Detection and Purification of Isoaccepting $tRNA^{Phe}$ Species Containing Y Base by Affinity Chromatography on Columns of Anti-Y Antibodies†

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ABSTRACT: Rat liver $tRNA^{Phe}$ contains a modified nucleoside which is similar in structure to the yeast Y-nucleoside. This peroxy Y-nucleoside is located immediately adjacent to the anticodon. Antibodies against yeast $tRNA^{Phe}$ were shown to be specific to the Y-nucleoside. These antibodies bind to rat liver $tRNA^{Phe}$ with a lower affinity than to yeast $tRNA^{Phe}$. Affinity chromatography on anti-Y antibody immobilized on Sepharose was used to determine the amount of rat liver $tRNA^{Phe}$ species containing the peroxy Y-nucleoside. Unfractionated rat liver tRNA was charged with phenylalanine. In order to preserve the aminoacyl-tRNA ester bond, the phenylalanyl-tRNA was acylated with *N*-hydroxysuccinimide acetyl ester to yield *N*-acetyl[^{3}H]phenylalanyl-tRNA. When this preparation was applied to the antibody column, between 7 and 10% of the radioactivity was not bound by the column indicating the absence of peroxy Y-nucleoside in a minor isoaccepting $tRNA^{Phe}$ species. The bound material comprising 90% of the radioactivity was recovered from the column by elution with 1 *M* NaCl. On the other hand, yeast $tRNA^{Phe}$ was tenaciously held to the column and could not be eluted even with 5 *M* NaCl. When the peroxy Y base was excised from the rat liver tRNA none of the *N*-acetyl[^{14}C]phenylalanyl-tRNA was retained on the column, thus attesting to the antigenic specificity of the antibodies. The anti-Y-nucleoside column can also be used on a preparative scale, and rat liver $tRNA^{Phe}$ was purified at least 20-fold by this procedure.

Antibodies to rare nucleosides in tRNA were found to be a useful tool in detection of minute amounts of these nucleosides (Inouye et al., 1971, 1973; Littauer and Inouye, 1973; Hacker et al., 1972). A highly modified guanosine derivative (designated Y) was found to be located immediately adjacent to the anticodon of yeast $tRNA^{Phe}$ (RajBhandary et al., 1967). This nucleoside was shown to be the sole inducer of antibodies upon injection of glutaraldehyde conjugated yeast $tRNA^{Phe}$ with BGG[1] into goats (Fuchs et al., 1974). When the immunoglobulin fraction from such antiserum was immobilized on Sepharose, it retained the antibody activity. This column was found to bind specifically yeast $tRNA^{Phe}$, while immunoglobulins from normal goats failed to retain this tRNA species (Fuchs et al., 1974).

Mammalian $tRNA^{Phe}$ also contains a modified nucleoside that differs from the one from yeast by the presence of a hydroperoxide group on the β carbon of the side chain (Blobstein et al., 1973). Although the peroxy Y-nucleoside from rat liver $tRNA^{Phe}$ differs from the yeast Y-nucleoside in the four-carbon side chain, we find in this work that anti-yeast $tRNA^{Phe}$ antibodies cross-react with rat liver $tRNA^{Phe}$. This property enabled us to use a column of anti-yeast $tRNA^{Phe}$ antibodies immobilized to Sepharose to determine the amount of rat liver $tRNA^{Phe}$ species containing the peroxy Y-nucleoside, as well as to purify these tRNA species.

Experimental Section

Materials. L-[^{3}H]Phenylalanine (50.5 Ci/mmol) was obtained from Schwarz/Mann. L-[^{14}C]Phenylalanine (522 mCi/mmol) was purchased from Amersham. Proteinase K was from Merck Darmstadt. Sepharose 4B was from Pharmacia. Yeast $tRNA^{Phe}$ was a kind gift from Dr. F. von der Haar.

Methods. Induction of Goat Anti-Y-Nucleoside Antibodies. Anti-Y antibodies were prepared by injection of yeast $tRNA^{Phe}$ into goats as described (Fuchs et al., 1974). Anti-Y antibodies immobilized on Sepharose were prepared by conjugation of the immunoglobulin fraction to CNBr-activated Sepharose (Fuchs et al., 1974). The Sepharose bound antibodies were suspended in PS[1] and packed in a column (1 × 3 cm). The column could be regenerated repeatedly (at least 20 times). After each chromatographic analysis the column was washed with 5 ml of 5 *M* NaCl solution followed by 70 ml of PS.

