Unconventional Reading of the Glycine Codons*

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We have used a protein-synthesizing in vitro system programmed with the phage message MS2-RNA to investigate the ability of glycyl-tRNAs with different anticodons to read the glycine codons. Under conditions of no competition, when the glycyl-tRNA analyzed was the only source of glycine for protein synthesis, each of the isoacceptors tested, tRNA$^{\text{Gly}}$ (anticodon CCC), tRNA$^{\text{Gly}}$ (anticodon N/UCU), tRNA$^{\text{Gly}}$ (anticodon GCC) from Escherichia coli, and tRNA$^{\text{Gly}}$ (anticodon UCC) from Mycoplasma mycoides, could read all of the glycine codons in the MS2 coat protein cistron (GGU, GGC, GGA, and GGG). However, tRNA$^{\text{Gly}}$ seemed to have difficulties reading through the whole cistron. Experiments in which two glycyl-tRNAs competed for the same codon showed that the mycoplasma tRNA$^{\text{Gly}}$ (anticodon UCC) was almost as efficient in the unorthodox reading of the codons GGU and GGC as it was in conventional reading. It would seem to be the only tRNA$^{\text{Gly}}$ present in Mycoplasma mycoides and our results are consistent with this finding since the mycoplasma tRNA$^{\text{Gly}}$ appears to have been designed to read all four glycine codons with approximately equal efficiency. The competition experiments furthermore showed that E. coli tRNA$^{\text{Gly}}$ (anticodon CCC) reads the codon GGA more efficiently than it reads GGU and GGC suggesting that the mispair C-A between the wobble position of the anticodon and the third codon position might have appreciable stability.

Codon misreadings of a kind that automatically leads to translational errors could obviously not occur frequently in vivo or in protein-synthesizing systems in vitro capable of translating natural messengers into functional proteins. On the other hand, half of the codons in the genetic code belong to codon families, i.e. groups of four codons specifying the same amino acid, which have their first two nucleotides in common. In such families it makes no difference, as far as translational fidelity is concerned, how the third codon nucleotide is read since the first two are enough to specify the amino acid. The codon families should, therefore, be promising hunting ground for anyone interested in unconventional codon reading. To explore these possibilities we have used an in vitro protein-synthesizing system from Escherichia coli programmed with the phage message MS2-RNA and have observed a type of unconventional reading whereby a codon may be read by an anticodon that cannot, according to the wobble rules (1), form a stable base pair with the third codon nucleotide (2-5).

To interpret these findings it is necessary to consider the two basic principles of the classic codon-reading scheme. The first principle is embodied in the wobble rules that presumably govern the base pairing between codon and anticodon, while the second principle demands that an anticodon must form stable base pairs with all three nucleotides of the codon in order to read it. Clearly, one of these principles must be invalidated if we are to explain our experimental results. One could, for instance, assume that the wobble restrictions are not valid meaning that in the reading of the third codon position base pairs may be formed in violation of the wobble rules with a stability that is comparable to that of regular base pairs. Although this might be the case in some instances to be discussed below, as a more general principle it appears unlikely both because of the seemingly intractable structural problems it presents (6) and because it fails to explain how, on this assumption, the translational apparatus could discriminate against codon-anticodon interactions that we know are not compatible with translational fidelity. We have, therefore, suggested an alternative explanation, the two-out-of-three reading hypothesis (7) which assumes that the wobble rules are basically correct. According to this hypothesis a codon may be read by relying mainly on the Watson-Crick base pairs formed with the first two codon positions, while the mispaired nucleotides in the third codon and anticodon wobble positions make a comparatively small contribution to the total stability of the reading interaction. On the other hand, it is not implied that the nature of the mispair is without importance for the efficiency of the unconventional reading. In fact, the results of the present investigation illustrates the role of the mispair in this respect.

The hypothesis furthermore assumes that the probability of two-out-of-three reading is a function of the strength of the interaction between the anticodon and the first two codon nucleotides and that a G-C interaction is stronger than an A-U interaction. Therefore, the probability of such reading would be greatest for codons making only G-C interactions in these positions (G-C codons), intermediate for codons which make one G-C and one A-U interaction ("mixed" codons), and minimal for codons making only A-U interactions (A-U codons). When the genetic code was examined for the distribution of the three codon categories, it was immediately apparent that G-C codons were without exceptions restricted to the codon families while the A-U codons were always outside the families (7). This is the distribution to be expected as the result of an evolutionary process which selected for a code where the probability that reading by two-out-of-three should cause translational errors was at a minimum (8, 9). On the other hand, this tentative structural explanation leaves the mixed codons unaccounted for. It was noted, however, that the distribution of the mixed codons with respect to the codon families is not random. In the left half of the codon square all mixed codons appear in families while in the right half they are all outside the families (7). This nonrandom distribution
is not likely to be fortuitous and instead suggests the possibility that mixed codons in the left half are confined to the families because they give a greater risk of being read by two-out-of-three than mixed codons in the right half.

