Kinetics of Homologous and Heterologous Aminoacylation with Yeast Phenylalanyl Transfer Ribonucleic Acid Synthetase†

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ABSTRACT: Yeast phenylalanyl-tRNA synthetase (PRS) can aminoacylate highly purified phenylalanine tRNAs of *E. coli* yeast and wheat and *E. coli* $tRNA_1^{Val}$, $tRNA_{2A}^{Val}$, $tRNA_{2B}^{Val}$, $tRNA_M^{Met}$, $tRNA^{Ile}$, $tRNA_1^{Ala}$, $tRNA_2^{Ala}$, and $tRNA^{Lys}$. The sequences of the first eight tRNAs have been compared, and based, in part, upon their unique sequence similarities, it was proposed that two regions of the tRNA molecule are primarily involved in the PRS recognition site. The double-stranded region adjacent to the dihydrouridine loop and adenosine as the fourth residue from the 3′ end were proposed to be directly involved in the recognition of a tRNA by this particular synthetase (B. Roe and B. Dudock, *Biochem. Biophys. Res. Commun. 49*, 399 (1972)). In this paper we report: (a) the purification of PRS essentially to homogeneity; (b) the pH optima for the PRS aminoacylation reactions; and (c) the kinetics of aminoacylation with PRS for each of the 11 tRNAs mentioned above at pH 6.0 (cacodylate buffer) and pH 8.2 (Tris buffer). This study shows that these tRNAs differ only slightly (tenfold) in their K_m's for the PRS aminoacylation reaction but differ considerably (200-fold) in their V_{max} values. At pH 8.2 (Tris buffer), these 11 tRNAs fall into three distinct classes characterized by order of magnitude differences in their V_{max} for the PRS aminoacylation reaction. These three classes are a fast class (V_{max} = 0.4–0.5), an intermediate class (V_{max} = 0.01–0.09) and a slow class (V_{max} = 0.003–0.008). Each of these classes can be correlated primarily with two structural features of the tRNA molecule, specifically the size of the dihydrouridine loop (8 or 9 nucleotides) and the presence of an N^2-methylguanine or an unmodified guanine at position 10 from the 5′ end. It is further shown that *E. coli* $tRNA_2^{Ala}$, which can be aminoacylated by PRS, can act as a competitive inhibitor of the homologous aminoacylation of yeast $tRNA^{Phe}$ by PRS. Furthermore, under identical conditions, *E. coli* $tRNA_3^{Gly}$, which cannot be aminoacylated by PRS, cannot act as a competitive inhibitor of the homologous aminoacylation of yeast $tRNA^{Phe}$. *E. coli* $tRNA_3^{Gly}$ has the "correct" nucleotides in the double-stranded region adjacent to the dihydrouridine loop (diHU stem) but lacks adenosine at the fourth position from the 3′ end (it contains uridine at this position). This lack of competitive inhibition suggests that in an intact tRNA specific nucleotides in both the diHU stem region and adenosine as the fourth residue from the 3′ end are required for binding to PRS. The model of the PRS recognition site is discussed as well as its applicability to other synthetases.

One approach to the elucidation of the aminoacyl-tRNA synthetase recognition site is to compare the sequences of several tRNAs, all of which are aminoacylated by a single synthetase. Those regions which are common to these tRNAs are then most likely candidates to be involved in the recognition site for that synthetase. The more tRNAs which can be compared, *i.e.*, they are of known sequence and are all aminoacylated by a single synthetase, and the greater the differences among these tRNAs, the more confidence one can place in those unique regions observed.

Yeast phenylalanyl-tRNA synthetase[1] (PRS) can aminoacylate the phenylalanine tRNAs of *E. coli* yeast and wheat and *E. coli* $tRNA_1^{Val}$, $tRNA_{2A}^{Val}$, $tRNA_{2B}^{Val}$, $tRNA_M^{Met}$, $tRNA^{Ile}$, $tRNA_1^{Ala}$, $tRNA_2^{Ala}$, and $tRNA^{Lys}$, as shown in Table I (Roe and Dudock, 1972; Roe *et al.*, 1971; Dudock *et al.*, 1970; Taglang *et al.*, 1970). The aminoacylation of, for example, *E. coli* $tRNA_1^{Val}$ by PRS has been called "heterologous mischarging" for it refers to the instance in which a synthetase from one source aminoacylates an "incorrect" tRNA from another source. This type of "incorrect" aminoacylation reaction was first reported by Barnett and Jacobson (1964) who observed that *N. crassa* phenylalanyl-tRNA synthetase can aminoacylate *E. coli* $tRNA^{Val}$. Heterologous mischarging can be a most valuable tool in elucidating the nucleotides which are involved in aminoacyl-tRNA synthetase recognition sites, for it allows the comparison of very different tRNAs, all of which are aminoacylated by a single synthetase. Using this approach the sequences of the first eight tRNAs mentioned above were compared (see composite tRNA, Figure 1), and based, in part, upon their unique sequence similarities, it was proposed that two regions of the tRNA molecule are primarily involved in the PRS recognition site. Both the double-stranded region adjacent to the dihydrouridine loop (diHU stem) and adenosine, at the fourth position from the 3′ end, were proposed to be directly involved in the recognition of a tRNA by this particular synthetase (Roe and Dudock, 1972). The absolute requirement for adenosine at the fourth position from the 3′ end was further substantiated by the observation that three tRNAs (*E. coli* $tRNA_3^{Gly}$, *E. coli* $tRNA^{His}$, and

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[1] Abbreviations used are: A_{260} unit, the quantity of material contained in 1 ml of solution which has an absorbance of 1 at 260 nm when measured in a 1-cm light-path cell; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; diHU stem, the double-stranded region adjacent to the dihydrouridine loop of tRNA; PRS, yeast phenylalanyl-tRNA synthetase.

TABLE 1: tRNA Aminoacylated by Yeast Phenylalanyl tRNA Synthetase.[a]

tRNA	Acceptor Activity: Homologous Aminoacylation (nmol/A_{260}) pH 7.6	Heterologous Aminoacylation nmol/A_{260} pH 5.8	Heterologous Aminoacylation nmol/A_{260} pH 8.2	% Homologous pH 5.8	% Homologous pH 8.2
Yeast phenylalanine	1.2				
Wheat phenylalanine	1.2	1.2	1.2	100	100
E. coli phenylalanine	1.2	1.2	1.0	100	83
E. coli valine 1	1.4	1.25	0.84	89	60
E. coli valine 2 (A and B)	1.2	1.0	0.92	83	77
E. coli isoleucine	1.3	1.0	0.65	77	50
E. coli methionine (M)[b]	1.2	0.96	0.84	80	70
E. coli alanine 1	1.5	1.35	0.78	90	52
E. coli alanine 2	1.0	0.85	0.80	85	80
E. coli lysine	1.2	1.1	0.81	92	68

[a] Homologous aminoacylation reactions were performed as described in Materials and Methods using mix A, while mix B was used for the heterologous aminoacylation reactions with either potassium cacodylate (pH 5.8) or Tris-HCl (pH 8.2). [b] *E. coli* $tRNA_M^{Met}$ was distinguished from *E. coli* $tRNA_F^{Met}$ by the procedure of Schofield (1970).

