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The association of NADPH with the guanine nucleotide exchange factor from rabbit reticulocytes: A role of pyridine dinucleotides in eukaryotic polypeptide chain initiation

(eukaryotic initiation factor 2/fluorescence/protein synthesis regulation)

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ABSTRACT The guanine nucleotide exchange factor (GEF) was purified to apparent homogeneity from postribosomal supernatants of rabbit reticulocytes by chromatography on DEAE-cellulose and phosphocellulose, fractionation by glycerol gradients, and chromatography on Mono S and Mono Q (Pharmacia). At the Mono S step GEF is isolated as a complex with the eukaryotic polypeptide chain initiation factor 2 (eIF-2) and is separated from this factor by column chromatography on Mono Q. An emission spectrum characteristic of a reduced pyridine dinucleotide was observed when GEF was subjected to fluorescence analysis. By both coupled enzymatic analysis and chromatography on reverse-phase or Mono Q columns, the bound dinucleotide associated with GEF was determined to be NADPH. The GEF-catalyzed exchange of eIF-2-bound GDP for GTP was markedly inhibited by NAD⁺ and NADP⁺. This inhibition was not observed in the presence of equimolar concentrations of NADPH. Similarly, the stimulation of ternary complex (eIF-2-GTP-Met-tRNA_f) formation by GEF in the presence of 1 mM Mg²⁺ was abolished in the presence of oxidized pyridine dinucleotide. These results demonstrate that pyridine dinucleotides may be directly involved in the regulation of polypeptide chain initiation by acting as allosteric regulators of GEF activity.

The first step in eukaryotic polypeptide chain initiation is the formation of a ternary complex between the initiator tRNA, GTP, and the eukaryotic polypeptide chain initiation factor 2 (eIF-2). Upon formation of the 80S initiation complex, GTP is hydrolyzed and eIF-2 is released as the eIF-2-GDP binary complex (1, 2). In mammalian systems, this binary complex is stable in the presence of Mg²⁺ (3, 4) and a second factor, the guanine nucleotide exchange factor (GEF), is required for the exchange of bound GDP for GTP (4-7). It is at this point in the eIF-2 cycle that regulation of the initiation factor appears to occur. This regulation may occur as a result of the GTP/GDP ratio (3, 8), or by phosphorylation of the α subunit of the eIF-2 by cAMP-independent protein kinases, which includes the heme-sensitive (2, 9, 10), or the double-stranded RNA-induced (11, 12) kinases. Phosphorylation of eIF-2 by these kinases in reticulocyte lysates is associated with the cessation of protein synthesis and is due to the inability of GEF to catalyze the GTP/GDP nucleotide exchange reaction (13-15).

The rate of protein synthesis in cell-free extracts may also be affected by factors that prolong or restore protein synthesis. These factors include sugar phosphates (16-19), NADPH (16, 20, 21), and GTP (22, 23), as well as dithiothreitol, glutathione, and thiol-reducing systems (20, 21, 24,

25). Recent reports have indicated that these factors may be regulating the activity of the eIF-2 kinase (19, 20) or altering the redox state of sulfhydryl groups on eIF-2 (24).

In this report we provide direct evidence for the interaction of NADPH with GEF, and present evidence that the levels of NADPH and NADP⁺ may regulate the ability of eIF-2 to recycle during protein synthesis. GEF can be isolated as a complex with NADPH, and the strength of this interaction is stabilized by glycerol and dithiothreitol. NADP⁺ at 0.5-0.75 mM abolishes the ability of GEF to catalyze the GTP-dependent release of GDP from an eIF-2-GDP binary complex. A preliminary account of this work was reported (26).

