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Fidelity in the Aminoacylation of $tRNA^{Val}$ with Hydroxy Analogues of Valine, Leucine, and Isoleucine by Valyl-tRNA Synthetases from *Saccharomyces cerevisiae* and *Escherichia coli*

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ABSTRACT: Several analogues of valine, leucine, and isoleucine carrying hydroxyl groups in the γ- or δ-position have been tested in the aminoacylation of tRNA by valyl-tRNA synthetases from *Saccharomyces cerevisiae* and *Escherichia coli*. Results of the ATP/PP_i exchange and of the aminoacylation reactions indicate that the amino acid analogues not only can form the aminoacyl adenylate intermediate but are also transferred to tRNA. However, the fact that the reaction consumes an excess of ATP indicates that the misactivated amino acid analogue is hydrolytically removed. Thus, valyl-tRNA synthetase from *S. cerevisiae* shows a high fidelity in forming valyl-tRNA. Although the much bulkier amino acid analogues allo- and iso-γ-hydroxyvaline and allo- and iso-γ-hydroxyisoleucine are initially charged to tRNA, the misaminoacylated $tRNA^{Val}$ is enzymatically deacylated. This cleavage reaction is mediated by the hydroxyl groups of the amino acid analogues which are converted into the corresponding lactones.

Protein biosynthesis is remarkably error free; about one mistake is made in 3000 amino acids incorporated (Loftfield, 1963). A crucial step in this process is the aminoacylation of transfer RNA (tRNA) with the cognate amino acid, a reaction catalyzed by the aminoacyl-tRNA synthetases. This reaction can be divided into two separable steps: (i) the activation of the amino acid by ATP to an enzyme-bound aminoacyl adenylate (activation step) and (ii) the transfer of the aminoacyl residue to the cognate tRNA (transfer step).

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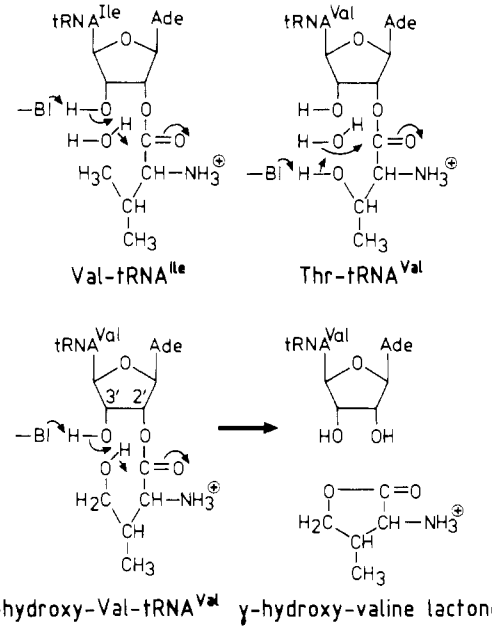

FIGURE 1: Schematic display of valine esterified to tRNA^{Ile} (above, left) with the proofreading hydrolysis due to chemical proofreading, of threonine esterified to tRNA^{Val} (above, right) exhibiting a hydrolytic proofreading without participation of the 3′-hydroxyl group of the terminal ribose, and of the γ-hydroxyvaline esterified to tRNA^{Val} (below) with the mechanism proposed for the lactone formation upon hydrolytic ester cleavage.

The first step is measured by the ATP/PP$_i$ exchange reaction, while the second step is monitored in the overall aminoacylation reaction. The high degree of accuracy in the overall reaction is due, in some cases, to a "proofreading" mechanism at either step of the overall reaction. Different models are currently proposed for the mechanisms of proofreading: "kinetic proofreading" (Hopfield, 1974), "double-sieve proofreading" (Fersht, 1980), and "chemical proofreading" (Cramer et al., 1979). In such proofreading mechanisms the wrong intermediate, e.g., valyl adenylate formed by isoleucyl-tRNA synthetase (pretransfer proofreading), or the wrong product, e.g., Val-tRNA^{Ile} (posttransfer proofreading), is destroyed by hydrolysis. In the normal aminoacylation reaction one molecule of ATP is sufficient for the amino acid to be activated to form aminoacyl-tRNA. However, in order to correct mistakes, additional ATP is needed (Hopfield, 1974; Fersht, 1977). In the case of valyl-tRNA synthetase both activation and transfer steps show fidelity in the same range as for the overall reaction [reviewed in Schimmel (1987) and Englisch et al. (1985)]. In the case of isoleucyl-tRNA synthetase, however, the initial discrimination of valine versus isoleucine is only 1:200 due to the small difference in binding energy between the incorrect and the correct substrate [reviewed in Freist (1988) and Fersht (1977)].

