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Anticodon-independent aminoacylation of an RNA minihelix with valine

(tRNA identity/valylation/valyl-tRNA synthetase)

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ABSTRACT **Minihelices mimicking the amino acid acceptor and anticodon branches of yeast $tRNA^{Val}$ have been synthesized by *in vitro* transcription of synthetic templates. It is shown that a minihelix corresponding to the amino acid acceptor branch and containing solely a valine-specific identity nucleotide can be aminoacylated by yeast valyl-tRNA synthetase. Its charging ability is lost after mutating this nucleotide. This ability is stimulated somewhat by the addition of a second hairpin helix that mimicks the anticodon arm, which suggests that information originating from the anticodon stem–loop can be transmitted to the active site of the enzyme by the core of the protein.**

It is at present well accepted that the specificity of tRNA aminoacylation by aminoacyl-tRNA synthetases is governed by positive as well as negative signals present on the tRNA (1–3). Positive signals are generally elicited by a limited set of nucleotides, the determinants, which trigger aminoacylation by the cognate aminoacyl-tRNA synthetases. These determinants, transplanted into another tRNA, confer the new identity to this tRNA, provided its conformation allows an optimal presentation of the determinants toward the synthetase. Negative signals (structural features including modified bases) prevent tRNAs from being efficiently recognized by noncognate aminoacyl-tRNA synthetases (3). Positive identity determinants were first explicitly defined for *Escherichia coli* $tRNA^{Ala}$ (4, 5) and further defined for an increasing number of tRNAs (e.g., ref. 2 and references therein; refs. 6–8). They occur either scattered over the three-dimensional structure of the nucleic acid such as in *E. coli* $tRNA^{Ser}$ (9), $tRNA^{Arg}$ (10, 11), and $tRNA^{Phe}$ (12) and also in yeast $tRNA^{Phe}$ (13) and $tRNA^{Asp}$ (6) or are concentrated within two specific regions—namely, the amino acid accepting arm and the anticodon loop—as found in *E. coli* $tRNA^{Gln}$ (7) and $tRNA^{Gly}$ (8). Finally, in the simplest cases, the identity set is concentrated in one of these two regions: the acceptor arm is important for $tRNA^{Ala}$ (4, 14, 15) and $tRNA^{His}$ (16, 17), and the anticodon loop is important for *E. coli* $tRNA^{Met}$ (18) and $tRNA^{Val}$ (18). As reflected by the variability in aminoacylation capacities of tRNA variants, the relative contribution of identity determinants on chargeability of a tRNA is more or less important. In general, it appears that the higher the number of identity nucleotides required to assess specificity of a tRNA, the lesser the importance of their individual contribution. On the contrary, when the number of identity nucleotides is very low, their strength is very high. In this case, when the strong identity elements are concentrated in the aminoacyl acceptor stem, the full-size tRNA can even be efficiently replaced by minihelices mimicking this region (15, 17). However, there are more complex cases where the existence of a combination of identity determinants of different strength was shown. Indeed, this occurs, for example, in the tRNA-like structure of turnip yellow mosaic virus, valylatable by yeast valyl-tRNA synthetase (ValRS) (19).

For tRNAs having scattered identity nucleotides of different strength, little is known about the interrelation between specificity elements and about the role of minor determinants. To approach this problem, the valine system constitutes a good experimental model. Valylation by yeast ValRS not only requires the presence of determinants in the anticodon for specific tRNA aminoacylation (19, 20) but also is sensitive to the presence of at least one other determinant located far away at the other extremity of the molecule—namely, at the discriminator position next to the amino acid accepting end. This is illustrated in the case of valylatable turnip yellow mosaic virus RNA by the specificity constants k_{cat}/K_m, which are reduced 30- to 300-fold upon mutation at the discriminator position and are reduced 20,000-fold upon mutation in the anticodon (19). This is also likely to be true for yeast $tRNA^{Val}$, since the kinetic valylation parameters are similar for both molecules (21).

