Site-directed Mutagenesis of Yeast eEF1A
VIABLE MUTANTS WITH ALTERED NUCLEOTIDE SPECIFICITY*

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Site-directed mutants of eEF1A (formerly eEF-1a) were generated using a modification of a highly versatile yeast shuttle vector (Cavallius, J., Popkie, A. P., and Merrick, W. C. (1997) Biochim. Biophys. Acta 1350, 345–358). The nucleotide specificity sequence NKMD (residues number 153–156) was targeted for mutagenesis, and the following mutants were obtained: N153D (DKMD), N153T (TKMD), D156N (NKMN), D156W (NKMW), and the double mutant N153T,D156E (TKNE). All of the yeast strains containing the mutant eEF1As as the sole source of eEF1A were viable except for the N153D mutant.

Most of the purified mutant eEF1As had specific activities in the poly(U)-directed synthesis of polyphenylalanine similar to wild type, although with a 1–2 orders of magnitude. The mutants showed a reduced rate of GTP hydrolysis, and most displayed misincorporation rates greater than wild type. The mutant NKMW eEF1A showed unusual properties. The yeast strain was temperature sensitive for growth, although the purified protein was not. Second, this form of eEF1A was 10-fold more accurate in protein synthesis, and its rate of GTP hydrolysis was about 20% of wild type. In total, the wild-type protein contains the most optimal nucleotide specificity sequence, NKMD, and even subtle changes in this sequence have drastic consequences on eEF1A function in vitro or yeast viability.

Eukaryotic elongation factor 1A (eEF1A) binds aminoacyl-tRNAs in a GTP-dependent manner and positions the bound aminoacyl-tRNA in the A site of the ribosome. After or concomitant with the proper recognition of codon and anticodon, GTP is hydrolyzed, and eEF1A-GDP is released from the ribosome allowing for peptide bond formation with the peptidyl-tRNA in the ribosomal P site.

Besides being involved in the synthesis of every peptide bond, eEF1A is an exceptionally abundant protein comprising 1–3% of the soluble protein in most eukaryotic cells. Elongation factor 1A amino acid sequences have been inferred from more than 100 different organisms including bacteria, archaeabacteria, plants, and animals. The relatively slow rate of change of the sequence of EF1A as it evolved into eEF1A has made it an excellent sequence for determining phylogenetic trees. In large measure, it would seem that the overall tertiary structure has probably been maintained given the 33% identity and 56% similarity of Escherichia coli EF1A (formerly EF-Tu) with human eEF1A, and a discussion of the evolution of the EF1A into the eEF1A sequence has been published (1).

Beyond its role in protein synthesis, eEF1A has also been of interest as a member of the G protein family, as the only crystal structures known are for EF1A (2), EF2 (formerly EF-G) (3), Ras (4), and transducin (5). Crystals, but no structure, have been reported for archaebacterial EF1A (6). The very high homology between EF1A and eEF1A has allowed us to use the available crystal structures for EF1A to model the changes we have made in the yeast eEF1A mutants. Many attempts have been made to mutate GTP utilizing proteins to make them use XTP (7–10), including the prokaryotic counterpart to eEF1A, EF1A (11, 12).

To study the nucleotide specificity of eEF1A, site-directed mutagenesis has been used to alter the binding pocket for the nucleotide in domain I of eEF1A. To do this, a highly efficient chromogenic selection system on a shuttle vector was used (13). We have made five mutants of yeast eEF1A at asparagine 153 and aspartic acid 156, the conserved amino acids in the nucleotide specificity sequence, NKMD. Several yeast strains grow exclusively on the mutant form of eEF1A, and the NKMN mutant eEF1A, when purified, uses XTP just as well as the wild type uses GTP. Curiously, the yeast strain for the NKMW mutant was temperature-sensitive, whereas the protein itself was not.

