Recombinant Subunits of Mammalian Elongation Factor 1 Expressed in Escherichia coli

SUBUNIT INTERACTIONS, ELONGATION ACTIVITY, AND PHOSPHORYLATION BY PROTEIN KINASE CKII*

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The first step in elongation requires two different activities; elongation factor (EF)-1α transfers aminoacyl-tRNA to the ribosome and is released upon hydrolysis of GTP, EF-1βγδ catalyzes exchange of GDP on EF-1α with GTP. To analyze the role of the individual subunits of EF-1 in elongation, the cDNAs for the β, γ, and δ subunits of EF-1 from rabbit were cloned, and proteins of 225, 457, and 280 amino acids, respectively, were expressed in Escherichia coli. The purified recombinant β subunit migrates as a dimer and the γ subunit as a trimer upon gel filtration, whereas the δ subunit forms a large aggregate. Complexes of βγ, γδ and βγδ were formed by self-association and eluted with a molecular mass of approximately 160, 530, and 670 kDa, respectively; no interaction was observed between β and δ. The activity of the recombinant subunits was determined with native EF-1α by measuring stimulation of the rate of elongation by poly(U)-directed polyphenylalanine synthesis. Recombinant β and δ alone stimulated the rate of elongation by 10-fold, with a ratio of 5α to 2β or δ. The βγδ complex stimulated EF-1α activity up to 10-fold with a ratio of 20α to 1βγδ. Phosphorylation of the β and δ subunits alone or in βγδ by protein kinase CKII had no effect on the rate of elongation.

Eukaryotic elongation factor (EF)1 consists of four subunits, EF-1α, β, γ, and δ. EF-1α (50 kDa) forms a ternary complex with GTP and aminoacyl-tRNA and transfers the aminoacyl-tRNA to the ribosome and is released upon hydrolysis of GTP. EF-1βγδ facilitates the exchange of the GDP bound to EF-1α for GTP, initiating another round of elongation (1, 2). EF-1β and δ contain the GTP exchange activity (3); γ is tightly associated to β and is removed only under denaturing conditions (4). The cDNAs for β and γ have been cloned and sequenced from a number of different organisms and tissues (5–13). Recently, EF-18 cDNA has been sequenced from human (14) and Xenopus (15), and partial amino acid sequences have been obtained from Artemia (14). A leucine zipper motif in the amino terminus of δ is found in all three species (14). EF-1β and δ have a highly homologous carboxyl terminus that contains the GDP/GTP exchange activity, whereas the amino-terminal domains differ and appear to be important for regulation of EF-1 activity. The function of the γ subunit is unknown, although there is evidence that γ can stimulate the nucleotide exchange activity of β (16). EF-1γ may anchor the complex to the membrane (16) and has been shown to contain a sequence homologous to glutathione S-transferase in the amino-terminal domain, which has been postulated to be involved in regulation of the assembly of multisubunit complexes (17).

Eukaryotic EF-1 has been isolated from a variety of organisms and tissues with molecular weights ranging from 50,000 to about 1 × 106. The low molecular weight form is EF-1α, the intermediate form is EF-1αβγδ, and the high molecular weight form is a complex of five polypeptides, valyl-tRNA synthetase (ValRS) and EF-1αβγδ (18–20).

Using purified subunits of EF-1 from Artemia, the formation of complexes of αβ (21), αβγ, αβγδ, and αδ (4) were observed under nondenaturing conditions. β and γ, separated under denaturing conditions, were unable to reassociate. Reconstitution of a γδ complex was also unsuccessful (4). With purified subunits prepared from rabbit EF-1ValRS, Bec et al. (22) were able to reconstitute the EF-1βγδValRS complex; EF-1δ was required for association of ValRS.

The β subunit of EF-1 from Artemia was shown to be phosphorylated by an endogenous CKII-like protein kinase that copurified with EF-1βγ. The phosphorylation site was Ser99 in the sequence GSDEDEEE. When EF-1βγδ was treated with alkaline phosphatase to remove the phosphate, the nucleotide exchange rate was almost twice that of phosphorylated EF-1βγδ (23). In other studies, EF-1δ from Artemia, rabbit and wheat germ was shown to be phosphorylated by CKII (24). Using recombinant EF-1β from rabbit, Ser96 and Ser132 in the sequence of DLFGS99DEDEES112EEA were phosphorylated by CKII (5).

