Effect of Heavy Water Substitution for Water on the $tRNA^{Val}$–Valyl-tRNA Synthetase System from Yeast†

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ABSTRACT: We present a comparative study of the effects of water (H_2O) and heavy water (^2H_2O) on the stability and on the kinetic parameters of various reactions catalyzed by the yeast valyl-tRNA synthetase. In both solvents the enzyme exhibits the same stability against thermal inactivation. The affinities of the various substrates $tRNA^{Val}$, adenosine 5′-triphosphate (ATP), and valine, as well as the rate constant of the inorganic [^{32}P]pyrophosphate–ATP isotope-exchange reaction, remain unaffected, whereas the steady-state rate constant of the overall aminoacylation reaction decreases by a factor of ~5–6 in the presence of ^2H_2O at pL 7.2 (L ≡ ^{1}H or ^{2}H). In both solvents, the dissociation of the end products is rate determining at the steady state of the aminoacylation reaction; ^2H_2O affects the rates of both the first catalytic cycle (the transfer step of the activated amino acid to tRNA) and the dissociation of the end products. These results are discussed in relation with the rate-determining step of the aminoacylation reaction. The rate constants of both the chemical and the enzymatic [either adenosine 5′-monophosphate (AMP) and inorganic pyrophosphate (PP_i) independent or dependent] deacylation reactions are decreased after substitution of H_2O by ^2H_2O. Consequently, the level of aminoacylation obtained under standard aminoacylation conditions in the presence of ^2H_2O is higher than in the presence of H_2O. Finally, the equilibrium of the overall aminoacylation reaction was found to be more displaced toward the end products in the presence of H_2O than in the presence of ^2H_2O. This study indicates that heavy water, although affecting the catalytic process, does not alter significantly the stability of the enzyme and its interaction with tRNA, justifying structural studies of this system in heavy water solvent by neutron scattering.

Several chemical reactions involving proton-transfer steps have been studied in heavy water (^2H_2O) solvent (Jenks, 1969). Such steps, obviously, are involved in many enzymatic reactions, but isotope effects on enzyme-catalyzed reactions have been examined in relatively few instances [e.g., Schmidt et al. (1979)]. This is because the isotope effects might be multiple (i.e., effects on the reaction rate, on the association of the ligands to the enzyme, solvent effects, difficulty in discriminating between nonexchangeable and rapidly exchangeable protons, and acidity of the incubation medium) which makes the interpretation of results difficult [for reviews, see Jenks (1969) and Cleland et al. (1976)]. In recent years, however, neutron scattering has been introduced as a promising physical method for studying structural parameters of macromolecular systems [for a review, see Jacrot (1976)]. This method takes advantage of the very different scattering properties of hydrogen and deuterium and involves replacement of hydrogen by deuterium in the solvent as well as in the macromolecules. Structural data have been obtained in this way on several nucleic acid–protein complexes [i.e., Baldwin et al. (1975), Stuhrmann et al. (1977), Jacrot et al. (1977), Moore et al. (1977), Dessen et al. (1978), Koch et al. (1978), and Zaccaï et al. (1979)]. An implicit assumption of these studies is that ^2H_2O does not significantly perturb the system under investigation. As a first approximation, this can be admitted to be true; microorganisms can grow on ^2H_2O media (Ehresmann et al., 1971), and active ribosomes can be reconstituted with deuterated proteins (Moore et al., 1977). No systematic study of the effect of ^2H_2O on the biochemical properties of systems investigated by neutron-scattering methods, however, has been undertaken so far.

The interactions of various tRNAs with yeast valyl-tRNA synthetase have been studied by small-angle neutron scattering (Giegé et al., 1977; Zaccaï et al., 1979). In the present work, we report biochemical experiments exploring the effect of ^2H_2O substitution for H_2O on the $tRNA^{Val}$–valyl-tRNA synthetase interaction and on the $tRNA^{Val}$ valylation reaction. The results show that there are ^2H_2O effects on the kinetic parameters of the enzymatic reaction; however, the interaction of the two macromolecules appears not to be perturbed. It suggests that there are no important structural differences between the valyl-tRNA synthetase system in H_2O and in ^2H_2O.

Experimental Procedures

Materials. Valyl-tRNA synthetase (EC 6.1.1.9) was prepared from baker's yeast (Kern et al., 1975). It has a molecular weight of 130 000 and an extinction coefficient $E_{mg/(mL\cdot cm)}^{280nm}$ of 1.79. Its specific activity is 4000 units/mg [1 unit being the amount of enzyme which catalyzes the incorporation of 1 nmol of valine into crude yeast tRNA per min under standard assay conditions (Kern et al., 1975)].

Crude brewer's yeast tRNA was from Boehringer (Mannheim, Germany). Pure $tRNA^{Val}$ (major species) was purified by countercurrent distribution (Dirheimer & Ebel, 1967), followed by conventional chromatographic techniques. Concentrations of tRNAs, crude or valine-accepting species, were calculated by assuming that 1 absorbance unit at 260 nm corresponds respectively to 40 and 35 μg mL^{-1} of tRNA.