The chemical proofreading hypothesis for hydrolysis of misaminoacylated tRNAs (Figure 1) was based on experiments with 3′-deoxyadenosine tRNAs as amino acid acceptors (von der Haar & Cramer, 1975, 1976, 1978). In the case of yeast Val-tRNA^{Ile} the 3′-hydroxyl groups is necessary for the hydrolysis, whereas in the case of Thr-tRNA^{Val} (Igloi et al., 1977) this hydroxyl group is not essential.

In order to simulate the water molecule necessary for hydrolysis we synthesized the four isomers of γ-hydroxyvaline and γ-hydroxyisoleucine as analogues of valine and isoleucine (Figure 2). Hydrolysis of these analogues, resulting in proofreading, should lead to lactonization of the γ-hydroxy amino acids (Figure 1). As these lactones can be easily detected, we decided to undertake this study to examine the proofreading by valyl-tRNA synthetases from *E. coli* and *S. cerevisiae*.

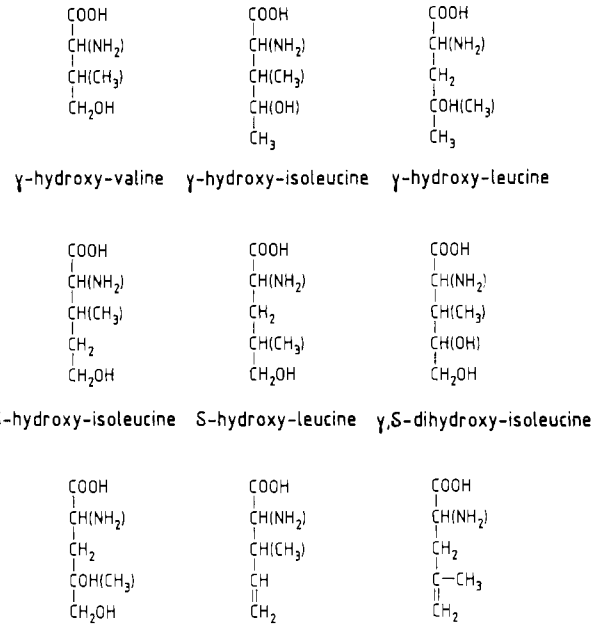

FIGURE 2: Structural formulas of the amino acid analogues used in this work.

Materials and Methods

Materials. Radioactivity was measured in a Berthold betazine BF 8000 liquid scintillation counter, HPLC was carried out with a Du Pont 850 liquid chromatograph, and UV measurements were done in a Zeiss PMQ3 spectrophotometer.

[^{14}C]Valine (specific activity 336 mCi/mmol), [^{14}C]ATP (specific activity 55 mCi/mmol), and [^{32}P]pyrophosphate (specific activity 11.1 mCi/mmol) were from Amersham-Buchler (Braunschweig, FRG). Homocysteine was purchased from Merck (Darmstadt, FRG). All chemical substances were of ultrapure grade.

Isolation of Valyl-tRNA Synthetase and tRNA^{Val}. Valyl-tRNA synthetase from *S. cerevisiae* was purified to homogeneity according to the procedures of Igloi et al. (1977) and von der Haar (1973, 1979) to a specific activity of 3990 units/mg. The *E. coli* enzyme was purified to a specific activity of 493 units/mg (Söder, 1983; von der Haar 1973, von der Haar 1979). Yeast tRNA^{Val} was purified by the procedure of Schneider et al. (1972) from unfractioned tRNA from bakers' yeast (Boehringer, Mannheim, FRG) to a valine acceptance of 1.47 nmol/A_{260} unit tRNA. *E. coli* tRNA^{Val} was purified from unfractionated *E. coli* tRNA (Boehringer, Mannheim, FRG) by the procedure of Hjertin et al. (1975) and finally by HPLC using derivatized reverse-phase material (Bischoff et al., 1983), resulting in a valine acceptance of 1.32 nmol/A_{260} unit tRNA.