In this study, the cDNA for rabbit EF-1δ has been cloned and sequenced. Recombinant EF-1β, γ, and δ subunits were expressed in Escherichia coli, purified and reconstituted to form complexes of βγ, βγδ, and γδ, but not βδ, as analyzed by gel filtration. The activity of the individual subunits and reconstituted complexes was measured by stimulation of elongation with native EF-1α, and the effects of phosphorylation of the β and δ subunits and EF-1βγδ on elongation were analyzed.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP was purchased from ICN. [35S]Labeled nucleotides and [2,3,4,5,6-3H]phenylalanine (127 Ci/mmol) were from Amersham Corp. The cDNA for EF-1α from human was a gift from Dr. Wim Moller, University of Leiden, The Netherlands. CKII was purified from rabbit reticulocytes as described previously (25) and provided by Wil-
Cloning and Sequencing of the cDNA for EF-1α from a Rabbit Spleen cDNA Library—To isolate the cDNA for EF-1α from a rabbit spleen library, human EF-1α cDNA was digested with PstI to obtain a 374-bp amplified region and to use as the probe. The cDNA fragment was labeled with the random primer labeling method (26) and used to screen the cDNA library by in situ plaque hybridization. After the third screening, several positive clones were identified and these cDNAs were transformed into E. coli XL1-Blue cells. Reconstitamts plasmids purified from single colonies were cleaved with EcoRI and XhoI followed by hybridization with the radiolabeled probe. The cDNA containing the longest insert was selected for cDNA sequencing and a set of nested deletions (27) was constructed. The longest insert was selected for cDNA sequencing and a set of nested deletions (27) was constructed. The EcoRI/XhoI and XhoI/KpnI sites were chosen for the construction of sense and antisense cDNA deletion clones, respectively. Sequencing of individual cDNA was carried out using the dideoxynucleotide chain termination method (28) with T7 and T3 primers.

Construction of Expression Vectors for EF-1α, γ, and δ—Rabbit EF-1α was cloned from a rabbit spleen cDNA library, sequenced, and expressed in E. coli as described by Chen and Traugh (5). The cDNA for EF-1α, cloned and sequenced from the rabbit spleen cDNA library (13), and the cDNA for rabbit EF-1α were subcloned into the pT7–7 expression vector. Synthetic oligonucleotides were prepared that contained a gene of interest in the 5′-end sense primer (5′-TTTGCAGGATCGCCGCGGGA23-3′) for γ and (5′-AAGCGGATCCAATGACGAGCTTCTAGT7-3′) for δ. The restriction sites are indicated by an underline and the start codon ATG is indicated as bold. The sense and antisense primers were used for amplification of the cDNA by the polymerase chain reaction for 30 cycles (29). The amplified DNA products were analyzed by agarose gel electrophoresis. The purified cDNA fragment corresponding to EF-1α was digested with NdeI and HindIII restriction enzymes to produce the sites for subcloning of the cDNA coding region into the pT7–7 vector. The recombinant plasmids were subcloned into competent BL21(DE3) cells by the CaCl2 precipitation method (30).

Expression of EF-1α, γ, and δ in E. coli—Expression of recombinant EF-1α and γ in E. coli was induced with 0.4 mM IPTG for 2 h at 37 °C, and the supernatant and pellet were prepared as described by Chen and Traugh (5).

An overnight culture of BL21(DE3) cells transformed with pT7–7 containing E. coli containing EF-1α or γ was chromatographed on a DEAE-cellulose column equilibrated with buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.02% NaN3, 1 mM diithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride) as described (5). Elution was carried out with a linear gradient of 0–0.5 M NaCl (200 ml) in buffer for EF-1α, and 0–0.3 M NaCl (200 ml) for EF-1γ. EF-1α or γ was purified further by FPLC on a Mono Q HR5/5 column as described (5) with a 10-mL gradient of 0–1.0 M NaCl in buffer. Aliquots (20 μl) of the fractions were analyzed on a 10% gel by SDS-PAGE and stained with Coomassie Blue. The remainder of the fractions were harvested and the pellet and supernatant were obtained as described above.