Heavy water (^2H_2O) had an isotopic purity of 99.95%. It was distilled under argon before use. Radioactive valine (^{3}H or ^{14}C labeled, respectively, 30 Ci/mmol and 250 mCi/mmol) and [^{32}P]PP_i were from the Commissariat à l'Energie Atomique (Saclay, France). Inorganic pyrophosphatase (PP_i) was from Sigma Chemical Co. (1 unit catalyzes the formation of 1 μmol of inorganic phosphate/min at 25 °C). All other

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Table I: Equilibrium Constants of the Aminoacylation Reaction of $tRNA^{Val}$ in H_2O and ^2H_2O

		final concentrations of reactants[b]						
expt[a]		ATP (mM)	valine (mM)	$tRNA^{Val}$ (μM)	AMP (mM)	PP_i (mM)	Val-$tRNA^{Val}$ (μM)	K_{eq}[c]
H_2O	1	1.98	0.08	1.17	0.14	0.14	16.12	1.70
	2	1.99	0.09	3.10	0.26	0.26	14.22	1.73
	3	1.99	0.09	12.47	1.05	1.05	4.85	2.39
^2H_2O	4	1.99	0.09	4.12	0.26	0.26	7.85	0.72
	5	2.00	0.10	7.85	0.50	0.50	4.12	0.66
	6	2.00	0.10	10.47	1.01	1.01	1.50	0.73

[a] The equilibria were obtained either in the presence of H_2O (expt 1-3) or in the presence of ^2H_2O (expt 4-6) as described under Experimental Procedures. [b] The concentrations of the various reactants at the equilibrium were determined as follows: the [^{14}C]Val-$tRNA^{Val}$ by trichloroacetic acid precipitation of 40-μL aliquots and the remaining uncharged $tRNA^{Val}$ by substracting the concentration of the Val-$tRNA^{Val}$ from that of the $tRNA^{Val}$ initially present (17.3 μM). The final ATP and valine concentrations were obtained by substracting those consumed for the synthesis of the Val-$tRNA^{Val}$ from those initially present (2 and 0.1 mM, respectively). The final AMP and PP_i concentrations were obtained by adding those generated by the ATP consumption to those initally present (0.125 mM of both substrates in expt 1, 0.250 mM in expt 2 and 4, 0.50 mM in expt 5, and 1.0 mM in expt 3 and 6). [c] $K_{eq} = [AMP][PP_i][\text{Val-}tRNA^{Val}]/([ATP][Val][tRNA^{Val}])$.

chemicals were of the highest purity commercially available.

Buffer Conditions. The ^2H_2O buffers were prepared as described previously (Zaccai et al., 1979), and the acidity (pL) of the solutions was determined by using the relation

$$pL = \text{pH meter reading} + \Delta pH$$

where L is ^2H or ^1H and $\Delta pH = 0.3314n + 0.0766n^2$, where n is the ^2H_2O fraction (Covington et al., 1968). Comparative studies in H_2O and ^2H_2O buffers were made at the same pL values.

Thermal Stability of the Enzyme. Thermal stability was determined by incubating for various times the enzyme (0.13 mg/mL) at 40 °C and by measuring at 28 °C the remaining [^{32}P]PP_i–ATP isotope-exchange and tRNA aminoacylation activities under standard conditions (see below). The H_2O and ^2H_2O incubation media contained 50 mM KOH–Hepes,[1] pL 7.2, 5 mM β-mercaptoethanol, and 0.1 mg of bovine serum albumin per mL.

Binding of $tRNA^{Val}$ to Valyl-tRNA Synthetase. Binding was investigated by using the filtration method through nitrocellulose membranes (Yarus & Berg, 1967; Bonnet & Ebel, 1975).

[^{32}P]PP_i–ATP Isotope-Exchange Reaction. The experiments were conducted as described previously (Kern & Giegé, 1979) (specific activity of [^{32}P]PP_i 1300 cpm/nmol).

Aminoacylation Reaction. The incubation mixtures contained 50 mM KOH–Hepes buffer, pL 7.2 (unless otherwise indicated), 20 mM $MgCl_2$, 6 mM ATP, 30 mM KCl, 5 mM β-mercaptoethanol, 0.2 mM ^{14}C or ^{3}H labeled valine (specific activity respectively 65 and 2400 cpm/pmol), 0.1 mg of bovine serum albumin/mL, and appropriate concentrations of enzyme and tRNA. The enzyme dilutions were performed with the same buffer as that used for the study of the thermal stability. Incubation times and temperatures as well as the percent of ^2H_2O in the media are indicated in the text and legends of figures. The Val-$tRNA^{Val}$ was measured by liquid scintillation counting as a trichloroacetic acid precipitate (Kern et al., 1975). Large-scale preparations of [^{14}C]Val-$tRNA^{Val}$ were performed as described by Bonnet & Ebel (1975).

For the determination of the equilibrium constants of the overall aminoacylation reaction, the incubation mixtures (of a total volume of 200 μL) contained 50 mM KOH–Hepes, pL 7.2, 20 mM $MgCl_2$, 30 mM KCl, the various substrates and products (ATP, [^{14}C]Val-$tRNA^{Val}$, AMP, and PP_i) as indicated in Table I, 0.28 μM enzyme in the presence of H_2O, and 0.50 μM enzyme in the presence of ^2H_2O. Aliquots of 40 μL were removed after various incubation times at 37 °C. Stable aminoacylation plateaus were reached in each case after a maximal incubation time of 10 min. It was verified that, under the experimental conditions used, the $tRNA^{Val}$ was charged to a 100% extent in the absence of added AMP and PP_i.