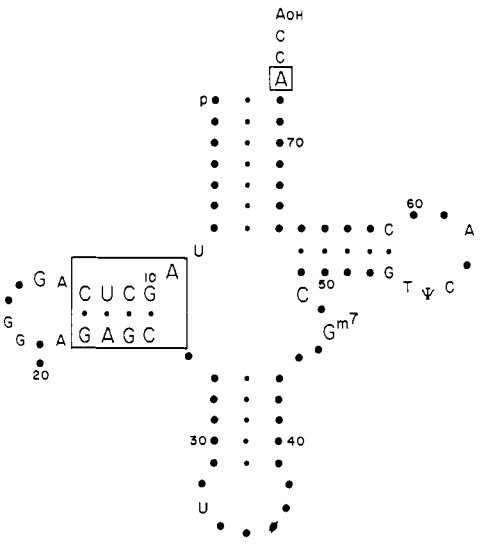

FIGURE 1: Composite tRNA for yeast phenylalanyl tRNA synthetase. Those nucleotides that are not the same in the first eight tRNAs of Table I are shown in the composite with a dot. Those nucleotides that are found in the same position in all tRNAs are shown in the composite in light type. Those nucleotides that are uniquely common to the first eight tRNAs of Table I are shown in boldface. The extra dot near the dihydrouridine loop denotes the fact that four tRNAs (*E. coli* $tRNA_{2A}^{Val}$, *E. coli* $tRNA_{2B}^{Val}$, *E. coli* $tRNA_M^{Met}$, and *E. coli* $tRNA^{Ile}$) have one extra nucleotide in the dihydrouridine loop. The central nucleotide of the anticodon of the eight tRNAs upon which this composite is based is adenosine. This has been eliminated from the composite since, based on the genetic code, it could not be present in that position in any species of alanine tRNA. Indeed, *E. coli* $tRNA_1^{Ala}$ has a guanosine at that location (R. Williams, W. Nagel, B. Roe, and B. Dudock, manuscript in preparation).

yeast $tRNA^{Lys}$) whose sequences are extremely similar or identical in all respects with that shown in the composite tRNA (Figure 1), except that they have nucleosides other than adenosine at the fourth position from the 3′ end, were not aminoacylated by PRS (Roe and Dudock, 1972). In addition to confirming the requirement for adenosine at the fourth position from the 3′ end, these experiments also show the high degree of specificity which is present in these PRS aminoacylation reactions (Roe and Dudock, 1972). Indeed, in order to use heterologous mischarging as a tool to probe the synthetase recognition site, the specificity of the aminoacylation reaction must be maintained. It has recently been shown (Kern *et al.*, 1972) that tRNA synthetases markedly lose specificity under certain conditions, particularly in the presence of denaturing agents such as dimethyl sulfoxide. Under these conditions relatively little information can be obtained about a synthetase recognition site. Therefore, we have used conditions under which a high degree of specificity is maintained.

In order to understand more completely the PRS recognition process, the aminoacylation kinetics with this synthetase have now been studied. The aminoacylation reaction of a tRNA with phenylalanine by PRS can be described by the following simplified equations.

$$\text{PRS} + \text{Phe} + \text{ATP} \rightleftharpoons \text{PRS} - \text{Phe} \sim \text{AMP} + \text{PP}_i \quad (1)$$

$$\text{PRS} - \text{Phe} \sim \text{AMP} + \text{tRNA}^{AA} \rightleftharpoons \text{intermediate complex} \rightleftharpoons \text{PRS} + \text{AMP} + \text{Phe} - \text{tRNA}^{AA} \quad (2)$$

Equation 1 describes the amino acid activation while eq 2 represents the formation of the intermediate complex and its subsequent breakdown into the aminoacyl-tRNA. At present we are concerned with the reactions described in eq 2. An intermediate complex must be formed during the aminoacylation reactions involving PRS, and it is in the formation of this complex and subsequent release of product that the synthetase recognition site on a tRNA has its role. In a simplified sense, once a steady state is achieved, the binding constant for the complex can be characterized by determination of the overall K_m, while the rate of breakdown of the complex to product can be described by V_{max}.

In this paper, we show these kinetic parameters for the 11 tRNAs which are all acceptable substrates for PRS and phenylalanine. For this study each of the tRNAs was obtained in highly purified form and the synthetase, PRS, was also purified essentially to homogeneity. We will discuss: (a) the purification of PRS, (b) the pH optima for the PRS aminoacylation reaction, and (c) the kinetics of aminoacylation with PRS for each of the 11 tRNAs mentioned above at pH 6.0 (cacodylate buffer) and pH 8.2 (Tris buffer).

Materials and Methods

The purification of wheat germ $tRNA^{Phe}$ has been reported (Dudock *et al.*, 1969). The purification of *E. coli* tRNAs was accomplished by chromatography on benzoylated DEAE-cellulose (Gillam *et al.*, 1967), reversed-phase Plaskon columns (Pearson *et al.*, 1971), DEAE-Sephadex (Nishimura, 1971), and by phenoxyacetylation (Gillam *et al.*, 1968) and has been described in detail elsewhere (Roe and Dudock, 1972; Roe *et al.*, 1971; Dudock *et al.*, 1970). Highly purified yeast

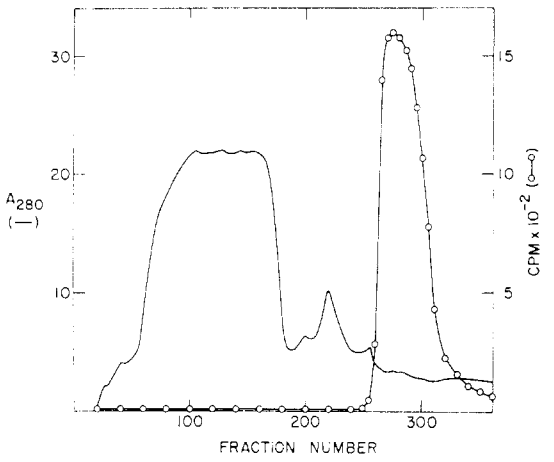

FIGURE 2: DEAE-cellulose chromatography in the purification of yeast phenylalanyl-tRNA synthetase. The supernatant (70,000 A_{280} units) from the 10,000g centrifugation of the disrupted yeast cells was applied to a DEAE-cellulose column (2.5 × 100 cm) previously equilibrated in buffer A. The column was then washed with 500 ml of buffer A and at tube 180, a 3-l. linear gradient (0.0–0.3 M KCl, both in buffer A) was generated. The flow rate was maintained at 12 ml/5 min per tube. Protein was monitored at A_{280} and PRS activity was measured as described in Methods.