Purification of Recombinant EF-1α and γ by DEAE-cellulose Chromatography and FPLC on Mono Q—The supernatant obtained from 220 ml of culture containing E. coli containing EF-1α or γ was chromatographed on a DEAE-cellulose column equilibrated with buffer (50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.02% NaN3, 1 mM diithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride) as described (5). Elution was carried out with a linear gradient of 0–0.5 M NaCl (200 ml) in buffer for EF-1α, and 0–0.3 M NaCl (200 ml) for EF-1γ. EF-1α or γ was purified further by FPLC on a Mono Q HR5/5 column as described (5) with a 10-mL gradient of 0–1.0 M NaCl in buffer. Aliquots (20 μl) of the fractions were analyzed on a 10% gel by SDS-PAGE and stained with Coomassie Blue.

Purification of Recombinant EF-1α—E. coli (500 ml) containing the pT7–7–δ insert were grown at 28 °C for 19 h and harvested, and the supernatant was chromatographed on a DEAE-cellulose column (2.5 × 15 cm). The column was washed with 120 ml of 200 mM NaCl in buffer, and δ was eluted in a single step with 700 mM NaCl in buffer. EF-1α (0.5 ml) was chromatographed on a Superose 12 column equilibrated with buffer containing 50 mM KCl; 0.35-mL fractions were collected. EF-1α was stored in aliquots at −70 °C for further analysis.

Isolation of the EF-1βγ Complex—Equimolar amounts of the β (400 μg) and γ (815 μg) subunits were mixed in a volume of 2.5 ml which was incubated with 1.5 ml of affinity purified δ for 2 h at 37 °C and the mixture was dialyzed 5-fold with buffer to reduce the conductivity. The complex was isolated by chromatography on a DEAE-cellulose column (1.2 × 8.0 cm) and eluted with a 50-mL gradient of 0–500 mM NaCl in buffer. Fractions containing βγ were pooled, and the complex was purified by gel filtration as described above.

Isolation of the EF-1βγδ Complex—Purified EF-1α (30 μg), β (100 μg), and δ (40 μg) in a final volume of 0.6 ml, and purified EF-1γ (50 μg) and δ (65 μg) in a final volume of 1.5 ml were incubated on ice for 30 min. Each sample was analyzed separately by FPLC on Mono Q with a 10-ml gradient of 0–1.0 M NaCl in buffer. Fractions containing the highest amounts of βγδ or γδ were selected for further analysis by gel filtration.

Analysis of Individual Subunits and Complex Formation on Superose 12—EF-1γ and γ (0.5 ml) purified by chromatography on Mono Q were analyzed separately on a Superose 12 column equilibrated with buffer containing 100 or 500 mM NaCl. Fractions of 0.35 ml were collected. Aliquots of 20 μl were analyzed by SDS-PAGE. The protein standards for gel filtration were blue dextran (Vb), thyroglobulin (670 kDa), IgG (150 kDa), β-lactoglobulin (30 kDa), and cytochrome C (12 kDa).

Aliquots (0.5 ml) of the EF-1γδ complex isolated by DEAE-cellulose chromatography and EF-1βγδ and γδ isolated by FPLC on Mono Q were analyzed separately on a Superose 12 column at 4 °C. EF-1γ (40 μg) and δ (17 μg) incubated together (0.5 ml) on ice were also analyzed for complex formation. The column was equilibrated with 200 mM KCl in buffer. Fractions of 0.35 ml were collected. Aliquots (20 μl) from each fraction were analyzed by gel electrophoresis, and the proteins were visualized by staining with Coomassie Blue.

The stained proteins in the gels were scanned with a LKB laser scanner and quantified. Similar results were obtained when the ratio of each subunit in the complexes was determined according to the content of basic residues (lysine, arginine, and histidine) in each subunit (32). EF-1α contained 29 basic residues, γ contained 62, and δ contained 44.