Here we consider the specific role of the minor valine identity determinant at the discriminator position on the chargeability of $tRNA^{Val}$ by yeast ValRS. This will be approached by studying the aminoacylation properties of a minihelix mimicking the acceptor stem of $tRNA^{Val}$. In addition we will investigate the functional interrelation between this minor identity element with the major one present in the anticodon by using a second hairpin helix mimicking the anticodon region.

MATERIALS AND METHODS

Materials. Yeast ValRS (22), yeast $tRNA^{Val}$ (major species) (22), and T7 RNA polymerase (23) were purified according to established procedures. The DNA templates with the upstream consensus promoter sequence (positions −17 to −1) of T7 RNA polymerase were synthesized on an Applied Biosystems 381A DNA synthesizer by using the phosphoramidite method and repurified by HPLC on a Nucleosil 120 (5 μm pore size) C_{18} column (Bischoff, Leonberg, Federal Republic of Germany). T4 polynucleotide kinase was purchased from New England Biolabs. L-[^{3}H]Valine and [γ-^{32}P]ATP (3200 Ci/mmol; 1 Ci = 37 GBq) were from Amersham.

Preparation of RNA Minihelices. Minihelices derived from yeast $tRNA^{Val}$ were synthesized by *in vitro* transcription of single-stranded synthetic templates (15, 24). Transcriptions were performed for 4 hr (at 37°C) in a mixture containing 40 mM Tris·HCl (pH 8.1), 5 mM dithiothreitol, 5 mM spermidine, bovine serum albumin at 50 μg/ml, 40 mM $MgCl_2$, each nucleoside triphosphate at 4 mM, 5 mM GMP, each DNA

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Abbreviations: ValRS, valyl-tRNA synthetase; TCA, trichloroacetic acid; MetRS, methionyl-tRNA synthetase.

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strand (17-mer for the plus strand and 42- and 52-mers for the minus strands) at 400 nM, and 3000 units of T7 RNA polymerase. Transcription of the acceptor minihelix template was markedly enhanced by addition of spermidine substitutes into the transcription mixture (M.F., C.F., R.G., and W. Hosseini, unpublished results). Full-length transcripts were purified on a 15% preparative denaturing polyacrylamide/8 M urea gel. Concentrations of RNA solutions were determined by using extinction coefficients at 260 nm calculated according to ref. 25. The sequence and two-dimensional folding of RNA transcripts were verified with enzymatic probes (26).

Valylation Assays. Aminoacylation assays of minihelices were conducted at 20°C in 25 mM Tris·HCl at pH 7.5 containing 7.5 mM $MgCl_2$, 0.5 mM ATP, bovine serum albumin at 0.1 mg/ml, 100 μM L-[^{3}H]valine (1925 cpm/pmol), 7 μM minihelix (heated to 60°C and slowly cooled down to room temperature in 1 mM $MgCl_2$), and variable amounts (0.21 μM–3.2 μM) of yeast ValRS (22). Since the small RNA fragments used in this work could not be quantitatively precipitated in 5% trichloracetic acid (TCA) on 3MM Whatman filters, the amount of charged RNA could not be determined in the classical manner. This was circumvented either by spotting aliquots (10 μl from a total mixture of 75 μl) of the incubation mixtures on DE 81 Whatman filters on which minihelices are fully retained (unbound amino acid was removed by three washes in 5% TCA and one wash in ethanol) or by isolating the charged RNA on DEAE-Sephacel (Pharmacia) columns. In the latter case, aminoacylation was performed in 100-μl samples with internally ^{32}P-labeled minihelices. After an 80-min incubation, mixtures were adjusted to 15 mM EDTA and deposited on the DEAE-Sephacel columns. The columns (1 ml), equilibrated in 10 mM sodium acetate, pH 7.0/10 mM NaCl, were washed after the deposit with the same buffer containing 100 mM NaCl and resolved with a linear NaCl gradient from 100 to 1200 mM in 10 mM sodium acetate (pH 7.0 to pH 6.0). The flow rate was 5 ml/hr, and the fraction volume was 170 μl; the columns were run at 4°C, and the different buffers contained 3 mM EDTA in order to minimize enzymatic deacylation (27, 28) during column elution. Fractions were deposited on Whatman 3MM papers and dried, and ^{3}H and ^{32}P radioactivities were determined simultaneously by liquid scintillation spectroscopy.