EXPERIMENTAL PROCEDURES

Yeast Strains—The yeast strains M214 and M213 were generous gifts from Drs. M. G. Sandbaken and M. R. Culbertson, and both had the following genotype: Muta leu2-3, 112 Dde1::LEU2 tef2-D2 lys2-20 his3-1734 sole to indicate this fact.

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Heat shock for 15 min was performed at 42 °C in a water bath. The cells mM EDTA was added and incubated for 30 min at 30 °C with agitation. The cells was thawed on ice and mixed with up to 5
was plated on selective supplemented minimal medium (SMM) plates
were made using the Promega Wizard Minipreps DNA purification
of 2.5, concentrated by centrifugation (15 min at 4,000 x g), and stored
as a cell pellet at liquid nitrogen vapor temperature until purification was performed according to a scheme modified from Curvalho et al. (16). After
several passages on 5-FOA plates (except for the N153D, JC2, mutant),
the mutant yeast strains were grown in standard YPD media to an A600
mM MgCl2, 10% glycerol, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, pH 8.0, 0.1 μM aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation at 12,000 x g for 20 min. The supernatant was added to DEAE-cellulose (DE52, Whatman) pre-equilibrated with buffer 1 (20 mM Tris-HCl, pH 7.5, 25% glycerol, 1 mM DTT, 0.1 mM EDTA, pH 8.0, 0.1 μM aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride) with 100 mM KCl. The unbound material and the wash were then added to phosphocellulose (P-11, Whatman) pre-equilibrated with buffer 1 with 100 mM KCl. The phosphocellulose-bound protein was washed with buffer 1 with 100 mM KCl and then made 0.5 mM KCl by adding solid KCl. The released material and wash was dialyzed overnight to yield a
dialyzed solution that was 50 mM with respect to KCl. The supernatant was applied to a CM-cellulose column (CM52, carboxymethyl cellulose, Whatman) pre-equilibrated with buffer 1 with 50 mM KCl. The column was eluted with a linear salt gradient (total of 10 x column volume) from 50 to 300 mM KCl in buffer 1. Purity, as checked by SDS-polyacrylamide gel electrophoresis, was found to be approximately 98%.
The DKMD mutant (JC2), purified as described above, was dialyzed overnight in buffer 1 with 100 mM KCl and 1 mM Mg(CH3CO2)2. The dialyzed solution was mixed for 1 h at 4 °C with GTP-agarose pre-equilibrated with buffer 1 with 100 mM KCl and 1 mM Mg(CH3CO2)2. The slurry was packed into a 0.9 x 14-cm tube. The run-through was collected and reapplied to the column at a flow rate of 5 ml/h. The column was washed with buffer 1 with 100 mM KCl and 1 mM Mg(CH3CO2)2. The wash-through, containing the mutant JC2, DKMD, was concentrated on a phosphocellulose column pre-equilibrated with buffer 1 with 100 mM KCl. Protein was eluted from the column with 200 mM KCl. The eluate was dialyzed against a 100 mM KCl

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The target for site-directed mutagenesis was the NKMD yeast eEF1A, and the yeast strains were analyzed for growth on nonselective media, where both the wild-type and mutant eEF1A would be expressed, and on selective media (5-FOA), where the mutant eEF1A would be the sole source of the yeast eEF1A.

RESULTS

The target for site-directed mutagenesis was the NKMD sequence of eEF1A, known as the nucleotide specificity sequence. Fig. 1A shows how in eEF1A the hydrogen bonding pattern was tested by Hwang and Miller (11) who changed the NKMD sequence to NKXN. As illustrated in Fig. 1A, this allows for the same network of hydrogen bonds but with the nucleotide XTP, not GTP. In their studies, the EF1A NKMD mutant had the same affinity for XTP as the wild-type protein did for GTP. However, the NKXN mutant of EF1A was unable to support the growth of _E. coli_ as the sole source of the elongation factor.