METHODS

Factor Preparation. Rabbit reticulocyte lysates were obtained from Green Hectares (Oregon, WI). eIF-2 and GEF were prepared in a buffer containing 100 mM KCl, 20 mM Tris-HCl (pH 7.5), 0.05 mM EDTA, 2 mM dithiothreitol, and 10% (vol/vol) glycerol (buffer A). eIF-2 was purified from the 0.5 M KCl wash of rabbit reticulocyte ribosomes by chromatography on DEAE-cellulose and phosphocellulose as described (7). Fractions containing eIF-2 activity were further purified by gradient elution from Mono Q (eluted with 315 mM KCl) and Mono S (eluted with 250 mM KCl) ion-exchange columns (Pharmacia). GEF was purified from postribosomal supernatants by chromatography on DEAE-cellulose and phosphocellulose, fractionation on glycerol gradients (6, 8), and chromatography on Mono S (eluted with 210-260 mM KCl) and Mono Q (eluted with 400 mM KCl). The Mono S fraction of GEF contained both eIF-2 and GEF, and these factors were separated upon chromatography on Mono Q. The Mono Q fraction was used for the fluorescence studies and NADPH determination. For activity assays and storage, the GEF fraction was made 50% in glycerol by the addition of glycerol with buffer A. This preparation was 98% pure as judged by NaDodSO₄/polyacrylamide gel electrophoresis (27) and staining with Coomassie brilliant blue R-250. It consisted of five polypeptides of M_r 82,000, 65,000, 55,000, 40,000, and 34,000, for an aggregate M_r of 276,000. Extensive dialysis of GEF or chromatography of the protein on Mono Q with buffer lacking glycerol and dithiothreitol resulted in the loss of bound NADPH. After elution from Mono Q, the fractions containing NADPH-depleted GEF were made 10% in glycerol and 2 mM in dithiothreitol. eIF-2 activity was assayed by ternary complex (eIF-2-GTP-³⁵S]Met-tRNA_f) formation and GEF activity by the release

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Abbreviations: eIF-2, eukaryotic polypeptide chain initiation factor 2; GEF, guanine nucleotide exchange factor.
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of [^3H]GDP from an eIF-2-[^3H]GDP binary complex as described (7, 15).

Fluorescence and Light-Scattering Measurements. Fluorescence spectra were obtained by using a Perkin Elmer MPF-44A spectrofluorimeter connected to a Nicolet 2090-III-206 digital oscilloscope (Nicolet, Madison, WI). The excitation slit was 4 nm and the emission slit was 6 nm. Background subtraction was accomplished by using the differencing function of the Nicolet oscilloscope. Light-scattering results were obtained with the Perkin Elmer spectrofluorimeter with excitation and emission wavelengths set at 400 nm. Changes in light scattering were monitored after addition of an equimolar amount of NADPH to 50 μl of buffer containing GEF (0.1 mg/ml). The time course of the reaction was recorded with the Nicolet oscilloscope.

RESULTS

GEF Fluorescence and Light Scattering. When purified fractions of GEF were analyzed by fluorescence spectroscopy, a significant but variable amount of intrinsic fluorescence was observed. In Fig. 1, the fluorescence of the tyrosyl and tryptophanyl residues in GEF is illustrated. The fluorescence emission at 335 nm resulting from excitation at 250 nm may represent tyrosine emission red-shifted from the typical 305-nm maximum (28, 29). Tryptophan fluorescence in GEF is shifted from the normal maximum at 348 nm for the free amino acid to ≈ 325 nm, a common value for tryptophan in polypeptides (28). This blue shift indicates that the tryptophanyl residues, which are sensitive to solvent polarity, are in a hydrophobic environment (28–30).

A fluorescence maximum near 435 nm was observed when GEF was excited at 350 nm. Protein-bound reduced pyridine dinucleotides typically emit at 425–450 nm, blue-shifted from the 475-nm emission maximum of the free dinucleotide (29, 31). This result suggested the presence of NADH or NADPH bound to GEF. When the emission was monitored at 400 and 440 nm (Fig. 2), the excitation profiles further indicated the presence of NADH or NADPH and suggested that tryptophan emission potentially contributes to the blue emission above 400 nm, evidenced by the excitation maximum at 285 nm. Fig. 3 illustrates a three-dimensional energy-corrected contour plot of total fluorescence for GEF. A broad fluorescence excitation at 335 nm with an emission maximum around 435 nm is clearly seen. This is consistent with the presence of a reduced pyridine dinucleotide in GEF.

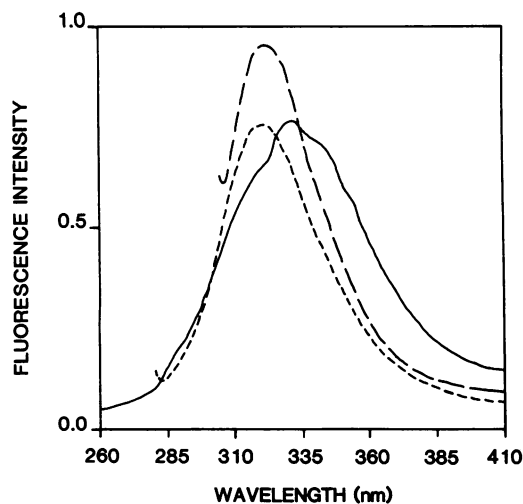


FIG. 1. Fluorescence emission spectra of tryptophan and tyrosine in GEF. The uncorrected spectra have had the buffer background subtracted. Excitations were 250 nm (—) with $\times 100$ scaling; 275 nm (---) with $\times 10$ scaling; 300 nm (— · —) with $\times 30$ scaling.