ATP/PP$_i$ Exchange. The ATP/PPi exchange reaction contained in a total volume of 0.1 mL: 2 mM [^{32}P]pyrophosphate, 0.5–20 mM amino acid, 2 mM ATP in 150 mM Tris/HCl, pH 8.0, containing 150 mM KCl, 10 mM $MgSO_4$, and 5 mM β-mercaptoethanol. The reaction was started by addition of 5 μg of valyl-tRNA synthetase and incubated at 37 °C. Aliquots of 10 μL were taken after 2, 4, 6, 8, 10, 20, 30, 40, and 60 min and spotted onto charcoal filter disks. The disks were washed twice for 10 min in 1.5% trichloracetic acid containing 40 mM unlabeled pyrophosphate and once for 5 min in water and dried and the radioactivity was counted

(Simlot & Pfaender, 1973; Igloi et al., 1978, 1979).

AMP Formation. AMP formation was determined by incubating purified enzyme in the total volume (0.1 mL) with either 2 mM [^{14}C]ATP or 2 mM [^{14}C]ATP plus 0.5–5 mM amino acid or 2 mM [^{14}C]ATP plus 0.5–5 mM amino acid plus 30 μM $tRNA^{Val}$ in 150 mM Tris/HCl, pH 8.0, containing 150 mM KCl, 10 mM $MgSO_4$, and 5 mM β-mercaptoethanol. The reaction was started by the addition of 6 μg of enzyme. Within 1–120 min, 10-μL aliquots were withdrawn and spotted onto PEI cellulose plates containing a fluorescent indicator (254 nm). Ascending chromatography was carried out in 1 M LiCl + 1 mM acetic acid/2-propanol 2:1 (v/v) with ATP, ADP, and AMP as references (Igloi et al., 1978; Jakubowski & Fersht, 1981). The nucleotide spots were identified with UV light and cut out and the radioactivity was counted.

Aminoacylation. Aminoacylation assays contained (in a total volume of 0.1 mL) 300 μM [^{14}C]amino acid, 10 mM ATP, and 1 mg/mL unfractionated tRNA from yeast or *E. coli* in 150 mM Tris/HCl, pH 8.0, containing 150 mM KCl, 10 mM $MgSO_4$, and 5 mM β-mercaptoethanol. The reaction was started by addition of 5 μg of valyl-tRNA synthetase (Igloi et al., 1978, 1979; Jakubowski & Fersht, 1981) and incubated at 37 °C. After 2, 5, and 10 min, 10-μL aliquots were withdrawn and spotted onto 3-mm filter disks. The disks were washed three times for 10 min with 5% trichloroacetic acid, 5 min with ethanol, and 2 min with ether and then the radioactivity was counted. For aminoacylation with unlabeled amino acid analogues, 0.5–20 mM concentrations of the analogues were used.

Back Titration. Back titration was carried out essentially as described for aminoacylation assays. With the beginning of back titration, new enzyme (5 μg) was added and [^{14}C]-valine was added in surplus according to Igloi et al. (1978, 1979).

γ-Hydroxyvaline. The isomers of γ-hydroxyvaline [L-allo (*SR*), D-allo (*RS*), L-iso (*SS*), and D-iso (*RR*)] and their lactones were prepared by a modified Erlenmeyer synthesis (Englisch-Peters, 1989); the diastereomers were separated by column chromatography, the enantiomers by D- or L-amino acid oxidase (Englisch-Peters, 1989).

γ-Hydroxyisoleucine and γ-Hydroxyleucine. These analogues were synthesized according to Faulstich et al. (1972) by photochlorination of isoleucine or leucine and purified by Soxhlet extraction of the corresponding lactones. The diastereoisomers of γ-hydroxyisoleucine were separated by HPLC (Englisch, 1984).

