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MIXED-MODE CHROMATOGRAPHIC MATRICES FOR THE RESOLUTION OF TRANSFER RIBONUCLEIC ACIDS

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SUMMARY

Modification of approximately 65% of the amine groups of an aminopropylsilyl bonded-phase silica high-performance liquid chromatographic anion exchanger (APS-Hypersil) with organic acids containing *n*-alkyl moieties of different chain lengths, results in mixed mode chromatographic matrices of varying hydrophobic character. These stationary phases result in high resolution of transfer ribonucleic acids (tRNAs) when used with a decreasing gradient of ammonium sulfate. The observed resolution appears to be primarily a function of interfacial precipitation effected by the high initial salt concentration of the mobile phase and the hydrophobic character of the stationary phase, followed by selective resolubilization during the decreasing salt gradient. After resolubilization, adsorptive processes may additionally contribute to resolution. The residual amine groups decrease the hydrophobic character of the stationary phase compared to a pure reversed-phase support. The mixed-mode matrices are useful for the resolution of aminoacylated tRNAs from non-aminoacylated tRNAs.

INTRODUCTION

Owing to their variety and similar molecular weights, transfer ribonucleic acids (tRNAs) are not well resolved by techniques such as polyacrylamide gel electrophoresis. Pure species have been isolated primarily by chromatographic techniques. Initially, pure tRNAs were obtained by using liquid–liquid countercurrent distribution[1,2]. However, this technique has been largely replaced by column chromatography on anion exchangers, such as DEAE-cellulose[3] or DEAE-Sephadex[4]. Chromatography on the RPC-5 matrix[5,6] has also been successful for the isolation of pure tRNA species. Interfacial precipitation and subsequent elution by decreasing salt gradient has been described with a Sepharose 4B column[7–9] and more recently with a weakly hydrophobic silica-based high-performance liquid chromatographic (HPLC) column[10].

Since nucleic acids, such as the tRNAs, are polyanions containing hydrophobic nucleobases, we have been attempting to design various mixed-mode chromatographic matrices with which both ionic and hydrophobic interactions can occur. In one

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approach, a reversed-phase octadecylsilyl bonded-phase silica HPLC column was coated with a tetraalkylammonium salt to introduce the desired sites for ionic interaction of the hydrophobic matrix[11]. Using a mobile phase of high salt concentration, the ammonium salt is essentially irreversibly bound to the matrix. This matrix can be used successfully for the resolution of tRNA mixtures[11,12]. In the present report various organic acids containing *n*-alkyl groups of varying hydrophobicities have been covalently bound to an HPLC grade anion exchanger. The resulting mixed-mode chromatographic matrices have been used with either increasing or decreasing salt gradients for the resolution of native or aminoacylated tRNAs.

MATERIALS

APS-Hypersil, ODS-Hypersil, MOS-Hypersil and APS-Hypersil WP (300 Å pore diameter) and 5-μm particle size have been purchased from Shandon Southern (Runcorn, U.K.). $tRNA^{Val}$-C-C and valyl-tRNA synthetase (baker's yeast) were gifts from Dr. H. Sternbach (Göttingen, F.R.G.). $tRNA^{Asp}$-C-C and aspartyl-tRNA synthetase (baker's yeast) were gifts from Dr. B. Lorber (Strasbourg, France). $tRNA^{Ser}$-C-C and $tRNA^{Phe}$-C-C (baker's yeast) have been isolated according to ref. 13. tRNA-nucleotidyltransferase (10,000 U/μl) was a gift from Dr. H. Sternbach (Göttingen, F.R.G.). L-[U-^{14}C]valine and L-[U-^{14}C]aspartic acid (100 mCi/mmol; 50 μCi/ml) have been purchased from Amersham Buchler (Braunschweig, F.R.G.). Ammonium sulfate (ultra pure) was purchased from BRL (Neu-Isenburg, F.R.G.). Adenosine-5'-triphosphate was purchased from Sigma (München, F.R.G.). All other analytical grade chemicals were purchased from Merck (Darmstadt, F.R.G.).