Enzymatic and Chemical Deacylation Reactions of $tRNA^{Val}$. For the measurements of the enzymatic (AMP- and PP_i-independent) and chemical deacylation reactions, the incubation mixtures contained 100 mM KOH–Hepes, pL 7.2, 30 mM KCl, 20 mM $MgCl_2$, 20 μM ATP, 0.18 μM $tRNA^{Val}$, 50 μM L-[^{14}C]valine, 1.5 μM valyl-tRNA synthetase, and 2 units of inorganic pyrophosphatase/mL. An aminoacylation plateau was obtained at 37 °C in either H_2O or 85% ^2H_2O media after about 5 min of incubation; this reflects the establishment of an equilibrium between the aminoacylation and the various deacylation reactions (Bonnet & Ebel, 1972). After the disruption of this equilibrium which corresponds to the ATP depletion, the first-order rate of decay of [^{14}C]-Val-$tRNA^{Val}$ was measured. It corresponds to the sum of the chemical and enzymatic deacylation rates. In a control experiment done with preformed [^{14}C]Val-$tRNA^{Val}$, the chemical deacylation rate constant was measured under the same experimental conditions, except that the enzyme was omitted.

The reverse of the aminoacylation reaction (the AMP- and PP_i-dependent deacylation of Val-$tRNA^{Val}$) was measured at 37 °C in an incubation mixture containing 100 mM KOH–Hepes, pL 7.2, 10 mM $MgCl_2$, 2 mM PP_i, 10 mM AMP, 3.7 μM [^{14}C]Val-$tRNA^{Val}$ (272 cpm/pmol), and 0.025–0.1 μM valyl-tRNA synthetase. The results were corrected for the chemical and enzymatic (AMP- and PP_i-independent) deacylations.

Results

Thermal Stability of Valyl-tRNA Synthetase. No differences were found in the half-life of valyl-tRNA synthetase activities in H_2O and ^2H_2O. Indeed, when heated at 40 °C in both solvents, these half-lives were found respectively equal to 11.5 and 16.4 min for the aminoacylation and the [^{32}P]-PP_i–ATP exchange activities.

Binding of $tRNA^{Val}$ to Valyl-tRNA Synthetase. The association between the two macromolecules was studied kinetically as well as by direct-interaction experiments. Figure 1 represents the Scatchard analysis of the saturation curves of the enzyme by Val-$tRNA^{Val}$ and the Lineweaver–Burk plots

[1] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Val-$tRNA^{Val}$, transfer ribonucleic acid aminoacylated with valine.

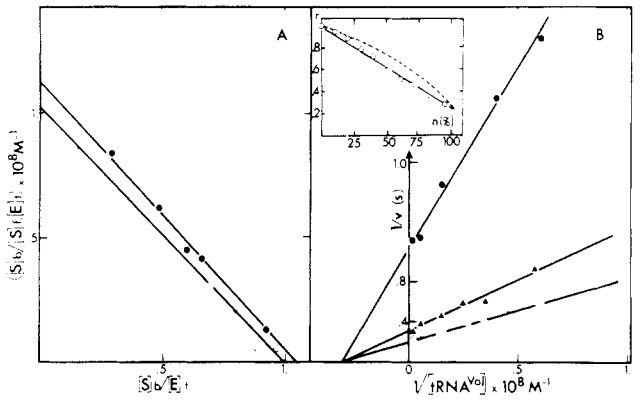

FIGURE 1: Determination of binding parameters and kinetic constants of the $tRNA^{Val}$–valyl-tRNA synthetase system. (A) Nitrocellulose disk filtration of the complex. The reaction mixture contained 50 mM potassium phosphate, pL 6.3, 5 mM β-mercaptoethanol, 0.01 mg of bovine serum albumin, 5.6×10^{-8} M enzyme, $(1–25) \times 10^{-8}$ M [^{14}C]Val-$tRNA^{Val}$ (280 cpm/pmol), and either 0% ^2H_2O (○) or 80% ^2H_2O (●). $[E]_t$, $[S]_b$, and $[S]_f$ represent the concentrations of the enzyme (free and bound), of the bound substrate, and of the free substrate, respectively. The yield of retention of the enzyme–[^{14}C]Val-$tRNA^{Val}$ complex was determined as described by Bonnet & Ebel (1975). (B) Kinetic analysis of the aminoacylation reaction. The conditions were as described under Experimental Procedures: the pL was of 7.2, the $tRNA^{Val}$ concentration varied between 1.1×10^{-8} and 66.6×10^{-8} M, the enzyme concentration was 0.07×10^{-8} M, and the incubation temperature was 25 °C. The solvents were either 0% ^2H_2O (○), 40% ^2H_2O (▲), or 90% ^2H_2O (●). The results are analyzed according to the Lineweaver–Burk representation. In both sets of experiments, reactions in heavy water were performed with the enzyme and tRNA previously dissolved in a 100% ^2H_2O buffer to ensure exchange of labile hydrogen by deuterium atoms. The inset figure shows the dependence of the ratio (r) between the aminoacylation rate constant in H_2O and that in ^2H_2O with the ^2H_2O concentration (n). The solid line is a linear fit for the experimental points; the theoretical curve (broken line) was calculated for a model where the isotopic effect would arise from a sulfhydryl group giving a proton [for details, see Schowen (1976)].

of the aminoacylation reactions in both solvents. It can be seen that similar association-constant values ($K_a \simeq 10^8$ M^{-1} at pL 6.8) and Michaelis constant values ($K_m = 3.3 \times 10^{-8}$ M at pL 7.2) were found in both solvents.

Affinities of ATP and L-*Valine for Valyl-tRNA Synthetase.* The ATP- and valine-concentration dependence of the [^{32}P]PP_i–ATP isotope exchange was found to be the same in both solvents. It was shown, for example, that in the presence of a half-saturating concentration of one of these ligands [corresponding to the K_m values determined in H_2O, 0.2 and 0.05 mM for ATP and valine, respectively (Kern & Giegé, 1979)], the other ligand and PP_i being present at saturating concentrations, the isotope exchange occurs with the half-maximal rate. Thus, both ligands exhibit a similar affinity for the enzyme in H_2O and ^2H_2O.