δ-Hydroxyisoleucine and γ-Hydroxyleucine. These analogues were prepared by 1,4-Michael addition (Englisch-Peters, 1989); the diastereomers of δ-hydroxyisoleucine were separated by HPLC.

γ,δ-Dehydroisoleucine and γ,δ-Dehydroleucine. These analogues were prepared by Claisen rearrangement according to Bartlett et al. (1982).

γ,δ-Dihydroxyisoleucine and γ,δ-Dihydroxyleucine. These analogues were obtained from γ,δ-dehydroisoleucine and γ,δ-dehydroleucine by oxydation with sodium chlorate and OsO_4 (Brewster et al., 1973). They were purified by Soxhlet extraction of the corresponding lactones. All analogues were obtained in high purity and were characterized by melting points and ^{1}H NMR (Peters, 1981; Englisch, 1984).

Preparative Aminoacylation of $tRNA^{Val}$ with δ-Hydroxyvaline. Preparative aminoacylation reactions contained in a final volume of 1 mL: 5 mM amino acid, 10 mM ATP, and 1 mg of $tRNA^{Val}$ in 150 mM Tris/HCl, pH 8.0, containing 150 mM KCl and 10 mM $MgSO_4$. The reaction was started by addition of 25 μg of enzyme and incubated at 37 °C. After 20 min the reaction was stopped by addition of 0.2 mL of 2 M sodium acetate, adjusted to pH 5.0, and applied at 4 °C to a DEAE A 25 column equilibrated at pH 5.0 with a buffer of 20 mM sodium acetate, pH 5.2, containing 10 mM $MgSO_4$ and 0.3 M NaCl. After washing with 0.3 and 0.5 M NaCl the aminoacyl-tRNA was eluted with 1 M NaCl and desalted over a P2 column (Fersht & Kaethner, 1976; Sprinzl et al., 1977).

Detection of Lactones by Thin-Layer Chromatography. From aminoacylation assays (started by addition of enzyme) 10-μL aliquots were taken after 0, 5, 10, 20, 40, 60, 120, 180, and 240 min and spotted onto silica thin-layer chromatography plates. The chromatogram was run in 2-butanol/acetic acid/water (4:1:1) (v/v/v) for 5 h, and then the plates were dried. The spots were visualized by ninhydrin spray (Jakubowski & Fersht, 1981; Jakubowski et al., 1977).

Enzymatic and Spontaneous Hydrolysis of Aminoacyl-$tRNA^{Val}$. The reaction was carried out in the aminoacylation buffer with or without 6 μg of enzyme. At 10-min intervals aliquots were withdrawn and the content of deacylated $tRNA^{Val}$ was measured by back titration (Igloi et al., 1977, 1979).

Table I: Kinetic Constants for the Amino Acids in the ATP/PP_i Pyrophosphate Exchange by Valyl-tRNA Synthetase Using Various Amino Acids and Analogues

	AMP formation in the exchange[a,b,c]			
	yeast enzyme		*E. coli* enzyme	
amino acids	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
allo-γ-hydroxyvaline	5.3	2.2	1.5	0.69
iso-γ-hydroxyvaline	4.7	1.1	3.2	0.9
allo-δ-hydroxyisoleucine	–	–	3.8	0.27
iso-δ-hydroxyisoleucine	–	–	2.9	0.22
γ,δ-dehydroisoleucine	–	–	4.1	0.41
homocysteine	8.9	0.75	3.1	3.7
valine	0.25	10	0.15	8.7

[a] The AMP formation was measured in the presence and in the absence of tRNA. In the absence of tRNA no activity was found. [b] A substance is called a substrate if the reliable pyrophosphate exchange kinetics are measurable for a substance concentration up to 20 mM (10 times the ATP concentration in the assay). The limit is based on the physiological concentration of other amino acids. A dash means that the substance was not a substrate. [c] The values given are average values from five independent measurements. The accuracy falls within a factor of ±1.5 of the data shown.

