Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 51–54, January 1988
Biochemistry

Phosphorylation of the guanine nucleotide exchange factor from rabbit reticulocytes regulates its activity in polypeptide chain initiation

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

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Communicated by Myron K. Brakke, September 14, 1987 (received for review July 29, 1987)

ABSTRACT **We have demonstrated that the purified guanine nucleotide exchange factor (GEF) may be isolated as a complex with NADPH. Complete inhibition of the GEF-catalyzed exchange of eukaryotic initiation factor 2-bound GDP for GTP was observed in the presence of either 0.5–0.75 mM NAD^+ or $NADP^+$. Incubation of GEF with ATP results in the phosphorylation of its M_r 82,000 polypeptide. This phosphorylation is strongly inhibited by heparin but is not affected by heme or H8 {*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride}, an inhibitor of cAMP- and cGMP-dependent protein kinases and protein kinase C. The purification of GEF was modified to eliminate any contaminating kinase activity and the isolated protein appears to be homogeneous as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis and silver staining. The M_r 82,000 subunit of GEF is phosphorylated only upon addition of ATP and casein kinase II. The extent of phosphorylation is ≈0.55 mol of phosphate per mol of GEF, and this results in a 2.3-fold increase in the guanine nucleotide exchange activity. Following treatment of the phosphorylated GEF with alkaline phosphatase, the activity of the protein is reduced by a factor of 5. Rephosphorylation of GEF increases its specific activity to that of the phosphorylated protein. The results of this study suggest that phosphorylation/dephosphorylation of GEF plays a role in regulating polypeptide chain initiation.**

During the initiation of protein synthesis in eukaryotic cells, the formation of a ternary complex [eIF-2·GTP·Met-$tRNA_f$; where eIF-2 is eukaryotic initiation factor (eIF) 2] is followed by the transfer of this complex to a 40S ribosomal subunit (1, 2). Upon joining the 60S subunit to give the complete 80S initiation complex, GTP is hydrolyzed, and eIF-2 is released as the eIF-2·GDP binary complex (3, 4). In mammalian systems, this binary complex is stable in the presence of Mg^{2+} and is functionally inactive (5–7). Regeneration of the eIF-2·GTP·Met-$tRNA_f$ species requires the guanine nucleotide exchange factor (GEF), which facilitates nucleotide exchange and recycling of eIF-2 (7–10). Phosphorylation of the α subunit of eIF-2 [eIF-2(α)] by either the heme-controlled repressor (4, 11) or the double-stranded RNA induced kinase (12, 13) is associated with the cessation of protein synthesis and is due to the inability of GEF to catalyze the GDP/GTP exchange from eIF-2(α-P)·GDP (8, 10, 14). Studies from this laboratory have demonstrated that the redox state of the cell may also influence GEF activity (15).

Phosphorylation plays a major role in the regulation of eukaryotic protein synthesis. Initiation factors, ribosomal proteins, messenger ribonucleoprotein particles, and aminoacyl-tRNA synthases have been shown to be modified by phosphorylation (16–22). In addition to eIF-2(α), the phosphorylation of other initiation factors [eIF-2(β), eIF-4B, and eIF-4F] has been reported *in vivo* under conditions of heat shock (19) and of nutrient deprivation (20) and *in vitro* by cAMP-independent kinase (21). In contrast to eIF-2(α) phosphorylation, which inhibits protein synthesis, the alteration in activity of the other initiation factors due to phosphorylation is not well understood.

The covalent modifications of the GEF either *in vivo* or *in vitro* have not been studied. We demonstrated (15) that GEF contains tightly bound NADPH and that oxidized pyridine dinucleotides at 0.5–0.75 mM abolish the ability of the factor to catalyze the exchange of eIF-2-bound GDP for GTP. In this communication, we report that the phosphorylation of the M_r 82,000 subunit of GEF by ATP and casein kinase II results in an increase in the activity of the factor. Following treatment of the phosphorylated GEF with alkaline phosphatase, the activity of the protein is reduced by a factor of 5. A preliminary account of this study was reported (23).