METHODS

The chromatographic separations were performed on a DuPont 850 liquid chromatograph equipped with a variable-wavelength UV detector and a thermostatically controlled oven.

Approximately 65% of the amine groups present on APS-Hypersil were modified[12] as acetic acid, *n*-butanoic acid, *n*-hexanoic acid or *n*-octanoic acid amides which resulted in the modified matrices APS-C_2, APS-C_4, APS-C_6 and APS-C_8, respectively.

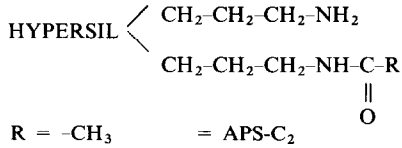
Preparation of aminoacyl-tRNAs

The 100-μl reaction mixture contained 80 μ*M* tRNA-C-C, 0.2 *M* potassium chloride, 0.2 *M* Tris–HCl pH 7.6, 20 m*M* magnesium sulphate, 5 m*M* ATP, 200 μ*M* L-[^{14}C]amino acid (approximately 100,000 cpm/nmol), 200 U/ml tRNA nucleotidyltransferase and 130 μg/ml aminoacyl-tRNA synthetase. After incubating the mixture for 30 min at 37°C, 1 μl was spotted on a Whatman 3 MM filter disk, washed three times with 5% trichloroacetic acid, washed with ethanol and dried. Radioactivity was measured in a liquid scintillation counter in a xylene-based scintillator. The aminoacyl tRNA was precipitated with two volumes of absolute ethanol for 30 min at −20°C and isolated by centrifugation (approximately 35,000 *g*). The dried pellet was dissolved in water and used for HPLC analysis.

Acetylation of the α-amine group of the amino acid bound to the tRNA was carried out according to ref. 14.

RESULTS

The present mixed-mode (ionic–hydrophobic) matrices have been prepared by reacting an anion exchanger (APS-Hypersil) with various organic acid anhydrides or chlorides of different alkyl chain lengths[12].



R = $-CH_3$ = APS-C_2

R = $-(CH_2)_2-CH_3$ = APS-C_4

R = $-(CH_2)_4-CH_3$ = APS-C_6

R = $-(CH_2)_6-CH_3$ = APS-C_8

With this procedure 65% of the primary amine groups are modified. The remaining unmodified amine groups will give the modified chromatographic matrix an anion-exchange character, the extent of which will be dependent upon the pH of the mobile phase. At lower pH values more extensive protonation of the amine groups takes place, and the columns have a higher anion-exchange character. This effect decreases as the pH of the mobile phase increases. The hydrophobic (or reversed-phase) character of the columns is a function of the *n*-alkyl moieties of the modifying organic acid anhydrides or chlorides. Increasing hydrophobic character occurs with increasing *n*-alkyl chain length. The extent of hydrophobic interactions can additionally be controlled by variations of the organic solvent in the mobile phase.

pH and salt concentrations

Using small oligonucleotide solutes, we have previously shown[12] that resolution on these modified matrices is a result of both ionic and hydrophobic interactions. However, the use of the columns with a similar mobile phase, involving an increasing salt gradient for the resolution of larger solutes such as tRNAs, was not successful. Pure tRNA species were eluted from the columns with poor peak shape. That both ionic and hydrophobic interactions occurred with the support in the chromatography of tRNAs was indicated by the observation that retention decreased with increasing pH of the mobile phase and that retention increased with increasing hydrophobicity of the column (data not shown).