Results

Proofreading depends on the hydrolysis of the incorrectly activated or mischarged amino acid. An amino acid carrying a hydroxyl group should be in a favorable position to participate in such a hydrolysis. Therefore, we synthesized hydroxyl analogues of valine, isoleucine, and leucine in order to examine the accuracy of amino acid discrimination by *E. coli* and *S. cerevisiae* valyl-tRNA synthetases. For our planned studies we synthesized the following amino acids: γ-hydroxyvaline, γ-hydroxyisoleucine, γ-hydroxyleucine, δ-hydroxyisoleucine, δ-hydroxyleucine, γ,δ-dihydroxyisoleucine, γ,δ-dihydroxyleucine, γ,δ-dehydroisoleucine, and γ,δ-dehydroleucine (Figure 2). This was accomplished by classical methods (Englisch-Peters, 1989). We then determined the K_M and k_{cat} values in the activation step (measured by ATP/PP_i exchange) with these amino acid analogues. As can be seen in Table I, in the reaction with yeast valyl-tRNA synthetase the K_M values ($K_M \sim 5$ mM) are ten times higher and the k_{cat} values ($k_{cat} \sim 2$ s^{-1}) are ten times lower than those of valine ($K_M = 0.25$ μM; $k_{cat} = 10$ s^{-1}). Besides valine, the yeast valyl-tRNA synthetase accepts three amino acid analogues in the pyrophosphate ex-

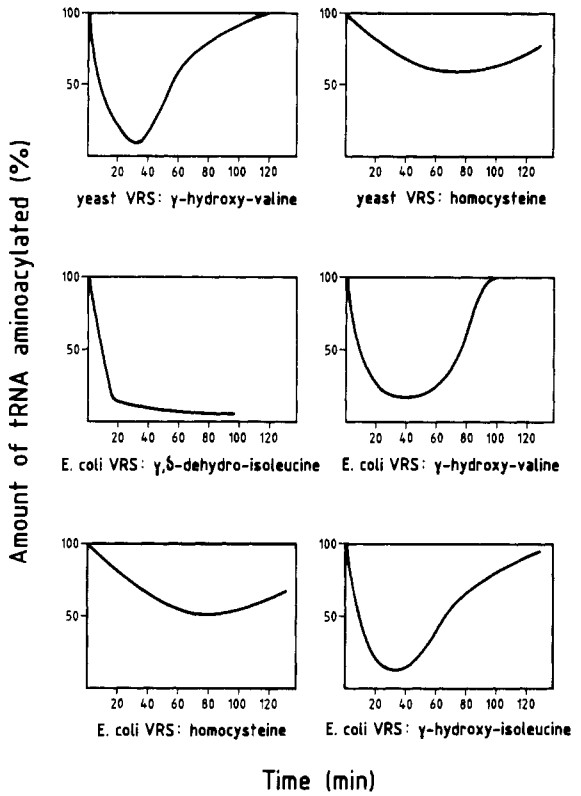

FIGURE 3: Amount of aminoacylation of $tRNA^{Val}$ with amino acid analogues, measured as the level of remaining $tRNA^{Val}$ estimated by back titration with [^{14}C]valine and valyl-tRNA synthetase. (For γ-hydroxyvaline and -isoleucine no differences between allo and iso have been found.)

change, while the *E. coli* synthetase shows a reaction with six amino acids.

As it is difficult to detect the level of the aminoacyl-tRNA when unlabeled amino acid is used, we determined the amount of aminoacylation of $tRNA^{Val}$ with nonradioactive amino acid analogues by back titration of $tRNA^{Val}$ with [^{14}C]valine. In this assay the level of aminoacylation of $tRNA^{Val}$ by an amino acid analogue (established in a slow reaction) is measured by aminoacylating the remaining free $tRNA^{Val}$ with [^{14}C]valine (in a fast reaction). As seen in Figure 3 both the yeast and *E. coli* valyl-tRNA synthetases are able to transfer the activated amino acid analogues onto tRNA. After 30 min the tRNA is completely charged with the γ-hydroxy analogues of valine and isoleucine and almost fully with γ,δ-dehydroisoleucine. In contrast, acylation with homocysteine never exceeds the 50% level. Thus, these amino acid analogues do get linked to tRNA.