Expression of Recombinant EF-1γδ and γδ—Extraction of recombinant EF-1γδ (1.0 μg) was incubated in 0.07 ml reaction mixtures containing 50 mM Tris-HCl, pH 7.4, 50 mM KCl, 10 mM MgCl2, 0.14 mM γ-[32P]ATP (2,000 cpm/pmol) in the presence and absence of 30 units of CKII at 30 °C for 30 min. Phosphorylation of β and δ was determined by autoradiography following SDS-PAGE and 32P was quantified by excising the proteins and counting in a scintillation counter. The radiolabeled ATP was replaced with unlabeled ATP for analysis of elongation activity by poly(U)-dependent polyphenylalanine synthesis. The samples were kept on ice prior to assaying for elongation activity.

Analysis of EF-1 by Poly(U)-dependent Polyphenylalanine Synthesis—The activity of native EF-1α alone and in combination with the β, γ, and δ subunits was analyzed by poly(U)-dependent H1polyphenylalanine synthesis as described by Venema et al. (20). The specific activity of [γ32P]polyphenylalanine RNA was 9,100 cpm/pmol. Native EF-1α was preincubated alone and with the indicated subunits at 30 °C for 10 min, then added to the elongation assay mixture. Similar results were obtained by preincubation at 0 °C for 30 min.

Protein Determination—Protein concentrations of α, β, γ, and δ were determined by the Bradford method (33) with bovine serum albumin as a standard.

RESULTS

Molecular Cloning and Sequence Analysis of the cDNA for EF-1α from Rabbit—The cDNA for EF-1α isolated from a rabbit spleen library was 958 bp and contained the entire 840-bp coding region for EF-1α, a 57-bp 5′-untranslated region, a 43-bp 3′-untranslated region and a poly(A) tail of 18 nucleotides. The derived amino acid sequence consisted of 280 amino acids with a calculated molecular weight of 31,075 and pl of 4.91 (Fig. 1) and was 91% homologous with human EF-1α, which contained an additional asparagine at position 164. Comparing the sequences of EF-1α from rabbit with those from human, Xenopus, and the partial sequence from Artemia, the carboxyl terminus containing the nucleotide exchange activity (3) was more highly conserved between species than the amino terminus, which contained a leucine zipper domain. The homologous carboxyl-terminal region started at residue Glu157 followed by a region rich in acidic amino acids. The conserved portion of the amino terminus contained a leucine zipper from Leu80 to Leu115 consisting of 6 leucine residues. A site on EF-1α, Thr122, shown to be phosphorylated in Xenopus by p34cdc2 kinase (34) was not present in rabbit and human, which contained a glutamic acid at the corresponding residue 139 (Fig. 1).

Expression and Purification of Recombinant EF-1α, γ, and δ—The cDNA for EF-1α was cloned into the pT7–7 vector and expressed in E. coli. Following induction with IPTG for 2 h at 37 °C, the protein was present in significant amounts, primar-
Recombinant Subunits of EF-1

Amino acid sequence comparison of EF-1β from rabbit, human, Xenopus laevis, and Artemia salina. The protein sequences deduced from the cDNA sequences of human (14), Xenopus (15), and the partial sequences from Artemia (14) were compared with the rabbit sequence. Identical amino acids are marked with (.), and gaps with (−). X indicates undetermined. * indicates the leucine residues comprising the leucine zipper motif. Underlined is the threonine site in the EF-1α subunit. Recombinant EF-1β was expressed almost exclusively as a soluble protein at 37 °C.

**Analysis of the Recombinant Subunits by Gel Filtration**—When recombinant EF-1β (24.8 kDa) was analyzed by gel filtration on Superose 12, the protein eluted with a molecular mass of about 50 kDa (Fig. 4A). The same elution pattern was observed at 50, 100, and 500 mM NaCl. Recombinant EF-1β (31.0 kDa) migrated as a large aggregate in the void volume at 50, 200, and 600 mM NaCl. The addition of 0.1% Triton X-100 with 600 mM NaCl had no effect on the state of aggregation (Fig. 4B). Unlike the β and δ subunits, the salt concentration during gel filtration was important for the γ subunit. Recombinant EF-1γ (50.0 kDa) migrated as a large aggregate when analyzed on Superose 12 at 100 mM NaCl (Fig. 5, top panel). At 500 mM NaCl, the γ subunit migrated as a distinct peak of about 140 kDa (Fig. 5, bottom panel).