METHODS

Factor Preparation. Rabbit reticulocyte lysates were obtained from Green Hectares (Oregon, WI). eIF-2 and GEF were prepared in buffers containing 20 mM Tris·HCl (pH 7.5), 0.05 mM EDTA, 2 mM dithiothreitol, 10% (vol/vol) glycerol, and KCl as indicated (buffer A). eIF-2 was purified to apparent homogeneity from the 0.5 M KCl wash of rabbit reticulocyte ribosomes as described (24). GEF was purified from the postribosomal supernatants by chromatography on DEAE-cellulose and phosphocellulose, fractionation on glycerol gradients, and chromatography on Mono S and Mono Q ion-exchange columns (Pharmacia) as described (15). The protein was applied to phosphocellulose in buffer A containing 0.1 M KCl, and GEF activity was eluted with buffer A containing 1 M KCl (method 1). To eliminate any contaminating kinase activity, the protein eluting from the DEAE-cellulose column was applied to phosphocellulose in buffer A where 25 mM potassium phosphate, pH 7.2, was substituted for Tris·HCl (method 2). The column was washed with this buffer, and the adsorbed protein was successively eluted with buffer containing 0.1, 0.5, and 1 M KCl. Under these conditions, casein kinase II did not adsorb to the column (25), and GEF activity was eluted with buffer containing 0.5 M KCl. Purified casein kinase II was either from *Artemia* (26) or rabbit reticulocytes (a gift from Erwin Reimmann, Department of Biochemistry, Medical College of Ohio, Toledo). The heme-controlled repressor was purified as described (27).

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Abbreviations: GEF, guanine nucleotide exchange factor; eIF, eukaryotic polypeptide chain initiation factor; eIF-2(α) and eIF-2(β), α and β subunits of eIF; H8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride.

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Protein kinase C was generously provided by Katherine Albert from the laboratory of Paul Greengard (The Rockefeller University, New York).

Assays. Phosphorylation of GEF by casein kinase II (26), heme-controlled repressor (27), or protein kinase C (28) was carried out with [γ-^{32}P]ATP (2000 cpm/pmol) as described. The phosphorylated samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by autoradiography (26). The gels were either silver stained (29) or stained with Coomassie brilliant blue R-250 (24). For quantitation of phosphorylation, the polypeptide bands were excised and soaked in Uni/verse LSC mixture (J. T. Baker Chemical), and radioactivity was measured in a liquid scintillation counter (26). Radioactive nucleotides were purchased from DuPont/New England Nuclear. GEF activity was assayed by monitoring the release of [^{3}H]GDP from an isolated eIF-2·[^{3}H]GDP binary complex (15). The dephosphorylation of GEF (10 μg) was carried out in a reaction mixture of 30 μl containing 20 mM Tris·HCl (pH 7.5), 0.1 M KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, and mammalian alkaline phosphatase at 20 units/ml (Sigma). The protease inhibitors, phenylmethylsulfonyl fluoride, pepstatin, leupeptin, and chymostatin were added at final individual concentrations of 0.3 mM. After a 30-min incubation at 37°C, the reaction was either terminated and analyzed as described for the kinase assay or applied to a Mono S column. The column was washed with five column volumes of buffer A containing 0.1 M KCl and eluted with buffer A containing 0.3 M KCl. Alkaline phosphatase and ATP do not absorb to Mono S and are easily separated from GEF. Similarly, phosphorylated GEF was also reisolated by chromatography on a Mono S column. Protein was determined by the method of Bradford (30) by using the Bio-Rad protein assay reagent and bovine serum albumin as the standard.

RESULTS

Incubation of GEF purified as described (15) with [γ-^{32}P]ATP resulted in the phosphorylation of its M_r 82,000 polypeptide (Fig. 1). A 2.3-fold increase in GEF activity was observed when the phosphorylation of the protein was first carried out and then followed by the guanine nucleotide exchange assay (Table 1). The GEF-catalyzed release of eIF-2-bound GDP is dependent on the presence of added guanine nucleotides (GDP or GTP), and the addition of ATP alone had no effect on the release of GDP (Table 1).