Aminoacylation assays of yeast tRNA^{Val} for competition experiments in the presence of minihelices were conducted at 30°C in 50 mM Tris·HCl at pH 7.5 containing 25 mM $MgCl_2$, 60 mM KCl, 70 mM 2-mercaptoethanol, 50 mM ATP, and 8.8 μM L-[^{3}H]valine (1820 cpm/pmol).

RESULTS AND DISCUSSION

The two regions of yeast tRNA^{Val} that contain the identity nucleotides have been synthesized by *in vitro* transcription of single-stranded templates. They correspond to a minihelix mimicking the amino acid acceptor limb and to a hairpin helix mimicking an extended anticodon arm of the tRNA (Fig. 1). Here we show the aminoacylation ability of the amino acid acceptor minihelix and the effect of the anticodon hairpin helix on this aminoacylation. As a prerequisite to this study, it was shown that the two dissected parts of tRNA^{Val} bind to ValRS. Nitrocellulose filter binding (31) performed at pH 6.0 indicated one binding site and dissociation constants values of about 250 ± 50 nM for both molecules (Fig. 2*A*). These values are about 400-fold higher than that of tRNA^{Val} measured under the same conditions (27). The binding of the amino acid accepting minihelix to ValRS was further confirmed by competition experiments of tRNA^{Val} aminoacylation. They showed, in addition, that the interaction of the minihelix with the enzyme occurs at the tRNA binding site since the inhibition is competitive (Fig. 2*B*). Competition experiments performed with the anticodon hairpin helix gave a more complex inhibition pattern (see below). It should be noted that under conditions favoring the specificity of valylation (i.e., at pH 7.5 and 60 mM salt concentration) the affinity of the minihelix to ValRS derived from the K_i values is about 5 times lower than that obtained under the nitrocel-