**Complex Formation with the β, γ, and δ Subunits**—To determine whether recombinant EF-1β and γ were able to form a complex similar to that of native EF-1βγ, equimolar amounts of purified β and γ were incubated together for 5 min at 4 °C. Both subunits co-chromatographed at 200 mM NaCl on DEAE-cellulose (data not shown). On Superose 12, the βγ complex eluted with an apparent molecular mass of about 160 kDa (Fig. 6A). The ratio of β and γ subunits in the complex was 1:1 as determined by densitometric scanning of the polyacrylamide gel following electrophoresis. Because the calculated molecular mass for βγ is 74.8 kDa, the results suggested a heterodimer was formed.

Purified EF-1γ and δ were combined in a ratio of 1:2 and incubated at 4 °C for 15 min. The proteins co-chromatographed during FPLC on Mono Q, eluting at 600 mM NaCl (data not shown). Upon gel filtration (Fig. 6B), γ and δ migrated as a large molecular mass of ~530 kDa but not in the void volume as observed with δ alone (Fig. 4B). The ratio of γδ subunits was 2:1. The calculated molecular mass of γδ complex was 131 kDa, considerably smaller than the size determined by gel filtration, indicating the γδ complex was at least a heterodimer (γδ2) and possibly larger. Recombinant EF-1βγ and δ, combined in a ratio of 3:1 and incubated at 4 °C for 15 min, showed no interaction (Fig. 6C). EF-1δ migrated in the void volume as a large aggregate as observed with the pure protein, whereas all of β migrated around 50 kDa apparently as a dimer.

For reconstitution of the βγδ complex, the subunits were combined at a molar ratio of 1:2:1, respectively, and incubated for 15 min at 4 °C. To remove any free subunits, the βγδ complex was isolated by FPLC on Mono Q (data not shown). The peak of the complex chromatographing at 600 mM NaCl was selected for further analysis by gel filtration to confirm the existence of the subunit interactions and to determine the
approximate size of the complex. EF-1γδ migrated as a large complex of ~670 kDa (Fig. 6D). The ratio of the β, γ, and δ subunits was 1β:2γ:1δ, as determined by densitometric scanning following SDS-PAGE. With a calculated molecular mass of 155.8 kDa for the complex, the results of gel filtration suggested that βγδ was a multimer.

Stimulation of the Elongation Activity of EF-1α by EF-1β, δ, and βγδ—The activity of native EF-1α from rabbit reticulocytes was measured by poly(U)-directed polyphenylalanine synthesis. With 10–40 pmol of EF-1α (0.5–2.0 μg), the elongation rate was linear over a 30-min incubation period (data not shown).

To measure the effect of the reconstituted βγδ complex on EF-1α activity, changes in the rate of elongation upon addition of the nucleotide exchange complex were monitored using 10 pmol of EF-1α and increasing concentrations of recombinant EF-1γδ (0.1–0.9 pmol). In the absence of EF-1γδ, 1.4 pmol of polyphenylalanine were synthesized (Fig. 7). A maximal stimulation of approximately 10-fold was observed at 0.5 pmol of EF-1γδ with the molar ratio of 20α:1βγδ. Thus, the reconstituted βγδ complex was functionally active in stimulating elongation.

EF-1β and δ were analyzed individually for the ability to stimulate elongation as shown in Fig. 8. Polyphenylalanine synthesis was stimulated 10-fold with EF-1β. EF-1δ also stimulated polyphenylalanine synthesis by 10-fold. However, the amount of the individual β and δ subunits required for stimulation of elongation was significantly greater (5α:2β or 2α:1δ) than the optimal concentration of EF-1βγδ.

Effects of Phosphorylation of β and δ by Protein Kinase CKII on Elongation—CKII has been shown to phosphorylate the β and δ subunits of EF-1 (35). When the purified recombinant subunits were examined separately as substrates for CKII, 0.6 and 0.8 mol/mol of phosphate were incorporated into the β and δ subunits, respectively. When these subunits were analyzed for the ability to stimulate the elongation activity of the α subunit, little or no effect of phosphorylation was observed over that obtained with the non-phosphorylated subunits (Table I).