To characterize the kinase responsible for phosphorylating GEF, the effect of various inhibitors was studied (Fig. 1). The phosphorylation of GEF was not affected by 50 μM heme or H8 {*N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride}, an inhibitor of cAMP-dependent (K_i, 1.2 μM) and cGMP-dependent (K_i, 0.48 μM) protein kinases, and protein kinase C (K_i, 15 μM) (31). It was, however, strongly inhibited by heparin, suggesting that a casein kinase II may be a contaminant of the GEF preparation. Quantitation of the [γ-^{32}P]phosphate associated with the M_r 82,000 polypeptide of GEF indicated that heparin at 0.5 μg/ml results in 40% inhibition of GEF phosphorylation, whereas at 1 μg/ml complete inhibition is observed. The purification procedure of the factor was modified to eliminate the contaminating kinase activity. When the protein was applied to the phosphocellulose column in phosphate buffer lacking KCl, 80–90% of the rabbit reticulocyte casein kinase activity did not adsorb to the phosphocellulose, and the remaining kinase activity was eluted with a buffer containing 0.7 M KCl (25). Under these conditions, GEF adsorbed to phosphocellulose and was eluted with buffer containing 0.5 M KCl, thus separating it from the casein kinase activity. GEF prepared by method 2 appeared to be homogeneous as judged by NaDodSO$_4$/polyacrylamide gel electrophoresis and silver staining (Fig. 2). The incubation of the purified factor with [γ-^{32}P]ATP did not result in its phosphorylation (Fig. 2), confirming that the kinase activity was removed by the modified procedure. GEF prepared by method 2 was used in the remainder of the studies.

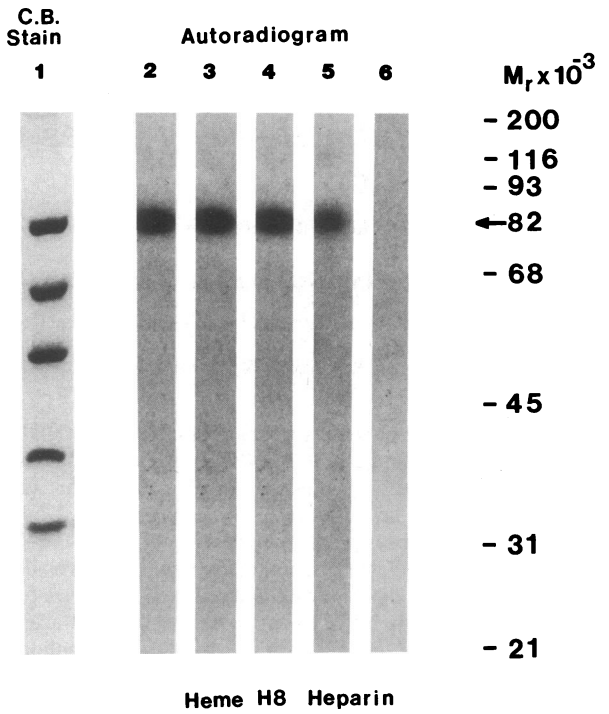

FIG. 1. Effect of various inhibitors on GEF phosphorylation. GEF (4 μg) purified as described (16) (method 1) was added with 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) to a reaction mixture (20 μl) containing 20 mM Tris·HCl (pH 7.5), 0.1 M KCl, 10 mM $MgCl_2$, and 2 mM dithiothreitol. Each reaction was incubated for 15 min at 30°C, either in the absence of added inhibitors (lane 2) or presence of 50 μM heme (lane 3), of 50 μM H8 (lane 4), or of heparin at 0.5 (lane 5) or at 1 (lane 6) μg/ml. Incubations were terminated by the addition of 20 μl of NaDodSO$_4$ sample buffer (26), and samples were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis followed by autoradiography. Lane 1 shows a Coomassie brilliant blue R-250 stained gel, and lanes 2–6 show an autoradiogram of the gel.

