1979) and *E. coli* tyrosyl-tRNA synthetase (Hountondji et al., 1986b) but not with the phenylalanyl-tRNA synthetases from yeast (Baltzinger et al., 1979) or *E. coli* (Hountondji et al., 1987). In the latter two cases, inactivation of the aminoacylation reaction but not the ATP–PP$_i$ exchange reaction was observed. In all of these examples, the inactivation was due to covalent cross-linking, presumably via the formation of a Schiff base between one of the 3′-aldehydes on the oxidized tRNA^{Ala} and the ϵ-amine of one or more lysines (Fayat et al., 1979). This is in contrast to the noncovalent, partial interference of some cognate tRNAs with the adenylate synthesis reaction (Buonocore & Schlesinger, 1972; McNeil & Schimmel, 1972).

In our work, the specificity of the affinity label was investigated by testing for interference with the inactivation process by native tRNA^{Ala}. This experiment revealed the presence of competition between native tRNA^{Ala} and the dialdehyde derivative (Figure 1A), suggesting that the affinity label binds to a functionally important site. The slow inactivation obtained in the presence of only the native tRNA^{Ala} species (Figure 1A) was not due to the formation of covalent synthetase–tRNA^{Ala} complexes (Figure 2, lane 1) but may represent nonspecific sodium cyanoborohydride reduction of functionally important amino acid side groups.

The identity of the amino acid residue responsible for the formation of the major peptide–tRNA^{Ala} complex was determined by peptide sequence analysis to be Lys-73. This identity was based on a peptide sequence that corresponded, in the majority of residues, with an alanyl-tRNA synthetase peptide that extends from Ala-70 to Arg-85 (Putney et al., 1981b). In this peptide, tryptic cleavage at Lys-73 was prevented due to the inherent modification of this base in the cross-linking procedure. We therefore concluded that Lys-73 is located in the region of alanyl-tRNA synthetase responsible for binding the 3′ end of tRNA^{Ala} and may be at least partially responsible for this functional interaction.

Peptide sequences potentially responsible for the association of three other *E. coli* aminoacyl-tRNA synthetases with the 3′ end of their cognate tRNAs have been identified. In the case of methionyl-tRNA synthetase, one minor and two major peptides were labeled with oxidized initiator tRNA^{Met} (Hountondji et al., 1985), the location of which can be determined from comparison with the crystallographic structure (Zelwer et al., 1982; Blow et al., 1983; Brunie et al., 1987). All of these peptides are located in the amino-terminal domain, which extends to residue 360 and includes the mononucleotide binding fold (Rossmann et al., 1977; Risler et al., 1981). The two major peptides contain Lys-61 and Lys-335, respectively,

both of which are in connecting sequences between an α-helix and a β-sheet in this fold (Brunie et al., 1987; Starzyk et al., 1987). Substitution of Lys-335 with glutamic acid results in complete inactivation of methionyl-tRNA synthetase, although it is not clear from the published data whether this mutation blocks adenylate synthesis or inhibits aminoacylation at a subsequent step (Brunie et al., 1987). The minor peptide, containing Lys-142, -147, and -149, lies in a long connective polypeptide that joins two β-sheet segments of the nucleotide fold.

In the case of tyrosyl-tRNA synthetase, three lysine residues (Lys-229, -234, and -237) were found to be labeled by oxidized $tRNA^{Tyr}$ (Hountondji et al., 1986b). These lysines correspond to three lysines in a highly conserved sequence in the same enzyme from *Bacillus stearothermophilus* (Winter et al., 1983), two of which have been shown by mutagenesis experiments to be important in the synthesis of tyrosyladenylate (Bedouelle & Winter, 1986; Fersht et al., 1988). On the basis of this information, and by analogy with the known three-dimensional structure of *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (Bhat et al., 1982; Blow et al., 1983), Lys-229, -234, and -237 in the *E. coli* enzyme are most likely located in or near the nucleotide binding fold.

Affinity labeling studies with phenylalanyl-tRNA synthetase have also identified potential $tRNA^{Phe}$ binding peptide sequences in the amino-terminal region of this enzyme's large α subunit (Hountondji et al., 1987). In this case, peptides containing Lys-2, -61, and -106 were shown to be almost equally capable of complexing with the dialdehyde derivative of $tRNA^{Phe}$. The orientation of these lysines in the three-dimensional structure of phenylalanyl-tRNA synthetase remains to be determined.