Kinetics of the Overall $tRNA^{Val}$ Valylation Reaction. Although ^2H_2O does not affect the association constant of the $tRNA^{Val}$–valyl-tRNA synthetase system, it induces a significant decrease in the rate of aminoacylation (Figure 1B). This decrease was found to be a linear function of the $^2H_2O/H_2O$ ratio in the incubation mixture (Figure 1B inset) and was observed over a pL range from 6.5 to 8.0, the aminoacylation rate being faster at alkaline than at acidic pL. The decrease of the aminoacylation rate in the ^2H_2O medium is not linked to the presence of nonsaturating ATP and/or valine concentrations, since increasing the concentration of these substrates by a factor of 10 does not increase the aminoacylation rate.

The ^2H_2O effect is also visualized in the Arrhenius analysis of the valylation reaction (Figure 2) which shows an ap-

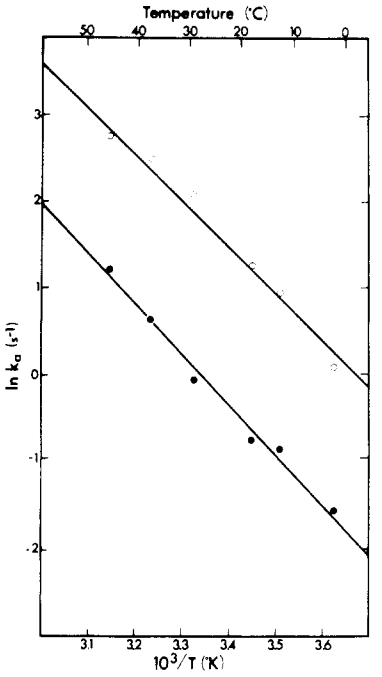

FIGURE 2: Effect of temperature on the aminoacylation of $tRNA^{Val}$ by valyl-tRNA synthetase in the presence of 0% ^2H_2O (○) or 90% ^2H_2O (●). The experimental conditions were as described under Experimental Procedures: the enzyme and tRNA concentrations were 0.076×10^{-8} and 10^{-5} M, respectively. Enzyme and tRNA stock solutions were prepared as described under the legend to Figure 1. Initial rates were measured and the results were analyzed according to the Arrhenius representation.

proximatively sixfold decrease of the aminoacylation rate in 90% ^2H_2O as compared to that in H_2O at pL 7.2 in the temperature range from 4 to 45 °C. This implies that in both solvents the activation energies of the overall aminoacylation reaction are the same; for instance, at 25 °C they are 11 kcal mol^{-1}.

Finally, it was shown that the ^2H_2O-induced effect on the reaction is reversible. After incubation of the enzyme in a ^2H_2O aminoacylation medium, it recovered very quickly its initial properties when transferred into an H_2O valylation medium (Figure 3). Similar behavior was observed when the enzyme was transferred from an H_2O aminoacylation medium into a ^2H_2O medium (Figure 3).

Kinetics of the Partial Steps of the $tRNA^{Val}$ Valylation Reaction. It has been shown in a H_2O medium that the aminoacylation of $tRNA^{Val}$ by yeast valyl-tRNA synthetase occurs via a two-step mechanism and that the dissociation of the end products (probably Val-$tRNA^{Val}$) is rate determining at the steady state (Kern et al., 1978). In this section the ^2H_2O effect on these various steps has been examined.

The [^{32}P]PP_i–ATP exchange occurs at the same maximal rate ($k_{exch} = 60$ s^{-1}) in the absence or in the presence of $tRNA^{Val}$ (Kern & Giegé, 1979) and in both solvents either without or after preincubation of the enzyme (for 1 h at 4 °C) in the corresponding solvent under exchange conditions. When the aminoacylation of $tRNA^{Val}$ was measured in the presence of high amounts of enzyme, allowing the detection of the first catalytic cycles, biphasic kinetics were obtained in both solvents; the decrease in the rates occurred after the first catalytic cycle of the enzyme (Figure 4). The biphasic kinetics were obtained either without or after preincubation of the enzyme in the corresponding solvent (Figure 4). Consequently, in both solvents, the rate-determining step at the steady state follows catalysis and most probably reflects the dissociation of the end-product complex. Under these experimental conditions,

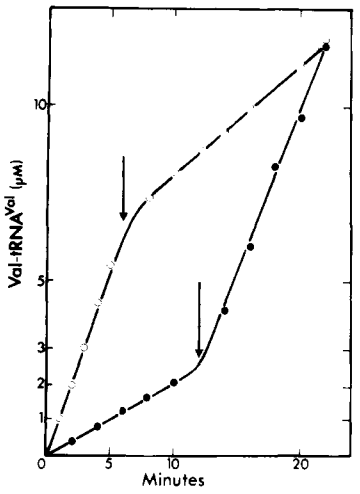

FIGURE 3: Induction of the ^2H_2O effect on the valylation reaction of tRNA^{Val} and reversion by H_2O. A catalytic amount of valyl-tRNA synthetase (0.3×10^{-8} M) and an excess of tRNA^{Val} (15×10^{-6} M) were first incubated in a valylation mixture containing either 0% ^2H_2O (○) or 90% ^2H_2O (●) (the other conditions being those described under Experimental Procedures). Enzyme and tRNA stock solutions were prepared as described in the legend to Figure 1. After 6 or 12 min of incubation at 30 °C (see arrows), aliquots of the H_2O and ^2H_2O media were respectively transferred into fresh ^2H_2O and H_2O valylation media (devoid of enzyme) so that the final concentrations of heavy water were 80% (○) and 7% (●), respectively. The amounts of Val-tRNA synthesized after the transfer were normalized for the same enzyme concentration as in the first incubation.