The effect of the heme-controlled repressor, protein kinase C, and casein kinase II on GEF phosphorylation was next investigated with the purified factor. As illustrated in Fig. 2, the M_r 82,000 subunit of GEF was phosphorylated only by casein kinase II and not by either the heme-controlled repressor or protein kinase C. This phosphorylation was again strongly inhibited by heparin but not by heme or H8. Treatment with mammalian alkaline phosphatase results in the dephosphorylation of the phosphorylated GEF (Fig. 2, lanes 5 and 6). The amount of alkaline phosphatase required for the complete dephosphorylation was determined by varying the enzyme concentration and using a fixed amount of the phosphorylated GEF as substrate (data not presented). The phosphorylation of GEF by casein kinase II was studied as a function of time. It may be seen in Fig. 3 that 0.8 mol of phosphate per mol of GEF was incorporated after a 45-min incubation when the factor was dephosphorylated. No additional phosphorylation was observed upon addition, after a 30-min incubation, of 2 μg of casein kinase II. The extent of phosphorylation of several GEF preparations purified from various rabbit reticulocyte lysates varied from 0.4 to 0.65 mol of phosphate per mol of GEF. After phosphorylation by casein kinase II the activity of GEF increased 2.6-fold when assayed either in a two stage assay or after reisolation of the factor on a Mono S column (Table 1). The higher activity of the phosphorylated form of GEF is due to an ≈3-fold increase

Table 1. Effect of phosphorylation of GEF on the release of [^{3}H]GDP from the eIF-2·GDP binary complex

Addition(s)		[^{3}H]GDP released, mol/mol of GEF	Relative activity, fold
Stage 1	Stage 2		
	Experiment 1		
GEF	–	0	–
GEF/ATP	–	0	–
GEF	GDP	4.3	1.9
GEF/ATP	GDP	9.9	4.3
GEF	GTP	4.4	1.9
GEF/ATP	GTP	10.5	4.6
	Experiment 2		
GEF	GDP	4.6	2.0
GEF/CK II	GDP	4.6	2.0
GEF/ATP	GDP	4.6	2.0
GEF/CK II/ATP	GDP	11.1	4.8
	Experiment 3		
Isolated GEF	GDP	4.6	2.0
Phosphorylated GEF	GDP	11.1	4.8
Dephosphorylated GEF	GDP	2.3	1.0
Rephosphorylated GEF	GDP	11.1	4.8

In the absence of GEF, the nucleotides had no effect on the exchange reaction, and there was no exchange catalyzed by GEF in the absence of added GDP or GTP. GEF used in experiment 1 was purified as described (16) (method 1); GEF for experiments 2 and 3 was purified by the modified procedure (method 2). A two-stage assay was used for measuring GEF activity. For stage 1, GEF (1.5 μg, 5.25 pmol) was incubated 30 min at 30°C in a 20-μl reaction mixture containing 20 mM Tris·HCl (pH 7.5), 0.1 M KCl, 2 mM dithiothreitol, 10 mM Mg^{2+}, 20% (vol/vol) glycerol, 100 μM ATP, and 0.5 μg of casein kinase II (CK II) as indicated. One microliter (0.26 pmol of GEF) of this reaction mixture was assayed in the second stage (75 μl) for the release of [^{3}H]GDP from the eIF-2·[^{3}H]GDP binary complex (5.0 pmol, 6000 cpm/pmol) in the presence of either 4 μM GDP or 100 μM GTP as indicated. The dephosphorylation of GEF was carried out and was rephosphorylated as in stage 1. For experiment 3, the covalently modified GEF was reisolated by chromatography on a Mono S column. Nucleotide exchange activity ([^{3}H]GDP release) was determined with an excess of isolated eIF-2·[^{3}H]GDP binary complex and plotted against GEF concentration (in the range 0.1–0.4 pmol). The exchange rate was linear under these conditions, and the specific activity of GEF (mol of [^{3}H]GDP released per mol of GEF) was taken as the slope of the resulting line. Relative activity, normalized to the dephosphorylated form of GEF, is the mean of three independent experiments.

in the V_{max} of the guanine nucleotide exchange reaction (data not presented). Treatment with alkaline phosphatase of the phosphorylated GEF decreased its specific activity ≈5-fold, and rephosphorylation by casein kinase II restored it to the level of phosphorylated factor (Table 1).