Upon the identification of lysine residues most reactive with the 3′-acceptor end of their cognate tRNAs in *E. coli* methionyl- and tyrosyl-tRNA synthetases, it was noted that similarities exist among their adjacent peptide sequences (Hountondji et al., 1986b). This led to a systematic computer analysis to search for similarities between these peptide sequences and those in other synthetases with known primary structure (Hountondji et al., 1986a). The relevant amino acid sequence KMSKS was found to be highly conserved in the primary structures of six aminoacyl-tRNA synthetases, with similar short peptides noted in five others. *E. coli* alanyl-tRNA synthetase is among the enzymes containing a similar short peptide, consisting in this case of Ala-815–Lys-819 (AGVSK) in the carboxyl-terminal region. This sequence was noted with reservation as to its significance because it shares only 3 amino acid identities and 1 conservative replacement out of 11 compared residues. Moreover, it should be noted that the entire sequence of *E. coli* alanyl-tRNA synthetase has little or no similarity with that of any other synthetase for which the primary structure has been determined (Schimmel, 1987).

In order to test the significance of Lys-73 in the functional alignment of the 3′-acceptor end of $tRNA^{Ala}$ with alanyl-tRNA synthetase, we introduced a single amino acid substitution at this position by site-directed mutagenesis. The substitute of choice was glutamine, in order to eliminate the positive charge yet maintain closely the steric and hydrophilic properties of lysine. With this mutation, we established that Lys-73 is not essential for the aminoacylation of $tRNA^{Ala}$, because the mutant protein [875N(KQ73)] was able to complement the growth of an *alaS* deletion strain. However, the substitution of glutamine at position 73 did prevent cross-linking at the major contact site with oxidized $tRNA^{Ala}$ (Figure 3), thus confirming our peptide sequence analysis.

The functional importance of Lys-73 in alanyl-tRNA synthetase was assessed in a comparison of the steady-state apparent kinetic parameters obtained with both the wild-type and the mutant enzymes (Figure 4). By studying apparent K_m and k_{cat} values determined in both the aminoacylation and ATP–PP_i exchange reactions, we noted that the most significant effect of the KQ73 mutation was a 12-fold decrease in the apparent affinity of alanyl-tRNA synthetase for $tRNA^{Ala}$ (Table II). When considered in the context of the aminoacylation reaction, this mutation resulted in a 98% decrease in the catalytic efficiency of this enzyme. We thus conclude that the side chain of Lys-73, with its inherent positive charge, is partially responsible for the functional alignment of the 3′-acceptor end of the cognate tRNA but is not essential for catalysis of the aminoacylation reaction. The precise role of this residue in catalysis may be determined upon the substitution of other amino acids at position 73. Furthermore, because crystals of an alanyl-tRNA synthetase fragment containing Lys-73 have recently been obtained (Frederick et al., 1988), we anticipate detailed structural information on the orientation of this amino acid to be available in the near future.

In contrast to the aminoacylation reaction, the KQ73 mutation introduced relatively small alterations in the ability of this enzyme to catalyze the ATP–PP_i exchange reaction. Specifically, we noted only an approximate 2-fold increase in the apparent K_m for alanine with no significant change in the other values determined. Other mutations in *E. coli* alanyl-tRNA synthetase have also been shown to affect the affinity of this enzyme for alanine [see Theall et al. (1977) and references cited therein], but the precise locations of these mutations in the *alaS* gene sequence have not been determined. Such mutations are possibly in a region containing a mononucleotide binding fold, as is thought to be the case for the methionyl-tRNA synthetase from yeast. In this enzyme, a single amino acid change in the carboxyl-terminal part of the putative mononucleotide binding fold apparently elevates the K_m for methionine in vivo and causes the enzyme to be labile and inactive in vitro (Chatton et al., 1987).

It is not surprising that a mutation in the region of alanyl-tRNA synthetase responsible for binding the 3′ end of $tRNA^{Ala}$ slightly interferes with alanine binding (Table II), because both components must at some time be brought into close proximity in order for aminoacylation to occur. On the other hand, the finding that the KQ73 mutation affects mostly $tRNA^{Ala}$ interactions portrays the substrate specificity of Lys-73 in this multisubstrate enzyme. Previous work in our laboratory showed that an amino acid substitution (Ala-409 → Val) on the carboxyl-terminal side of the adenylate synthesis domain alters the enzyme–$tRNA^{Ala}$ interaction (Ho et al., 1985; Regan et al., 1988). However, to our knowledge, Lys-73 is the first amino acid identified in the adenylate synthesis domain of alanyl-tRNA synthetase that is involved specifically in the interaction of this enzyme with its cognate tRNA.