In the present investigation we have used the MS2 in vitro protein-synthesizing system to investigate the reading of the glycine codons and test the predictions of the two-out-of-three hypothesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

MS2 RNA—The growth of phage MS2 and the preparation of MS2 RNA has been described previously (2).

S30 extract—E. coli ts48 that has a ts mutation in its glycine tRNA ligase (10) was a gift from Dr. N. P. Fil, Copenhagen, Denmark. It was grown at 30°C in L broth (10 g of Tryptone, 5 g of yeast extract, and 16 g of NaCl per liter) and the cells were harvested in medium log phase and stored frozen at −80°C. The S30 extract was prepared as described previously (2).

tRNAs—tRNA from E. coli was obtained from Boehringer Mannheim, Mannheim, West Germany. tRNA$^{\text{Gly}}$, tRNA$^{\text{Asp}}$, and tRNA$^{\text{Ser}}$ were separated by chromatography on benzoylated DEAE-cellulose (11) as described in Ref. 2. The tRNA$^{\text{Gly}}$ fractions were further purified by reverse chromatography on benzoylated DEAE-cellulose using the same conditions.

Mycoplasma mycoides sp. capri was a gift from Professor E. A. Freundt, Aarhus, Denmark. It was grown in a medium containing per liter 1 g of heart infusion broth, 25 g of yeast extract, 1 g of taileurate, 24 mg of DNA, 200 mg of horse serum, and 500,000 international units of penicillin (12). Cells were harvested after growth at 37°C for 48 h and stored frozen at −80°C. tRNA$^{\text{Gly}}$ was isolated by extraction of 50 g of cells, suspended in 100 ml of 0.1 M sodium acetate buffer, pH 4.5, with 100 ml of phenol saturated with the same buffer. The phenol phase was washed twice with the same buffer and the combined water phases were precipitated with 3 volumes of cold ethanol after the addition of NaCl to a concentration of 1.5 M. The precipitate was centrifuged and dissolved in water. A crude tRNA fraction was obtained by chromatography on DEAE-cellulose using a linear NaCl gradient from 0.25 to 1.5 M. Fractions containing tRNA were combined, precipitated with 2 volumes of ethanol, and finally dissolved in water. tRNA$^{\text{Gly}}$ was further purified by chromatography on benzoylated DEAE-cellulose as described above.

tRNA$^{\text{Ser}}$, tRNA$^{\text{Phe}}$, and tRNA$^{\text{Asp}}$ from E. coli and tRNA$^{\text{Gly}}$ from M. mycoides were esterified with labeled glycine, and the glycyl-tRNAs were recovered from the aminoacylation mixture as described previously (2). The E. coli glycyl-tRNA$^{\text{Gly}}$ gave a single homogeneous peak when they were analyzed by chromatography on an RPC-5 column (13). M. mycoides tRNA$^{\text{Gly}}$ also gave a single homogeneous peak on both benzoylated DEAE-cellulose and RPC-5, in agreement with the findings of Kilpatrick and Walker (14). The primary structure of tRNA$^{\text{Gly}}$ (15), tRNA$^{\text{Asp}}$ (16), and tRNA$^{\text{Ser}}$ (17) from E. coli as well as M. mycoides tRNA$^{\text{Gly}}$ (14) has been determined.

**Purification of Glycine-tRNA Ligase**—Glycine-tRNA ligase was purified either from E. coli cells obtained from the Applied Microbiology and Research, Porton Down, England, or from cells of E. coli CH 574 pLC1-3 grown in xylene minimal medium as described by McDonald et al. (18). The strain CH574 pLC1-3 which carries a hybrid ColEl plasmid containing gly S, the structural gene for glycine-tRNA ligase, was a gift from Dr. G. M. Nagel, Fullerton, California. The enzyme was purified using ammonium sulfate fractionation and DEAE-cellulose chromatography as described previously (19). Further purification was achieved by chromatography on blue dextran-Sepharose according to McDonald et al. (18) and by gel filtration using G-100 with 0.1 M potassium phosphate buffer, pH 7.0, 30% glycerol, 10 mM mercaptoethanol as elution buffer.