$tRNA^{Phe}$ (1.2 nmol/A_{260} unit[1]) was purchased from Boehringer Mannheim.

DEAE-cellulose (standard grade number 70, Schleicher and Schuel) was pretreated before use as follows: 100 g of dry DEAE-cellulose was added to 3 l. of 0.1 M NaOH in a 4-l. glass beaker. The mixture was heated to 60–70° with gentle stirring and kept at this temperature for 1 hr. The mixture was then allowed to cool down to room temperature and, after settling, the supernatant was decanted off. The cellulose was then washed three times with 3 l. of distilled water and then added to 3 l. of 0.2 M acetic acid and stirred for 15 min. After settling, the supernatant was decanted off. The cellulose was then washed and defined five times with 3 l. of distilled water in a 4-l. graduated cylinder.

Hydroxylapatite (Bio-Gel HTP) was mixed in a ratio of 4 g of HTP/1 g of Whatman powdered cellulose. The mixture was defined five times before the slurry was used to pack the column. Phosphocellulose was Whatman P11 prepared as described by Burgess (1969).

Tris was obtained from Sigma, cacodylic acid from Baker, and Hepes, Mes, and dithiothreitol from Calbiochem. ^{14}C labeled amino acids were (Stan Star, 50 Ci/mol) purchased from Schwarz/Mann. All other chemicals were analytical reagent grade.

Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard or by using the A_{280}/A_{260} method of Warburg and Christian (1941) with the table of Layne (1957). RNA was determined by measuring the A_{260} assuming 1 mg of RNA in 1 ml of solution gives an A_{260} of 20.

Several sets of incubation mixtures were used in this study. Homologous tRNA acceptor activity was assayed using an aminoacylation mixture (mix A) containing (in 1.0 ml) 50 mM Tris (pH 7.6), 0.5 mM EDTA, 20 mM $MgCl_2$, 2.5 mM potassium ATP, 0.05 mM [^{14}C]amino acid (10 Ci/mol), an appropriate amount of tRNA (0.02–0.2 A_{260} unit of pure tRNA, larger amounts were used with impure tRNAs), and a saturating amount of crude tRNA synthetase prepared as previously described (Roe *et al.*, 1973). Homologous reactions were incubated at 30° for 20 min. For enzyme isolation assays an appropriate amount of column effluent (1–10 μl) was used with an incubation time of 2, 5, or 10 min.

Heterologous reactions were performed in an incubation mixture (mix B) containing (in 1.0 ml) 50 mM potassium cacodylate (pH 5.8), 0.5 mM EDTA, 40 mM $MgCl_2$, 2.5 mM potassium ATP, 0.05 mM [^{14}C]phenylalanine (10 Ci/mol), an appropriate amount of tRNA, and a saturating amount of either 200-fold purified PRS prepared as described (Dudock *et al.*, 1970) or pure PRS prepared as described below. When pure PRS was used 0.4 mg of bovine serum albumin was included in the reaction. These reactions were incubated at 30° for 2 hr, at which time at least 80% heterologous mischarging is obtained for all of the tRNAs used in this study as shown in Table I.

All kinetic studies were done using an incubation mixture (mix C) containing (in 1.0 ml) either 50 mM potassium cacodylate (pH 6.0) or 50 mM Tris (pH 8.2), 40 mM $MgCl_2$, 2.5 mM potassium ATP, 0.05 mM [^{14}C]phenylalanine (50 Ci/mol), 0.4 mg of bovine serum albumin, an appropriate amount of pure tRNA, and either 0.5 or 0.05 μg of pure PRS. Incubation was at 30° for 2, 5, 10, 15, 30, or 60 min depending upon the reaction studied. In all cases, ranges of incubation times were used to show linear incorporation of [^{14}C]phenylalanine.

The pH studies were done using incubation mixture (mix C) as used in the kinetic studies, except that the 50 mM buffer was either potassium cacodylate, Hepes–KOH, Mes–KOH, Tris-HCl, or glycine–KOH.

All aminoacylation reactions were terminated by the addition of cold 10% trichloroacetic acid (2 ml) and the mixtures were immediately filtered on a GF/A filter (previously moistened with cold 2% trichloroacetic acid) and then washed six times with cold 5-ml portions of 2% trichloroacetic acid, dried, and counted in omnifluor–toluene. In some studies aliquots of 0.10 ml were removed from the 1.0 ml incubation mixture at various times, precipitated with cold 10% trichloroacetic acid, and treated as above. All results were obtained in duplicate and corrected for zero time controls.

Results

Enzyme Isolation. Yeast phenylalanyl-tRNA synthetase (PRS) was obtained 600-fold purified from Fleishmann's yeast cakes using the following procedure. Unless otherwise stated, all work was done at 0–4°. Yeast cells were broken by a modification of the procedure of Hoskinson and Khorana (1965). Yeast (100 g) was placed in a 500-ml Virtis cup together with 100 g of acid-washed glass beads and 100 ml of a solution containing 0.02 M Tris (pH 8.0), 0.01 M $MgCl_2$, 0.001 M dithiothreitol, and 20% glycerol (buffer A). The yeast was disrupted in a Virtis homogenizer, using three Teflon blades in a no. 16-301 Virtis extender, by using two cycles of 3 min at a setting of 90% maximum and 3 min at 10% maximum. The mixture was allowed to settle, the supernatant was decanted off, the glass beads were extracted three times with 50-ml portions of buffer A, and the combined extracts and supernatant were then centrifuged at 10,000g for 30 min. For large scale isolation of PRS this complete grinding cycle was repeated three additional times so that a total of 400 g of yeast cells were processed giving a total of 2200 ml of solution. This crude extract was then absorbed onto a DEAE-cellulose column (2.5 × 100 cm) built and equilibrated in buffer A. The column was washed with 500 ml of buffer A and PRS was then eluted in a linear KCl gradient as shown in Figure 2. The PRS activity eluted as a single peak at approximately 0.20 M KCl. Peak tubes (270–300 of Figure 2) were pooled

TABLE II: Purification of Yeast Phenylalanyl-tRNA Synthetase.

Fraction	Protein (mg)	Total Activity[a] (EU)	Sp Act. (EU/mg)	Purification	Recovery (%)
I. Crude extract	19,800	18,700	0.94		100
II. DEAE-cellulose fraction	1,200	4,860	4.05	4.3	26
III. Phosphocellulose fraction	13.6	1,940	143	152	10
IV. Hydroxylapatite fraction	2	1,140	570	606	6

[a] One enzyme unit (EU) is defined as that amount of enzyme which catalyzes the incorporation of 1 nmol of phenylalanine into tRNA in 1.0 min at 30° under standard homologous conditions (mix A).

giving 450 ml of solution which was then dialyzed against three changes of 4 l. each of 0.02 M Tris (pH 8.0), 0.002 M EDTA, 0.0005 M dithiothreitol, and 20% glycerol (buffer B).