We have decided to try a similar experiment in eukaryotic cells to see if it was possible to isolate and purify mutant eEF1A proteins. As a number of investigators have been unable to express eEF1A in soluble form in _E. coli_, we chose to use the yeast protein and purify the expressed proteins from yeast. In addition, we have attempted to alter the nucleotide specificity of the eEF1A based upon the observation of some differences in the NKXN nucleotide specificity sequence that have been reported for a number of proteins that utilize GTP (24). These changes are indicated in Table I. The wild-type NKMD and the mutant NKXN sequences were discussed above. The NKXW sequence is found in two enzymes, phosphoenolpyruvate carboxykinase and GTP:AMP phosphotransferase. Both of these proteins will use ITP as well as GTP. The TKXW sequence is found in the enzyme guanyltransferase. Here, one could imagine that because the glutamic acid residue is one methylene group longer than aspartic acid, a compensatory shortening would have to occur at the position normally occupied by asparagine. In this instance, the threonine residue provides the hydroxyl group, and this group is one methylene group closer to the main chain. Thus, one would expect to maintain the GTP specificity, although the possible loss of one or more of the hydrogen bonds labeled G, F, or H in Fig. 1A, might result in a slight increase in the _K_d for GTP. Finally, it was thought that the substitution of an aspartic acid in place of the asparagine would weaken the binding for GTP and perhaps even allow a hydrogen bond between the aspartic acid and the 6-amino group of ATP. Thus, these substitutions were made in yeast eEF1A, and the yeast strains were analyzed for growth on nonselective media, where both the wild-type and mutant eEF1A would be expressed, and on selective media (5-FOA), where the mutant eEF1A would be the sole source of the eEF1A.

Table II presents the characteristics of the yeast strains with mutant eEF1A species. All of the yeast strains that contained both host and mutant eEF1A were viable, indicating that none

with the ribose and phosphate moieties, but these are not shown as they do not contribute to nucleotide specificity. The numerous hydrogen bond possibilities likely account for the fact that the _K_d for GTP in G proteins (GTP-binding proteins associated with signal transduction) is often about 1 _μM_, whereas that for ATP-utilizing proteins is in the 50–500 _μM_ range. A proof of the hydrogen bonding pattern was tested by By analogy to reovirus guanylyltransferase (28).

**GTPase Activity and Stimulation of GTPase Activity by Aminoacyl-tRNA and Ribosomes—GTPase activity was measured as described by Merrick (19) with the following modifications. Hydrolysis of ['gamma-32P]GTP was performed in a 20-μl reaction containing 25 mM Hepes, pH 7.5, 125 mM KCl, 8.5 mM MgCl₂, 1 mM DTT, 6.25% glycerol, 15 pmol of eEF1A, 100 _μM_ GTP, and, when indicated, 10 pmol of aminoacyl-tRNA (all 20 amino acids) and/or 0.7 _A_₂₆₀ units of sucrose cushion ribosomes and/or 0.15 _A_₂₆₀ units of poly(U). The reaction mixture was incubated for 15 min at 37 °C and then placed on ice; the following additions were then made in order: (a) 0.5 ml of 20 mM silicotungstate in 20 mM H₂SO₄; (b) 1.2 ml of 1 M K₂HPO₄, pH 7.0; (c) 0.5 ml of 5% ammonium molybdate in 4 M H₂SO₄; and (d) 0.3 ml of 5% trichloroacetic acid:acetone (1:1). After these additions, the free ['gamma-32P]GTP was extracted with 2 ml of isobutanol:benzene (1:1), and the liquids were mixed by vortexing for 30 s. After centrifugation for 3 min at 1500 rpm in a Beckman model TJ-6 centrifuge at 4 °C, a 1-ml aliquot of the organic (upper) phase was removed and mixed with CytoScint from ICN. The amount of hydrolyzed ['gamma-32P]GTP was determined by liquid scintillation spectrometry.