Recently[10], tRNA mixtures have been resolved on a weakly hydrophobic trimethylsilyl bonded-phase silica by using a decreasing gradient of ammonium sulfate. Under mobile-phase conditions involving a decreasing ammonium sulfate gradient in the presence of 0.05 *M* potassium dihydrogen phosphate pH 6.0, tRNAs could be eluted from the present modified columns with markedly improved peak shape. We then examined elution of a specific tRNA from the unmodified APS matrix, the four modified matrices and a C_8 reversed-phase matrix (MOS-Hypersil) by using a decreasing gradient of ammonium sulfate. Under these mobile-phase conditions, yeast $tRNA^{Val}$ was not retained on the unmodified APS support. However, retention was observed on the modified supports and was related to the alkyl chain length of the modifying *n*-alkyl moiety. Elution occurred at progressively lower concentrations of

ammonium sulfate (*i.e.* longer retention time) for the APS-C_2, APS-C_4 and APS-C_6 columns, respectively. The tRNA could not be eluted from APS-C_8. Additionally no elution was observed from the unmodified C_8 reversed-phase MOS-Hypersil column. Finally, we examined an APS-C_6 column in which only 32% of the amine groups had been acylated. However, we were unable to elute the tRNA from it.

It was unclear whether this phenomenon was solely a result of the hydrophobic interactions or in part due to the amine groups available for ionic interaction. Using the mobile-phase conditions described above, the chromatographic analyses were repeated at pH 4.6 and pH 7.0 (Fig. 1). Chromatography with a decreasing ammonium sulfate gradient exhibited only a small pH dependence, Noteably, $tRNA^{Val}$ was eluted from APS-C_8 at pH 7 but could not be eluted by a mobile phase of pH 6.0 or pH 4.6.

Previous examination of these matrices has indicated that with increasing salt gradients mixed-mode chromatography occurs[12]. Therefore, using the mobile phase containing ammonium sulfate, we compared the elution of $tRNA^{Val}$ from the various columns with both increasing and decreasing salts gradients (Fig. 2). Using the increasing salt gradient, $tRNA^{Val}$-C-C was eluted from the APS support at a concentration of 0.6 *M* ammonium sulfate. Using the APS-C_2 and APS-C_4 columns and the increasing salt gradient, surprisingly, two peaks were eluted. The retention times of the earlier appeared dependent upon the modification of the matrix and those of the second peaks appeared to be related only to the salt concentration of the mobile phase. With the APS-C_6 column a single peak was again observed and no elution was observed from the APS-C_8 column. In all cases where the solute was eluted from

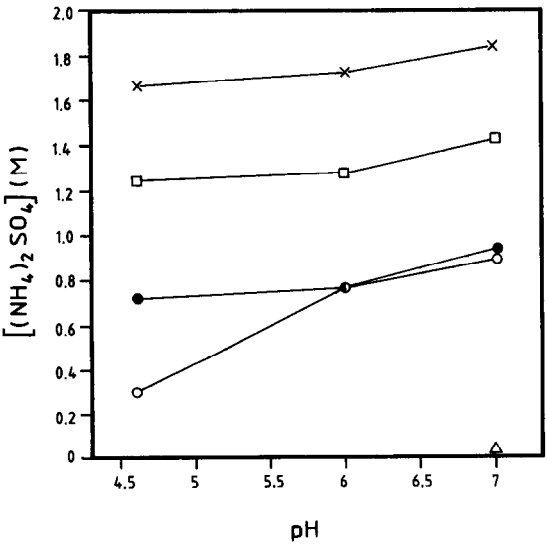


Fig. 1. Concentration of ammonium sulfate at which yeast $tRNA^{Val}$-C-C is eluted when a decreasing salt gradient at various pH values is used. Buffer A, 0.05 *M* potassium dihydrogen phosphate; buffer B, 0.05 *M* potassium dihydrogen phosphate containing 2.0 *M* ammonium sulphate. Gradient: 100–0% buffer B in 60 min. Flow-rate: 1 ml/min. Temperature: 35°C. Column: 4.6 × 150 mm I.D. of APS-C_2 (×), APS-C_4 (□), APS-C_6 (●), APS-C_6 wide pore (300 Å) (○)and APS-C_8 (△).