When the reconstituted βγδ complex was phosphorylated by CKII, approximately 1.0 mol of phosphate was incorporated per mol of β and 0.7 mol/mol of δ subunit. The effects of phosphorylation were monitored with the polyphenylalanine synthesis
assay over a 30-min time period, using native EF-1α and phosphorylated and nonphosphorylated EF-1βγδ (Fig. 9). Polyphenylalanine synthesis with EF-1α alone was linear for up to 30 min with 1.8 pmol of polyphenylalanine synthesized at 15 min. With the addition of βγδ, polyphenylalanine synthesis was linear for 15 min. At that time, the elongation rate was 5-fold higher than that observed with EF-1α alone. Phosphorylated EF-1βγδ showed the same degree of stimulation of EF-1α as the nonphosphorylated complex. Thus, phosphorylation of EF-1βγδ by CKII appeared to have no effect on elongation.

Fig. 5. Gel filtration of EF-1γ expressed in E. coli. The recombinant γ subunit of EF-1 expressed in E. coli was purified as described under “Experimental Procedures” and analyzed by FPLC on Superose 12. Aliquots (20 μl) of the fractions were analyzed on 10% polyacrylamide gels, and the protein was stained with Coomassie Blue. Purified EF-1γ (0.5 ml from Mono Q) was eluted with buffer containing 100 mM NaCl (upper panel). EF-1γ was eluted with buffer containing 500 mM NaCl (lower panel).

Fig. 6. Analysis of complex formation by gel filtration. Purified subunits of EF-1 were combined as described under “Experimental Procedures,” and complex formation was analyzed by gel filtration on Superose 12 containing 200 mM KCl. Aliquots (20 μl) from each fraction were analyzed on a 10% SDS-polyacrylamide gel and the protein was visualized by staining with Coomassie Blue. Panel A, β and γ subunits; panel B, γ and δ subunits; panel C, β and δ subunits; panel D, β, γ, and δ subunits.

Table II. The properties of the α, β, γ, and δ subunits of EF-1 from rabbit derived from the cDNA sequences are summarized in Table II. The α subunit is basic with an isoelectric point of 9.71. The β and δ are acidic with pl of 4.33 and 4.91, respectively, whereas γ is relatively neutral. The amino acid sequences of EF-1α, β, γ, and δ from rabbit are compared with those of human (9, 12, 14, 36), the homology is 100, 94, 98, and 91%, respectively. Recombinant EF-1α expressed in E. coli is present only in inclusion bodies. Addition of GDP or GMP-PNP during

DISCUSSION

The properties of the α, β, γ, and δ subunits of EF-1 from rabbit derived from the cDNA sequences are summarized in Table II. The α subunit is basic with an isoelectric point of 9.71. The β and δ are acidic with pl of 4.33 and 4.91, respectively, whereas γ is relatively neutral. When the amino acid sequences of EF-1α, β, γ, and δ from rabbit are compared with those of human (9, 12, 14, 36), the homology is 100, 94, 98, and 91%, respectively. Recombinant EF-1α expressed in E. coli is present only in inclusion bodies. Addition of GDP or GMP-PNP during
denaturation/renaturation of EF-1α from inclusion bodies, results in a soluble protein that can be highly purified by CM-Sephadex chromatography; however, the protein is inactive (37). Recombinant β and γ expressed as soluble proteins in E. coli at 37°C have been highly purified by chromatography on DEAE-cellulose and Mono Q. When analyzed by gel filtration on Superose 12, EF-1β elutes at ~50 kDa under conditions of low and high salt. In contrast, EF-1γ elutes as a large aggregate at low salt (100 mM) and ~140 kDa at high salt (500 mM). EF-1δ expressed in E. coli at 37°C is present primarily in inclusion bodies; at 28°C, the majority of the recombinant δ is soluble, but elutes in the void volume upon gel filtration.