**Table I**

| Amino acid sequence | Expected nucleotide specificity | Strain name |
|---------------------|-------------------------------|-------------|
| NKMD#               | GTP                           | Wild type   |
| NKXN#               | XTP                           | JC6         |
| NKXXW#              | GTP or ITP                    | JC33        |
| TKXX#               | GTP                           | JC5 and JC32|
| DXKX#               | ATP                           | JC2         |

# This is the wild-type eEF1A sequence (25).
# By analogy to the crystal structure and experiments involving EF1A (11, 12).
# By analogy to cytosolic phosphoenolpyruvate carboxykinase (26) and GTP:AMP phosphotransferase (27).
# By analogy to reovirus guanylyltransferase (28).

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**Fig. 1. Amino acid residues in the nucleotide binding pocket adapted from Kjeldgaard and Nyborg (2).** Illustrated are the putative hydrogen bonds associated with the guanine ring (A) as determined in the crystal structure of EF1A (2). Each of the hydrogen bonds was given a letter (bold italics). Panel B illustrates the alternative hydrogen bond possible between the NKMN mutant eEF1A and XTP. Note that for this mutant eEF1A, the binding of GTP would occur with 1 less hydrogen bond and possibly some steric hindrance between the amide group of asparagine and the amino group of GTP. Further, note that many additional hydrogen bonds to the ribose or phosphates would be expected to be the same for the mutants and wild-type eEF1A.

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**TABLE I**

**Anticipated nucleotide specificity**

Nucleotide binding specificity sequences from different proteins and their found or expected nucleotide specificity. The letter X indicates that any of the 20 amino acids could be in this position.

| Amino acid sequence | Expected nucleotide specificity | Strain name |
|---------------------|-------------------------------|-------------|
| NKMD#               | GTP                           | Wild type   |
| NKXN#               | XTP                           | JC6         |
| NKXXW#              | GTP or ITP                    | JC33        |
| TKXX#               | GTP                           | JC5 and JC32|
| DXKX#               | ATP                           | JC2         |
of the mutant eEF1As were behaving as dominant negative mutants. When grown on selective media, only the DKMD mutant, JC2, failed to grow, although the NKMW mutant, JC33, did display a temperature-sensitive phenotype with wild-type growth rates at or below 18 °C and no growth at elevated temperatures. Within experimental error, the yeast strains containing just the mutant form of eEF1A grew in liquid media at wild-type growth rates. Similarly, growth on alternate carbon sources (glycerol, lactose, or sucrose) did not show any difference between yeast with the wild-type or mutant eEF1As (data not shown).

To examine the nucleotide specificity of the eEF1A species, the proteins were purified as described previously except for the DKMD mutant eEF1A. As yeast containing only this form of eEF1A were not viable, the yeast containing both the mutant and wild-type eEF1A were grown. The eEF1A was purified, and the wild-type protein was separated from the mutant by GTP-agarse chromatography. The first test of the mutant eEF1As was their ability to direct the synthesis of polyphenylalanine on poly(U)-programmed ribosomes. An Eadie-Hofstee plot for the wild-type eEF1A is shown in Fig. 2A. The apparent scatter in the data likely reflects that for polyphenylalanine synthesis, there are two GTP-requiring enzymes, eEF1A and eEF2. eEF2 promotes the translocation of the mRNA and the anticodon ends of the tRNAs during polypeptide synthesis (for an overview, see Ref. 20). In contrast, plots of the enzymatic activity of the mutant eEF1As yielded linear plots with little error (Fig. 2B and Table III). In large measure, this reflects the fact that the $K_m$ values for the mutant eEF1As were well above the wild-type $K_m$ (estimated to be about 0.14 $\mu$M)$^2$ ranging from 2.7 to 13.1 $\mu$M.

Of the mutant eEF1As examined, most of the mutant eEF1As had a specific activity equivalent to wild type (i.e., as seen as $V_{max}$). The TKMD mutant eEF1A was about 50% as active as wild type. However, if additional protein was added, it was possible to achieve the same high rate of protein synthesis possible with wild-type protein. In contrast, the NKMW mutant eEF1A was quite different. With the highest concentration of the NKMW mutant eEF1A tested, only about 35% of the maximal rate of polyphenylalanine synthesis was achieved. Possible causes for the reduced rates of protein synthesis are examined below.