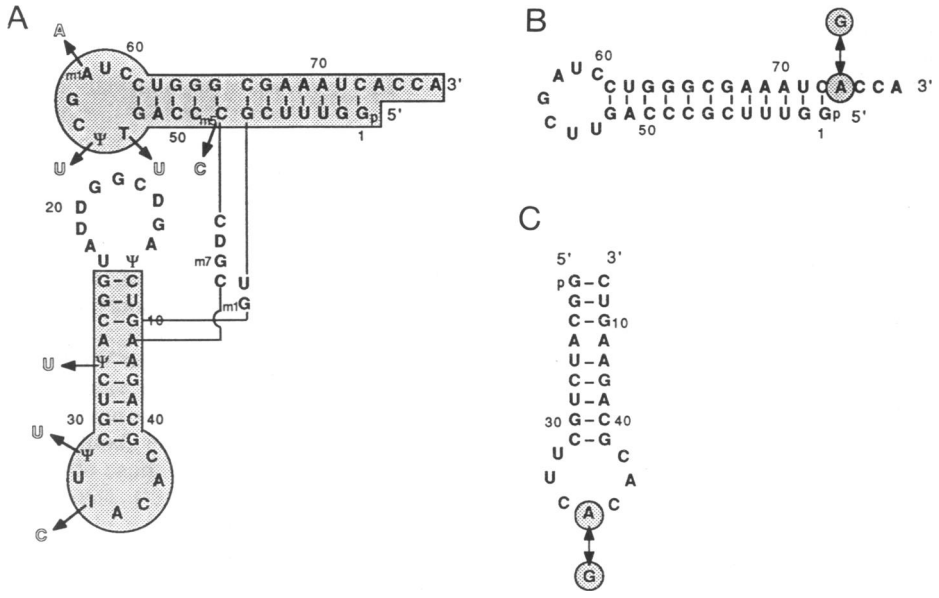

FIG. 1. Sequences of minihelices derived from yeast tRNA^{Val}. (*A*) L-shaped representation of tRNA^{Val} emphasizing regions corresponding to the minihelices synthesized by *in vitro* transcription of synthetic templates (15, 29). Arrows indicate the nucleotides incorporated in place of the modified nucleotides during transcription (a cytidine has been incorporated at the wobble position, position 34, of the anticodon hairpin helix by analogy to the anticodon in the tRNA-like structure from turnip yellow mosaic virus). (*B*) Sequences of the amino acid acceptor minihelices composed of the TΨ stem and loop and the acceptor stem. (*C*) Sequences of the anticodon arm minihelices that contain the anticodon stem and loop, the tertiary interaction residues 26–44, as well as three base pairs from the D stem, which ensure an efficient start of transcription of the corresponding synthetic template. Numbering is according to classical tRNAs (30). Variants of both minihelices with mutations at position 73 or 35 have also been synthesized.

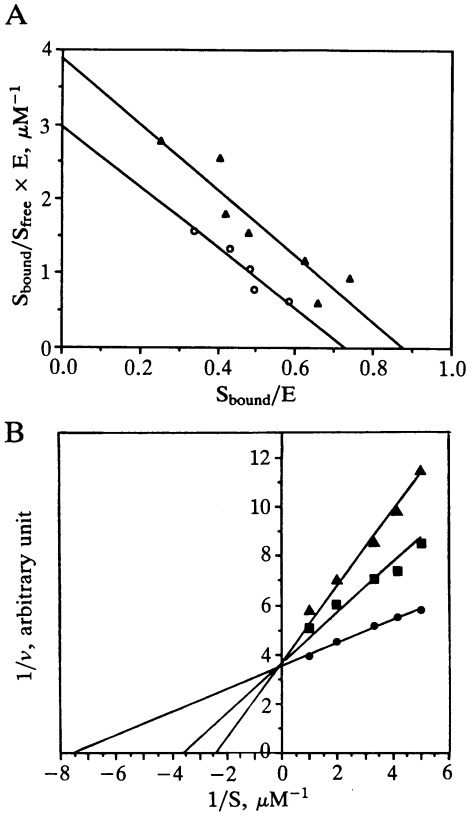

FIG. 2. Interaction of the transcripts with ValRS. (*A*) Binding analyzed according to Scatchard. K_d values were derived from the slopes of the straight lines (○, acceptor minihelix; ▲, anticodon hairpin helix); intercepts with the abscissa give *n*, the number of RNA binding sites on the enzyme. (*B*) Competition experiments of yeast $tRNA^{Val}$ charging by acceptor stem minihelices. Aminoacylation of $tRNA^{Val}$ in the absence of minihelix (●) or in the presence of 5 μM (■) or 15 μM (▲) wild-type minihelix is shown. Aminoacylation was performed at 30°C in the presence of 0.3–1 μM $tRNA^{Val}$ and 0.7 nM ValRS. Bound amino acid was measured by liquid scintillation spectroscopy after precipitation of the nucleic acids on 3MM Whatman filters. K_i values measured for both concentrations of the wild-type minihelix were similar: 7 ± 2 μM and 10 ± 2 μM, respectively. S, substrate; E, enzyme.