In order to check on the proofreading in the aminoacylation reaction and to get further evidence on the chemical proofreading mechanism, we need to determine the total consumption of ATP during this reaction, which should reveal the number of ATP molecules hydrolyzed per correctly formed Val-$tRNA^{Val}$. None of the amino acid analogues catalyzed AMP formation in the absence of tRNA; there is no measurable hydrolysis of the aminoacyl adenylate. In contrast, in the presence of $tRNA^{Val}$ almost all analogues bring about the release of AMP; the misaminoacylated tRNA is subject to enzymatic hydrolysis with net cleavage of ATP. An exception is γ,δ-dehydroisoleucine where the hydrolysis reaction shows a roughly ten-fold lower k_{cat} compared to the reaction of the other analogues (Table II).

Misaminoacylated tRNAs undergo slow spontaneous hydrolysis but are hydrolyzed faster enzymatically in a proof-

Table II: Kinetic Constants for the Amino Acids of AMP Formation in the Aminoacylation Assay by Valyl-tRNA Synthetases from Yeast and *E. coli* Using $tRNA^{Val}$ and Various Amino acid Analogues

	AMP formation in the assay[a]			
	yeast enzyme		*E. coli* enzyme	
amino acids	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
allo-γ-hydroxyvaline	3.4	1.3	5.5	0.6
iso-γ-hydroxyvaline	3.5	1.3	5.5	0.6
allo-γ-hydroxyisoleucine	–	–	5.0	0.5
iso-γ-hydroxyisoleucine	–	–	5.0	0.5
γ,δ-dehydroisoleucine	–	–	3.7	0.07
homocysteine	5.1	1.0	4.5	0.64
valine	0.17	8.5	0.125	7.9

[a] Footnotes as in Table I.

reading event. To trace both possible pathways, we prepared tRNAs aminoacylated with γ-hydroxyvalyl-$tRNA^{Val}$ (allo and iso) and compared the spontaneous and enzymatic hydrolysis (by measuring the back titration). The γ-hydroxyvalyl-$tRNA^{Val}$ was isolated by chromatography and enzymatically hydrolyzed at an enzyme/tRNA ratio of $1:10^3$. A $t_{1/2}$ value of about 20 min was found, whereas spontaneous hydrolysis in buffer needed more than 5 h to reach the same extent (data not shown).

The purpose of using hydroxy amino acids in thee reactions was the assumption that the hydroxyl group would facilitate the hydrolysis and engage in lactone formation in this process. Thus, γ-hydroxyvalyl-$tRNA^{Val}$ should rapidly hydrolyze in solution to form a cyclic five-ring lactone. When yeast and *E. coli* valyl-tRNA synthetase samples were incubated with γ-hydroxyvaline, ATP, and $tRNA^{Val}$, TLC analysis of the reactions clearly showed the formation of the corresponding lactone. The lactone increases with time until the spot has the same intensity as that of the free amino acid. This lactonization is strongly dependent on the presence of tRNA. Thin-layer chromatography of aliquots of the phyrophosphate exchange assay did not show any lactonization of the analogues.

Discussion

The necessary high fidelity of aminoacyl-tRNA formation can only be maintained in some cases by an enzyme-catalyzed proofreading mechanism resulting in the hydrolysis of noncognate products. Such mechanisms work either at the level of the aminoacyl adenylate, the pretransfer proofreading (Hopfield, 1974; Fersht, 1980), or at the level of misaminoacylated tRNA, posttransfer proofreading (Cramer et al., 1979; von der Haar & Cramer, 1975). Our studies showed that the valyl-tRNA synthetases from yeast and *E. coli* use a posttransfer proofreading mechanism to hydrolyze the misactivated and misacylated amino acid analogues used in this study.