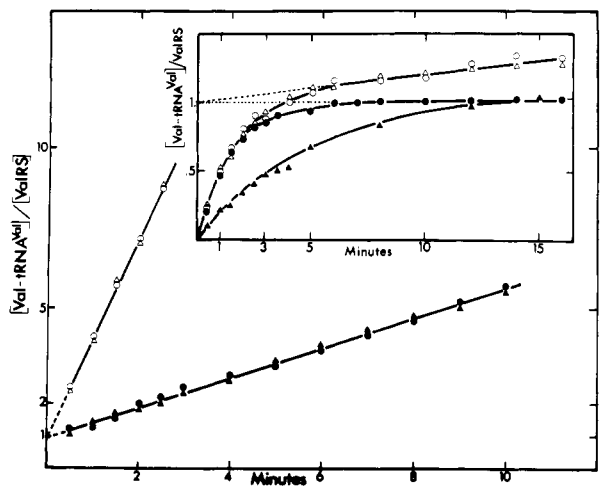

FIGURE 4: Kinetics of aminoacylation of tRNA^{Val} in the presence of high enzyme concentrations. The reactions were conducted at 10 °C in the presence of 50 mM KOH–Mes, pL 6.0, 30 mM KCl, 20 mM $MgCl_2$, and 1.11 μM enzyme, the various ligands being present at saturating concentrations [tRNA^{Val}, 32 μM; ATP, 5 mM; L-[^{14}C]valine, 0.1 mM (70 cpm/pmol)], and in the presence of either 0% ^2H_2O (○ and △) or 90% ^2H_2O (● and ▲), without preincubation (○ and ●) or after preincubation (△ and ▲) of the enzyme. The experiments shown in the inset figure are conducted at −15 °C in the presence of the same enzyme and substrate concentrations but with the following solvents: 0% ^2H_2O and 30% glycerol (○ and △) or 55% ^2H_2O and 30% glycerol (● and ▲) without preincubation (○ and ●) or after preincubation (△ and ▲) of the enzyme. The preincubation mixtures contained 5 mM KOH–Hepes, pL 7.2, 30 mM KCl, 20 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2.22 μM valyl-tRNA synthetase, and as solvents either 0% ^2H_2O (○) or 90% ^2H_2O (●). After preincubations for 1 h at 4 °C (during which time no enzyme inactivation occurs), the medium was diluted by adjusting substrates, solvents, and buffer concentrations as well as the pL to the above given values. The valylation process was initiated at either 10 or −15 °C by the addition of tRNA^{Val} to the media.

however, the rates of Val-tRNA^{Val} synthesis (the first catalytic cycles) are, in both solvents, too fast to be measured. Nevertheless, indications of a ^2H_2O effect on this step were obtained by decreasing its rate (in the prescence of glycerol at −15 °C). The inset of Figure 4 shows similar behavior of the enzyme in both solvents during the first cycle of aminoacylation, when the enzyme was not preincubated in the presence of ^2H_2O. However, when the enzyme was equilibrated against ^2H_2O (for 1 h at 4 °C) before starting the reaction in this solvent, a two- to threefold decrease of the rate of the first catalytic cycle was observed, as compared to that of the parallel experiment performed in H_2O (inset of Figure 4).

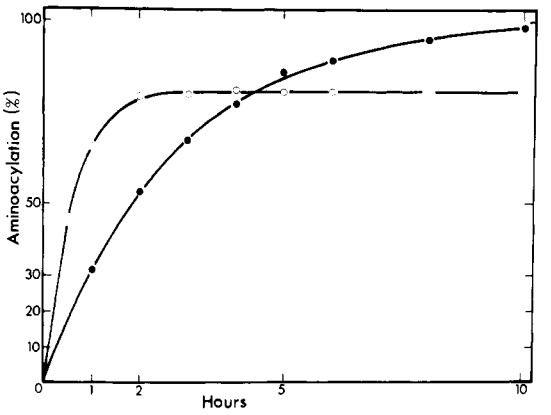

FIGURE 5: Kinetics of aminoacylation of tRNA^{Val} in the presence of catalytic enzyme concentrations. The reactions were conducted at 37 °C in the presence of 100 mM KOH–Hepes, pL 7.2, 30 mM KCl, 20 mM $MgCl_2$, 1.28 μM tRNA^{Val}, 50 μM L-[^{3}H]valine (600 cpm/pmol), 5 mM ATP, 2 units of inorganic pyrophosphatase/mL, 0.05 nM enzyme, and as solvent either 0% ^2H_2O (○) or 85% ^2H_2O (●). The lines represent theoretical curves calculated according to the relation $[aa\text{-}tRNA] = [tRNA]_0 v_{a_0}/(v_{a_0} + v_{b_0} + v_{c_0} + v_{d_0})\{1 - \exp[-[(v_{a_0} + v_{b_0} + v_{c_0} + v_{d_0})/[tRNA]_0]t]\}$ established by Dietrich et al. (1976) where [aa-tRNA] and $[tRNA]_0$ represent respectively the concentration of the aminoacylated tRNA at the time t and that of the total tRNA at $t = 0$, v_{a_0} and v_{b_0} represent respectively the initial rates of aminoacylation (rate constants k_a in H_2O = 10 s^{-1} and k'_a in ^2H_2O = 2.8 s^{-1}) and of the chemical deacylation when [aa-tRNA] = $[tRNA]_0$ (rate constants k_b in $H_2O = 0.12 \times 10^{-3}$ s^{-1} and k'_b in ^2H_2O was considered as negligible), v_{c_0} and v_{d_0} represent the initial rates of the enzymatic deacylations (also when [aa-tRNA] = $[tRNA]_0$) respectively AMP and PP_i independent (rate constants k_c in $H_2O = 0.48 \times 10^{-3}$ s^{-1} and k'_c in $^2H_2O = 0.26 \times 10^{-3}$ s^{-1}) and AMP and PP_i dependent. This last reaction was neglected in our systems because pyrophosphatase was added to the incubation mixtures. The v_0 values were calculated according to Dietrich et al. (1976).