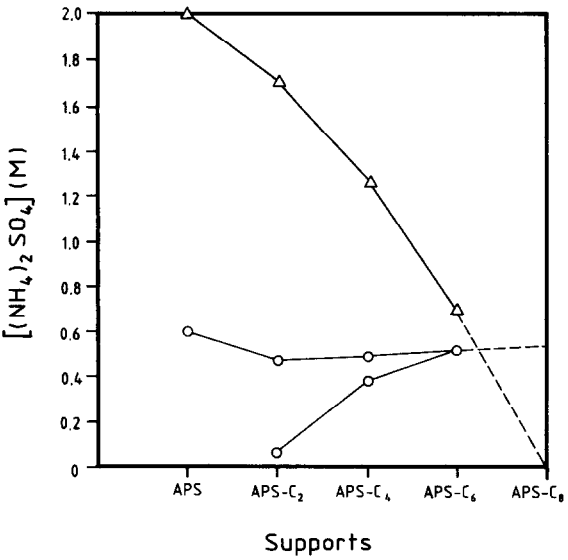


Fig. 2. Concentration of ammonium sulfate at which yeast $tRNA^{Val}$-C-C is eluted when an increasing (○) or decreasing (△) gradient of ammonium sulfate in 0.05 *M* potassium dihydrogen phosphate (pH 6.0) is used on various mixed-mode chromatographic columns. Buffers are those described in the legend to Fig. 1. Increasing salt gradient: 0–100% buffer B in 60 min. Decreasing salt gradient: 100–0% buffer B in 60 min.

the column, elution occurred at a higher salt concentration when the decreasing ammonium sulfate gradient was used (Fig. 2).

Effect of pore size

In the case of the APS-C_6 column we additionally examined the effect of pore size. A 300-Å pore size APS-Hypersil matrix was modified to the same extent with hexanoic acid anhydride. The results of the decreasing ammonium sulfate gradient at various pHs using yeast $tRNA^{Val}$ is additionally plotted in Fig. 1. Retention on the large-pore matrix was much more strongly affected by low pH than had been observed with the normal-pore (80–110 Å) matrix. It is also noteworthy that when the increasing salt gradient at pH 4.6 is used, $tRNA^{Val}$ is eluted at 0.5 *M* ammonium sulfate in comparison to 0.3 *M* ammonium sulfate, observed when the decreasing gradient is used.

Effect of the organic solvent

In addition to changing the hydrophobic interactions available on a given support by varying the chain length of the acylating moiety, it is also possible to vary the hydrophobic interactions by addition of an organic solvent to the mobile phase. We have examined the change in retention characteristics when different concentrations of 2-propanol are used in conjunction with the decreasing ammonium sulfate gradient for the APS-C_6, APS-C_8 and C_8 reversed-phase (MOS-Hypersil) columns (Fig. 3). The MOS unmodified C_8 reversed-phase column appears to have the highest hydrophobic character, as would be expected, and $tRNA^{Val}$ is not eluted from the column with less than 2% 2-propanol in the mobile phase. Additionally, it exhibits

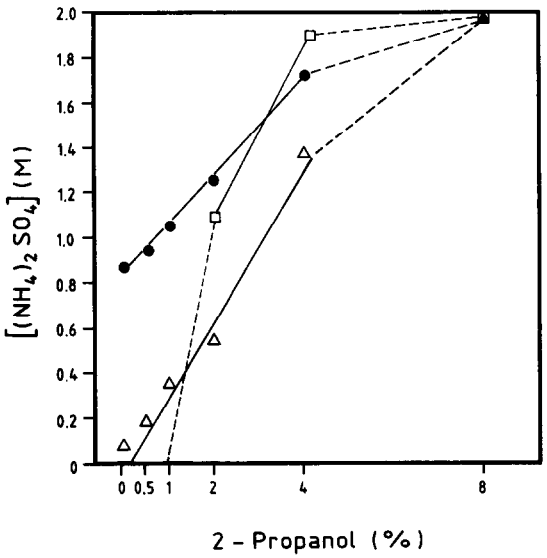


Fig. 3. Concentration of ammonium sulfate at which yeast $tRNA^{Val}$-C-C is eluted. A decreasing gradient of ammonium sulfate in 0.05 *M* potassium dihydrogen phosphate at pH 7.0 with various concentrations of 2-propanol is used. Column: 4.6 × 150 mm I.D. column of APS-C_6 (●), APS-C_8 (△), or C_8 reversed-phase (MOS-Hypersil) (□). Other chromatographic conditions as described in the legend to Fig. 1.