The observation that ATP–PP_i exchange activity was partially inhibited upon cross-linking of alanyl-tRNA synthetase with the 3′ end of $tRNA^{Ala}$ (Figure 1B) must now be understood in light of the kinetic data obtained with the mutant enzyme. Because Lys-73 is not required for aminoacyl-adenylate synthesis to occur, it is possible that the partial inactivation observed represents steric interference by the large, covalently bound $tRNA^{Ala}$ molecule.

Our studies on the nature of one region of the $tRNA^{Ala}$ binding site on alanyl-tRNA synthetase provide another example of the complementary use of a chemical modification technique and site-directed mutagenesis. We utilized an af-

finity label to identify Lys-73 as an amino acid residue that probably exists in the region of alanyl-tRNA synthetase responsible for binding the 3′-acceptor end of its cognate tRNA. However, on the basis of the affinity label studies alone, the significance of Lys-73 in the catalytic function of this synthetase could not be assessed. Site-directed mutagenesis provided a mutant enzyme with which we established that Lys-73 in alanyl-tRNA synthetase is important for a $tRNA^{Ala}$-dependent step that is subsequent to the synthesis of the aminoacyladenylate intermediate.

ACKNOWLEDGMENTS

We thank M. Toth for guidance with the oligonucleotide-directed mutagenesis procedures, S. Blackman and P. Matsudaira for peptide sequencing, and F. Fasiolo and R. Starzyk for their critical reading of the manuscript.

Registry No. Lys, 56-87-1; Gln, 56-85-9; ATP, 56-65-5; Ala, 56-41-7; alanyl-tRNA synthetase, 9031-71-4; aminoacyl-tRNA synthetase, 9028-02-8.

REFERENCES

Baltzinger, M., Fasiolo, F., & Remy, P. (1979) *Eur. J. Biochem. 97*, 481–494.

Bedouelle, H., & Winter, G. (1986) *Nature (London) 320*, 371–373.

Bhat, T. N., Blow, D. M., Brick, P., & Nyborg, J. (1982) *J. Mol. Biol. 158*, 699–709.

Biggin, M. D., Gibson, T. J., & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3963–3965.

Blow, D. M., Bhat, T. N., Metcalfe, A., Risler, J. L., Brunie, S., & Zelwer, C. (1983) *J. Mol. Biol. 171*, 571–576.

Breton, R., Sanfacon, H., Papayannopoulos, I., Biemann, K., & Lapointe, J. (1986) *J. Biol. Chem. 261*, 10610–10617.

Brunie, S., Mellot, P., Zelwer, C., Risler, J.-L., Blanquet, S., & Fayat, G. (1987) *J. Mol. Graphics 5*, 18–21.

Buonocore, V., & Schlesinger, S. (1972) *J. Biol. Chem. 247*, 1343–1348.

Calendar, R., & Berg, P. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) pp 384–399, Harper and Row, New York.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835–840.

Chatton, B., Winsor, B., Boulanger, Y., & Fasiolo, F. (1987) *J. Biol. Chem. 262*, 15094–15097.

Fayat, G., Hountondji, C., & Blanquet, S. (1979) *Eur. J. Biochem. 96*, 87–92.

Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., & Hartley, B. S. (1975) *Biochemistry 14*, 1–4.

Fersht, A. R., Knill-Jones, J. W., Bedouelle, H., & Winter, G. (1988) *Biochemistry 27*, 1581–1587.

Frederick, C. A., Wang, A. H.-J., Rich, A., Regan, L., & Schimmel, P. (1988) *J. Mol. Biol. 203*, 521–522.

Ho, C., Jasin, M., & Schimmel, P. (1985) *Science 229*, 389–393.

Hoben, P., Royal, N., Cheung, A., Fumiaki, Y., Biemann, K., & Soll, D. (1982) *J. Biol. Chem. 257*, 11644–11650.

Hountondji, C., Fayat, G., & Blanquet, S. (1979) *Eur. J. Biochem. 102*, 247–250.

Hountondji, C., Blanquet, S., & Lederer, F. (1985) *Biochemistry 24*, 1175–1180.

Hountondji, C., Dessen, P., & Blanquet, S. (1986a) *Biochimie 68*, 1071–1078.

Hountondji, C., Lederer, F., Dessen, P., & Blanquet, S. (1986b) *Biochemistry 25*, 16–21.

Hountondji, C., Schmitter, J. M., Beauvallet, C., & Blanquet, S. (1987) *Biochemistry 26*, 5433–5439.

Hunkapiller, M. W., Lujan, E., Ostrander, F., & Hood, L. E. (1983) *Methods Enzymol. 91*, 227–236.