**Chemicals**—Cathepsin C and α-aspartylglycine were from Sigma, and trypsin was from the same company or from Serva Feinbiochemica, Heidelberg, West Germany. [14C]Glycine (113 mCi/mmol) and [1$^\text{H}$]glycine (20 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. LiChrosorb RP-18 (10 μm) was a product of E. Merck, Darmstadt, West Germany, and DEAE-cellulose was from Whatman Biochemicals Ltd., Springfield Mill, Kent, England (DE25 and DE22) or from Calbiochem (Cellul D); Benzoylated DEAE-cellulose was prepared from DE22 cellulose (11). Sephadex G-100, G-75, CNBr-activated Sepharose 4B, and blue dextran was from Pharmacia. The preparation of blue dextran-Sepharose has been described by Ryan and Vestling (20).

**Methods**

**Conditions for In Vitro Protein Synthesis and Isolation of Glycine-containing CoA Protein Peptides**—Conditions for in vitro protein synthesis were as described by Nirenberg (21) with the modifications of the basic protocol indicated in Ref. 2. For the experiments described in Table 1 the S30 extract (200 μg of protein) was preincubated at 37°C for 5 min. The final reaction mixture contained 1 A$^{260}$ unit of MS2 RNA, 0.6 A$^{260}$ unit of crude tRNA from E. coli, and 12 μM L-[14C]glycine (or 2 μM [14C]glycyl-tRNA) in a volume of 50 μl. Incubation was for 20 min at 37°C, the reaction was stopped by adding 1 ml of 0.4 M perchloric acid, and the sample was heated for 20 min at 90°C in order to solubilize any aminoacyl-tRNA. The sample was chilled and the precipitate collected on Whatman GF/C 2.5-cm glass filter discs and rinsed with 0.4 M perchloric acid. The radioactivity of the samples was determined as described previously (2).

For experiments where glycine-containing peptides were to be analyzed, the procedure described above was scaled up 100 times. In the competition experiments (Table II) the reaction mixture contained the competing glycyl-tRNAs in concentrations of 1.2 μM each. One of them was esterified with 3H-glycine (172 cpm/pmol), and the other with [1$^\text{H}$]glycine (807 cpm/pmol). The incubation was terminated by the addition of NaOH to a concentration of 0.1 M and incubation was for 20 min at 25°C. The coat protein was isolated by gel filtration and digested with trypsin, and the tryptic peptides were separated by paper chromatography and high voltage electrophoresis as described previously (22). The peptides contained labeled glycine were visualized by autoradiography as shown in Fig. 1.

For the isolation of tryptic peptides the following alternative procedure was also used. The incubation mixture was neutralized by the addition of acetic acid and dialyzed against 3 liters of water with one change of water. The sample was lyophilized, extracted with a total of 4 ml of 80% acetic acid, and 10 mg of carrier coat protein was added. After lyophilization 10 ml of 0.05 M NH$_4$CO$_3$ and 0.4 mg of trypsin were added, and the mixture was incubated for 5 h at 37°C. The sample was lyophilized and peptides soluble in 0.3 M pyridine, formic acid, pH 4.0, (buffer A) were separated by reverse-phase HPLC on a LiChrosorb RP-18 column (1.2 x 60 cm). Elution was carried out with a linear propanol gradient using 200 ml of buffer A in the mixing chamber and 200 ml of buffer A containing 25% 1-propanol in the reservoir. Fractions of 3.5 ml were collected and radioactivity was determined in 100-μl aliquots of each fraction. The relevant peptides (Fig. 2) were lyophilized and peptides 2 and 6 were further purified by high voltage paper electrophoresis. Peptide 6 was then visualized by spraying with ninhydrin while peptide 2 could be identified by its UV fluorescence. The peptides were cut out, eluted with 1 M acetic acid, and their [1$^\text{H}$/14C] ratios determined (2). The final

**Table I**

| Source of glycine | Glycine | tRNA ligase | MS2 RNA-dependent incorporation of glycine |
|------------------|---------|------------|-------------------------------------------|
|                  | pmol    |            | Yield                                     |
| [14C]Glycine     |         |            |                                           |
| [1$^\text{H}$]Glycine |         |            |                                           |
| Unfractionated E. coli [14C]Gly-tRNA$^{\text{Gly}}$ | 25.3    | 25.3       |                                           |
| E. coli [1$^\text{H}$]Gly-tRNA$^{\text{Gly}}$ | 6.0     | 6.0        |                                           |
| E. coli [14C]Gly-tRNA$^{\text{Gly}}$ | 20.6    | 20.6       |                                           |
| E. coli [1$^\text{H}$]Gly-tRNA$^{\text{Gly}}$ | 17.4    | 17.4       |                                           |
| M. mycoides [1$^\text{H}$]Gly-tRNA$^{\text{Gly}}$ | 19.2    | 19.2       |                                           |