Preparation of Rat Liver tRNA. Rat liver (40 g) was homogenized in 2 vol of 10 m*M* tris-HCl buffer (pH 7.5)–1 m*M* $MgCl_2$–0.1 m*M* Na_2EDTA–20 m*M* β-mercaptoethanol and 10 mg/ml Macaloid. Homogenization was performed for 15 sec in an Ultra Turrax high speed homogenizer. The homogenate was mixed with an equal volume of water-saturated phenol (pH 7.0); after 15 min at 23° the phases were separated by centrifugation. The phenol layer was extracted once more with 0.5 vol of the above buffer. Both aqueous layers were combined and deproteinized once more with an equal volume of phenol and 0.1 vol of chloroform. The RNA was precipitated from the aqueous phase with 2 vol of ethanol, the precipitate was washed with ethanol, and the tRNA was solubilized with 0.2 ml of 1 *M* NaCl–1 m*M* $MgCl_2$–20 m*M* β-mercaptoethanol. This extraction was repeated once more and the RNA precipitated with ethanol. The precipitate was dissolved in a solution containing 0.5 mg/ml proteinase K–10 m*M* Tris-HCl (pH 8.0)–20 m*M* β-mercaptoethanol and then incubated for 60 min at room temperature. The solution was mixed with 0.2

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[1] Abbreviations used are: IgG, immunoglobulin G; BGG, bovine γ globulin; PS, 5 m*M* phosphate buffer (pH 7.0) and 75 m*M* NaCl; Y, tricyclic imidazopurine to which is attached a complex four-carbon side chain.

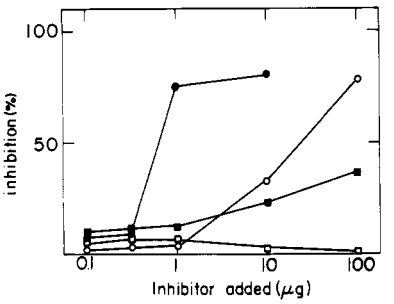

FIGURE 1: Inhibition of the binding of rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA to anti-Y antibodies by various tRNAs: (●) yeast tRNA^{Phe}; (○) unfractionated yeast tRNA; (■) unfractionated rat liver tRNA; (□) *E. coli* tRNA. The inhibitor was incubated with 0.5 mg of the immunoglobulin fraction for 15 min at 4°. Rat liver *N*-acetyl-[^{3}H]phenylalanyl-tRNA (2 μg, with 2000 cpm) was then added and the reaction mixtures were incubated for an additional hour at 4°. The bound antigen was precipitated and estimated as described by Fuchs et al. (1974).

vol of 5 *M* $NaClO_4$, 0.5 vol of water-saturated phenol, and 0.1 vol of chloroform and the tRNA recovered by ethanol precipitation (Littauer, 1971). This treatment served to eliminate trace amounts of contaminating nucleases from the tRNA preparation. The assay for nuclease contamination was as previously described (Littauer, 1971). The tRNA was dissolved in 2.5 ml of H_2O and applied to a Sephadex G-100 column (2.5 × 30 cm) equilibrated with 1 *M* NaCl–1 m*M* $MgCl_2$–20 m*M* β-mercaptoethanol. The tRNA was then eluted from the column with the equilibration buffer.

Preparation of Rat Liver Aminoacyl-tRNA Synthetase. Aminoacyl-tRNA synthetases were partially purified from a 105,000*g* supernatant by phase partition (Babinet, 1967). Phase II was found to contain the phenylalanyl-tRNA synthetase. The enzyme preparation catalyzed the aminoacylation of tRNA^{Phe} species, both containing and lacking the peroxy Y-nucleoside.

Aminoacylation of Rat Liver tRNA^{Phe}. The incubation mixture (0.15 ml) for the preparative aminoacylation of tRNA with phenylalanine contained 40 m*M* Tris-HCl (pH 7.5), 2 m*M* ATP, 10 m*M* $MgCl_2$, 140 m*M* KCl, 3 m*M* reduced glutathione, 1.5 μCi of L-[^{3}H]phenylalanine or 0.75 μCi of L-[^{14}C]phenylalanine, 40–60 μg of RNA, and 10 μg of aminoacyl-tRNA synthetase preparation. The incubations were carried out at 30° for 15–20 min and then halted by chilling and the addition of 0.1 vol of 1 *M* sodium acetate buffer (pH 4.5) and 2 vol of ethanol.