The dialyzed protein was then chromatographed on a phosphocellulose column (2.5 × 48 cm) in a manner analogous to that described by Burgess (1969), for RNA polymerase. The phosphocellulose column was washed with 50 ml of buffer B and then eluted with a linear KCl gradient as shown in Figure 3. PRS eluted as a sharp peak at approximately 0.5 M KCl. Peak tubes (164–172 of Figure 3) were pooled giving 35 ml of enriched PRS solution which was then dialyzed 2 hr against 4 l. of 0.01 M potassium phosphate (pH 7.0), 0.002 M EDTA, 0.0005 M dithiothreitol, and 20% glycerol (buffer C) and applied to a hydroxylapatite column (1 × 15 cm). This column, which was run essentially as described by Schmidt *et al.* (1971), was washed with 10 ml of buffer C and eluted with a linear potassium phosphate gradient as shown in Figure 4. PRS eluted as a sharp peak at approximately 0.2 M potassium phosphate; peak tubes (70–76 of Figure 4) were combined to give a final yield of 2 mg in 10 ml. The enzyme has been stored in 50% glycerol at −20° for at least 9 months without noticeable loss of activity. The purification scheme and yield are shown in Table II.

The PRS prepared by this three-step chromatographic procedure is at least 90% pure based upon the following criteria. Only one major band was obtained using various concentrations of enzyme on disc gel electrophoresis (7.5% acrylamide) in the position expected for PRS (Fasiolo *et al.*, 1970). A purification of 460–610-fold has been previously demonstrated by Schmidt *et al.* (1971) and Fasiolo *et al.* (1970) to give a homogeneous preparation of PRS, and the kinetic data for our preparation give similar V_{max} to those reported for yeast PRS (Fasiolo *et al.*, 1970).

Aminoacylation Kinetics. In addition to PRS, several other aminoacyl-tRNA synthetases have been shown to incorrectly aminoacylate tRNAs. These synthetases include *N. crassa* phenylalanyl-tRNA synthetase (Holten and Jacobson, 1969), *E. coli* valyl-tRNA synthetase (Giege *et al.*, 1971, 1973; Strickland and Jacobson, 1972), and *E. coli* isoleucyl-tRNA synthetase (Mertes *et al.*, 1972; Yarus, 1972). In these studies several different aminoacylation conditions have been employed. In previous studies of PRS we found that the use of the reaction mixture (mix B) including 50 mM potassium cacodylate (pH 5.8), 40 mM $MgCl_2$, an excess of 200-fold purified PRS, and a 2-hr 30° incubation gave greater than 80% heterologous mischarging of several purified *E. coli* tRNAs as shown in Table I (Roe and Dudock, 1972). In a similar reaction mixture containing 50 mM Tris (pH 8.2) instead of 50 mM potassium cacodylate (pH 5.8), 50–80% heterologous mischarging was obtained for these purified

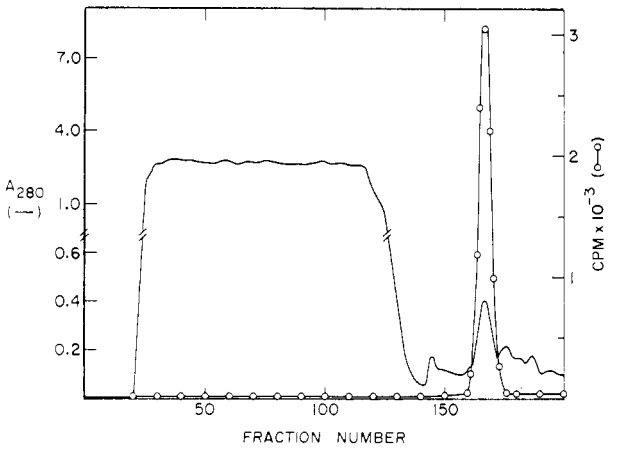

FIGURE 3: Phosphocellulose chromatography in the purification of yeast phenylalanyl-tRNA synthetase. The pooled and dialyzed PRS peak (tubes 270–300) from the DEAE-cellulose column (1600 A_{280} units in 500 ml) was applied to a phosphocellulose column (2.5 × 48 cm) previously equilibrated in buffer B. The column was washed with 50 ml of buffer B, and at tube 90, a 500-ml linear gradient (0.0–0.8 KCl, both in buffer B) was generated at which point the flow rate was maintained at 4 ml/5 min per tube. Protein was monitored at A_{280} and PRS activity was measured as described in Methods.

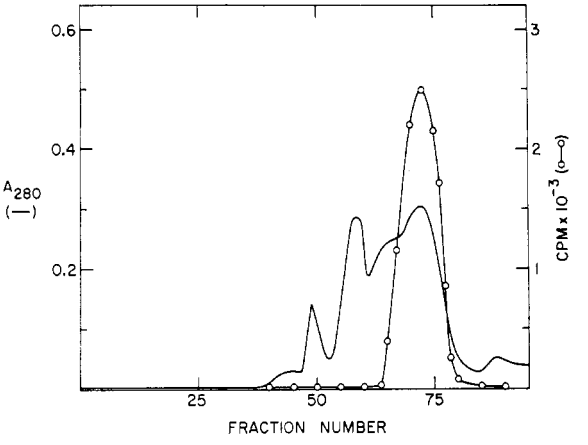

FIGURE 4: Hydroxylapatite chromatography in the purification of yeast phenylalanyl-tRNA synthetase. The pooled and dialyzed PRS peak (tubes 169–172) from the phosphocellulose column (20 A_{280} units in 40 ml) was applied to a hydroxylapatite column (1 × 15 cm) previously equilibrated in buffer C. The column was then washed with 10 ml of buffer C and at tube 15 a 50-ml linear gradient (0.1–0.3 M phosphate buffer C) was generated. The flow rate was 1 ml/2 min per tube. Protein was monitored at A_{280} and PRS activity was measured as described in Methods. Tubes 68–78 contained PRS with a specific activity of 570 EU/mg (see Table II).