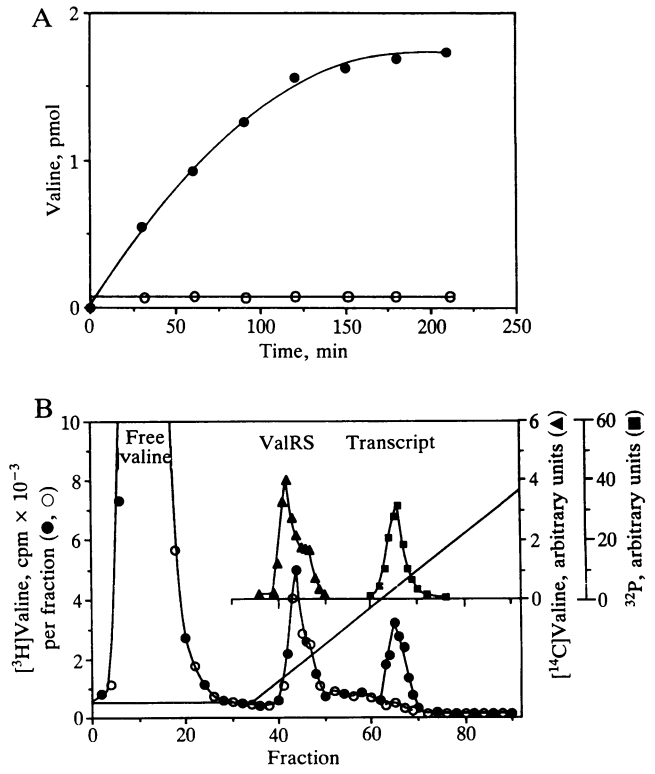

FIG. 3. (*A*) Time course of valylation of the wild-type (●) and mutated (○) acceptor minihelices as measured by precipitation of the nucleic acids contained in 10-μl aliquots (from 75-μl assays) on DE 81 Whatman filters. (*B*) Chromatograms on DEAE-Sephacel columns of the aminoacylation mixtures of the wild-type (●) and mutated (○) acceptor minihelices after an 80-min incubation. The ^{32}P peak localizes the transcripts (■), and ValRS (▲) was detected by aminoacylation assays conducted with bulk yeast tRNA (for the sake of simplicity, only fractions above background are represented). In the presence of the mutant acceptor minihelix, the ^{3}H radioactivity at the level of the third peak (○) disappears.

lulose binding conditions. Because the conditions at pH 7.5 are more stringent for specific aminoacylation, they were used for the aminoacylation analysis of the minihelix.

The wild-type acceptor minihelix, with the minor identity determinant A73 (19), is chargeable by yeast ValRS. This was first shown by the retention of labeled valine on DE 81 Whatman papers after precipitation of the nucleic acids contained in the aminoacylation mixtures by TCA (Fig. 3*A*). Because the levels of radioactivity incorporated by the minihelix were low and could have been due to artificial trapping of the amino acid, the reality of aminoacylation was demonstrated by the isolation of the charged RNA fragment by chromatography on DEAE-Sephacel columns. As shown in Fig. 3*B*, labeled valine coelutes with the minihelix. Controls in which an anticodon hairpin helix was the substrate in the aminoacylation reaction did not reveal any radioactivity coeluting with the RNA. Radioactivity that coelutes with the enzyme was shown to be due to adenylate. Adenylate is not retained on DE 81 Whatman papers treated with TCA. Thus, the radioactivity shown in Fig. 3*A* corresponds unambiguously to covalent fixation of valine to the amino acid acceptor minihelix of yeast $tRNA^{Val}$. Varying the enzyme and substrate concentrations led to valylation of up to 2.5% of the input minihelices. Thus it clearly appears that a minihelix corresponding to the amino acid acceptor arm of $tRNA^{Val}$ can be aminoacylated.