Deacylation Reactions of Val-tRNA^{Val}. The rate constants of the chemical (k_b) and of the enzymatic (k_c) AMP- and PP_i-independent (Bonnet, 1974) deacylation reactions of Val-tRNA^{Val} were found to be decreased in ^2H_2O solvent. At pL 7.2 and 37 °C these rate constants are $k_b = 0.12 \times 10^{-3}$ s^{-1} and less than 0.17×10^{-5} s^{-1} and $k_c = 0.48 \times 10^{-3}$ s^{-1} and 0.26×10^{-3} s^{-1} in H_2O and 85% ^2H_2O, respectively. Similarly, the rate constant (k_d) of the reverse of the aminoacylation (AMP- and PP_i-dependent enzymatic deacylation) was also found reduced in the ^2H_2O medium (k_d = 0.12 s^{-1} and 0.03 s^{-1} at pL 7.2 and 37 °C in H_2O and 85% ^2H_2O, respectively).

Aminoacylation Plateaus. It is shown that the extent of aminoacylation of tRNA at the plateau results from an equilibrium between the aminoacylation reaction and the various deacylation reactions of the charged tRNA (Bonnet & Ebel, 1972). Since in the presence of catalytic enzyme concentrations and in the absence of end products, the chemical deacylation is mainly responsible for the existence of incomplete aminoacylation plateaus (Bonnet & Ebel, 1972), it can be expected that, under similar experimental conditions, the extent of tRNA^{Val} charging will be higher in the presence of

^2H_2O than in the presence of H_2O. Figure 5 verifies this prediction: at the beginning of the kinetics the amount of Val-tRNA^{Val} synthetized is higher in the H_2O than in the ^2H_2O medium, as expected from the aminoacylation rate constants; for longer incubation times, the chemical deacylation reaction starts to predominate in the H_2O medium, thus leading to a tRNA^{Val} charging extent lower than in the ^2H_2O medium, where chemical deacylation remains negligible.

The equilibrium constant of the overall aminoacylation process was determined in both solvents. A difference of a factor of ~ 3 was found, the reaction being more displaced toward the end products in the presence of H_2O than in that of ^2H_2O [$K_{eq} = 1.74 \pm 0.39$ and 0.70 ± 0.04 in H_2O and 85% ^2H_2O, respectively (see Table I)].

Discussion

Solvent isotope effects on enzymic reactions have been discussed succinctly by Schowen (1976). Their most direct application is in the testing of mechanistic models for a catalytic process, since the effect of ^2H_2O–^1H_2O exchange on many chemical reactions is well-known. A detailed mechanistic model for the action of aminoacyl-tRNA synthetases has not been proposed yet; however, our concern in this study has been to interpret the observed solvent isotope effects in terms of the general protein–tRNA interaction, as well as to set limitations on possible catalytic mechanisms.

Exchanging ^1H_2O by ^2H_2O appears to have a stabilizing effect on certain macromolecular interactions. This is probably due to the greater structural order in ^2H_2O as suggested by its thermodynamic parameters (Nemethy & Scheraga, 1964), which should also lead to more stable hydrophobic interactions. Interactions which involve proton transfers between sites of different bond strengths are also likely to have a different stability in ^2H_2O. For instance, the greater stability of bovine heart ATPase (Tuena de Gomez-Puyou et al., 1978) and bacterial ATPase (Satre & Zaccaï, 1979) in ^2H_2O has been attributed to stronger hydrophobic interactions between the subunits of these enzymes. In this line Lewin & Stow (1971) and Lewin & Williams (1971) have reported that ^2H_2O enhances the helical stability of nucleic acids and, in particular, of tRNA, for which the melting temperature increases by several degrees in ^2H_2O solvents. The common experience of investigators who have performed neutron-scattering experiments is that aggregation effects are greatly enhanced in ^2H_2O buffers, and it appears to be a general rule that if a protein, for example, has a tendency to aggregate, it will do so more readily in ^2H_2O. Solvent conditions can usually be found to minimize such aggregation, by varying ionic strength.

The fact that the thermal stability of yeast valyl-tRNA synthetase and of its interactions with tRNA and the smaller substrates is not affected significantly by ^2H_2O must be compared to results on yeast aspartyl-tRNA synthetase which was found to be significantly more stable against thermal denaturation in ^2H_2O (R. Giegé and D. Kern, unpublished experiments). Aspartyl-tRNA synthetase is a dimeric enzyme (Dietrich et al., 1980), and these experiments suggest that subunit interactions which are more stable in ^2H_2O are necessary to its catalytic action. Valyl-tRNA synthetase, on the other hand, is a monomer (Kern et al., 1975), so that either the stability of the protein tertiary structure is not increased in ^2H_2O buffer or the maintenance of a rigid tertiary structure is not a prerequisite for the aminoacylation reaction. Zaccaï et al. (1979) have discussed briefly the evidence for flexible protein sequences in RNA–protein interactions. A second consequence of the thermal stability experiments is that the protein–tRNA binding cannot involve predominant interactions which would have a different stability in ^2H_2O, such as hydrogen bonds of significantly different strength than those in water, or hydrophobic interactions.