TABLE III: Kinetic Parameters for tRNAs Aminoacylated by Yeast Phenylalanyl-tRNA Synthetase.[a]

tRNA	Class	Tris (pH 8.2)		Cacodylate (pH 6.0)	
		K_m ($\times 10^7$)	V_{max}	K_m ($\times 10^7$)	V_{max}
Yeast phenylalanine	Fast	0.83	0.45	0.89	0.0063
Wheat phenylalanine		0.56	0.53	0.10	0.0059
E. coli phenylalanine	Intermediate	5.8	0.091	4.5	0.134
E. coli valine 1		3.7	0.046	3.1	0.125
E. coli alanine 1		4.2	0.022	1.8	0.022
E. coli lysine[b]		5.2	0.011	4.5	0.013
E. coli alanine 2[b]	Slow	3.4	0.0077	2.2	0.0044
E. coli valine 2 (A and B)		1.5	0.0057	13	0.0330
E. coli isoleucine		1.3	0.0033	3.3	0.0020
E. coli methionine		1.0	0.0031	9.0	0.0018

[a] Kinetic experiments were performed as described in Materials and Methods using mix C. All experiments were done in duplicate and corrected for zero time controls, and the data were analyzed by extrapolation of the double reciprocal plots. K_m is μM tRNA, V_{max} is μmol/mg per min. [b] *E. coli* $tRNA^{Lys}$ and *E. coli* $tRNA_2^{Ala}$ are placed in the intermediate and slow class, respectively, based upon their kinetic data. Since the sequences of these two tRNAs are unknown, the line between the intermediate and slow class is somewhat arbitrary at present.

tRNAs (Table I). The reasons for incomplete aminoacylation have recently been discussed by Bonnet and Ebel (1972).

To further examine these PRS aminoacylation reactions the pH optima were studied using conditions in which the reaction is linearly dependent upon enzyme concentration. Figure 5a shows the pH curve over the range 5.4–9.4 for yeast $tRNA^{Phe}$ with a limiting amount of pure PRS. An identical pH curve (not shown) is obtained for wheat germ $tRNA^{Phe}$. As shown in Figure 5a, a broad pH optima is observed for both these tRNAs over the range 7.2–8.4 using mix C (*i.e.*, 40 mM $MgCl_2$). There is also a small but reproducible pH optima at pH 5.8 with approximately a sevenfold lower rate than that obtained at pH 8.2.

Figures 5b and c show the pH optima under the same conditions for *E. coli* $tRNA_1^{Val}$ and *E. coli* $tRNA_2^{Val}$, respectively. As seen in these two figures, a biphasic curve was obtained with two pH optima, one at pH 6.0 and the other at pH 8.2–8.8. We also observed this biphasic curve with optima at pH 6.0 and pH 8.2–8.8 for the aminoacylation of *E. coli* $tRNA^{Phe}$ and *E. coli* $tRNA_M^{Met}$ with phenylalanine by PRS (graphs not shown). A biphasic curve with two pH optima has also been observed by Ritter *et al.* (1970) for the aminoacylation of *E. coli* $tRNA^{Val}$ by *N. crassa* phenylalanyl-tRNA synthetase with phenylalanine.

Since two pH optima were obtained in these studies the kinetic parameters were determined for the different tRNAs at both of these pH's. The double reciprocal plots for all of the tRNAs in Table I were determined, and these studies show that the tRNAs which are aminoacylated with phenylalanine by PRS fall into three distinct classes based upon order of magnitude differences in their V_{max} at pH 8.2. These classes all have similar K_m's but widely varied V_{max}'s and these kinetic parameters are shown in Table III. Yeast $tRNA^{Phe}$ and wheat germ $tRNA^{Phe}$ fall into a class of tRNAs which have the largest V_{max} at pH 8.2 and about a 100-fold lower V_{max} at pH 6.0. *E. coli* $tRNA^{Phe}$, $tRNA_1^{Val}$, $tRNA_1^{Ala}$, and $tRNA^{Lys}$ have about a tenfold lower V_{max} compared to yeast and wheat $tRNA^{Phe}$ at pH 8.2 and have a greater V_{max} than yeast and wheat $tRNA^{Phe}$ at pH 6.0. *E. coli* $tRNA_2^{Ala}$, $tRNA^{Ile}$, $tRNA_2^{Val}$, and $tRNA_M^{Met}$ all have a 100-fold lower V_{max} compared to yeast and wheat $tRNA^{Phe}$ at pH 8.2 and a similar low V_{max} at pH 6.0.

As shown in Table I, PRS can aminoacylate homologous yeast $tRNA^{Phe}$ and ten heterologous tRNAs with phenylalanine. To show that the homologous and heterologous tRNAs both bind to the same site on PRS, we undertook competitive inhibition experiments. *E. coli* $tRNA_2^{Ala}$ (a heterologous mischarging tRNA of the slow class) was examined and found to act as a competitive inhibitor of the homologous aminoacylation of yeast $tRNA^{Phe}$ by PRS. Incubation was in mix C for 3 min and the results are given in Figure 6. It can be seen that increasing concentrations of *E. coli* $tRNA_2^{Ala}$ have no effect on the V_{max} but do cause an increase in the K_m for the homologous reaction, indicating competitive inhibition. When the results are analyzed using a Dixon ($1/v$ *vs.* i) plot (figure not shown) the K_i for *E. coli* $tRNA_2^{Ala}$ is 5.6×10^{-7}, which is similar to the K_m for this tRNA in the heterologous mischarging reaction. Therefore, both the homologous yeast $tRNA^{Phe}$ and the heterologous tRNAs bind to the same site on PRS.

It has been proposed that two regions of the tRNA molecule are primarily involved in the PRS recognition site, specifically the nucleotides of the diHU stem region and adenosine at the fourth position from the 3′ end (Roe and Dudock, 1972). *E. coli* $tRNA_3^{Gly}$ contains the "correct" nucleotides in the diHU stem region but instead of adenosine at the fourth position from the 3′ end, it contains a uridine (Squires and Carbon, 1971) and is not aminoacylated in the heterologous mischarging reaction by PRS at either pH 6.0 or 8.2. Although *E. coli* $tRNA_3^{Gly}$ is not aminoacylated by PRS it is possible that this tRNA binds to PRS and, because of the lack of adenosine at the fourth position, not be an acceptable substrate for the aminoacylation reaction. To test this possibility, we have studied the inhibition of the homologous reaction (yeast $tRNA^{Phe}$) with PRS by highly purified *E. coli* $tRNA_3^{Gly}$ using mix C. Various concentrations of *E. coli* $tRNA_3^{Gly}$ were used in an attempt to inhibit the homologous reaction. No inhibition was observed even when the molar ratio of *E. coli* $tRNA_3^{Gly}$ to yeast $tRNA^{Phe}$ was 20:1, a concentration which shows competitive inhibition of the homologous reaction with *E. coli* $tRNA_2^{Ala}$. This suggests that in an intact tRNA nucleotides in the diHU stem region and adenosine at the fourth position from the 3′ end are required for a tRNA to bind to PRS.