1 The abbreviations used are: HPLC, high-pressure liquid chromatography; N, an unknown derivative of uridine; sU, 2-thio-5-methylcytosineuridine or, in the case of tRNA$^{\text{Gly}}$, an unknown derivative of 2-thiouridine.
Reading of Glycine Codons

**TABLE II**

Table: Efficiency of different glycine tRNAs in reading the codons GGU, GGA, and GGC

Two different glycine tRNAs, esterified with $[^3]$H- and $[^14]$C-glycine, respectively, were incubated together in an MS2-RNA programmed in vitro system as described under "Experimental Procedures." By comparing the ratio of $^3$H to $^{14}$C in the peptide glycine residues corresponding to the different codons with the isotopic ratio of the glycyll-tRNAs incubated, the relative efficiency of the competing tRNAs in the reading of a certain codon could be calculated. The reading efficiency corresponding to the codon GGU was calculated using a mean value of the isotope ratios obtained from peptides 2 and 6, and the reading efficiencies of codons GGA and GGC were obtained from the isotope ratios of peptides 1A and 1B (Fig. 1), respectively.

| Competing tRNAs                  | Relative reading efficiency* |
|----------------------------------|-----------------------------|
|                                  | GGU  | No. of experiments | GGA  | No. of experiments | GGC  | No. of experiments |
| M. mycoides tRNA$^{Gy}$/E. coli tRNA$^{Gy}$ | 0.42 ± 0.05 | 6     | 33 ± 15 | 5     | 0.36 ± 0.07 | 6     |
| E. coli tRNA$^{Gy}$/E. coli tRNA$^{Gy}$   | 0.24 ± 0.06 | 8     | 55 ± 17 | 2     | 0.18 ± 0.08 | 6     |
| E. coli tRNA$^{Gy}$/E. coli tRNA$^{Gy}$   | 0.018 ± 0.003 | 6    | 3.6 ± 1.2 | 6     | 0.04 ± 0.02 | 3     |
| E. coli tRNA$^{Gy}$/M. mycoides tRNA$^{Gy}$ | 0.36 ± 0.05 | 5     | 1.8 ± 0.5 | 4     | 0.36 ± 0.10 | 5     |
| E. coli tRNA$^{Gy}$/E. coli tRNA$^{Gy}$   | 0.11 ± 0.02 | 3     | 0.11 ± 0.01 | 3     | 0.11 ± 0.01 | 2     |
| E. coli tRNA$^{Gy}$/M. mycoides tRNA$^{Gy}$ | 0.09 ± 0.04 | 3     | 0.15 ± 0.05 | 3     |            |

*Mean ± S.D.

**Fig. 1.** Glycine-containing tryptic peptides from MS2 coat protein. Upper left, separation of glycine-containing tryptic peptides from MS2 coat protein by a combination of chromatography and electrophoresis. Upper right, autoradiogram of $[^14]$C-glycine-containing tryptic peptides from MS2 coat protein. Bottom, Sequences of glycine-containing tryptic peptides from MS2 coat protein and the corresponding sequences in MS2 RNA.

The yield of labeled peptides 2 and 6 was approximately 100 pmol. The identity of peptides obtained from the HPLC chromatography was checked by determination of the amino acid composition of each peptide.

Analysis of Peptide 1—Peptide 1 (approximate 100 pmol of radioactive material) was digested with cathepsin C (dipeptidyl transferase, dipeptidyl aminopeptidase I) as described by Callahan et al. (22). The lyophilized peptide was dissolved in 200 μl of 16 mM HCl, 0.10 M pyridine, 52 mM acetic acid, 15 mM mercaptoethanol, 0.5 mM EDTA, pH 5.0. Cathepsin C, 0.16 mg of protein in 20 μl of 0.15 M NaCl, 0.5 mM EDTA, was added, and the reaction mixture was incubated for 4 h at 37 °C, diluted with 1 ml of water, and lyophilized. The digestion products were dissolved in 1 M acetic acid and separated by high voltage paper electrophoresis (2). Under the conditions used (pH 3.5) peptide 1A (aspartylglycine, Figs. 1 and 3) migrates toward the anode, while peptide 1B (Figs. 1 and 3) migrates toward the cathode.
FIG. 2. Separation of glycine-containing tryptic peptides by reverse-phase HPLC. Coat protein was synthesized with unfraccionated \textsuperscript{3}H\textsuperscript{2}glycyl-tRNA\textsubscript{Y} from E. coli as the source of glycine and was digested with trypsin. Peptides were separated by reverse-phase HPLC on a LiChrosorb RP-18 column. Details are described under "Experimental Procedures." The glycine-containing peptides (1, 2, and 6 in Fig. 1) used in the determination of codon reading efficiencies are indicated in the figure.