Preparation of *N*-Acetylphenylalanyl-tRNA. The ethanolic precipitate of phenylalanyl-tRNA was dissolved in 0.02 ml of 0.01 *M* sodium acetate (pH 5.0). To this solution 0.06 ml of dimethyl sulfoxide containing 5 mg of *N*-hydroxysuccinimide acetyl ester was added and the mixture was incubated for 10 hr at room temperature and the *N*-acetylphenylalanyl-tRNA was precipitated with 0.2 ml of ethanol (Salomon and Littauer, 1974). For some experiments the phenylalanyl-tRNA was acetylated with acetic anhydride (Haenni and Chapeville, 1966).

Results

In all the experiments to be described, we have used labeled *N*-acetylphenylalanyl-tRNA, since N-substituted aminoacyl-tRNA derivatives have been shown to be considerably more resistant to hydrolysis than the corresponding unblocked aminoacyl-tRNA molecules (cf. Daniel et al., 1970).

Table I: Inhibition of the Binding of Rat Liver *N*-Acetyl[^{3}H]phenylalanyl-tRNA to Anti-Y Antibodies by Oligonucleotides.[a]

Inhibitor	Amount of Inhibitor Added (A_{260} Units)		
	2×10^{-2}	2×10^{-3}	2×10^{-4}
Gm-A-A-Y	86	87	36
Gm-A-A-Rib	0	0	0
Y-Ap	87	83	62
Y>p	89	85	85

[a] The numbers in the table represent the percentage inhibition obtained for each inhibitor concentration. Inhibition experiments were carried out as described in the caption to Figure 1. The oligonucleotides were derived from yeast tRNA^{Phe} and kindly supplied by Dr. F. von der Haar. Gm-A-A-Rib was prepared by acid treatment of Gm-A-A-Y.

Cross-Reaction of Rat Liver tRNA with Antibodies against Yeast Y-Nucleoside. Antibodies reacting with the yeast Y-nucleoside were induced by immunizing goats with yeast tRNA^{Phe}–BGG conjugate (Fuchs et al., 1974). These antibodies were found to cross-react with rat liver tRNA. Figure 1 shows an inhibition experiment in which the antibodies were mixed with various tRNA preparations and then incubated with labeled rat liver *N*-acetylphenylalanyl-tRNA. The fraction bound to the antibody was precipitated and its radioactivity determined. The binding of radioactive rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA by the antibodies was completely inhibited by preincubation with 1 μg of yeast tRNA^{Phe} and about 100 μg of unfractionated yeast tRNA was required to reach the same inhibition level. Unfractionated rat liver tRNA at the same concentration was less inhibitory, while *Escherichia coli* tRNA, which does not contain a Y-nucleoside, had no effect at all. By comparing the slope of the inhibition curves for the unfractionated tRNAs it was deduced that yeast tRNA binds to its homologous antibody at least 10 times stronger than the heterologous rat liver tRNA.

The specificity of the cross-reaction of the antibodies with rat liver tRNA was further examined by measuring the ability of several oligonucleotides derived from yeast tRNA^{Phe} to inhibit the reaction. As demonstrated in Table I, the Y-containing oligonucleotides Gm-A-A-Y and Y-Ap as well as Y>p were potent inhibitors, whereas Gm-A-A-Rib which has lost the Y-base by mild acid treatment did not inhibit at all. Thus the cross-reaction of the antibodies with rat liver tRNA is due to the presence of a peroxy Y-nucleoside in this preparation (Li et al., 1973).

Analysis of Rat Liver N-Acetylphenylalanyl-tRNA on Anti-Y Antibody Columns. Antibodies against yeast Y-nucleosides were conjugated to CNBr-activated Sepharose and packed in a column. It was previously shown that the Sepharose-bound material retained its antibody activity (Fuchs et al., 1974), and affinity chromatography on such a column was used to determine the amount of rat liver tRNA^{Phe} species containing the peroxy Y-nucleoside.