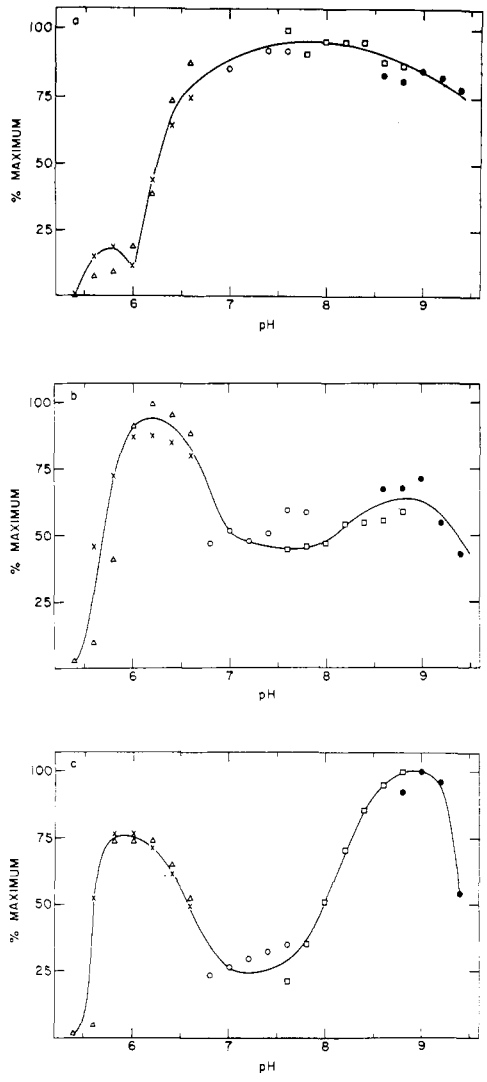

FIGURE 5: pH optima studies with PRS. (a) Yeast $tRNA^{Phe}$ was incubated in mix C and 50 mM buffer, as indicated, for 2 min at 30° with 0.05 μg of pure PRS. The buffers used were: (△) cacodylate; (×) Mes; (○) Hepes; (□) Tris; (●) glycine. (b) *E. coli* $tRNA_1^{Val}$ was incubated in mix C and 50 mM buffer, as indicated, for 15 min at 30° with 0.5 μg of pure PRS. (c) *E. coli* $tRNA_2^{Val}$ was incubated in mix C and 50 mM buffer, as indicated, for 45 min with 0.5 μg of pure PRS.

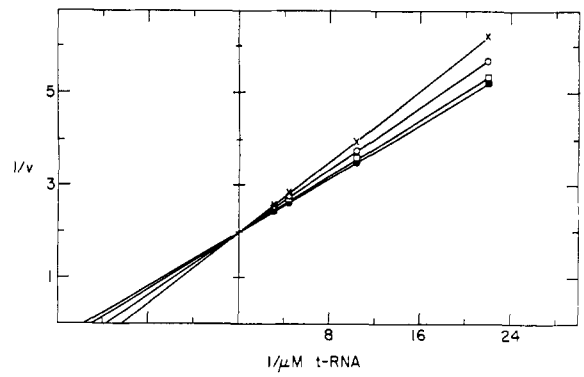

FIGURE 6: Competitive inhibition of the homologous aminoacylation of yeast $tRNA^{Phe}$ by *E. coli* $tRNA_2^{Ala}$. Incubations were performed in mix C containing 50 mM Tris-HCl (pH 8.2) and 0.05 μg of pure PRS as described in Methods using various concentrations of pure *E. coli* $tRNA_2^{Ala}$. The concentrations of *E. coli* $tRNA_2^{Ala}$ (μmol/ml of incubation mixture) were: none (●); 0.215 M (□); 0.430 M (○); and 0.861 M (×).

Discussion

Several approaches to determine the specific nucleotides and regions of the tRNA molecule primarily involved in a specific tRNA synthetase recognition site appear fruitful. Specific chemical modification such as employed by Chambers (1971) and by Schulman and Goddard (1973), the isolation and characterization of tRNA mutants of altered amino acid specificity (Shimura *et al.*, 1972; Hooper *et al.*, 1972), and the use of extensive sequence comparisons, especially involving heterologous mischarging, are three general approaches to the synthetase recognition site problem. Each of these methods, and several others not discussed, have distinct advantages and disadvantages. In the long run, several methods will probably aid in determining specific nucleotides and regions of tRNA molecules primarily involved in several synthetase recognition sites. We have employed the method of sequence comparison involving heterologous mischarging to probe the regions of the tRNA molecule primarily involved in the PRS recognition site. To use this approach several criteria should be fulfilled. (1) Each of the tRNAs studied, as well as the synthetase, should be highly purified, in order to absolutely confirm which isoaccepting tRNA species accepts the incorrect amino acid. (2) The aminoacylation conditions should be consistent with the maintenance of enzymatic specificity. (3) The kinetics of the homologous and heterologous aminoacylation reactions should be studied so that the system is clarified. In the case of PRS each of these criteria has been fulfilled.

Yeast phenylalanyl-tRNA synthetase can aminoacylate the 11 tRNAs shown in Table I. To be certain which tRNA is accepting the amino acid, each of the tRNAs in Table I has been extensively purified. The synthetase, PRS, has also been obtained in highly purified form. Based upon the sequences of these tRNAs (see composite tRNA, Figure 1), two regions of the tRNA molecule have been proposed to be primarily involved in the recognition of a tRNA by this particular synthetase. These consist of a group of nine nucleotides located in the double-stranded region adjacent to the diHU loop and adenosine as the fourth residue from the 3′ (Roe and Dudock, 1972). This proposal is supported by the observation that three tRNAs (*E. coli* $tRNA_3^{Gly}$, *E. coli* $tRNA^{His}$, and yeast $tRNA^{Lys}$), whose sequences are very similar to the composite tRNA (Figure 1) but which have nucleosides other than adenosine at the fourth position from the 3′ end, are not aminoacylated by PRS. In addition, tRNAs which have adenosine at the fourth position from the 3′ end but which lack the specific nucleotides in the diHU stem region, such as *E. coli* $tRNA^{fMet}$ and wheat germ $tRNA_1^{Gly}$, are not aminoacylated by PRS (Roe and Dudock, 1972), showing that specific nucleotides in both regions are required for aminoacylation by PRS. The proposed PRS recognition site is in agreement with recent sequence determinations of tRNAs aminoacylated by PRS, such as *E. coli* $tRNA_1^{Ala}$ (R. Williams, W. Nagel, B. Roe, and B. Dudock, manuscript in preparation), with chemical modifications of tRNAs which are aminoacylated by PRS (Litt and Greenspan, 1972; Schmidt *et al.*, 1970; Samuelson and Keller, 1972; Thiebe *et al.*, 1972; Streeck and Zachau, 1971; Kumar *et al.*, 1973) and with enzymatic methylation of tRNAs which are aminoacylated by PRS (B. Roe, M. S. Michael, and B. Dudock, manuscript in preparation).