### Table II

| Mutant yeast amino acid no.$^a$ | Expected nucleotide specificity | In yeast$^b$ | Doubling time as % of wild type$^c$ | Yeast strain |
|-------------------------------|-------------------------------|-------------|-------------------------------|-------------|
| Wild type [eEF1A on URA-3 YCpMS29] | NKMD | No | Yes | 100 | M213 |
| Wild type [eEF1A on TRP-1 YCpMS41] | No change (NKMD to TKMD) | No | Yes | 100 | M214 |
| N153T, D156E | GTP to ATP (NKMD to DKMD) | No | No | JC2 |
| N153D | GTP to ATP (NKMD to DKMD) | No | Yes | 114 | JC5 |
| D156W | GTP to ITP/GTP (NKMD to NKMW) | No | Yes | 113$^d$ | JC33 |
| D156N | GTP to XTP/GTP (NKMD to NKMN) | No | Yes | 107 | JC6 |

$^a$ Mutant designation: wild-type amino acid-residue number-mutant amino acid

$^b$ TS, temperature-sensitive. "Lethal" indicates whether the presence of the mutant eEF1A causes the yeast to not grow (i.e., dominant negative phenotype). "Viable" indicates whether the mutant eEF1A allows growth of the yeast as the sole source of eEF1A.

$^c$ Doubling time of wild type was 135 min at 30 °C.

$^d$ Wild-type doubling time at 18 °C was 310 min.

An examination of the ability of the various mutant forms of eEF1A to use alternate nucleotides indicated that the NKMW mutant utilized XTP with the same affinity as the wild-type protein had for GTP (Table IV), as was also observed with the equivalent substitution into EF1A (19). However, the NKMW protein was also capable of utilizing GTP, but with a much higher $K_m$ value. This ability to use GTP explains why this mutant could serve as the sole source of eEF1A in the absence of an XTP pool in yeast. With the normal intracellular concentration of GTP in cells in the hundreds of micromolar range, the protein would be well saturated with GTP. At the same time,
TABLE III
Km and Vmax for wild-type and five mutant eEF1As

*Km* and *Vmax* values were derived from poly(U) assays using 10 pmol of eEF1A (except for mutants NKMN and TKME, where 15 pmol were used) and calculated in an Eadie-Hofstee plot (see Fig. 1).

| eEF1A source | Km (μM) | Vmax (pmol Phe) |
|--------------|---------|-----------------|
| Wild type    | 0.14    | 2.4             |
| JC2, DKMD    | 2.7     | 2.4             |
| JC5, TKMD    | 6.0     | 1.1             |
| JC6, NKMN    | 13.1    | 2.6             |
| JC32, TKME   | 10.3    | 2.5             |
| JC33, NKMW   | 4.2     | 0.3             |

TABLE IV
Km and Vmax for the NKMN mutant eEF1A

*Km* and *Vmax* values were derived from poly(U) assays using 15 pmol of eEF1A and calculated in an Eadie-Hofstee plot (see Fig. 1).

| Nucleotide | Km (μM) | Vmax (pmol Phe) |
|------------|---------|-----------------|
| GTP        | 13.1    | 2.6             |
| XTP        | 0.18    | 2.5             |

As indicated above, most of the mutant forms of eEF1A were capable of sustaining wild-type growth rates and could achieve the same maximal velocity in polyphenylalanine synthesis as the wild-type protein. The single exception is the NKMW mutant of eEF1A, which yielded a yeast strain that was temperature sensitive and a purified protein that was only about 13% of the wild-type rate of polyphenylalanine synthesis (Table III). As this form of eEF1A came from a temperature-sensitive yeast strain, it was possible that this form of eEF1A was less stable to purification; however, efforts to improve the recovery of activity by increasing the glycerol concentration during purification were unsuccessful. Therefore, a direct test of the thermotolerance of the protein was made. As is evident from Fig. 4, the NKMW mutant eEF1A was actually more thermostable than the wild-type protein.