This low level of aminoacylation can be accounted for by the kinetic characteristics of the valylation process comprising acylation in equilibrium with deacylation reactions. Assuming that the deacylation reactions of a minihelix occur with similar kinetics as the deacylation reactions of yeast $tRNA^{Val}$, an estimate of the theoretical optimal chargeability (27, 28) of the minihelix can be given a value of 2%, which is comparable with the experimental determination. The moderate charging level is mainly due to unfavorable kinetic parameters of the forward acylation reaction. Since the binding of the minihelices to ValRS is weak, it is extremely difficult to derive precise values of K_m and k_{cat} from a Michaelis analysis. We have nevertheless estimated K_m to be about 1 mM and k_{cat} to be about 0.01 s^{-1}. These values are markedly different from those obtained for $tRNA^{Val}$ (K_m = 0.13 μM and k_{cat} = 0.75 s^{-1}). Thus, the catalytic efficiency of minihelix valylation is reduced about 6 × 10^5-fold as compared to that of canonical $tRNA^{Val}$. It must however be stressed that the minihelix interaction with ValRS is efficient enough to overcome the transition state for acylation. Only an additional 7–8 kcal/mol (estimated according to ref. 32) is required as compared to the canonical reaction, an energetic cost much lower than that required for the uncatalyzed reaction (>11–12 kcal/mol, assuming k_{cat} values of $<10^{-5}$ s^{-1} corresponding to aminoacylation plateaus of <0.01%).

To verify that the valylation of the minihelix was triggered by the identity element at position 73 and was not the

consequence of faint effects brought about by discrete structural elements embedded within the minihelix, we synthesized a variant in which the discriminator nucleotide was mutated (Fig. 1*B*). This minihelix also behaves as a competitive inhibitor for $tRNA^{Val}$ aminoacylation (results not shown) but does not show any detectable valylation within the experimental limits of our assays (Fig. 3). Thus, a single nucleotide within the minihelix is sufficient to activate the catalytic site of the enzyme and to confer a valylation identity to this structure.

These results suggest the possibility that simplified versions of tRNAs were the first substrates of synthetases but that for biological necessity (i.e., to better ensure acylation specificity and adaptor function) tRNAs became more sophisticated and most of them acquired additional identity elements. Thus, even if their effect is moderate in some canonical tRNAs, the identity elements found in the simplified versions of active tRNAs are likely to be those that appeared first during evolution.

What then is the effect of the other determinants? In present day $tRNA^{Val}$, the most efficient valylation identity determinants are located within the anticodon loop (18, 19). We show here that upon addition of a hairpin helix mimicking the anticodon arm of yeast $tRNA^{Val}$ to the aminoacylation medium, valylation of the acceptor minihelix is stimulated (Fig. 4*A*). This suggests that part of the effect on valylation contributed by the anticodon identity nucleotides is transmitted to the active site of the enzyme by the core of the protein. This is in agreement with previous observations that showed that the 3′ terminal adenosine or CCA of a tRNA can be charged, provided the aminoacylation mixture is supplemented with the tRNA lacking its 3′ terminus (33). The transfer of information from the anticodon to the active site, 75Å away, is specific and directly linked to the presence of at least one identity nucleotide in the anticodon hairpin helix, since the replacement of nucleotide 35 cancels the effect (Fig. 4*B*). The level of stimulation is dependent on the concentration of anticodon hairpin helix (Fig. 4*A Inset*) and of enzyme (data not shown). This stimulation (up to 3-fold under the best conditions) is not as strong as one could expect according to the high contribution to specificity of the central position of the anticodon triplet (19). This is not astonishing since we are in the presence of an artificial and not optimized system in which three macromolecules with relatively week binding affinities are involved. Both amino acid accepting minihelix and anticodon hairpin helix have to interact efficiently and simultaneously with the synthetase in order to present their identity elements in the correct way to the enzyme. Interestingly, the anticodon hairpin helix mutated at the central nucleotide is a competitive inhibitor of $tRNA^{Val}$ aminoacylation (inhibition constant K_i of 25 μM; results not shown). The effect of the wild-type anticodon hairpin helix on aminoacylation of $tRNA^{Val}$ is not typical and possibly reflects an equilibrium between inhibition and stimulation. Clear inhibition of $tRNA^{Met}$ aminoacylation was however observed with a hairpin helix mimicking the anticodon of this tRNA (34). Control experiments conducted with a nonrelated anticodon hairpin helix (anticodon region of yeast $tRNA^{Asp}$) did not have any stimulatory effect on minihelix valylation. Also the possibility that valine specificity is dependent on additional identity nucleotides not included in the minihelices cannot be excluded. However, our results clearly demonstrate that an anticodon nucleotide potentiates the action of the primary determinant located in the acceptor minihelix.