DISCUSSION

In the present study we have demonstrated that GEF isolated from rabbit reticulocytes may be phosphorylated by casein kinase II. Phosphorylation results in an increase in the specific activity of GEF. This activation is reversed by treatment of GEF with alkaline phosphatase. The increase in activity upon phosphorylation has not been reported for any other polypeptide chain initiation factor. GEF is equally phosphorylated by casein kinase II isolated from either rabbit reticulocytes or *Artemia*. Although GEF is size-fractionated by glycerol-gradient centrifugation during purification, the kinase is nevertheless associated with the factor preparation (Fig. 1), suggesting a possible association of the kinase with its substrate. The specific activity of the dephosphorylated GEF is half that of the isolated factor and one-fifth of the phosphorylated form. These results, with the observations that the stoichiometry of GEF phosphorylation increases

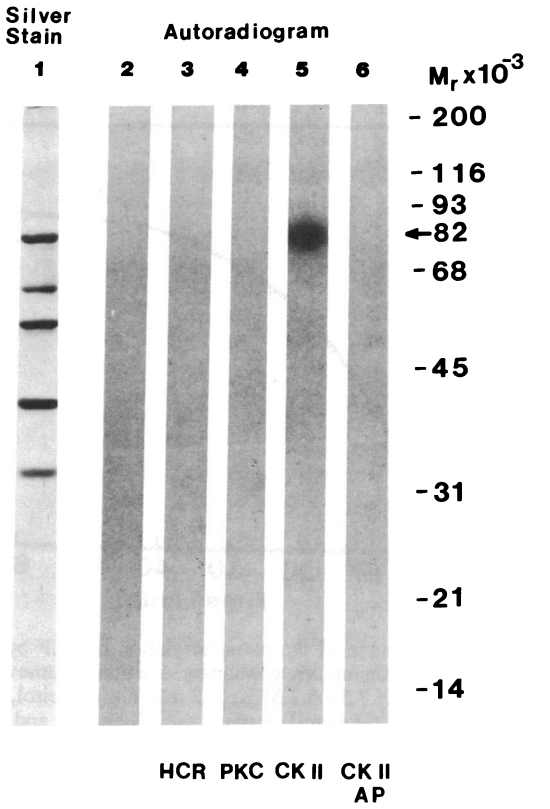

FIG. 2. Phosphorylation of GEF by various kinases. GEF (2 μg) purified by the modified procedure (method 2) was phosphorylated with [γ-^{32}P]ATP (2000 cpm/pmol). Each reaction mixture was incubated either in the absence (lane 2) or the presence of 2 μg of the heme-controlled repressor (HCR) (lane 3), of 1 μg of protein kinase C (PKC) (lane 4), or of 0.15 μg of casein kinase II (CK II) (lane 5). The reaction mixture for lane 6 contained GEF phosphorylated with casein kinase II, which was further incubated with 2 units of mammalian alkaline phosphatase for 30 min at 37°C. The incubations were terminated and analyzed as described in Fig. 1. Lane 1 shows a silver-stained gel, and lanes 2–6 show an autoradiogram of the gel.

after its dephosphorylation, suggest that the factor is partially phosphorylated when isolated from reticulocyte lysates.

Casein kinase II has been identified in the nucleus and the cytoplasm of a wide variety of eukaryotic organisms and shown to phosphorylate a number of endogenous substrates (16, 25). A role for the kinase in integrating cell metabolism was postulated on the basis that the enzyme may regulate the phosphorylation of a number of proteins in different metabolic pathways (25). The enzyme is inhibited by heparin (25) and stimulated by monovalent cations and polyamines. The characterization of GEF as a phosphoprotein represents further evidence that translation may be regulated by the phosphorylation state of the initiation factor. Other polypeptide initiation factors modified by phosphorylation include eIF-2, eIF-4B, and eIF-4F. The inhibition of protein synthesis resulting from eIF-2(α) phosphorylation has been extensively investigated (4, 8, 10, 11). eIF-4B and eIF-4F are dephosphorylated during heat shock (19, 32) and under other conditions of translational repression (19, 20), implying that phosphorylation of these factors may be required for optimum activity. Similarly, the change in the phosphorylation state of the β subunit of eIF2 [eIF-2(β)] under conditions of heat shock (19) and nutrient deprivation (20) has also been reported. The nature of the kinase phosphorylating eIF-4F and the effect of this phosphorylation is still being explored (32–34). eIF-2(β) is phosphorylated *in vitro* by casein kinase II (21, 22, 24, 26), but little is known about the role of this phosphorylation (22). The modification of GEF and eIF-2(β)