The radioactive *N*-acetylphenylalanyl-tRNA was applied to the antibody column and after washing with PS the bound material was eluted with PS containing 1 *M* NaCl. Figure 2 shows that the major fraction of the radioactive tRNA^{Phe} was strongly bound to the column and eluted with 1 *M* NaCl, probably due to the presence of the peroxy Y-nucleoside. About 7% of the radioactivity emerged from the

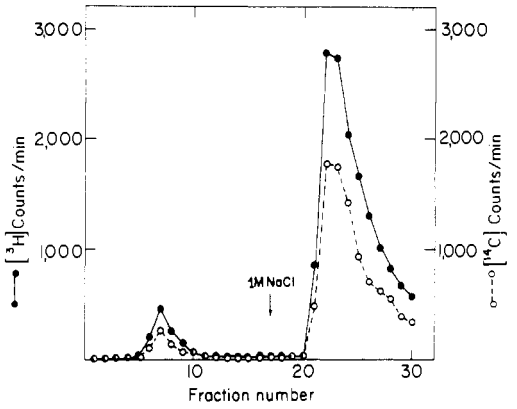

FIGURE 2: Elution profile of rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA together with *N*-acetyl[^{14}C]phenylalanyl-tRNA on an anti-Y Sepharose column. Anti-Y Sepharose was packed into a column (1 × 3 cm) and equilibrated with PS. A mixture of rat liver *N*-acetyl-[^{3}H]phenylalanyl-tRNA (20 μg, 22,000 cpm) and *N*-acetyl[^{14}C]phenylalanyl-tRNA (20 μg, 14,000 cpm) in 60 μl of PS was applied to the column. The column was eluted with 15 ml of PS, followed by 15 ml of PS containing 1 *M* NaCl. The elution was carried out at 23°; 1-ml fractions were collected, precipitated with 5% trichloroacetic acid, filtered through glass filters (Whatman GF/C), washed, and counted.

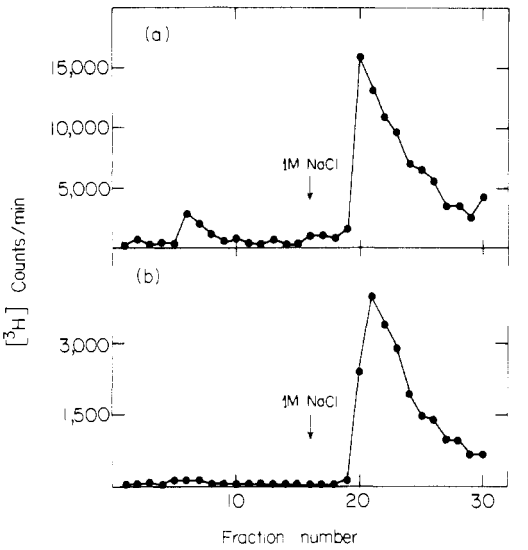

FIGURE 3: Separation and rechromatography of the 1 *M* NaCl fraction of rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA. Rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA (60 μg, 108,000 cpm) was chromatographed on an antibody column as described in Figure 2. Aliquots of 0.1 ml were removed from each tube and counted (a). The 1 *M* NaCl fractions were pooled and extracted with phenol–chloroform, 0.4 mg of *E. coli* tRNA carrier was added, and the RNA was precipitated by 2 vol of ethanol. This material was rechromatographed on a second column (b).

column without noticeable retardation, suggesting the absence of peroxy Y-nucleoside in a small fraction of rat liver $tRNA^{Phe}$. We have also attempted to fractionate yeast $tRNA^{Phe}$ on this column. However, in contrast to rat liver $tRNA^{Phe}$, in which about 95% of the applied material could be recovered from the column, the yeast $tRNA^{Phe}$ was tenaciously bound to the column and could not be eluted with 5 *M* sodium chloride or 3 *M* sodium thiocyanate. This is probably due to the higher affinity of the antibodies to the homologous antigen.

Several control experiments were carried out in order to determine the specificity of the antibody column. It was found that there is no isotope effect on the distribution pattern, since both *N*-acetyl[^{3}H]phenylalanyl-tRNA and *N*-acetyl[^{14}C]phenylalanyl-tRNA showed an identical elution profile (Figure 2). In addition, the same percentage of unbound $tRNA^{Phe}$ was obtained with crude tRNA preparations that were not subjected to NaCl extraction and Sephadex G-100 chromatography. On rechromatography there is also no change in the elution profile of the bound material. Thus, rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA was chromatographed on the antibody column (Figure 3a); the radioactive material that eluted with 1 *M* NaCl was collected, deproteinized by phenol–chloroform extraction, dialyzed, and rechromatographed on the column. As shown in Figure 3b very little if any of the recovered material was found in the PS wash and was eluted only with 1 *M* NaCl. This result shows that the tRNA was not changed by the affinity chromatography and that the appearance of some of the $tRNA^{Phe}$ chains in the column wash is probably due to the lack of peroxy Y-nucleoside and not a result of an inherent property of the column. In another control experiment, rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA was chromatographed on a column of normal goat immunoglobulins bound to Sepharose; in this case all the radioactive material emerged from the column without any retardation.