Preliminary kinetic studies indicated that the reaction rates of the tRNAs which were acceptable substrates for PRS varied over several orders of magnitude. In an attempt to

correlate these varied rates with structural differences, more extensive kinetic studies were undertaken. These studies show that the K_m's for the PRS aminoacylation reactions for these tRNAs vary only over a tenfold range, whereas the V_{max}'s for the reactions vary over a 200-fold range. A similar observation of a relatively small change in K_m and a large change in V_{max} has been observed for *E. coli* valyl-tRNA synthetase (Giege *et al.*, 1973). At pH 8.2 the tRNAs aminoacylated by PRS fall into three distinct classes. These are a fast class (V_{max} = 0.4–0.5), an intermediate class (V_{max} = 0.01–0.09), and a slow class (V_{max} = 0.003–0.008). By examining the sequences of the tRNAs in these classes, we can correlate them primarily with two structural features of the tRNA molecule, specifically the size of the diHU loop (8 or 9 nucleotides) and the presence of an N^2-methylguanine or an unmodified guanine at position 10 from the 5′ end.

Role of the Size of the diHU Loop. Of the five tRNAs in the slow class (see Table III) four are of known sequence (all except *E. coli* $tRNA_2^{Ala}$) and all four contain nine nucleotides in their diHU loops. Of the six tRNAs in the fast and intermediate classes, five of them are of known sequence (all except *E. coli* $tRNA^{Lys}$) and all five contain eight nucleotides in their diHU loops. Thus there is a complete correlation of the size of the diHU loop and the V_{max} for the PRS aminoacylation reaction. This correlation strongly suggests that an increase in the size of the diHU loop significantly affected the kinetics of the PRS reaction. This correlation is also strengthened by the observation that modifications of the diHU loop of yeast $tRNA^{Phe}$ by a small molecule, glyoxal, had little effect on the PRS aminoacylation reaction, whereas a similar modification by the larger molecule, kethoxal, was found to inhibit the PRS aminoacylation reaction (Litt, 1971).

Role of N^2-Methylguanine. As shown above, the presence of an extra nucleotide in the diHU loop (nine nucleotides as opposed to eight) can be correlated with the presence of a tRNA in the slow class of Table III. To explain the difference between the tRNAs of the fast and intermediate classes of Table III, we first looked primarily at those nucleotides which were previously shown to be directly involved in the PRS recognition site. It is apparent that the two tRNAs in the fast class have an N^2-methylguanine at position 10 (which is located in the diHU stem region, see Figure 1), whereas the three tRNAs of known sequence in the intermediate class have an unmodified guanine at that site. Thus it appears that the presence of a specific methylated base, N^2-methylguanine, strongly enhances the kinetics of aminoacylation with this particular synthetase. We have examined this proposal and have found it to be valid. In experiments to be described in detail elsewhere (B. Roe, M. S. Michael, and B. Dudock, manuscript in preparation), highly purified *E. coli* $tRNA^{Phe}$ (an intermediate class tRNA) was enzymatically methylated with highly purified rabbit liver N^2-methylguanine methylase, specifically converting guanine at position 10 to N^2-methylguanine. This specifically methylated *E. coli* $tRNA^{Phe}$ was aminoacylated by highly purified PRS with a tenfold higher V_{max} than unmodified *E. coli* $tRNA^{Phe}$. A similar result was observed with *E. coli* $tRNA_1^{Ala}$. These experiments confirm the correlation observed in the kinetics of the role of this methylated base in the aminoacylation of a tRNA by PRS. In addition, these results further confirm the role of the nucleotides of the diHU stem region in the PRS recognition site.

The observation that *E. coli* $tRNA_2^{Ala}$, which is aminoacylated by PRS (see Table I), can act as a competitive inhibitor of the homologous aminoacylation of yeast $tRNA^{Phe}$ by PRS shows that both the homologous and heterologous tRNAs bind to the same site on PRS.

Since the V_{max} for the PRS reaction varied over a 200-fold range, depending upon the tRNA (see Table III), it was possible that our enzyme preparation, although essentially homogeneous on disc gel, was a mixture of several forms of the same enzyme, each form reacting with a specific class of tRNAs. The observation of competitive inhibition described above eliminates this possibility.

The observation that *E. coli* $tRNA_3^{Gly}$, which is not aminoacylated by PRS, cannot act as a competitive inhibitor is significant. This tRNA is very similar to the composite tRNA of Figure 1 differing from it only in the absence of adenosine as the fourth residue from the 3′ end. All of the "correct" nucleotides in the diHU stem region are present in *E. coli* $tRNA_3^{Gly}$. The observation that this tRNA cannot act as a competitive inhibitor of the homologous aminoacylation of yeast $tRNA^{Phe}$ suggests that in an intact tRNA nucleotides in both the diHU stem region and adenosine, as the fourth residue from the 3′ end, are required for a tRNA to bind to PRS. It has recently been shown that a $tRNA^{Phe}$ fragment, lacking the terminal seven nucleotides of the 3′ end, can bind to PRS (Horz and Zachau, 1973). Thus it appears that the fourth residue from the 3′ end may act as a discriminator site, with the wrong nucleotide in that position preventing binding to PRS. The role of the fourth nucleotide from the 3′ end as a general discriminator site for tRNA synthetase recognition purposes has been proposed (Crothers *et al.*, 1972). It is currently unknown whether in the three-dimensional structure of a tRNA the nucleotides in these two regions can form a single "active site," although further X-ray analysis of tRNA crystals may soon permit a definite answer. It is also not known if the nucleotides in these two regions of the molecule are recognized directly by PRS or if these specific nucleotides engender a particular three-dimensional configuration, perhaps in a localized region, which in turn is recognized by PRS.

Model of the PRS Recognition Site. The current model of the PRS recognition site is that it consists primarily of specific nucleotides in both the diHU stem region and adenosine at the fourth position from the 3′ end. In addition, these nucleotides must be in a particular three-dimensional structural framework to be recognized since denatured yeast $tRNA^{Phe}$ is not aminoacylated by PRS (Streeck and Zachau, 1971). Although these two regions of the molecule must be present for recognition, and since the K_m's for these reactions are similar, we conclude that these two regions are primarily involved in the recognition and formation of the enzyme–aa–tRNA–AMP complex. However, the breakdown of this complex through such steps as the formation of the aa–tRNA and the release of product from the intermediate complex depends on several additional factors. We propose that two structural parameters involved in this rate-determining step are the size of the dihydrouridine loop and the presence of a modified base N^2-methylguanine instead of an unmodified guanine as the tenth residue from the 5′ end.