The L enantiomers of γ-hydroxyvaline (both iso and allo diastereomers) are activated by either enzyme, although they are bulkier than the cognate substrate valine. Even more surprising is the activation of the hydroxyisoleucine analogues by the *E. coli* enzyme, because besides the hydroxy group an additional methylene group is present. Also, γ,δ-dehydroisoleucine is activated by this enzyme. However, γ- and δ-hydroxyleucine and δ-hydroxyisoleucine are not activated at all, whereas homocysteine is again activated by both enzymes. Inspection of the model of γ-hydroxyvaline indicates that the hydroxy group might be fixed via a hydrogen bridge to the carboxyl group and thus γ-hydroxyvaline might look much more like valine than like isoleucine (Figure 4). Other investigators have reported that threonine, alanine, serine, me-

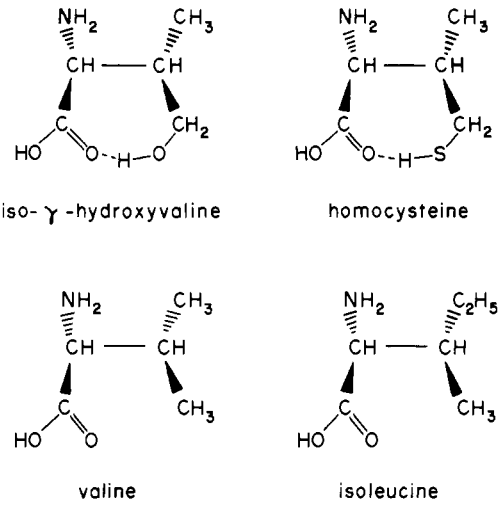

FIGURE 4: Projections of γ-hydroxyvaline and homocysteine in comparison with allo-isoleucine, isoleucine, and valine in aqueous solution at pH 7.6. The proposed hydrogen bonding is depicted by dots.

thionine, cysteine, and isoleucine were misactivated, including not only smaller amino acids but also amino acids that are bulkier (Jakubowski, 1980; Owen & Bell, 1970; Kern et al., 1981; Fersht & Dingwall, 1979b). Earlier investigations with leucyl-tRNA synthetases and phenylalanyl-tRNA synthetases and other substrates revealed the opposite behavior of the enzymes from yeast and *E. coli*. The *E. coli* enzyme is the more accurate species in the initial substrate recognition and with less elaborate proofreading, compared to the less specific substrate discrimination and more efficient proofreading of the eucaryotic synthetase (Englisch et al., 1986; Gabius et al., 1983).

The back-titration experiments clearly indicate a transient formation of misaminoacylated $tRNA^{Val}$, followed by hydrolysis until the ATP pool is exhausted due to proofreading. An exception is γ,δ-dehydroisoleucine, which, after being charged, is not hydrolyzed at all. In the AMP-forming assay the presence of tRNA is absolutely necessary for hydrolysis of misactivated γ-hydroxyvaline, again indicating a transient transfer to $tRNA^{Val}$ prior to proofreading. Our observation supports earlier studies which showed that proofreading of valyl-tRNA synthetases from yeast, *E. coli*, and *Bacillus stearothermophilus* predominantly occurs at the posttransfer level with the amino acid analogues tested in this work (Igloi et al., 1977, 1978; Fersht & Kaethner, 1976; Fersht & Dingwall, 1979a,b). Moreover thiolactonization was detectible with homocysteine in the presence of $tRNA^{Val}$ under conditions used in the assay. Also, for some other synthetases there are examples where the AMP formation is dependent on tRNA, suggesting that the aminoacyl residue is transferred to tRNA prior to hydrolysis (Hopfield, 1974).

This is in contrast to the proofreading of different hydroxy amino acids by the leucyl-tRNA synthetases from yeast and *E. coli* (Englisch et al., 1986) or of serine by alanyl-tRNA synthetase (Tsui & Fersht, 1981), where sequential combination of pre- and posttransfer proofreading occurs. Additionally, AMP formation by isoleucyl-tRNA synthetase from *E. coli* (Jakubowski & Fersht, 1980) and valyl-tRNA synthetase from lupin seeds (Jakubowski, 1980) occurs significantly in the absence of tRNA.