In order to elucidate the role of the rat liver peroxy Y-nucleoside in the antigenic specificity, the following experiments were performed. Rat liver tRNA was subjected to mild acid treatment that resulted in the excision of the peroxy Y-base (Thiebe and Zachau, 1968). We first examined the effect of this treatment on the aminoacylation capacity of the tRNA. Figure 4 shows that the phenylalanine acceptance of rat liver tRNA lacking the peroxy Y-base ($tRNA_{-Y}$) was considerably lower than that of the untreated tRNA. The phenylalanine acceptor capacity of the rat liver $tRNA_{-Y}$ was not enhanced by heating at 50° for 1 min, in the presence of 40 m*M* Tris-HCl (pH 7.5)–4 m*M* $MgCl_2$ or by heating under the conditions described by Wittig et al. (1974). It is possible that the incomplete charging of $tRNA^{Phe}{}_{-Y}$ may be due to an increased rate of deacylation of the aminoacylated molecule. It appears that the acid treatment specifically affects the aminoacylation capacity of $tRNA^{Phe}$, since the histidine acceptance of this material was hardly impaired. The chromatographic properties of $tRNA_{-Y}$ on the antibody column were then examined. *N*-Acetyl[^{14}C]phenylalanyl-$tRNA_{-Y}$ was chromatographed together with peroxy Y-containing *N*-acetyl-[^{3}H]phenylalanyl-tRNA on an antibody column. As demonstrated in Figure 5 excision of the peroxy Y-base abolishes the binding of rat liver tRNA to the column. Similarly, when tRNA was first converted to the *N*-acetylphenylalanyl-tRNA and only then treated with acid, 85% of this material came off with the column wash. This result serves to show that the excision of the peroxy Y-base affected the chromatographic properties of all the $tRNA^{Phe}$ chains.

It seems that only rat liver $tRNA^{Phe}$ is retarded on the column. This was deduced from the finding that aminoacylation of rat liver tRNA with a mixture of 19 ^{3}H-labeled amino acids in the presence of excess unlabeled phenylalanine resulted in a radioactive material that was not bound to the column. The specificity of the column was, therefore, exploited for the purification of rat liver $tRNA^{Phe}$ species which contain a peroxy Y-nucleoside. In a typical experiment rat liver tRNA (0.38 mg) was mixed together with 0.064 mg of *N*-acetyl[^{3}H]phenylalanyl-tRNA and applied onto an antibody column. The column was eluted with PS and then with PS containing 1 *M* NaCl. Figure 6 demon-

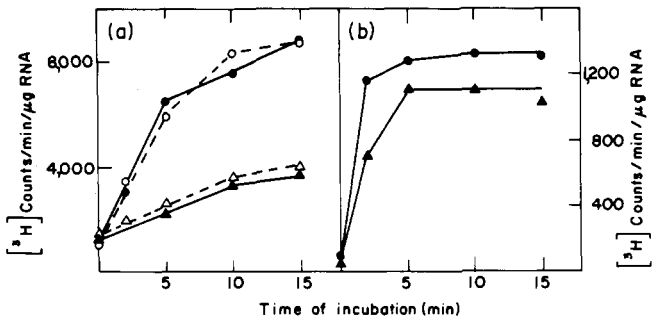

FIGURE 4: Time course of aminoacylation of acid-treated rat liver tRNA. (a) Phenylalanine acceptance of: (●—●) rat liver tRNA; (○ - - - ○) rat liver tRNA obtained after heating at 50° for 1 min in the presence of 40 m*M* Tris-HCl (pH 7.5) and 4 m*M* $MgCl_2$; (▲—▲) rat liver tRNA after acid treatment; (△ - - - △) acid-treated tRNA after heating to 50° for 1 min. (b) Histidine acceptance of: (●—●) rat liver tRNA; (▲—▲) rat liver tRNA after acid treatment.