At ordinary temperatures, there were no significant differences between the equilibrium constants in ^1H_2O and ^2H_2O for the interaction of valyl-tRNA synthetase with its substrates, and the activation energies for tRNA aminoacylation were found to be similar in both solvents. The overall reaction constants also showed similar dependences on pL in the different isotopic solvent mixtures. This shows that, inasmuch as they are relevant to the catalytic process, the conformations of the reactants are not altered in ^2H_2O solvents.

Substantial solvent isotope effects have been observed on the reaction rates, affecting the equilibrium constant for the overall aminoacylation process. The rate of the tRNA valylation reaction was found to be reduced a few-fold in ^2H_2O. By analogy with other reactions for which the rates were reduced in ^2H_2O, this suggests a proton (deuteron) transfer in the rate-limiting steps of the reaction (Jencks, 1969). An analysis of the reaction rate as a function of the $^2H_2O/^1H_2O$ ratio is called a "proton inventory" by Schowen (1976), who shows that it can put limitations on the type of hydrogen bonds involved. The plot for the valylation reaction rate as a function of the $^2H_2O/^1H_2O$ ratio is linear, which indicates that the isotopic fractionation factors ϕ_i of the hydrogens involved in the rate-limiting step are close to 1. A ϕ_i of unity indicates the net binding of a proton to be equal to that in bulk water. A value greater than 1 would indicate a preferential accumulation of deuterium, implying a stronger bond than in bulk water, and a value less than 1 would indicate a preferential accumulation of hydrogen, implying a weaker bond than in water. Since the ϕ_i depends only on the functional group with which the hydrogen is associated, it excludes from the rate-limiting step in the reaction mechanism of valyl-tRNA synthetase groups such as the sulfhydryl group, for example, whose hydrogen has a $\phi_i \simeq 0.4$. However, with almost no exceptions, the activity of aminoacyl-tRNA synthetases is sensitive to the addition of sulfhydryl reagents (Söll & Schimmel, 1974); in valyl-tRNA synthetase, for instance, six sulfhydryl groups can be titrated (Potier, 1979). The inset in Figure 1 shows the experimental points and the curve which would have resulted if a sulfhydryl-group hydrogen were involved in the rate-limiting step of the reaction.

The rates for the partial steps of the aminoacylation process were found either to be reduced or to remain unaffected by solvent ^2H_2O–^1H_2O exchange. This is illustrated first by the equilibrium constant measurements of the overall aminoacylation reaction. Because the values were found to be different in H_2O and ^2H_2O, it indicates that one or several partial steps are affected differently by the isotopic composition of the solvents. For instance, the steps which involve ATP, PP_i, and their complexes with Mg^{2+} could be responsible for the stronger displacement of the aminoacylation reaction toward the end products in H_2O, since the reactivity of these ligands has been found to be strongly dependent upon their ionization (Cole & Schimmel, 1970b; Lui et al., 1978; Kern & Lapointe, 1980). Since the ϕ_i of hydrogens bonded to phosphate groups is $\simeq 0.5$ (Schowen, 1976), this likely implies different association constants of Mg^{2+} with PP_i and ATP, so that the concentration of active substrates $MgATP^{2-}$ and $MgP_2O_7^{2-}$ (Cole & Schimmel, 1970b) will differ in the ^2H_2O and ^1H_2O solvents.

At saturating substrate concentrations the [^{32}P]PP_i–ATP exchange remains unaffected by ^2H_2O. Since this reaction reflects the rate of amino acid activation and its reversal (Cole

& Schimmel, 1970a; Höller, 1978), it might be concluded that the substitution of ^1H by ^2H in the protein and the participating substrates does not affect the catalytic site responsible for this process.

The transfer of the activated amino acid to tRNA appears to be affected only after preincubation of the enzyme in the presence of ^2H_2O. This can be interpreted in two ways: either an exchangeable proton is directly involved in the catalysis and its exchange with a deuteron results in a decrease of the reaction rate or the catalytic center is transconformed by discrete modifications of one or several strategic points after substitution of hydrogen by deuterium atoms, leading also to a decrease of the reaction rate. These proton–deuteron exchanges can occur either during the catalysis at pL 7.2 and 30 °C (as shown by the induction and reversion experiments in Figure 3) or during the single preincubation of the enzyme at 4 °C in the corresponding solvent. Chemical and enzymatic reactions involving proton transfers are often found to be reduced by a factor of ~4–6 after substitution of H_2O by ^2H_2O. The partial reaction described here is decreased by a factor of 2–3, but taking into account that the experiments were performed in the presence of 60% ^2H_2O and 30% glycerol, a stronger decrease can easily be expected in the presence of 100% ^2H_2O. However, the question remains open as to whether one or more protons are directly or indirectly involved in the catalytic step of transfer of the activated amino acid to tRNA.