retention characteristics which are most strongly dependent upon the 2-propanol concentration. At 1% 2-propanol the tRNA is not eluted and 4% 2-propanol the tRNA is only minimally retained on the column. On the other hand, retention of the tRNA on both modified supports is less dependent upon the 2-propanol concentration. At all 2-propanol concentrations below 8%, $tRNA^{Val}$ is more strongly retained on the APS-C_8 column than on the APS-C_6 column.

Selected applications

Using a decreasing gradient of ammonium sulfate in the presence of 2% 2-propanol, native tRNA species can be resolved on the APS-C_6 support into well defined peaks, as illustrated for the tRNAs specific for the amino acids valine, serine, and phenylalanine (Fig. 4). A slight increase in resolution was observed when the APS-C_8 column was used, but 3% 2-propanol was required in the mobile phase to obtain similar retention characteristics. Under the chromatographic conditions of Fig. 4, the retention times for yeast tRNAs, specific for valine, serine and phenylalanine were 19.8, 30.0 and 35.7 min, respectively, when the APS-C_6 column was used. With the APS-C_8 column and similar chromatographic conditions, but using 3% 2-propanol in the mobile phase, similar peak shape but retention times of 25.0, 38.5 and 51.2 min were observed for the valine, serine, and phenylalanine tRNAs, respectively.

These mixed-mode matrices are also useful for separating aminoacylated tRNAs from non-aminoacylated tRNAs. In this respect, two tRNAs have been examinated, $tRNA^{Val}$ and $tRNA^{Asp}$. The former is aminoacylated with the relatively hydrophobic amino acid valine, and the latter is specific for the hydrophilic amino

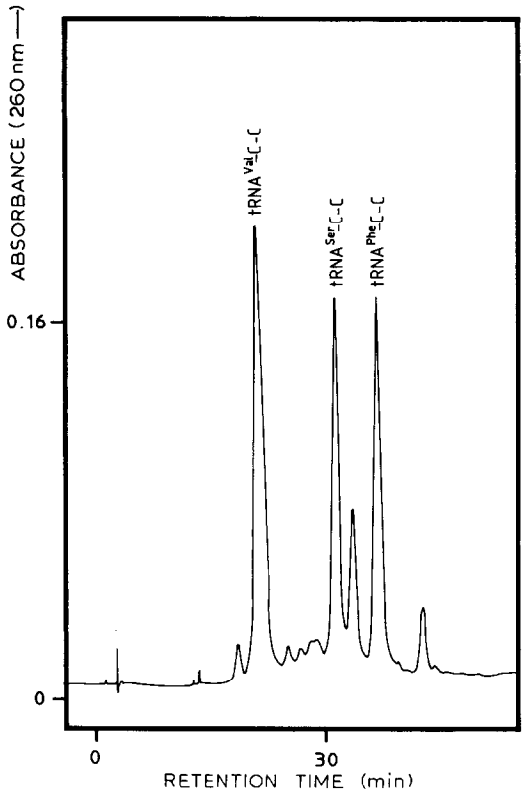


Fig. 4. Resolution of three tRNAs from baker's yeast. Column: 4.6 × 150 mm I.D. APS-C_6. Buffer A: 0.05 *M* potassium dihydrogen phosphate (pH 7) containing 2% 2-propanol. Buffer B: 0.05 *M* potassium dihydrogen phosphate (pH 7) containing 2% 2-propanol and 2.0 *M* ammonium sulfate. Gradient: 100–0% buffer B in 60 min. Flow-rate: 1 ml/min. Temperature: 35°C.