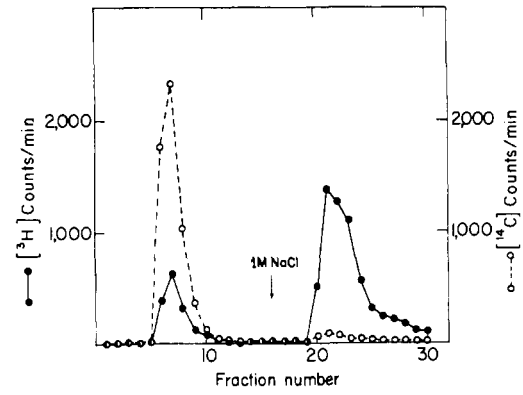

FIGURE 5: Elution profile of rat liver *N*-acetyl[^{14}C]phenylalanyl-tRNA$_{-Y}$ together with *N*-acetyl[^{3}H]phenylalanyl-tRNA. A mixture of rat liver *N*-acetyl[^{14}C]phenylalanyl-tRNA$_{-Y}$ (10 μg, 5000 cpm) and *N*-acetyl[^{3}H]phenylalanyl-tRNA (10 μg, 14,000 cpm) was chromatographed on an antibody column as described in Figure 2.

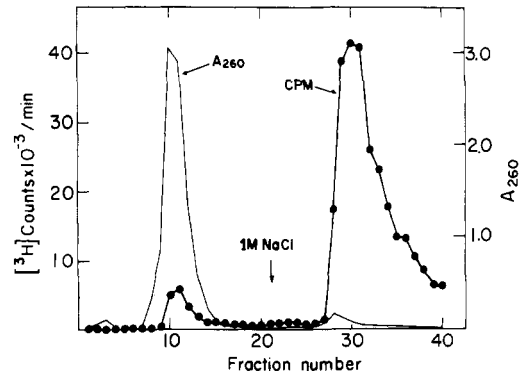

FIGURE 6: Purification of Y-containing rat liver tRNA^{Phe}. A mixture of rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA (64 μg, 282,000 cpm) and uncharged rat liver tRNA (380 μg) was applied on an antibody column (1 × 6 cm). The column was eluted with 20 ml of PS, followed by 20 ml of PS containing 1 *M* NaCl; aliquots of 0.1 ml were removed from each tube and counted. The PS fractions as well as the PS containing 1 *M* NaCl fractions were pooled separately, dialyzed against water, extracted with phenol–chloroform, and concentrated by evaporation to a volume of 1 ml. The radioactivity and A_{260} of the two concentrated fractions were then measured.

strates that the bulk of A_{260} was eluted with PS while most of the radioactivity was recovered with 1 *M* NaCl. The specific activity of the initial mixture amounted to 6,350 cpm/10 μg of RNA while the 1 *M* NaCl fraction reached a value of 129,000 cpm/10 μg of RNA, indicating a 20-fold purification of the tRNA^{Phe}. On the other hand, the material that eluted with PS alone had a low specific activity of 500 cpm/10 μg of RNA. In order to obtain biologically active tRNA^{Phe} we have purified the γ-globulins by DEAE-cellulose column chromatography, to recover the IgG fraction (Levy and Sober, 1960). The antibodies thus obtained did not cause any noticeable degradation of the tRNA^{Phe}. Using a collmn of IgG fraction bound to Sepharose, the peroxy Y-containing tRNA^{Phe} species was purified 28-fold and its phenylalanine acceptance activity amounted to 800 pmol/A_{260}.

Discussion

Antibodies made against yeast tRNA^{Phe} were previously shown (Fuchs et al., 1974) to be directed toward the Y-nucleoside present in the anticodon loop of yeast tRNA^{Phe}. Since the Y-nucleoside or its modified form was found to be present in tRNA molecules isolated from other eukaryotic cells such as wheat germ (Yoshikami et al., 1968), *Torulopsis utilis* (Kasi et al., 1971), beef, rat, and chicken liver (cf. Li et al., 1973; Blobstein et al., 1973), it seemed of interest to test the cross-reactivity of tRNA^{Phe} from these species with the antibodies to the yeast tRNA^{Phe}. Indeed, cross-reaction has been shown previously with wheat germ tRNA^{Phe} and now with rat liver tRNA^{Phe}.