Previously, the native EF-1βγδ complex from Artemia and the EF-1-ValRS complex from rabbit were dissociated into βγ and δ by chromatography on a phenyl-Sepharose (3) and hydroxylapatite (19), respectively. Artemia EF-1β and γ subunits were tightly associated and could be dissociated only under denaturing conditions. β and γ dissociated under these conditions were unable to reform a βγ complex (4). EF-1δ purified from the reticulocyte EF-1-ValRS complex showed aggregation of the δ subunit when analyzed by gel filtration (22). It was postulated that the leucine zipper motif may be involved in the self-association of EF-1δ. However, we replaced leucine L94, the third leucine of six present in the leucine zipper domain of rabbit with alanine; the mutant protein still formed high molecular weight aggregates upon gel filtration (data not shown).

The recombinant β and γ subunits readily formed a βγ complex that elutes at 160 kDa upon gel filtration on Superose 12 (Fig. 6A). Since equimolar amounts of β and γ are present, the

### Table I

| Subunits of EF-1 | Polyphenylalanine synthesized | Stimulation |
|-----------------|-------------------------------|-------------|
| None            | 1.91 ± 0.11 (n = 3)           | 1.0         |
| β               | 14.02 ± 0.78 (n = 3)          | 7.3         |
| β + CKII        | 11.59 ± 1.26 (n = 3)          | 6.1         |
| δ               | 10.15 ± 0.67 (n = 3)          | 5.3         |
| δ + CKII        | 9.92 ± 0.35 (n = 3)           | 5.2         |

Native EF-1α (0.5 μg, 10 pmol) was assayed for 15 min as a control and was assayed in the presence and absence of recombinant EF-1βγδ (0.5, 2.0, and 0.9 pmol) or δ (1.0, 2.0, and 4.0 pmol) for 10 min. Polyphenylalanine synthesis was carried out for 15 min. Data from three experiments are shown.

### Table II

| Subunit | EMBL accession no. | Number of amino acids | Molecular weight | Isoelectric point | Overall charge |
|---------|--------------------|-----------------------|------------------|------------------|---------------|
| EF-1α   | U09823             | 462                   | 50,140           | 9.71             | +11           |
| EF-1β   | X74728             | 225                   | 24,803           | 4.33             | −18           |
| EF-1γ   | X68142             | 437                   | 50,048           | 6.72             | −3            |
| EF-1δ   | U47663             | 280                   | 31,074           | 4.91             | −12           |

Properties of the α, β, γ, and δ subunits of EF-1 from rabbit calculated from the cDNA sequence cDNA sequences were analyzed with the Pepsort program in the GCG package.
data suggest the complex may be a βγ dimer. EF-1βγ isolated from EF-1-ValRS complex migrated with a molecular mass around 270 kDa when analyzed on Superose 6 (22), which is consistent with the βγ complex being a tetramer.

A direct interaction is also observed between recombinant γ and δ (Fig. 6B), but not between β and δ as shown by gel filtration (Fig. 6C). The molar ratio of γ and δ is 2:1 and eluted at ~530 kDa on gel filtration. Thus, the γδ complex appears to be at least a dimer of γδ. The large aggregate form of recombinant EF-1βγ is able to readily interact with recombinant γ and βγ to form γδ and the βγδ complex. The reconstituted βγδ complex elutes with a molecular mass of ~670 kDa (Fig. 6D), similar to that observed with the reconstituted native EF-1γδβ (700 kDa) from rabbit reticulocytes (22). The ratio of βγδ to EF-1βγδ complex requires EF-1γ to interact with the β and δ subunits.

Stimulation of EF-1α activity by EF-1β and βγ purified from rabbit (38), pig (39, 40), and Artemia (16) as assayed by phenylalanine synthesis has been reported previously. The molar ratio of EF-1α to βγ analyzed in these reports was 2:3 for rabbit, 1:3 for pig and 2:1 for Artemia, with a 2- to 7-fold maximal stimulation of EF-1α activity. The activities of βγ and βγδ were comparable when analyzed on a molar basis (16, 40); thus, γ seemed to have little effect on the activity of β, and β could substitute for βγ in stimulating the elongation (40). We have shown that recombinant β alone can stimulate elongation at a ratio similar to that observed by others with β and βγ (16, 40). A similar degree of stimulation is also observed with the recombinant δ subunit.