FIG. 3. Separation of products after digestion of peptide 1 with cathepsin C. \textsuperscript{3}H\textsuperscript{2}Glycine-labeled peptide 1 was digested with cathepsin C and the products separated by high voltage paper electrophoresis as described under "Experimental Procedures." The paper was cut into strips of 0.5 cm which were transferred to scintillation vials. Peptides were eluted with 0.2 ml of 1 M acetic acid, and radioactivity was determined.

cathode and intact peptide 1 remains at the point of application. Peptide 1A was identified by its comigration with a commercial sample of α-aspartylglycine. Peptides 1A and 1B were cut out, eluted with 1 M acetic acid, and their \textsuperscript{3}H/\textsuperscript{14}C ratios determined. The final yield of labeled peptides 1A and 1B was from 10 to 50 pmol.

RESULTS

For the present study of glycine codon reading we have used the same in vitro protein-synthesizing system programmed with the viral messenger MS2-RNA as in previous investigations (2-5). This system largely synthesizes the MS2 coat protein, whose amino acid sequence as well as the corresponding messenger RNA sequence are known (23).

Of the glycine codons only GGU is located in the coat protein cistron in such a way that its reading can be monitored directly by the isotope incorporation in the tryptic peptides (peptides 2 and 6, Fig. 1) separated by a two-dimensional combination of paper chromatography and high voltage electrophoresis or alternatively, by using reverse-phase HPLC chromatography as described under "Methods" (Fig. 2).

The glycine codons GGA and GGC, on the other hand, are represented by glycine residues that are both present in the same peptide (peptide 1 in Fig. 1). In order to measure the reading of these codons peptide 1 must be cleaved and the pertinent glycines separated from each other. The proteolytic enzyme cathepsin C removes dipeptides from the NH\textsubscript{2}-terminal end of polypeptides but will not cleave bonds involving proline residues. Consequently, in the case of peptide 1, which contains a proline in the fourth position from its NH\textsubscript{2}-terminal, the enzyme will only remove the dipeptide aspartyl glycine (peptide 1A in Fig. 1) leaving the rest of peptide 1 intact. The glycine in peptide 1A corresponds to the codon GGA while the glycine in the remaining part of peptide 1 (peptide 1B in Fig. 1) is coded for by GGC. Peptides 1A, 1B, and 1C were separated by paper electrophoresis (Fig. 3) and the reading properties of codons GGA and GGC were monitored individually. The unfavorable position of the codon GGG in the coat protein cistron made it impossible to analyze its reading properties separately. Thus, the codons that for technical reasons were not included in this investigation are: GCC corresponding to glycine residues 13 and 16; GGA corresponding to glycine 14; and GGG corresponding to glycine 28.

Reading of Glycine Codons under Noncompetitive Conditions—In one set of experiments (Table I) the reading of the glycine codons by the different tRNA\textsuperscript{Gly} isoacceptors investigated was determined in the absence of competition, i.e. in each experiment the glycyl-tRNA analyzed was the only aminoacylated tRNA\textsuperscript{Gly} present in the system and represented the only source of glycine for protein synthesis. This was made possible by using an S30 extract prepared from an E. coli mutant with a temperature-sensitive glycine-tRNA ligase (10). The enzyme was extremely heat labile and in our standard glycine:tRNA ligase assay we could not detect any activity in the S30 extract. Consequently, our protein-synthesizing system was strictly dependent on added glycyl-tRNA (Table I).

The reading properties of the following E. coli tRNA\textsuperscript{Gly} isoacceptors were determined: tRNA\textsuperscript{Gly} (anticodon CCC), tRNA\textsuperscript{Gly} (anticodon N/UCC where N is an unknown derivative of U), tRNA\textsuperscript{Gly} (anticodon GCC), and tRNA\textsuperscript{Gly} (anticodon UCC) from M. mycoides, the only tRNA\textsuperscript{Gly} known to be present in this organism. The tRNAs to be tested were esterified with labeled glycine and monitored individually for their ability to sustain protein synthesis in the MS2-RNA programmed system. The results showed that, with the exception of tRNA\textsuperscript{Gly}, the incorporation of glycine into protein from any of the isoacceptor tRNAs alone was comparable to that obtained with unfraccionated glycyl-tRNA from E. coli. Using glycyl-tRNA\textsuperscript{Gly}, however, the incorporation of labeled glycine into protein was only 20-25% of that obtained with unfraccionated glycyl-tRNAs (Table I). When the experiments were scaled up and the tryptic peptides of the protein products were analyzed it was found that in each case MS2 coat protein had been synthesized, based on the tryptic peptide pattern obtained. However, chromatography on Sephadex G-75 of the proteins synthesized indicated that, in addition to coat protein, an approximately equal amount of protein of lower molecular weight was synthesized in the experiments with tRNA\textsuperscript{Gly}. Work to establish this might be a truncated coat protein is in progress. To summarize, the results indicated that each of the isoacceptors could read all of the glycine codons in the coat protein cistron even if the tRNA\textsuperscript{Gly} seemed to have some difficulties reading through the whole cistron.