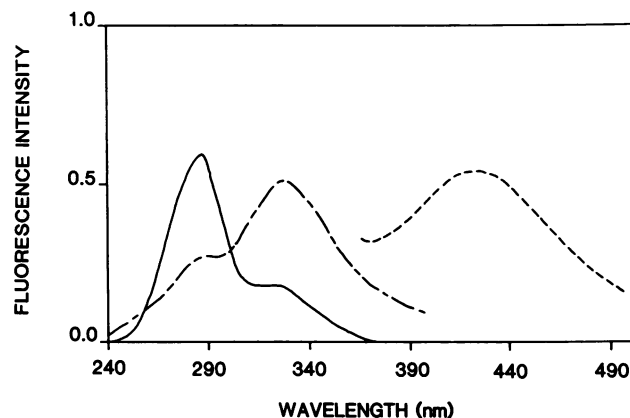


FIG. 2. Constant energy excitation and uncorrected emission spectra of GEF. Excitation scans: emission at 400 nm (—); at 440 nm (---). Emission scan: excitation at 350 nm (— · —).

The interaction of reduced pyridine dinucleotides with GEF were further examined by monitoring the emission spectra of the nucleotide-depleted GEF when either NADH or NADPH was added to the preparation. At approximately a 2-fold excess of pyridine dinucleotide, NADH quenched the tryptophan fluorescence $\approx 10\%$, whereas the NADPH produced a 60% reduction in the total fluorescence (Fig. 4). Furthermore, when GEF was added to buffer containing NADPH or NADH there was a 2-fold enhancement of NADPH emission, but no change in NADH emission was observed (data not presented).

The removal of glycerol from preparations of GEF yielded solutions that were visibly turbid, indicating significant aggregation of the factor. Addition of glycerol or equimolar concentrations of NADPH effectively eliminated sample turbidity. When monitored by changes in light scattering, the rate of disaggregation upon addition of NADPH was biphasic. Approximately 45% of the disaggregation occurred within a few seconds. The second phase was significantly slower, with $t_{1/2} = 120$ sec. Subsequent additions of NADPH quenched tryptophan fluorescence but did not further change light scattering.

Pyridine Dinucleotide Content of GEF. Two techniques were used to identify the fluorophore in GEF. The GEF was digested with proteinase K or extracted with 0.1 M KOH, and the sample was subsequently chromatographed on either a reverse-phase (32) or ion-exchange column (33) (Table 1). In each case, from the integrated dinucleotide peak, 2 pmol of NADPH per pmol of GEF were determined to be present. A

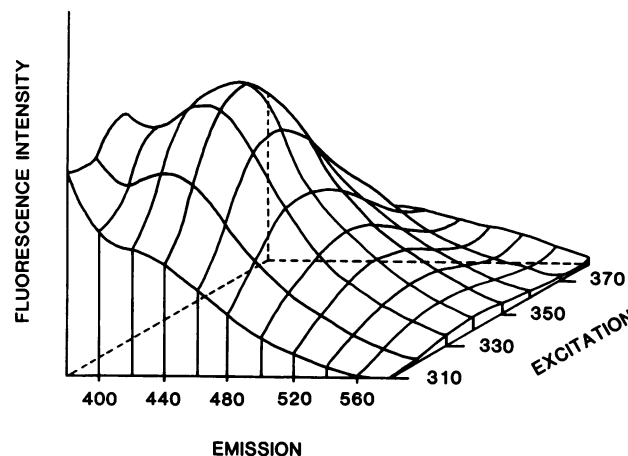


FIG. 3. Energy-corrected emission/excitation total fluorescence contour plot for GEF.