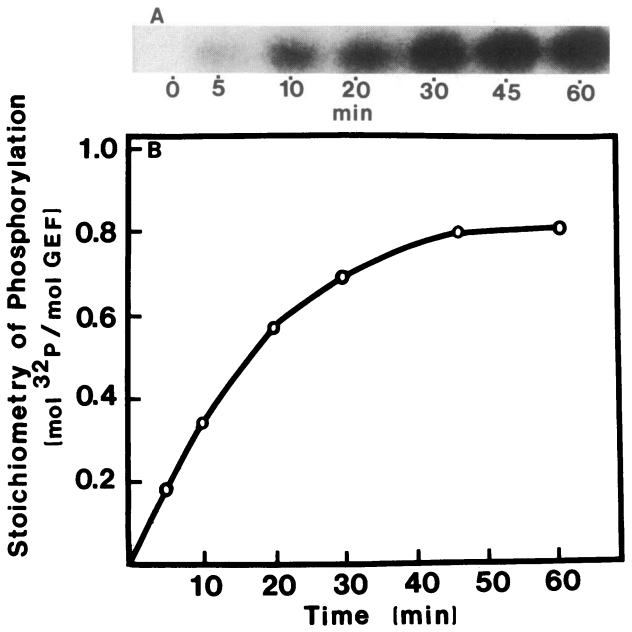

FIG. 3. Time course of the phosphorylation of GEF by casein kinase II. The reaction mixture containing, in a final volume of 70 μl, 20 mM Tris·HCl (pH 7.5), 0.2 M KCl, 2 mM dithiothreitol, 10 mM $MgCl_2$, 7 μg of GEF (24 pmol), 5 μg of casein kinase II, and 100 μM [γ-^{32}P]ATP (2000 cpm/pmol) was incubated at 30°C. A 10-μl aliquot was removed at 0, 5, 10, 20, 30, 45, and 60 min of incubation, mixed with an equal volume of $NaDodSO_4$ sample buffer, and electrophoresed on $NaDodSO_4$/polyacrylamide gels (26). The M_r 82,000 subunit of GEF was excised from the gel and soaked in Uni/verse LSC mixture (J. T. Baker Chemical), and radioactivity was measured in a liquid scintillation counter. (*A*) Autoradiogram of the gel corresponding to the M_r 82,000 subunit of GEF. (*B*) Stoichiometry of GEF phosphorylation.

by casein kinase II and eIF-2(α) by the heme-controlled repressor demonstrates the existence of two distinct phosphorylation/dephosphorylation mechanisms that have opposite effects on polypeptide chain initiation. The observation that both eIF-2(β) and GEF are phosphorylated by the same kinase may indicate a role for casein kinase II in the interaction of these two proteins. The results presented in this communication critically address only the effect of GEF phosphorylation on nucleotide exchange reaction. Experiments were designed to demonstrate that the increase in nucleotide exchange was not the result of eIF-2(β) phosphorylation. These controls were performed with GEF reisolated after phosphorylation and nucleotide exchange was assayed with GDP to prevent phosphorylation of eIF-2(β). Phosphorylation of the M_r 82,000 subunit of GEF may alter its association either with the other subunits of the GEF complex or with eIF-2. Specific antibodies to the M_r 82,000 subunit of GEF can be used to study the changes in the phosphorylation state of GEF under various conditions of translational repression. The phosphorylation/dephosphorylation of GEF may constitute an exciting unexplored locus of translational control.

We wish to thank Usha Verma and Anthony Jones for excellent technical assistance, Romie Brown for secretarial assistance, and Charles Woodley for critical reading of the manuscript. This investigation was supported in part by Grant GM 25451 from the National Institutes of Health.