Relative Reading Efficiency of Isoacceptor Glycine tRNAs—To evaluate the relative reading efficiencies of the different isoacceptor glycine tRNAs experiments were performed in which two tRNAs competed with each other for the same codon. In a competing pair one tRNA was esterified with \textsuperscript{3}H glycine and the other with \textsuperscript{14}C glycine and the tRNAs were incubated together in equimolar concentrations in the in vitro protein-synthesizing system. By comparing the ratio of \textsuperscript{3}H to \textsuperscript{14}C in the peptide glycine residues corresponding to the dif-
The two-out-of-three hypothesis does not imply that family codons in vivo are normally read by two-out-of-three even in situations where codon readings. Thus, (anticodon CCC) read both of tRNAs that were unusually inefficient in unconventional seem to be generally superior to the other tRNAGly isoacceptors in the reading of the glycine codons since it was only half of that of tRNACly is the only glycine isoacceptor the tRNA$^{\text{f}}_Y$ (anticodon GCC) read this codon with an efficiency which was only 2% of that of the conventional reader. On the other hand, mycoplasma tRNAGly in reading these codons was almost half of that of tRNA$^{\text{Gly}}$ (anticodon GCC) which reads GGU and GGC by using the classic codon reading scheme. On the other hand, mycoplasma tRNA$^{\text{Gly}}$ does not seem to be generally superior to the other tRNA$^{\text{Gly}}$ isoacceptors in the reading of the glycine codons since it was only half as efficient as tRNA$^{\text{Gly}}$ (anticodon N/UCC) in the reading of GGA. Instead one is left with the impression that mycoplasma tRNA$^{\text{Gly}}$ has a specifically enhanced ability to make unconventional codon readings. Such reading properties would indeed be required if this tRNA$^{\text{Gly}}$ is the only glycine isoacceptor tRNA in M. mycoides (assuming that all four glycine codons are actually used in this organism).

The tRNA$^{\text{Gly}}$ (anticodon N/UCC) made unconventional codon readings (codons GGU and GGC) with a relative reading efficiency comparable to that which we have previously observed for such readings in the valine codon family (9), the efficiency being of the order of 10-20% of that of the competing tRNA which reads the codons by conventional methods. However, the present investigation of the reading properties of the glycine tRNA isoacceptors has also yielded examples of tRNAs that were unusually inefficient in unconventional codon readings. Thus, tRNA$^{\text{Gly}}$ (anticodon CCC) read both GGU and GGC with an efficiency which was only 2-4% of that of the competing conventional reader. On the other hand, the reading of GGA by tRNA$^{\text{Gly}}$ was rather efficient indicating that the mispair C-A might have appreciable stability. In marked contrast to the efficiency of tRNA$^{\text{Gly}}$ in reading GGA, the tRNA$^{\text{Gly}}$ (anticodon CCC) read this codon with an efficiency which was only 2% of that of the conventional reader, tRNA$^{\text{Gly}}$.

**DISCUSSION**

The two-out-of-three hypothesis makes several predictions that can be tested experimentally. However, in this context it might be appropriate to consider also some predictions that have been mistakenly ascribed to the hypothesis. For instance, the hypothesis does not imply that family codons in vivo are normally read by two-out-of-three even in situations where the prerequisites for conventional codon reading are at hand. It only requires that in such circumstances the family codons should be read by two-out-of-three with a frequency that is not negligible, i.e. we assume it to be higher than the translational error frequency. Even on this limited assumption such reading would become a potential source of translational error and, therefore, also an important restriction in the evolution of the genetic code as pointed out above. Furthermore, the hypothesis does not necessarily require that two-out-of-three reading in vivo should be efficient enough to sustain protein synthesis on a level compatible with life in cases where the cell has lost an isoacceptor tRNA by mutation (24-26). In what follows we will discuss our results from protein synthesis in vitro in relation to the predictions of the two-out-of-three hypothesis and recent results from the analysis of tRNA genes and their gene products (27-29).