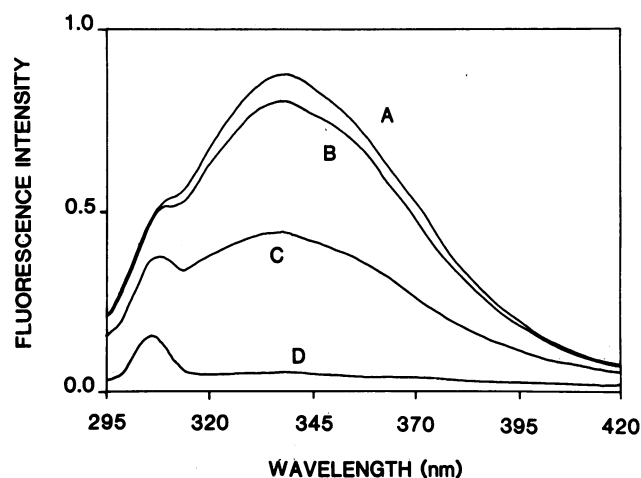


FIG. 4. Effect of pyridine dinucleotides on the tryptophan emission spectrum of GEF depleted of bound nucleotide. Excitation was at 275 nm. Each assay mixture contained 0.37 μ M GEF in 50 μ l of buffer A. Curve A, GEF; curve B, GEF plus 0.53 μ M NADH; curve C, GEF plus 0.53 μ M NADPH; curve D, background fluorescence observed in the absence of GEF.

NADPH-specific enzyme cycling assay with glucose-6-phosphate dehydrogenase and glutathione reductase (34) confirmed that the fluorophor was indeed NADPH. With most preparations of GEF, each assay method consistently indicated the presence of 2 mol of pyridine dinucleotide per mol of GEF. However, when GEF was dialyzed or chromatographed in buffers lacking dithiothreitol or glycerol, significantly less dinucleotide was recovered bound to GEF. For a given GEF preparation, the mol fraction of NADPH observed in the Mono S fraction (GEF·eIF-2) was the same as that found at the Mono Q step (GEF only).

Effect of Pyridine Dinucleotides on GEF Activity. GEF, which is isolated with bound NADPH, was assayed in the

Table 1. Quantitation of pyridine dinucleotide in the GEF

Technique	Mole fraction
A	2.2
B	2.1
C	1.5
D	0.7

Techniques: A, GEF (0.37 μ M) was digested with proteinase K (30 μ g/ml) in 50 μ l of buffer A adjusted to pH 9.0 with triethylamine. Incubation was for 1 hr at 30°C. The boiled reaction mixture was chilled, centrifuged, and chromatographed on an ALTEX Ultrasphere column (4.6 \times 250 mm) using 8% methanol (vol/vol) containing 0.1 M potassium phosphate (pH 6.5) (32). The extracted nucleotide eluted at the position corresponding to a NADPH standard, and no other nucleotide was observed. Quantitation was by comparison of the absorbance with NADPH standards. The value of 2.1 was also obtained when the digestion mixture was chromatographed on Mono Q. B, The GEF preparation was made 0.01% (wt/vol) in sodium dithionite, treated with 0.15 M KOH for 5 min at 60°C, neutralized with HCl, chilled in ice for 15 min, filtered through cellulose acetate (0.45 μ m), and chromatographed on a Pharmacia HR 5/5 Mono Q column (33). Quantitation was as described above. C, GEF was analyzed as in technique B, but reduction by dithionite was omitted. D, GEF was chromatographed on a Mono Q column in buffer A but lacking dithiothreitol and glycerol, and the peak eluting at the position corresponding to NADPH was quantitated by a NADP⁺ (H)-specific enzyme cycling assay (34). Incubation was for 60 min at 30°C. NADPH (1–20 pmol) was used as the standard. Protein was determined by both the method of Bradford (35) and by the use of the Pierce BCA protein reagent. Bovine serum albumin was used as the protein standard. The purity of the eIF-2 and GEF fractions was evaluated by electrophoresis of the factors in NaDodSO₄/10% polyacrylamide gels (27).

presence of oxidized and reduced pyridine dinucleotides for its ability to catalyze the release of [³H]GDP from a preformed isolated eIF-2·[³H]GDP binary complex (Table 2). Addition of NADH or NADPH had no effect on the exchange reaction. When either NAD⁺ or NADP⁺ was added to the assay mixture, complete inhibition of the GTP-dependent nucleotide-exchange reaction was observed at 0.5–0.75 mM dinucleotide. The inhibitory effects of NAD⁺ or NADP⁺ on GEF activity were not observed in the presence of equimolar amounts of NADPH (data not presented).

eIF-2 is usually isolated with bound GDP, and in the presence of Mg²⁺ this GDP is not released (4, 7) so that subsequent binding of Met-tRNA_f is reduced (36). Upon addition of GEF this inhibition is reversed (4–7). The effect of NADP⁺ and NADPH on the ability of GEF to relieve this Mg²⁺ inhibition of ternary complex formation is illustrated in Table 3. The addition of 1 mM Mg²⁺ reduced [³⁵S]Met-tRNA_f binding from 10,200 cpm to 2400 cpm and the presence of GEF partially reversed the effect of Mg²⁺ (7550 cpm bound). The addition of NADPH to 1 mM had no effect on ternary complex formation, whereas addition of NADP⁺ to 1 mM progressively reduced [³⁵S]Met-tRNA_f binding to nearly the level observed in the absence of GEF.