In the overall valylation reaction, the biphasic aminoacylation kinetics obtained in both solvents indicate the existence of a rate-determining step following the transfer step. A priori it appears to be the dissociation of the end products, probably the Val-tRNA^{Val} (because among the various end products it has the best affinity for the enzyme) as proposed after the discovery of similar kinetic behavior for the *Escherichia coli* isoleucine (Eldred & Schimmel, 1972) and the yeast arginine (Fersht et al., 1978) systems. The decrease of the rate of aminoacylation after the catalytic step, however, could also be related to a slow conformational change of the end-product complex, followed by a fast release of the end products.

The substitution of H_2O by ^2H_2O decreases strongly the rate-determining step of aminoacylation. Since binding experiments showed no modification in the association constant of the Val-tRNA^{Val}–valyl-tRNA synthetase system after the solvent substitution, the ^2H_2O-promoted decrease of the rate of this step cannot be related to a slower dissociation of the enzyme–Val-tRNA^{Val} complex. It appears, therefore, that ^2H_2O acts at another level. We postulate the existence of a transconformation step of the enzyme–Val-tRNA^{Val} end-product complex which is rate determining for the overall aminoacylation reaction and a ^2H_2O effect on some intra- or intermolecular interactions in the enzyme and/or in this complex which decreases the rate of this transconformation. The present study does not allow us to conclude whether AMP and Val-tRNA^{Val} dissociate before or after this putative transconformation step. However, as shown by the [^{32}P]-PP_i–ATP exchange rate, the PP_i dissociates much faster than this transconformation would occur, so that this ligand will dissociate before this step.

The ^2H_2O-promoted decrease in the rates of the chemical and enzymatic (AMP- and PP_i-independent) deacylation reactions is not surprising, because hydrolytic activities are implied. It was more surprising, however, to find that the enzymic deacylation is much less affected than the chemical one. Most probably this is a reflection of the more complex nature of this reaction. In addition, it suggests that the solvent substitution does not significantly affect the interaction between the tRNA and the synthetase. The reverse of the aminoacylation reaction is more affected than the enzymatic deacylation, after substitution of H_2O by ^2H_2O. Its rate decreased by about the same factor as that of aminoacylation. This again indicates that ^2H_2O has more of an effect on the catalytic mechanism than on the interaction between the ligands with the enzyme.

All the above results indicate inhibitory effects of ^2H_2O on reaction rates. It might be of interest to note that ^2H_2O can have, under certain circumstances, a stimulatory effect on the amount of Val-tRNA^{Val} synthesized as a consequence of the plateau theory of aminoacylation (Bonnet & Ebel, 1972) and of the decrease in the deacylation rates in heavy water. As a practical consequence, in systems where the reaction rates of aminoacylation are very low (as, for example, in tRNA mischarging systems or in systems involving crude enzymatic extracts and/or very unstable aminoacyl-tRNAs), ^2H_2O could be a good solvent, allowing an optimal aminoacylation extent.

The ^2H_2O effects described appear to be linked to isotope effects occurring during the catalytic mechanism of the enzyme. The protein itself, as well as its interactions with its substrates, is not significantly affected. Thus, structural parameters concerning either the free enzyme or enzyme–substrate complexes derived from physical measurements performed in solvents containing heavy water can be taken to reflect functional states reliably.

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Characterization of Protein Kinase Activity Associated with Rat Liver Polysomal Messenger Ribonucleoprotein Particles†

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ABSTRACT: Poly(adenylic acid)-containing rat liver polysomal messenger ribonucleoprotein particles (pmRNP) were isolated and found to contain protein kinase activity. The association of the enzyme(s) with the particles was confirmed by experiments showing that the protein kinase activity comigrated with the pmRNP on metrizamide gradients and bound to oligo(dT)–cellulose columns only under conditions where the pmRNP bound. The following properties were determined for the pmRNP-associated kinase(s). Casein and phosvitin were preferred substrates over histone and protamine. The optimal $MgCl_2$ and KCl concentrations were found to be 12.5 and 50 mM, respectively. $MnCl_2$ and $CaCl_2$ could not replace $MgCl_2$ and were inhibitory at low concentrations. The optimum pH range was 7.7–9.0, and the enzyme activity was cAMP independent. A molecular weight of 55 000–60 000 was determined for the kinase(s) by sucrose gradient analysis. The enzyme(s) was capable of phosphorylating proteins endogenous to the pmRNP. Membrane-bound pmRNP contained much less kinase activity than free pmRNP while pmRNP from hepatoma 7777 contained an elevated level of the enzyme(s). The relationship between the protein kinase activity and one of the pmRNP proteins of molecular weight 66 000 is discussed.

Although polysomal and nonpolysomal messenger ribonucleoprotein particles (mRNP) have been detected and isolated from a wide variety of cell types, little is known about the function of the associated proteins (Lindberg & Sundquist, 1974; Kumar & Pederson, 1975; Jeffery, 1977; Cardelli & Pitot, 1977). Some groups have reported that these proteins may play a role in the translation of mRNA either as initiation factors or as translational control factors (Bag & Sarkar, 1975; Barrieux & Rosenfeld, 1977; Liautard, 1977; Ilan & Ilan, 1977). It has been reported that one of the mRNP proteins may be identical with the initiation factor e-IF2 (Barrieux & Rosenfeld, 1977; Hellerman & Shafritz, 1975) and that isolated mRNP form initiation complexes with ribosomes in the absence of added initiation factors (Liautard, 1977; Cashion & Stanley, 1974). Consistent with these findings are data showing that some initiation factors can bind to isolated mRNA (Shafritz et al., 1976; Kaempfer et al., 1978). Other groups have reported that mRNP contain no initiation factor activity and are translated with the same efficiency as mRNA

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