Much additional information about this synthetase recognition site remains to be determined. As seen in the composite tRNA (Figure 1), there are nine boldface nucleotides in the diHU stem region which are uniquely common to the tRNAs aminoacylated by PRS. It is probable that not all nine of these nucleotides are required for a tRNA to be aminoacylated by PRS. Indeed, it may be that the nucleotides of only one strand of the double-stranded diHU stem region are directly involved in the recognition of a tRNA by this synthetase, with the

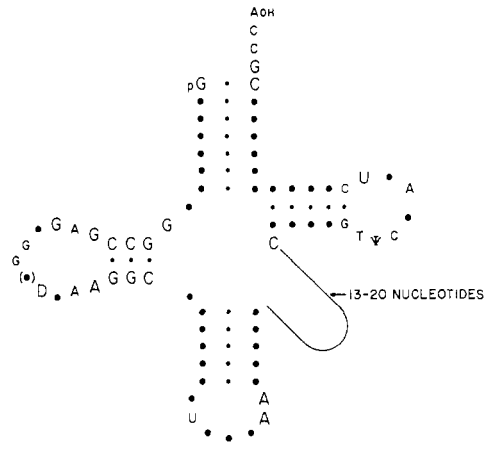

FIGURE 7: Composite tRNA for yeast seryl-tRNA synthetase. Composite tRNA based upon the sequences of the four known serine tRNAs (*E. coli* $tRNA_1^{Ser}$; *E. coli* $tRNA_3^{Ser}$; yeast $tRNA^{Ser}$, and rat liver $tRNA^{Ser}$). Those nucleotides that are not the same in these four tRNAs are shown in the composite with a dot. Those nucleotides that are found in the same position in all tRNAs are shown in the composite in small type. Those nucleotides that are uniquely common to these four tRNAs are shown in large type.

nucleotides of the other strand being fixed because of the rules of base pairing. The observation of the significant enhancement in the aminoacylation kinetics upon methylation of guanine at position 10 would tend to favor the upper strand (nucleotides 9–14) in the composite tRNA of Figure 1. However, information from further sequence comparisions and other experiments will have to be obtained before the role of the upper strand in the diHU stem region can be further clarified. See note added in proof.

Three other boldface nucleotides, at positions 15, 46, and 48 (see composite tRNA, Figure 1), remain to be discussed. These nucleotides are present at the same site in all tRNAs of known sequence aminoacylated by PRS. However, there is at present no tRNA which has all of the other boldface nucleotides as shown in Figure 1 but which lacks one or all of these three boldface nucleotides. Therefore, their role in the PRS recognition site remains somewhat unclear. 7-Methylguanine, residue 46, apparently is not required for the aminoacylation of a tRNA by PRS on the basis of chemical modification studies (Wintermeyer and Zachau, 1970). Finally, the effect of the size of the minor loop remains to be determined. All of the tRNAs of known sequence aminoacylated by PRS have exactly five nucleotides in their minor loop with 7-methylguanine at the apex. Here again the absence of a tRNA having all of the boldface nucleotides shown in Figure 1 but with a minor loop of a size other than five nucleotides precludes a definitive assignment of a specific role of the size of the minor loop. Although fragment studies show an intact minor loop is not required for aminoacylation by PRS (Wintermeyer and Zachau, 1970; Thiebe *et al.*, 1972), the size of the minor loop may affect the aminoacylation kinetics as was observed for the diHU loop.

Classes of tRNA Synthetase Recognition Sites. There now appears to be increasing evidence that the tRNA synthetase recognition site is not universal (Thiebe *et al.*, 1972; Crothers *et al.*, 1972; Chambers, 1971). Nevertheless, there may be other aminoacyl-tRNA synthetases that recognize tRNAs in a manner similar to that of PRS, *i.e.*, based primarily on specific nucleotides in the diHU stem region, a particular nucleotide at the fourth position from the 3′ end, and with the size of the diHU loop and perhaps of the minor loop as well significantly affecting the amino acylation reaction. The sequences of the four known serine tRNAs, *E. coli* $tRNA_1^{Ser}$ (Ishikura *et al.*, 1971), *E. coli* $tRNA_3^{Ser}$ (Yamada and Ishikura, 1973), yeast $tRNA^{Ser}$ (Zachau *et al.*, 1966), and rat liver $tRNA^{Ser}$ (Staehelin *et al.*, 1968), which are all aminoacylated by yeast seryl-tRNA synthetase (Yamada and Ishikura, 1973; Doctor and Mudd, 1963) are compared in Figure 7. It is apparent that there is a distinct similarity in the nucleotides of the diHU stem region, as suggested by Yamada and Ishikura (1973), and at the fourth position from the 3′ end, and in addition there is only a slight variability in the size of the diHU loops and a somewhat greater variability in the size of the minor loops (quite large in all four tRNAs). Thus there may be classes of tRNA synthetase recognition sites with yeast PRS and yeast seryl-tRNA synthetase members of the same class based upon the similar manner in which they recognize their cognate tRNAs. Much additional knowledge of tRNA synthetase recognition sites will have to be acquired before it can be determined if classes of tRNA synthetase recognition sites do indeed exist.

Added in Proof

The possible primary role of the upper strand of the diHU stem region in the yeast PRS recognition site is strengthened by the observation that *E. coli* $tRNA^{Arg}$ is aminoacylated by yeast PRS. This tRNA has all of the correct nucleotides in the upper strand but not in the lower strand of the diHU stem region. Specifically the residue at position 25 from the 5′ end is cytidine in the composite tRNA (Figure 1), and it is a uridine in *E. coli* $tRNA^{Arg}$.

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Synthesis and Characterization of Two Fluorescent Sulfhydryl Reagents†

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ABSTRACT: Two fluorescent sulfhydryl reagents, *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid (1,5-I-AEDANS) and the 1,8 isomer (1,8-I-AEDANS), were synthesized. Although sensitive to photocatalyzed degradation, these reagents readily react with thiol compounds and sulfhydryl groups in proteins yielding photostable covalent derivatives. The synthesis, fluorescence spectra, quantum yields, and lifetimes of model compounds in various solvents are presented in this paper. A following paper will report some of the general properties of the protein conjugates.

The use of fluorescent probes in protein studies is now well established. Since the introduction of fluorescein isocyanate (Creech and Jones, 1941) and dansyl chloride (Weber, 1952), a variety of covalent and noncovalent probes have been developed and applied to the elucidation of various properties of proteins (Steiner and Edelhoch, 1962; Stryer, 1968; Brand and Gohlke, 1972). With a few exceptions, however, the covalent probes have been nonspecific; *i.e.*, they react with several different amino acid side chains. This results in heterogeneous labeling and the subsequent measurements of fluorescent properties arise from, and are therefore averaged over, a number of different sites. This lack of specificity clearly limits the application and interpretation of the measurements with such probes.

We wish to report in this paper the synthesis and characterization of several new fluorescent reagents which combine the reactivity of iodoacetamide toward sulfhydryl groups with the spectral properties of the naphthalenesulfonic acids. The reagents are easily synthesized, water soluble, stable for long periods of time in crystalline form, and form stable conjugates with proteins. Like other naphthylaminesulfonic acids, they

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