DISCUSSION

Workers in several laboratories have established a link between sustained protein synthesis in cell-free reticulocyte lysates and the requirement for NADPH, sugar phosphates, a NADPH-generating system, or the addition of a reducing agent such as dithiothreitol (16–18, 20, 21). There is evidence that in the absence of these reducing equivalents or in the presence of oxidized glutathione (20, 37), the heme-controlled repressor is activated and eIF-2 is phosphorylated and is unable to recycle. However, in the absence of heme, NADPH and sugar phosphates are insufficient to protect against the activation of the heme-controlled repressor (20), and the addition of heme to lysates depleted of NADPH and sugar phosphates will also not protect against the cessation of protein synthesis (17, 21). Jagus and Safer (24) proposed that the NADPH/NADP⁺ ratio may influence the redox state of the sulfhydryl groups in eIF-2, thereby affecting the ability of the initiation factor to recycle. They also observed the transient inhibition of protein synthesis by 0.2–0.4 mM NADP⁺, which was not accompanied by the phosphorylation of eIF-2.

By spectrofluorimetric analysis of GEF, we have detected the presence of bound NADPH. The fluorescence spectra of GEF may be divided into two classes: intrinsic fluorescence due to tryptophan and tyrosine (Fig. 1), and fluorescence resulting from bound NADPH (Fig. 2). Excitation at 275 and

Table 2. Effect of pyridine dinucleotides on GEF-dependent release of [³H]GDP from the eIF-2-GDP binary complex

Concentration, mM	[³ H]GDP released, cpm			
	NAD ⁺	NADH	NADP ⁺	NADPH
0	4050	4060	4100	4080
0.25	4050	4070	2340	4385
0.50	2210	4100	1810	4365
0.75	0	4200	110	4400
1.00	0	4250	0	4300
2.00	0	4090	0	4040

Each reaction mixture contained in 75 μ l: 20 mM Tris·HCl (pH 7.8), 1 mM Mg²⁺, 1 mM dithiothreitol, 100 mM KCl, 0.2 mM GTP, 10 μ g of bovine serum albumin, 0.25 pmol of GEF, and 1.0 pmol of the isolated eIF-2·[³H]GDP (5000 cpm/pmol). The reactions were initiated by the addition of the preformed binary complex. In the absence of GEF, the pyridine dinucleotides had no effect on the nucleotide exchange reaction.

Table 3. Effect of NADP⁺ and NADPH on eIF-2-GTP-Met-tRNA_f ternary complex formation in the presence of GEF and Mg²⁺

Addition	Concentration, mM	[³⁵ S]Met-tRNA _f bound, cpm
None	0	7550
NADP ⁺	0.25	6400
	0.50	5030
	0.75	3500
	1.00	3000
NADPH	0.50	7350
	1.00	7700

Each reaction mixture contained in 75 μl: 20 mM Tris-HCl (pH 7.8), 100 mM KCl, 1 mM Mg²⁺, 1 mM dithiothreitol, 0.2 mM GTP, 10 μg of bovine serum albumin, 1.5 pmol of eIF-2, 0.4 pmol of GEF, 5 pmol of [³⁵S]Met-tRNA_f (10,000 cpm/pmol), and the pyridine dinucleotide as indicated. The assay was initiated by the addition of eIF-2. When GEF was not added, [³⁵S]Met-tRNA_f binding in the absence and presence of Mg²⁺ was 10,200 and 2400 cpm, respectively.

300 nm produces a fluorescence due primarily to tryptophan. Tryptophan fluorescence is highly sensitive to the solvent polarity (28–30, 38), and the 320-nm emission suggests a rather nonpolar environment (28). Excitation of tyrosine at 250 nm usually results in a weak emission at 305 nm, which is relatively insensitive to its environment (29). The observed red shift of tyrosine fluorescence in GEF to 335 nm may be due either to energy transfer to tryptophan and reemission at 335 nm (29) or to ionization of the amino acid residue to tyrosinate (28, 30, 39). Emission beyond 400 nm (Fig. 2) may have two sources. Maxima at 285 and 325 nm are observed when the excitation profile is examined for the 400- and 440-nm emissions. When liver alcohol dehydrogenase was excited at 287 nm, an observed 445-nm fluorescence maximum was attributed to an energy transfer from tryptophan to bound NADH (38). An excitation peak at 330 nm with a fluorescence maximum at 445 nm was also attributed to bound coenzyme.