Huynh, Q. K., Bauer, S. C., Bild, G. S., Kishore, G. M., & Borgmeyer, J. R. (1988) *J. Biol. Chem. 263*, 11636–11639.

Jasin, M., Regan, L., & Schimmel, P. (1983) *Nature (London) 306*, 441–447.

Jasin, M., Regan, L., & Schimmel, P. (1984) *Cell 36*, 1089–1095.

Jasin, M., Regan, L., & Schimmel, P. (1985) *J. Biol. Chem. 260*, 2226–2230.

Kato, H., Tanaka, T., Nishioka, T., Kimura, A., & Oda, J. (1988) *J. Biol. Chem. 263*, 11646–11651.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Leon, O., & Schulman, L. H. (1987a) *Biochemistry 26*, 1933–1940.

Leon, O., & Schulamn, L. H. (1987b) *Biochemistry 26*, 5416–5422.

Leon, O., & Schulman, L. H. (1987c) *Biochemistry 26*, 7113–7121.

Lorber, B., Giege, R., Ebel, J.-P., Berthet, C., Thierry, J.-C., & Moras, D. (1983) *J. Biol. Chem. 258*, 8429–8435.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning*, pp 365–373, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

McNeil, M. R., & Schimmel, P. R. (1972) *Arch. Biochem. Biophys. 152*, 175–179.

Mechulam, Y., Fayat, G., & Blanquet, S. (1985) *J. Bacteriol. 163*, 787–791.

Nickbarg, E. B., Davenport, R. C., Petsko, G. A., & Knowles, J. R. (1988) *Biochemistry 27*, 5948–5960.

Perona, J. J., Swanson, R., Steitz, T. A., & Soll, D. (1988) *J. Mol. Biol. 202*, 121–126.

Profy, A., & Schimmel, P. (1988) *Prog. Nucleic Acid Res. Mol. Biol. 35*, 1–26.

Putney, S. D., Melendez, D. L., & Schimmel, P. R. (1981a) *J. Biol. Chem. 256*, 205–211.

Putney, S. D., Royal, N. J., de Vegvar, H. N., Herlihy, W. C., Biemann, K., & Schimmel, P. (1981b) *Science 213*, 1497–1501.

Putney, S. D., Sauer, R. T., & Schimmel, P. R. (1981c) *J. Biol. Chem. 256*, 198–204.

Regan, L. (1986) Ph.D. Thesis, Massachusetts Institute of Technology.

Regan, L., Bowie, J., & Schimmel, P. (1987) *Science 235*, 1651–1653.

Regan, L., Buxbaum, L., Hill, K., & Schimmel, P. (1988) *J. Biol. Chem. 263*, 18598–18600.

Reilly, R. M., & RajBhandary, U. L. (1986) *J. Biol. Chem. 261*, 2928–2935.

Renaud, M., Fasiolo, F., Baltzinger, M., Boulanger, Y., & Remy, P. (1982) *Eur. J. Biochem. 123*, 267–274.

Risler, J. L., Zelwer, C., & Brunie, S. (1981) *Nature (London) 292*, 384–386.

Rossmann, M. G., Garavito, R. M., & Eventoff, W. (1977) in *Pyridine Nucleotide Dependent Hydrogenases* (Sund, H., Ed.) pp 3–30, DeGruyter, Berlin.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Schimmel, P. (1987) *Annu. Rev. Biochem. 56*, 125–158.

Schimmel, P. R., & Soll, D. (1979) *Annu. Rev. Biochem. 48*, 601–648.

Schoemaker, H. J. P., & Schimmel, P. R. (1974) *J. Mol. Biol. 84*, 503-513.
Schreier, A. A., & Schimmel, P. R. (1972) *Biochemistry 11*, 1582-1589.
Silberklang, M., Gillum, A. M., & RajBhandary, U. L. (1977) *Nucleic Acids Res. 4*, 4091-4108.
Starzyk, R., Webster, T. A., & Schimmel, P. (1987) *Science 237*, 1614-1618.
Theall, G., Low, K. B., & Soll, D. (1977) *Mol. Gen. Genet. 156*, 221-227.
Toth, M. J., & Schimmel, P. (1986) *J. Biol. Chem. 261*, 6643-6646.
Valenzuela, D., & Schulman, L. H. (1986) *Biochemistry 25*, 4555-4561.
Waye, M. M. Y., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1983) *EMBO J. 2*, 1827-1829.
Webster, T. A., Gibson, B. W., Keng, T., Biemann, K., & Schimmel, P. (1983) *J. Biol. Chem. 258*, 10637-10641.
Webster, T. A., Tsai, H., Kula, M., Mackie, G., & Schimmel, P. (1984) *Science 226*, 1315-1317.
Webster, T. A., Lathrop, R. H., & Smith, T. F. (1987) *Biochemistry 26*, 6950-6957.
Winter, G., Koch, G. L. E., Hartley, B. S., & Barker, D. G. (1983) *Eur. J. Biochem. 132*, 383-387.
Zelwer, C., Risler, J. L., & Brunie, S. (1982) *J. Mol. Biol. 155*, 63-81.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468-500.