The proofreading by the *E. coli* enzyme, however, is not in any case efficient enough to destroy the derivatives of all activated and transferred analogues before misaminoacylated tRNA is released from the tRNA/enzyme complex. Mischarging of $tRNA^{Val}$ with the γ,δ-dehydroisoleucyl analogue is about 10 times less efficient (AMP formation) than when using the yeast enzyme.

As established from the misactivation of valine in the yeast isoleucine system, the chemical proofreading model postulates the participation of the 3′-hydroxyl group of the ribose moiety and a suitably placed water molecule in the posttransfer hydrolysis (Figure 1) (Cramer et al., 1979). The fast enzymatic proofreading of threonine by valyl-tRNA synthetase from yeast, however, is dependent on the β-hydroxyl group of threonine and independent of the terminal 3′-hydroxyl group of $tRNA^{Val}$, whereas threonine analogues such as *O*-methylthreonine are very slowly hydrolyzed by the enzyme and only in the presence of the intact tRNA terminus (Igloi et al., 1977, 1978). The hydroxy analogues used in this study are able to form five-membered lactone rings, which are relatively stable. The proofreading and the lactone formation are absolutely dependent on $tRNA^{Val}$ and occur at the level of misaminoacylated tRNA, thus differing from the pretransfer hydrolysis of other synthetases. These experimental findings support the basic idea of the chemical proofreading; at least some enzymes, with appropriate tRNAs and amino acid analogues, use this sort of correction step. The predominant posttransfer proofreading of these enzymes has to compensate for the low initial discrimination of the substrate analogues, a process that requires high energy costs due to the hydrolysis of ATP.

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Transformation of Glucocorticoid Receptors Bound to the Antagonist RU 486: Effects of Alkaline Phosphatase†

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ABSTRACT: RU 486 is a synthetic steroid that binds avidly to glucocorticoid receptors without promoting their transformation into activated transcription factors. A significant part of this behavior has been shown to be due to a failure of the RU 486 bound receptor to be efficiently released from a larger (sedimenting at 8-9 S) multimeric complex containing the 90-kDa heat shock protein. Our studies have found that in vitro at 15 °C the RU 486-receptor was slowly released from the 8-9S complex and converted into a DNA binding protein by a process that could be blocked by sodium fluoride. Moreover, this transition was significantly accelerated by treatment with alkaline phosphatase. High-resolution anion-exchange chromatography showed that the profile of receptor subspecies released from the 8-9S complex (in the absence of phosphatase treatment) was different for the RU 486 bound receptor when compared to the receptor occupied by the agonist triamcinolone acetonide. Production of the earliest eluting receptor form (peak A) was inhibited with RU 486. Peak A had previously been shown to be the predominant form of the receptor possessing a capacity to bind DNA. Treatment of the RU 486-receptor with alkaline phosphatase increased the formation of the peak A subspecies as well as the capacity of receptor to bind DNA-cellulose. Taken together, the results indicate that phosphorylation of the receptor or a tightly bound factor contributes to defining the capacity with which individual steroids can promote dissociation of the 8-9S complex and conversion of the glucocorticoid receptor into a DNA-binding protein.

The steroid antagonist RU 486[1] binds avidly to glucocorticoid receptors (Jung-Testas & Baulieu, 1983; Bourgeois et al., 1984) but does not provoke the response normally manifested by agonist hormones at the level of gene expression (Baulieu, 1987; Becker et al., 1986; Chasserot-Golaz & Beck, 1984). This behavior is indicative of the antagonist's failure to promote one or more critical steps in the receptor's transition into an activated transcription factor (Baulieu et al., 1989). Therefore, RU 486 has the potential of helping to define the changes in receptor structure, which play an important role in converting the protein into its activated state. For example, early characterization of the effects of RU 486 found that it caused a lower (relative to agonists) amount of glucocorticoid receptor to translocate to the nucleus in vivo (Jung-Testas & Baulieu, 1983; Bourgeois et al., 1984). More recent in vitro

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[1] Abbreviations: BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; RU 486, 17β-hydroxy-11β-[4-(dimethylamino)phenyl]-17α-propynylestra-4,9-dien-3-one; TA, triamcinolone acetonide.

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