acid aspartic acid. In both cases, the tRNAs were isolated lacking the 3′-terminal adenosine. The 3′-termini were regenerated by using tRNA nucleotidyltransferase and ATP. The tRNAs were then aminoacylated by using the corresponding ^{14}C-labelled amino acid and aminoacyl-tRNA synthetase. Finally, the aminoacyl ester linkages were stabilized by acetylating the α-amine group of the bound amino acid with acetic acid-N-hydroxysuccinimide ester. None of the reactions were allowed to go to completion. Therefore, four tRNA species were present in the reaction mixtures. In the case of $tRNA^{Val}$ all four species could be resolved within 40 min (Fig. 5a). The species eluted first was $tRNA^{Val}$ lacking the 3′-terminal adenosine. With regeneration of the 3′-terminal C-C-A sequence a later retention time was observed. Aminoacylation with the relatively hydrophobic amino acid valine further increased the retention time for the tRNA. The species with the longest retention time was the N-acetyl-Val-$tRNA^{Val}$ (AcN-Val-$tRNA^{Val}$).

Chromatography of the mixture resulting from the tRNA specific for aspartic acid produced only three peaks (Fig. 5b) (the peak eluted at 40 min is a contaminent present in the $tRNA^{Asp}$ stock solution). Again, resolution between the tRNA-C-C and tRNA-C-C-A species was observed. However, aminoacylation with the hydro-