As an independent check of the activity of the various forms of eEF1A, a nucleotide-dependent binding of Phe-tRNA to ribosomes assay was performed with GTP and with GDPNP. The assay with GDPNP should determine the number of active molecules, as there is a one-to-one stoichiometry when GTP hydrolysis is blocked. In this assay, the eEF1A proteins were routinely 20–50% active, and there seemed to be only slight differences between the wild-type and mutant proteins except for the NKMW mutant, which displayed only about 25% of the wild-type activity (data not shown). When GTP was used as the nucleotide substrate, the binding increased by a factor of two or three, indicating that the eEF1A species were catalyzing more than a single binding event. However, the NKMW mutant of eEF1A showed no stimulation of binding under these conditions (data not shown). To more directly address the deficiency in the NKMW mutant eEF1A, all of the eEF1A species were assayed for their ability to catalyze GTP hydrolysis in the presence of activators (aminoacyl-tRNA, ribosomes, and poly(U)). The results in Table VI indicate that all of the mutant forms of eEF1A show a reduced rate of GTP hydrolysis. Although most of the mutants seemed to be about half as active, the NKMW mutant eEF1A was only about 20% as active. This very low level of GTP hydrolysis is consistent with the observation that the NKMW mutant eEF1A showed no stimulation in the Phe-tRNA binding assay when GTP was substituted for GDPNP.
A final test of the function of the eEF1A proteins was that of misincorporation. In this assay, equal molar amounts of \(^{[1}\text{H}]\text{Leu-tRNA}\) and \(^{[14}\text{C}]\text{Phe-tRNA}\) are added, and the ratio of \(^{[1}\text{H}]\text{Leu-tRNA}\) to \(^{[14}\text{C}]\text{Phe-tRNA}\) labeled protein is determined. The wild-type protein had a misincorporation rate of about 3%, a value similar to that reported previously in this assay using bacterial components (21). Most of the mutant proteins displayed misincorporation rates greater than wild type, although the maximal difference was less than 2.5-fold (Table VII). Surprisingly, the relatively inactive NKMW mutant eEF1A showed a misincorporation rate that was roughly 10% that of wild type, indicating that this form of eEF1A was about 10-fold more accurate than the wild-type protein.

In an attempt to correlate nucleotide affinity with error rate, the two eEF1A mutants that would use more than a single nucleotide were tested for their error rates. The idea here was that each of the eEF1A proteins might have a slightly different nucleotide specificity sequence. As this was done (see Table VIII), it was observed that the nucleotide with the lowest \(K_m\) value led to the least misincorporation. However, given the almost 2 order of magnitude difference in \(K_m\) values of the NKMN mutant eEF1A and only a 30% increase in misincorporation, it is clear that a direct linear correlation of nucleotide affinity and misincorporation rate does not exist.

**DISCUSSION**

Although the attempts at site-directed change in nucleotide specificity could have failed, as there is no endogenous pool of either ITP or XTP in yeast, the *in vitro* analysis of the purified proteins provided an answer as to why these efforts were successful. All of the mutant proteins were able to use GTP as the nucleotide triphosphate for the synthesis of polyphenylalanine. As expected from the studies with EF1A, the NKMN mutant eEF1A was able to use XTP as the nucleotide triphosphate and did so with the same apparent \(K_m\) for XTP as the wild-type protein exhibited for GTP. Additionally, this same protein was able to use GTP, although with reduced affinity. However, as the cellular concentration of GTP is in the hundreds of micromolar range, this mutant would have been fully saturated with GTP, as its \(K_m\) for GTP was 13 \(\mu\)M, about 2 orders of magnitude greater than the \(K_m\) for XTP and 1 order of magnitude less than the physiologic concentration of GTP. All of the mutants preferred GTP over other nucleotide triphosphates except for the NKMN mutant, which preferred XTP. Based upon studies where overexpression of eEF1A obviated the need for the recycling protein eEF1B (22), it is anticipated that all of the mutants will allow for growth in the absence of the recycling protein (this assumes that the \(K_m\) for GTP and GDP is the same as is observed with the wild-type protein). This hypothesis will be tested in the near future.