Role of the Histidine 176 Residue in Glyceraldehyde-3-phosphate Dehydrogenase As Probed by Site-Directed Mutagenesis†

A. Soukri,‡ A. Mougin,‡ C. Corbier,‡ A. Wonacott,§ C. Branlant,‡ and G. Branlant*,‡

Laboratoire d'Enzymologie et de Génie Génétique, UA CNRS 457, Faculté des Sciences, B.P. 239, 54506 Vandoeuvre les Nancy, France, and The Blackett Laboratory, Imperial College of Science and Technology, Prince Consort Road, London SW7 BZ, England

Received June 29, 1988; Revised Manuscript Received October 31, 1988

ABSTRACT: The catalytically essential amino acid, histidine 176, in the active site of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been replaced with an asparagine residue by site-directed mutagenesis. The role of histidine 176 as a chemical activator, enhancing the reactivity of the thiol group of cysteine 149, has been demonstrated, with iodoacetamide as a probe. The esterolytic properties of GAPDH, illustrated by the hydrolysis of *p*-nitrophenyl acetate, have been also studied. The kinetic results favor a role for histidine 176 not only as a chemical activator of cysteine 149 but also as a hydrogen donor facilitating the formation of tetrahedral intermediates. These results support the hypothesis that histidine 176 plays a similar role during the oxidative phosphorylation of glyceraldehyde 3-phosphate.

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] is a tetrameric enzyme, using NAD^+ as a cofactor. The reaction it catalyzes can be decomposed into an oxidoreduction step, with formation of an acyl-enzyme and NADH, and a phosphorylating step, including the nucleophilic attack of the acyl-enzyme by phosphate ion in the presence of NAD^+, giving rise to 1,3-diphosphoglycerate. The currently accepted mechanism (Harris & Waters, 1976), supported by extensive pre-steady-state (Trentham, 1971; Harrigan & Trentham, 1974) and steady-state kinetic experiments (Duggleby & Dennis, 1974; Meunier & Dalziel, 1978), is in favor of the formation of a thiohemiacetal intermediate involving two essential residues, Cys-149 and His-176. The His residue is postulated to act as a chemical activator by enhancing the reactivity of the thiol group of Cys-149, possibly through the formation of an ion pair with the imidazolium ring of His-176 (Polgar, 1975). The tetrahedral transition state for the hemithioacetal formation would also be stabilized by hydrogen bonding between the protonated imidazole N^ϵ of His-176 and the carbonyl oxygen atom of glyceraldehyde 3-phosphate, thus favoring a nucleophilic attack by the thiol group. A similar activation could also occur during the phosphorylating step, with the formation of a hydrogen bond between the imidazolium ring and the oxygen of the carbonyl group of the acyl-enzyme, facilitating the nucleophilic attack by the phosphate ion.

As postulated for certain dehydrogenases (Holbrook et al., 1976; Fersht, 1985), the His residue could also play a role as a base catalyst, facilitating hydride transfer, during the oxidoreduction step, in the case of GAPDH, from the thiohemiacetal intermediate toward the C4 position of the nicotinamidium ring of the coenzyme (Harris & Waters, 1976). According to the crystal structure of GAPDH from *Bacillus stearothermophilus* (Skarzynski et al., 1987; Skarzynski &

† This work was supported by Grant 84 V0670 of the Ministère de la Recherche et de la Technologie and le Centre National de la Recherche Scientifique, Essor des Biotechnologies.
* To whom correspondence should be addressed.
‡ UA CNRS 457, Faculté des Sciences.
§ Imperial College of Science and Technology.

[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); Tris, tris(hydroxymethyl)aminomethane; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; pNPA, *p*-nitrophenyl acetate; pNP, *p*-nitrophenol; IAM, iodoacetamide; G3P, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; P_i, inorganic phosphate; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms; *B. stearothermophilus*, *Bacillus stearothermophilus*; *E. coli*, *Escherichia coli*. Mutant is referred to as follows: His-176 → Asn-176 enzyme with asparagine at position 176.

0006-2960/89/0428-2586$01.50/0