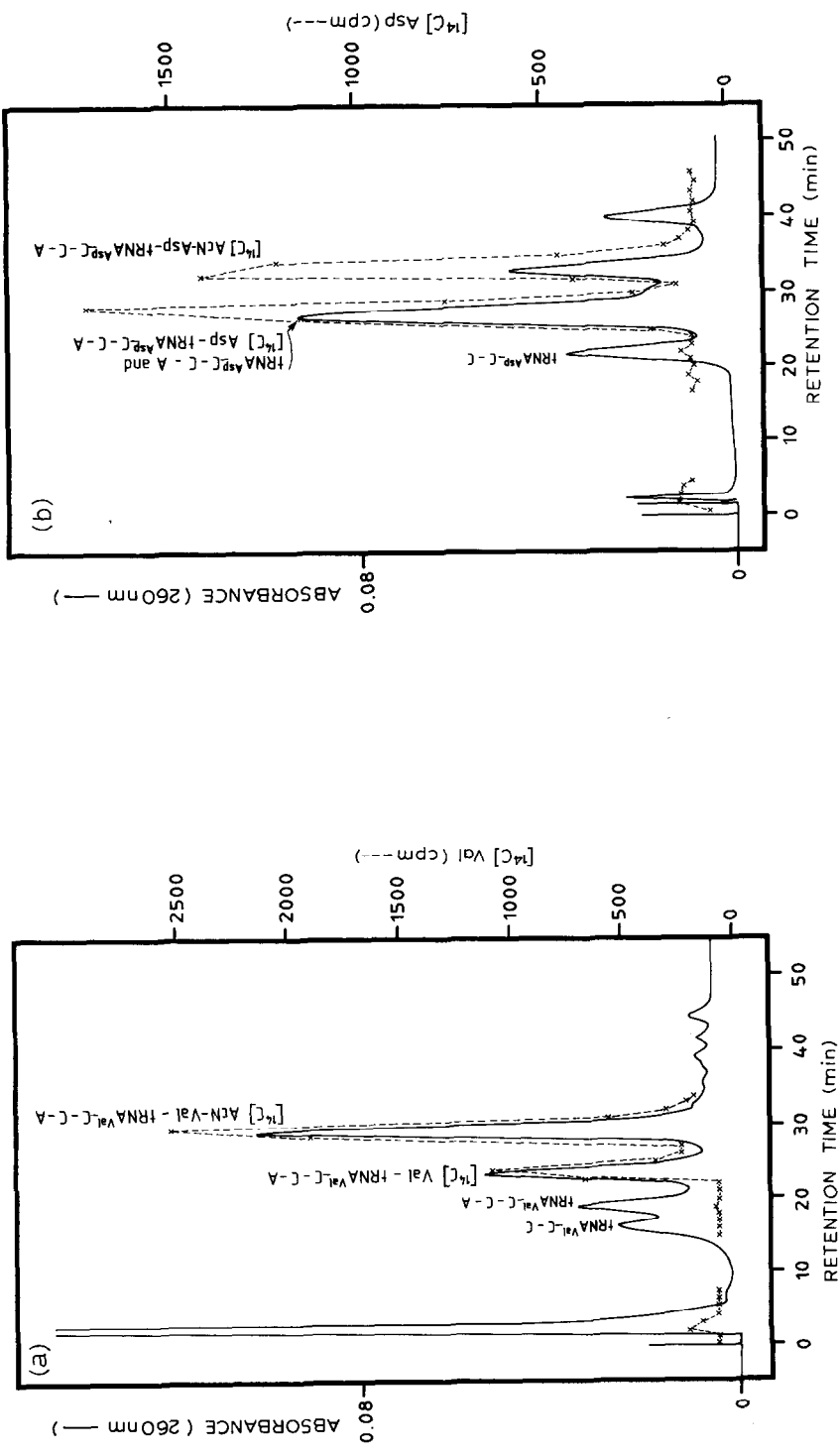


Fig. 5. Resolution of a tRNA mixture resulting from the aminoacylation and subsequent acetylation of (a) yeast $tRNA^{Val}$ and (b) yeast $tRNA^{Asp}$. Column and chromatographic conditions as described in the legend to Fig. 4.

philic amino acid did not allow separation of the aminoacylated from the non-aminoacylated tRNA. However, acetylation of the α-amine group of the amino acid did result in a longer retention time.

No significant hydrolysis of either the aminoacyl tRNAs or their acetylated derivatives was observed during the chromatography.

DISCUSSION

It is well known that macromolecular polyanions, such as nucleic acids, tend to be eluted in broad, tailing peaks when chromatographed on anion exchangers of high capacity. This effect has also been observed with the present mixed-mode matrices when a mobile phase with an increasing salt gradient is used. However, when the supports are used with a decreasing gradient of ammonium sulfate, resolution is dramatically improved.

Resolution of tRNAs by the decreasing salt gradient appears to be largely pH-independent in the pH range 4.6–7.0 (Fig. 1), although the protonation of amine groups on the present matrices will vary considerably within this pH range. Since protonation of the amine groups does not alter significantly the retention characteristics, it would appear that the amine groups are not directly involved in the retention mechanism. This suggests that the primary retention mechanism is that of interfacial precipitation of the tRNA induced by the hydrophobic *n*-alkyl chains at high salt concentrations of the mobile phase. This is also indicated from Fig. 1 where progressively lower concentrations of ammonium sulfate are necessary to elute the tRNAs with the increasing hydrophobicity of the stationary phases.

A comparison of retention characteristics in both increasing and decreasing salt gradients also indicates the importance of the hydrophobic effects and lack of direct ionic interactions (Fig. 2). When the decreasing salt gradient is used, the ammonium sulfate concentration necessary for elution of $tRNA^{Val}$ from the APS-C_2, APS-C_4 and APS-C_6 columns is in all cases higher than what is required for elution when the increasing salt gradient is used. Therefore, the salt concentration at which the tRNA is resolubilized after interfacial precipitation is still high enough that no significant ionic adsorption–desorption processes take place. The inability of the tRNA to be eluted from the APS-C_8 column may be a result of the "crossing over" of these two effects (Fig. 2). The APS-C_8 requires a very low concentration of ammonium sulfate so that resolubilization of the tRNA occurs. On the other hand, at this low salt concentration ionic adsorption occurs, and the tRNA is not eluted. This may also account for the observation that $tRNA^{Val}$-C-C was not eluted from the APS-C_6 matrix in which only 32% of the amine groups were acylated; with a lower extent of modification, increased ionic interactions will occur. At the low salt concentration necessary for resolubilization, irreversible ionic adsorption takes place.