Another *in vitro* characteristic of the mutant eEF1A proteins is that they all seemed to have similar specific activities (as judged in the poly(U) assay or the nitrocellulose filter assay) except for the NKMW mutant. As this eEF1A was derived from a temperature-sensitive strain of yeast, it was anticipated that the lower specific activity reflected a thermal denaturation of the protein during the purification process. However, a direct test of the thermolability of this eEF1A indicated that, if anything, this mutant eEF1A was somewhat more temperature stable than wild type (Fig. 4). Thus, at the present time we have no simple explanation for the temperature sensitivity of this yeast strain (JC33, D156W) nor the low specific activity of the NKMW mutant protein, although these effects may possibly be explained by the relatively low level of GTPase activity observed with this eEF1A (see Table VI).

As might be expected, it would seem that nature has selected the ideal eEF1A nucleotide specificity sequence. As this sequence is anticipated to be in a loop (based upon analogy with EF1A), it was thought that the site-directed mutants would not alter the tertiary structure of eEF1A to any large degree. Despite the anticipated subtle changes, all of the mutant eEF1As exhibited a considerable change in \(K_m\) for GTP. This change was partially anticipated based upon the disruption of the hydrogen bonding network that has been proposed for EF1A based on the crystal structure for either EF1A-GDP or EF1A-GDPNP (see Fig. 1). Further examination revealed that all of the mutant proteins also showed a reduced rate of GTP hydrolysis. The fact that only the NKMW mutant eEF1A displayed a reduced \(V_{max}\) in polyphenylalanine synthesis (*i.e.*, the synthesis rate could not be increased with increased protein

### Table VI

**GTPase activity in mol Pi/mol eEF1A**

Values are shown as mol of free phosphate released from GTP (background corrected) per mol of eEF1A in the assay (10 pmol of eEF1A for all except from mutants JC6 and JC32, where 15 pmol were used). The stimulation of the GTPase activity by the addition of aa-tRNA, ribosomes, and poly(U) is also shown. The free phosphate generation in the assay mixes without eEF1A were 2, 2, 2, and 31 pmol, with no additions, with aa-tRNA, with both aa-tRNA and ribosomes, and with aa-tRNA, ribosomes and poly(U), respectively.

| eEF1A source | Additions | None | aa-tRNA | aa-tRNA ribosomes | aa-tRNA ribosomes poly(U) |
|--------------|-----------|------|---------|-------------------|--------------------------|
| Wild type    |           | 0.6  | 2.1     | 10.1              | 16.7                     |
| JC2, DKMD    |           | 0.1  | 0.5     | 5.2               | 8.8                      |
| JC5, TKMD    |           | 2.8  | 2.3     | 4.5               | 9.8                      |
| JC6, NKMN    |           | 0.9  | 0.7     | 9.0               | 9.3                      |
| JC32, TKME   |           | 0.7  | 0.7     | 3.2               | 8.1                      |
| JC33, NKMW   |           | 0.6  | 0.9     | 2.0               | 4.0                      |

### Table VII

**Misincorporation for wild-type and five mutant eEF1As**

The misincorporation rates were derived from poly(U) assays (using 10 pmol of eEF1A for all except from mutants JC6 and JC32, where 15 pmol were used) using equal amounts of \(^{[1]}\text{H}\text{Leu-tRNA}\) and \(^{[14]}\text{C}\text{Phe-tRNA}\). Asterisk indicates that the observed value was not reliable as several hundred micromolar of GTP was needed to drive the reaction, and the commercial GTP was only 95% pure (possible GTP contamination, see "Results"). 100% corresponds to 3.0 leucines incorporated into a polypeptide chain for every 100 phenylalanines (see the legend to Table VII).