In mammals, all of the EF-1βγδ is associated with EF-1α and can be dissociated from α and ValRS by βγδ and γδ and by hydroxyapatite chromatography, but not as βγδ (19). Thus, the activity of the βγδ complex in nucleotide exchange and elongation has not been described previously. To address the question of EF-1βγδ activity, increasing amounts of βγδ were analyzed with native EF-1α, which has a low level of elongation activity. Recombinant EF-1βγδ stimulates the activity of EF-1α by up to 10-fold (Fig. 7). The ability of recombinant EF-1βγδ to stimulate EF-1α activity indicates the recombinant EF-1βγδ is a functional complex and produces a level of stimulation equivalent to or greater than that observed previously with β and βγ, and less of the βγδ is required.

Previously, Palen et al. (35) showed that GDP stimulated the phosphorylation of the β and δ subunits of native EF-1 by CKII. Such a stimulation was not observed with the recombinant subunits or reconstituted βγδ. The lack of an effect by GDP is consistent with the availability of β and δ in the reconstituted complex for phosphorylation by CKII as compared with EF-1. In the same study (35), polylysine was shown to stimulate phosphorylation of β in native EF-1 but not δ. With the recombinant subunits, polylysine stimulated phosphorylation of β but not the δ subunit (data not shown), which is consistent with the studies on native EF-1. The increased phosphorylation of β in the presence of polylysine was due primarily to phosphorylation of numerous other serine and threonine residues as confirmed by phosphopeptide mapping.

A model for Artemia has been proposed by Janssen et al. (4) and is based on a single γ subunit that binds both β and δ. A model for EF-1-ValRS from rabbit has been proposed by Bec et al. (22). This model is based on a core δ dimer with both β and δ binding to a single γ. From the data with the recombinant EF-1 subunits, we show that β and δ do not interact. A major difference between our data and that of the others is the number of γ subunits in the complex. We postulate a γ dimer core with the β and δ subunits binding to separate γ subunits. A model with additional supporting kinetic data will be published elsewhere.

Phosphorylation of dephosphorylated EF-1β from Artemia by CKII has been reported to reduce the nucleotide exchange activity approximately 2-fold (23). However, the site phosphorylated by CKII (Ser<sup>563</sup>) is not required for stimulation of the nucleotide exchange activity as shown with proteolytic fragments of the subunit; the activity resides in the carboxyl terminus of β (3). In contrast, phosphorylation of the guanine nucleotide exchange factor by CKII restores GTP binding and increases the activity of the factor by 4–5-fold (41).

In the studies herein, the rate of phosphorylation of the βγδ complex by CKII is addressed under kinetically valid conditions. The activity of native EF-1α is stimulated 5.3-fold by nonphosphorylated EF-1βγδ (Fig. 9). A similar degree of stimulation with phosphorylated βγδ (5.4-fold) is observed under conditions where phosphorylation of β is 1 and δ is 0.7 mol/mol. Thus, phosphorylation of βγδ by CKII has no effect on stimulation of α activity. Studies to examine the effects of phosphorylation on GDP/GTP exchange with the recombinant EF-1 from rabbit are currently underway.

Protein kinase CKII is a multipotential protein kinase that appears to function to integrate metabolism. Since the sites phosphorylated by CKII are almost fully phosphorylated in vivo, it has been suggested that CKII is required for maintaining normal physiological functions (42). Phosphorylation of EF-1 by CKII may be a silent phosphorylation event with little effect on EF-1, or it may have other functions such as mediating protein-protein interactions and targeting the EF-1 complex within the cell. Previously, EF-1 has been shown to be phosphorylated by protein kinase C (20, 43), p34<sup>cdc2</sup> (37, 44), and multipotential S6 kinase (45). Phosphorylation of EF-1 by protein kinase C and multipotential S6 kinase stimulates the rate of elongation by 2–3-fold. Thus, it is of interest to determine whether phosphorylation of EF-1 by CKII functions in conjunction with other protein kinases to regulate EF-1 activity.

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