Recently similar results have been obtained for another synthetase, namely *E. coli* methionyl-tRNA synthetase (MetRS), which charges, although very weakly, minihelices derived from $tRNA^{Met}$ (35). Together with the valylatable minihelix these are, to our knowledge, the first examples of synthetases that normally require several identity nucleotides scattered over the surface of their cognate tRNA that can aminoacylate minisubstrates that do not contain major identity determinants. As a consequence of the specific chargeability of the minihelices, minor determinants should be present in their structure, which could represent cryptic remains of primordial tRNAs. In the case of the valine system, such a role is played by the discriminator base, whereas in the methionine system, Martinis and Schimmel (35) have shown that the first three base pairs are involved in methionylation. Likewise, additional elements within the helical domain of the valine minihelix may be involved in valylation.

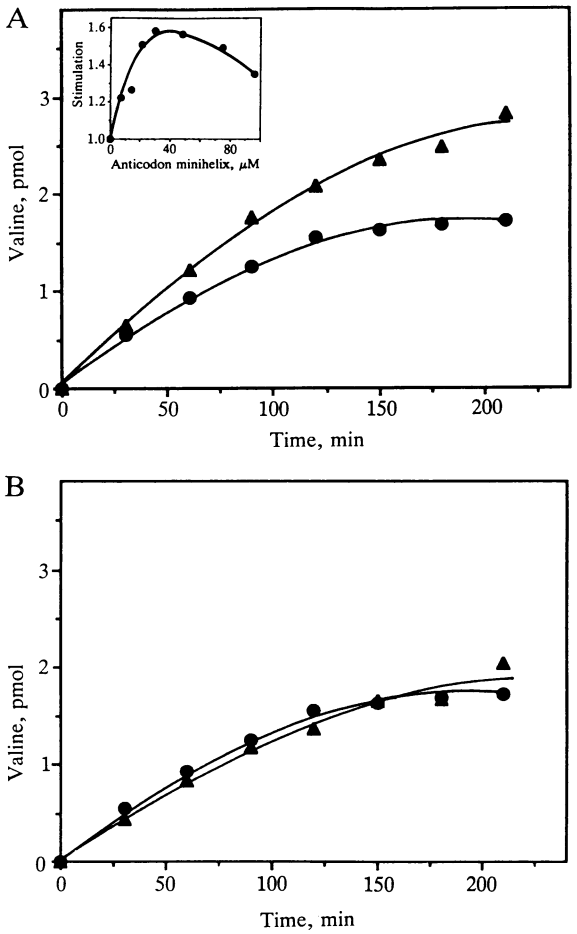

FIG. 4. Aminoacylation of the wild-type acceptor minihelix in the absence (●) and presence (▲) of wild-type (*A*) or mutant (*B*) anticodon hairpin helix. Assay conditions and detection of radioactivity were as in Fig. 3*A*; the anticodon hairpin helices were present at 30 μM. (*Inset*) Dependence of the aminoacylation level (after a 210-min incubation) as a function of wild-type anticodon minihelix concentration. In that case, the detection of radioactivity was as in Fig. 3*B*.

Interestingly, both ValRS and MetRS are class I synthetases (36). Thus, the ability to recognize and catalyze aminoacylation of small substrates is no longer restricted to class II synthetases as was the case for alanyl-tRNA synthetase (15) and histidinyl-tRNA synthetase (17). Moreover, in the case of the valine system, the major determinant is involved in the catalytic site activation even though it is presented to ValRS on a separate substrate. It would be of interest not only to test this possibility for other systems but also to investigate the possibility of engineering synthetases in the same way.

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