The results of the experiments involving glycine codon reading under noncompetitive conditions indicate that the isoacceptor glycine tRNAs tested, tRNA$^{\text{Gly}}$ (anticodon CCC), tRNA$^{\text{Gly}}$ (anticodon N/UCC), tRNA$^{\text{Gly}}$ (anticodon GCC), all from E. coli, and tRNA$^{\text{Gly}}$ (anticodon UCC) from M. mycoides, can each read all four glycine codons in the MS2 coat protein cistron under the present conditions of protein synthesis in vitro. However, in the experiments with tRNA$^{\text{Gly}}$ the in vitro system has a tendency to produce protein of lower molecular weight than coat protein. The reason for this is not clear, but one possible explanation would be that it has to do with the juxtaposition of two GGU codons in the coat protein cistron corresponding to amino acid residues 73 and 74 (23). The necessity of reading two consecutive GGU codons by an unconventional method might be accompanied by a high frequency of frameshift (+1) leading to a premature termination of the synthesized polypeptide chain when the first stop word is encountered in the new reading frame. Experiments to determine if this explanation is correct are in progress.

**Mispairs in Unorthodox Codon Reading**—The results of the competition experiments involving tRNA$^{\text{Gly}}$ (anticodon CCC) suggest a significant stability for the mispair C-A under our conditions of protein synthesis in vitro. While tRNA$^{\text{Gly}}$ is rather inefficient in the reading of the codons GGU and GGC, where the mispairs formed are C-U and C-C, respectively, it is more efficient in the unconventional reading of the codon GGA involving the formation of the mispair C-A between the third codon nucleotide and the reading anticodon. This result is in good agreement with the report by Stern and Schulman (30) that the tRNA$^{\text{Gly}}$ from E. coli, which contains N4-acetylcytidin in the wobble position and does not recognize the
isoleucine codon AUA, can be deacetylated and will now incorporate its amino acid in response to poly(A,U). They suggest that the acetylation of C in the tRNAs\textsubscript{Mec} is to prevent it from recognizing A in the third codon position. Our results would seem to support this conclusion. In the same vein, it is tempting to speculate that the tRNA\textsubscript{Gy} is so inefficient in the reading of the codons GGU and GGC because the mispairs C-U and C-C have very low stability and make no contribution, or perhaps even a negative one, to the overall stability of the codon-anticodon interaction. On the other hand, the low efficiency of tRNA\textsubscript{Gy} (anticodon GCC) in the reading of the codon GGA is difficult to explain as being caused by some particularly unfavorable quality of the mispair G-A since the same mispair occurs in the reading of the valine codon GUA by the tRNA\textsubscript{Y} (anticodon GAC) which is fairly efficient particularly unfavorable quality of the mispair G-A since the low efficiency of tRNA\textsubscript{Y} in the reading of the valine codon GUA by the tRNA\textsubscript{Y} (anticodon GAC) which is fairly efficient (3). Furthermore, our results with the glutamate and lysine codons (5) clearly indicate that the presence in an unconventional reading of the mispair C-A is not per se enough to ensure a high reading efficiency. This is illustrated by the experiments summarized in Table III where we have tried to assess the importance of the Watson-Crick base pairs between the anticodon and the first two codon nucleotides by comparing the efficiencies of readings that involve the same mispair between the wobble position and the third codon position, in this case the mispair C-A. It is immediately apparent from the results in Table III that the efficiency of the unconventional reading is a function of the ratio of G-C to A-U pairs in the interaction between the anticodon and the first two codon nucleotides as required by the two-out-of-three hypothesis. It is also obvious that an unconventional reading may involve the mispair C-A and still be very inefficient as in the reading of the lysine codon AAA by the tRNA\textsubscript{Gy} (anticodon CUU).

Unconventional Methods in the Cell's Normal Repertoire of Codon Readings—In its original version the two-out-of-three hypothesis regards such reading mainly as a potential source of translational errors and attempts to explain how it could have acted as a restriction in the evolution of the genetic code. One may then ask if there is any experimental evidence that two-out-of-three reading could cause translational errors in vivo. In this context it is important to emphasize that the most easily observed unconventional codon readings in vivo are those that actually lead to translational errors. It is possible to detect translational errors in the synthesized protein by two-dimensional gel electrophoresis provided that the translational error leads to an amino acid substitution that produces a difference in charge. For instance, substituting lysine for asparagine would result in a new protein spot on the gel, so-called stuttering. Using stuttering as an indication of translational error Parker and co-workers (31, 32) have shown that an asparagine auxotroph of \textit{E. coli}, containing a rel mutation, when starved for asparagine will incorporate lysine in response to the asparagine codons using what appears to be a two-out-of-three reading mechanism. It would, therefore, seem that at least in this particular genetic background, amino acid starvation can increase two-out-of-three reading to a point where it will produce a high error frequency \textit{in vivo} even in the reading of codons with the lowest probability of such reading. We may thus assume with some confidence that this alternative codon reading method can function as a potential error-producing mechanism \textit{also in vivo}. What, then, about the possibility that in some ecological niche it might actually sustain protein synthesis on a level compatible with life?