Raising the pH of the mobile phase to 7.0 allows elution of the tRNA from the APS-C_8 column at very low salt concentration (Fig. 1). This may reflect the decrease in ionic character of the matrix such that elution will occur at low ammonium sulfate concentration without significant ionic adsorption. On the other hand, this may simply indicate an increased solubility of the tRNA in the mobile phase as a result of higher pH[9].

The observation that the APS-C_2 and APS-C_4 columns yielded two peaks

when the increasing salt gradient was used is at present unclear. The peak eluted earlier appears to be related to hydrophobic interactions and the one eluted later is more dependent upon ionic interactions.

Increasing the pore size of the matrix alters retention characteristics only at low pH (Fig. 1). When the large-pore (300 Å) matrix was used, $tRNA^{Val}$ was eluted with a lower ammonium sulfate concentration (0.3 *M*) with the decreasing salt gradient than when the normal-pore (80–110 Å) column (0.7 *M*) was used. This suggests that when the tRNA is resolubilized after interfacial precipitation, additional adsorptive interactions are available as a result of higher accessibility to the larger pores.

Retention characteristics appear to be largely dependent upon the hydrophobic nature of the stationary phase. When the decreasing salt gradient at pH 7.0 is used, $tRNA^{Val}$ is more strongly retained on the APS-C_8 column than on the APS-C_6 column. On the more hydrophobic C_8 column (MOS-Hypersil) the tRNA remains irreversibly bound. These retention characteristics can, however, be altered with the addition of small amounts of organic solvent (2-propanol) to the mobile phase (Fig. 3). At low concentrations of 2-propanol the C_8 column exhibits the strongest retention characteristics and at higher concentrations (4% 2-propanol) the weakest. At least two explanations could account for these differences. At higher organic solvent concentrations additional interactions may be available on the mixed-mode columns. Although it seems unlikely that ionic interactions occur at the high salt concentrations present, there may be adsorptive effects after resolubilization of the tRNA as a result of the polar amine groups. It is also possible that at higher propanol concentrations a layer of organic solvent forms on the surface of the C_8 matrix and inhibits interfacial precipitation. This may not be possible to the same extent on the mixed-mode matrices as a result of the polar amine groups.

The elution of tRNAs from the mixed-mode columns appears largely dependent on hydrophobic interactions. The order of elution is roughly similar to the one we have observed on other mixed-mode columns[11]. $tRNA^{Phe}$, carrying the highly hydrophobic Y-base, is eluted relatively late in the gradient (Fig. 4). Both, the APS-C_6 and APS-C_8 column, resolve tRNA mixtures. Retention is stronger on the APS-C_8 column as a result of its higher hydrophobic character. Whether this is due to differences in interfacial precipitation or subsequent "adsorptive retardation[9]" after resolubilization of the tRNA cannot be determined from the present study.

That the hydrophobic nature of the tRNA is important for resolution is indicated by the resolution of the reaction mixtures of Figs. 5a and 5b. In both cases the tRNA-C-C-A is eluted later than the tRNA-C-C as a result of the hydrophobic terminal adenosine residue. In the case of the relatively hydrophobic amino acid valine a further increase in retention is observed. The inability to resolve $tRNA^{Asp}$ and Asp-$tRNA^{Asp}$ is related to the hydrophilic nature of the amino acid. The increase in hydrophobic character which occurs upon acetylation of the amino acid increases retention.

It is noteworthy that the aminoacylated tRNAs were stable to chromatography without acetylation of the α-amine group. It should therefore be possible to isolate aminoacyl tRNAs in high purity by using the present mixed-mode columns.

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