| eEF1A source | Misincorporation using GTP | Misincorporation using ITP | Misincorporation using XTP |
|--------------|---------------------------|---------------------------|---------------------------|
| Wild type    | 100                        | 102                       | 102                       |
| JC2, DKMD    | 234                        | 190                       | 190                       |
| JC5, TKMD    | 151                        | 11                        | 151                       |
| JC6, NKMN    | 190                        | 11                        | 190                       |
| JC32, TKME   | 11                         | 11                        | 11                        |
| JC33, NKMW   | 11                         | 11                        | 11                        |

### Table VIII

**Nucleotide-dependent misincorporation for NKMN and TKME mutant eEF1As**

The misincorporation rates were derived from poly(U) assays (using 15 pmol of eEF1A) using equal amounts of \(^{[1]}\text{H}\text{Leu-tRNA}\) and \(^{[14]}\text{C}\text{Phe-tRNA}\). Asterisk indicates that the observed value was not reliable as several hundred micromolar of GTP was needed to drive the reaction, and the commercial GTP was only 95% pure (possible GTP contamination, see "Results"). 100% corresponds to 3.0 leucines incorporated into a polypeptide chain for every 100 phenylalanines (see the legend to Table VII).

| eEF1A source | Misincorporation using GTP | Misincorporation using ITP | Misincorporation using XTP |
|--------------|---------------------------|---------------------------|---------------------------|
| Wild type    |                           |                           |                           |
| JC2, DKMD    | 102                       | 102                       | 102                       |
| JC5, TKMD    | 234                       | 190                       | 190                       |
| JC6, NKMN    | 190                       | 11                        | 190                       |
| JC32, TKME   | 151                       | 175                       | 183                       |
| JC33, NKMW   | 11                        | 11                        | 11                        |
added) suggests that in most instances the rate-limiting step in
the elongation cycle is likely to be the GTP-dependent translation
step catalyzed by eEF2. In this same vein, the wild-type
eEF1A seemed to be the most accurate form of eEF1A except
for the NKMW mutant eEF1A, which showed a 10-fold greater
accuracy and a 20-fold greater rate catalyzed by eEF2. In this same
vein, the wild-type eEF1A seems to be the most accurate form of eEF1A except
for the NKMW mutant eEF1A, which preferred XTP
over GTP by about 70-fold (Table IV). This failure most likely
reflects two things. First, there is no crystal structure for
eEF1A, and it is possible that there are slightly different con-
straints on the binding of GTP relative to EF1A. These subtle
differences might be anticipated as EF1A has a 100-fold pref-
erence for GDP over GTP, whereas eEF1A binds both nucleo-
tides with about the same affinity. Second, the nucleotide spec-
ificity sequences chosen as alternates were based upon
a crystal structure for
eEF1A, which preferred XTP
over GTP by about 70-fold (Table IV). This failure most likely
reflects two things. First, there is no crystal structure for
N. eEF1A, and it is possible that there are slightly different con-
straints on the binding of GTP relative to EF1A. These subtle
differences might be anticipated as EF1A has a 100-fold pref-
erence for GDP over GTP, whereas eEF1A binds both nucleo-
tides with about the same affinity. Second, the nucleotide spec-
ificity sequences chosen as alternates were based upon
enzymes that use GTP. As these enzymes nominally have $K_m$
values for GTP in the 50–200 μM range, it is possible that the
specificity for GTP does not involve the extensive hydrogen
bonding network evidenced in EF1A. Another view would be
that in the attempt to identify the nucleotide specificity se-
quence based upon consensus elements in the linear amino acid
sequence (24), the incorrect elements had been chosen. Future
studies that provide the crystal structure for each of the model
enzymes and the definition of the elements for GTP specificity
will provide the answer to this dilemma.

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