The affinity of the antibodies was found to be much weaker toward the heterologous peroxy Y-nucleoside from rat liver than toward the homologous yeast Y-nucleoside. It was reported that the antigenic determinant of nucleic acid accommodates tetra- or pentanucleosides in the polynucleotide chain (Levine and Stollar, 1968). However, the nucleotide sequence in the anticodon loop of yeast and mammalian tRNA^{Phe} are identical except for the Y-nucleoside side chain (RajBhandary et al., 1967; Keith et al., 1974). Thus, the weaker affinity to the antibody could be ascribed to the structural difference between the yeast Y-base and the rat liver peroxy Y-base and/or to differences in the three-dimensional conformation of the nucleotide sequence reacting with the antibody.

Nevertheless, the lower affinity of rat liver tRNA to the antibody is advantageous and allowed us to develop the present detection method. Whereas the homologous yeast tRNA^{Phe} binds very tenaciously to the antibody column and cannot be eluted easily, rat liver tRNA^{Phe} is retarded on the column and can be eluted with 1 *M* NaCl. The affinity chromatography on the antibody column can, therefore, be used to analyze the preparation of tRNA^{Phe} species that lack the peroxy Y-base. The chromatographic procedure is extremely rapid and takes less than 1 hr to complete. Another advantage of the antibody column is that it can be used repeatedly (more than 20 times). We have also found that the antibody can be used for the purification of peroxy Y-containing tRNA^{Phe} species.

The antibody column can be applied for the analysis of the percentage of tRNA^{Phe} species lacking the Y-base from other sources. It was recently reported (Mushinski, 1974; Grunberger et al., 1975) that there are structural differences between the tRNA^{Phe} from normal and rat hepatoma. These authors have shown that in contrast to rat liver tRNA^{Phe}, Morris rat hepatoma 7777 contains a relatively high percentage of tRNA^{Phe} species lacking the peroxy Y-nucleoside. We have now confirmed this result by analyzing the tRNA^{Phe} from Morris hepatoma 7777 (a gift from Dr. F. Mushinski) on the antibody–Sepharose column. Anti-Y

antibodies may, therefore, serve as a tool to search for such differences in many other tissues.

Acknowledgments

We wish to thank Professor M. Sela for very helpful discussions. This work was supported, in part, by U.S. National Cancer Institute Contract No. NOICP 33220 and by Grant No. 1 RO1 AI 11405-01 from the National Institutes of Health, United States Public Health Service.

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Thermolysin and Neutral Protease: Mechanistic Considerations†

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ABSTRACT: The pH dependence of the catalytic activities of thermolysin and neutral protease indicates that each enzyme is dependent upon the protonation of a single residue with a pK_a of 7.5. Chemical modification with ethoxyformic anhydride indicates that a single histidyl residue, with a pK_a of 7.6–7.9, is essential for the catalytic activity of each enzyme. The only free histidyl residue in the active site of thermolysin is His-231, which, in crystallographic studies, was found to be the binding site for several heavy-atom derivatives. The present study demonstrates that these heavy metal compounds inactivate the enzymes reversibly and protect them from covalent inactivation by ethoxyformic anhydride. These findings lead to the proposal that His-231 may serve as a proton donor during catalysis. Other structural considerations indicate that Glu-143 may be in the proper orientation to serve as an attacking nucleophile. The proposed mechanism resembles certain features of the mechanism of carboxypeptidase A on the one hand and of serine proteases on the other.

Detailed descriptions of the amino acid sequence of thermolysin (Titani et al., 1972) and of its three-dimensional structure (Matthews et al., 1972a,b; Colman et al., 1972) have established that this proteolytic enzyme is unique as it bears no structural resemblance to any of the four other sets of homologous proteases, i.e. the serine proteases, the sulfhydryl proteases, the metalloexopeptidases, and the acid proteases (Hartley, 1960; Walsh, 1975).

Two neutral proteases (A and B) isolated from *Bacillus subtilis* resemble thermolysin in specificity, metal dependence, and molecular weight (Tsuru et al., 1964; Matsubara and Feder, 1971; Pangburn et al., 1973b). Neutral proteases A and B differ threefold in their specific activity (Pangburn et al., 1973a) but no differences in amino acid composition or sequence are yet evident. Preliminary se-

† From the Department of Biochemistry, University of Washington, Seattle, Washington 98195. *Received May 9, 1975.* This work has been supported by a research grant (GM 15731) and a predoctoral training grant to M.K.P. (GM 00052) from the National Institutes of Health.

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