1. Pain, V. M. (1986) *Biochem. J.* **235**, 625–637.
2. Moldave, K. (1985) *Annu. Rev. Biochem.* **54**, 1109–1149.
3. Trachsel, H. & Staehelin, J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 204–208.
4. Clemens, M. S., Pain, V. M., Wong, S. & Henshaw, E. C. (1982) *Nature (London)* **292**, 93–95.
5. Walton, G. M. & Gill, G. N. (1975) *Biochim. Biophys. Acta* **390**, 231–245.
6. Panniers, R. & Henshaw, E. C. (1983) *J. Biol. Chem.* **258**, 7928–7934.
7. Mehta, H. B., Woodley, C. L. & Wahba, A. J. (1983) *J. Biol. Chem.* **258**, 3438–3441.
8. Siekierka, J., Mauser, L. & Ochoa, S. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2537–2540.
9. Salimans, M., Goumans, H., Amesz, H., Benne, R. & Voorma, H. O. (1984) *Eur. J. Biochem.* **145**, 91–98.
10. Goss, D. J., Parkhurst, L. J., Mehta, H. B., Woodley, C. L. & Wahba, A. J. (1984) *J. Biol. Chem.* **259**, 7374–7377.
11. Farrell, R. J., Balkow, K., Hunt, T., Jackson, R. J. & Trachsel, H. (1977) *Cell* **11**, 187–200.
12. Levin, D. H., Petryshyn, R. & London, I. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 832–836.
13. Ranu, R. S. (1980) *Biochem. Biophys. Res. Commun.* **97**, 252–262.
14. Matts, R. L., Levin, D. H. & London, I. M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2559–2563.
15. Dholakia, J. N., Mueser, T. C., Woodley, C. L., Parkhurst, L. J. & Wahba, A. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6746–6750.
16. Hathaway, G. M. & Traugh, J. A. (1981) *Curr. Top. Cell. Regul.* **21**, 101–112.
17. Krebs, E. G. & Beavo, J. A. (1979) *Annu. Rev. Biochem.* **48**, 923–959.
18. Cohen, P. (1982) *Nature (London)* **296**, 613–620.
19. Duncan, R. & Hershey, J. W. B. (1984) *J. Biol. Chem.* **259**, 11882–11889.
20. Duncan, R. & Hershey, J. W. B. (1985) *J. Biol. Chem.* **260**, 5493–5497.
21. Benne, R., Edman, J., Traut, R. R. & Hershey, J. W. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 108–112.
22. Tuazon, P. T., Merrick, W. C. & Traugh, J. A. (1980) *J. Biol. Chem.* **255**, 10954–10988.
23. Dholakia, J. N. & Wahba, A. J. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2270 (abstr.).
24. Dholakia, J. N. & Wahba, A. J. (1987) *J. Biol. Chem.* **262**, 10164–10170.
25. Hathaway, G. M. & Traugh, J. A. (1983) *Methods Enzymol.* **99**, 317–331.
26. Mehta, H. B., Dholakia, J. N., Roth, W. W., Parekh, B. S., Montelaro, R. C., Woodley, C. L. & Wahba, A. J. (1986) *J. Biol. Chem.* **261**, 6705–6711.
27. Woodley, C. L., Roychowdhury, M., MacRae, T. H., Olsen, K. W. & Wahba, A. J. (1981) *Eur. J. Biochem.* **117**, 543–551.
28. Albert, K. A., Walaas, S. I., Wang, J. K. T. & Greengard, P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2822–2826.
29. Wray, J., Boulikas, T., Wray, V. P. & Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203.
30. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
31. Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5050.
32. Duncan, R., Milburn, S. C. & Hershey, J. W. B. (1987) *J. Biol. Chem.* **262**, 380–388.
33. Rychlik, W., Gardner, P. R., Vanaman, T. C. & Rhoads, R. E. (1986) *J. Biol. Chem.* **261**, 71–75.
34. Rychlik, W., Lawson, T. G., Hagedorn, C. H., Thach, R. E. & Rhoads, R. E. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2185 (abstr.).