In this report, we provide evidence that oxidized and reduced pyridine dinucleotides may directly interact with the GEF and consequently establish a link between the redox state of the cell and the regulation of eIF-2 activity. This evidence is based on the following observations.

(i) GEF was isolated with bound NADPH. The amount of NADPH recovered was enhanced by the presence of high levels of glycerol, dithiothreitol, and limited dialysis. The bound NADPH was detected by direct fluorescence analysis. The identity of the reduced pyridine dinucleotide was confirmed after extraction of the NADPH from GEF and subsequent chromatography on reverse-phase or ion-exchange columns, as well as by a NADP⁺ (H)-specific enzyme assay.

(ii) When NADPH was added to GEF depleted of the pyridine dinucleotide, a fluorescence profile was obtained that is identical to that of the isolated GEF-NADPH complex. The result of adding NADPH and NADH to GEF initially depleted of the pyridine dinucleotide provides evidence for the specific binding of NADPH. The addition of a 2-fold excess of NADPH to GEF depleted of NADPH resulted in a quenching of tryptophan fluorescence by 60%, whereas the same level of NADH produced only a 10% reduction in tryptophan fluorescence. Judging by the relative amount of tryptophan fluorescence quenching in GEF, NADPH appears to form a tighter complex with GEF than does NADH (Fig. 4). A similar degree of quenching of tryptophan fluorescence was observed upon NADH or NADPH binding to dehydrogenases (40, 41). A 2-fold enhancement of fluores-

cence associated with the direct excitation of the reduced coenzyme was also observed (31, 38, 41).

(iii) Addition of NAD⁺ or NADP⁺ strongly inhibited the GEF-dependent exchange of GTP for eIF-2-bound GDP (Table 2), and the presence of NADPH protected the factor against this inhibition. The inhibition of nucleotide exchange activity was not linear with respect to the concentration of the oxidized pyridine dinucleotides, suggesting that this inhibition is not the result of simple competition with NADPH for a single binding site. This is consistent with the finding of 2 pmol of NADPH per pmol of GEF. In the absence of GEF, these pyridine dinucleotides had no effect on the release of GDP from the eIF-2-GDP binary complex. Similar results were obtained when NADP⁺ or NADPH was added to ternary complex assays containing 1 mM Mg²⁺. The addition of NADPH to these assays had no effect on the ability of GEF to reverse the Mg²⁺ inhibition of ternary complex formation. In contrast, 1 mM NADP⁺ effectively prevented GEF from exchanging eIF-2-bound GDP for free GTP and the subsequent binding of [³⁵S]Met-tRNA_f.

(iv) GEF that was depleted of NADPH was active in the nucleotide exchange reaction provided it was assayed immediately after its elution from the Mono Q column. This preparation did not retain activity upon freezing and thawing or prolonged storage. In the absence of glycerol, preparations of GEF became visibly turbid, indicating aggregation of the factor. The addition of stoichiometric levels of NADPH effectively dissociated GEF aggregates. This phenomenon, as monitored by changes in the light scattering of the solutions, strongly suggests that NADPH is important in the maintenance of the structural integrity of GEF. No change in light scattering was observed when NADPH was added to GEF preparations containing 10% glycerol.

Current evidence supports a model in which the metabolic state of a cell may influence protein synthesis directly at the level of nucleotide exchange and indirectly by regulating the activity of the heme-controlled repressor. In this communication, we provide a rationale for a mechanism in which the rate of protein synthesis is regulated by the reducing power of the cell. We propose that at physiological levels of Mg²⁺, NADPH binding to GEF prevents inhibition of GEF activity by NAD⁺ and NADP⁺. The observation that pyridine dinucleotides can directly influence GEF-dependent nucleotide exchange provides a basis for many earlier observations on the effects of NADPH and sugar phosphates on protein synthesis and the requirement of glycerol and dithiothreitol for the maintenance of GEF activity. Further work is required to elucidate the role of NADPH in the direct regulation of eIF-2 and GEF activity and the relationship of this mechanism to the regulation of the heme-controlled repressor by NADPH.

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