The analysis of the mitochondrial genome and its gene products from human cells as well as from yeast and \textit{Neurospora} has shown that in these organelles codon families are each read by only one tRNA. Such tRNAs have in the wobble position with the exception of the yeast tRNA\textsubscript{Gy} reading the arginine family CGN which has I (or possibly A) in this position. Outside the families, codons of the type NN\textsubscript{A} are read by tRNAs with U (or substituted U) in the wobble position, while NN\textsubscript{C} codons are read by tRNAs with G in this position (27–29). In all cases where we have information on the primary structure, tRNAs that read family codons have an unsubstituted U in the wobble position while the tRNAs that read nonfamily codons in yeast and \textit{Neurospora} have this U substituted. The initiator tRNAs are special in that they always contain C in the wobble position as do the methionine tRNAs from yeast and \textit{Neurospora}. These findings might indicate that a two-out-of-three reading mechanism operates in the mitochondrion (27, 28). However, it is equally possible, as pointed out by Heckman et al. (29), that in the mitochondrion U in the wobble position of the anticodon can form stable base pairs with both U and C in the third position of the codon (33).

It is not possible yet to decide what mechanism operates in unconventional codon reading in the mitochondrion, whether it is of the two-out-of-three or the \textit{"U reads all"} type or whether the actual method used incorporates elements of both these mechanisms. Are there any indications, then, that also in cytoplasmic protein synthesis \textit{in vivo} a codon family may be read by a single tRNA? Kilpatrick and Walker (14) have recently reported that \textit{M. mycoides} sp. \textit{capri} contains only one glycine tRNA and that this tRNA has an unsubstituted U in the wobble position. Nothing is known about codon usage in this \textit{Mycoplasma} but unless there is an absolute bias against the glycine codons GGU and GGC the mycoplasma glycine tRNA must be able to read these codons. We have tested this tRNA in our \textit{in vitro} protein-synthesizing system and have found that it was almost as efficient in the unorthodox reading of the codons GGU and GGC as it was in conventional reading (Table II). This, of course, the result to be expected for a tRNA that had been specifically designed to read all four codons in a family. The possibility that this tRNA might be generally superior to the competing glycine tRNAs from \textit{E. coli} in all codon readings and not only in the conconventional ones is ruled out by the finding that it was less efficient than the \textit{E. coli} tRNA\textsubscript{Gy}, with the anticodon N/ UCC, in the reading of GGA. This superior ability of the \textit{Mycoplasma} glycine tRNA to make unconventional codon readings is probably not inherent in the wobble nucleotide U per se since preliminary data using the mutant tRNA\textsubscript{Gy}, with

### Table III

| Codons | Mispair involved | Competing anticodons | Relative efficiency of unconventional reading as a percentage of normal reading | Mean value ± S.D. |
|--------|------------------|----------------------|---------------------------------------------------------------------------------|------------------|
| GGA    | C-A              | CCC                  | 15 ± 5                                                                          |                  |
| GGA    | C-A              | UCC                  | 11 ± 1                                                                          |                  |
| CAA    | C-A              | NCC                  | 2.8 ± 0.5                                                                       |                  |
| AAA    | C-A              | sUUU                 | 0.5 ± 0.25                                                                      |                  |

The data have been compiled from the present investigation and Ref. 5.
the anticodon UCC, which is derived from tRNA\textsuperscript{gly} by a change from G to U in the wobble position, indicate that this tRNA does not show the same reading properties as the mycoplasma tRNA\textsuperscript{gly}. Nor can the enhancement of unconventional codon reading displayed by the mycoplasma tRNA\textsuperscript{gly} possibly be explained by some property of the \textit{E. coli} ribosome. It would, therefore, seem that the structural context provided by the mycoplasma tRNA molecule enhances the ability of the anticodon to make unconventional codon readings. This is reminiscent of the report by Yarus and co-workers \citep{34,35} that the ability of an anticodon to suppress a nonsense mutation is influenced by its structural context in the tRNA molecule.

CONCLUDING REMARKS

The two-out-of-three hypothesis suggests that a codon may in certain instances be read by an anticodon that cannot make a good base pair with the third codon nucleotide. The hypothesis proposes that this type of unconventional codon reading, which is easily demonstrable \textit{in vitro}, could have acted as a restriction that in some respects guided the evolution of the genetic code. Thus, codons that are more amenable to reading by two-out-of-three would only be used in the codon families, while outside the families only codons which are less amenable to reading by two-out-of-three would only be used in the codon families, and our results with the alanine, glycine, and valine codons and the first two codon nucleotides but also the nature of the third nucleotide of the codon is of importance for the efficiency of the genetic code.

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