AppppA binds to several proteins in *Escherichia coli*, including the heat shock and oxidative stress proteins DnaK, GroEL, E89, C45 and C40

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The dinucleotide AppppA (5',5'''-P¹, P⁴-diadenosine tetraphosphate) is rapidly synthesized in cells exposed to heat stress or oxidative stress. Stress-induced AppppA accumulation has been observed in all cell types studied to date. In order to study the function(s) of AppppA, we created a mutation in the *Escherichia coli* gene that encodes the sole AppppN hydrolase (*apaH*). High levels of AppppA have subsequently been shown to affect many cellular processes, including expression of catabolite repressible genes and the ability to survive starvation, oxidative stress and near-UV irradiation. Nevertheless, the precise role of AppppA remains undefined. In order to better understand the mechanism by which AppppA exerts its effects, we attempted to determine which proteins bind to AppppA by synthesizing (α' -³²P) 8-N₃AppppA for use in photocrosslinking experiments with extract derived from cells with different genetic backgrounds and exposed to various stress conditions. We report here that several *E. coli* proteins bind AppppA, including the heat shock and oxidative stress proteins DnaK, GroEL, E89, C45 and C40. In addition, we show that *apaH* mutants, which have high basal levels of AppppA, are hypersensitive to killing by heat.

Key words: *apaH*/AppppA/heat shock/oxidative stress

Introduction

Small nucleotide derivatives have been proposed to act as cellular signals (alarmones) of various stress conditions (Bochner *et al.*, 1984). The cellular concentration of one such molecule, AppppA (5',5'''-P¹, P⁴-diadenosine tetraphosphate) increases rapidly when *Escherichia coli*, and all other cell types studied to date, are exposed to heat shock or oxidative stress (for reviews, see Varshavsky, 1983; Leveque *et al.*, 1990a). When *E. coli* cells are shifted from 28°C to 43°C, the intracellular AppppA concentration increases from ~1 μ M to 160 μ M (Farr *et al.*, 1989; Plateau *et al.*, 1987). Similar increases are observed following treatment with hydrogen peroxide, ethanol and cadmium chloride (VanBogelen *et al.*, 1987). Accordingly, these results led early investigators to propose that AppppA serves as a signal or 'alarmone' for induction of the heat shock or oxidative stress responses (Bochner *et al.*, 1984).

More recent evidence, however, has eliminated AppppA as the inducer of the oxidative stress or heat shock responses since overexpression of the gene encoding AppppN hydrolase (*apaH*) on a high copy plasmid does not affect the induction of heat shock proteins (Plateau *et al.*, 1987). Addi-

tionally, *apaH* mutants, whose basal level of AppppA is at least 100-fold higher than in wild type cells, do not constitutively express any heat shock or oxidative stress proteins (Farr *et al.*, 1989).

Nevertheless, while AppppA cannot serve as the inducing signal for the heat shock or oxidative stress responses, it may serve as a modulator of these responses, since AppppA inhibits the nucleotidase activity of DnaK, which in turn plays a crucial role in the down-regulation of the heat shock response (Tilly *et al.*, 1983a; Bochner *et al.*, 1986). In addition, λ C1^{ts} lysogens develop functional phage particles more slowly in *apaH* mutants than in wild type cells when exposed to 42°C (S.B. Farr, unpublished observations). Lambda DNA replication and morphogenesis require several heat shock genes, including DnaK and GroEL (reviewed by Friedman *et al.*, 1984).

Several other cellular functions of AppppA have been reported. In bacteria, AppppA overexpression in *apaH* mutants prevents the full induction of catabolite repressible genes, and, in conjunction with mutations in the *ksgA* gene, causes cell filamentation (Farr *et al.*, 1989; Leveque *et al.*, 1990b). In eukaryotes, *in vitro* studies have shown that AppppA inhibits poly(ADP-ribose)polymerase (Tanaka *et al.*, 1981), binds to and primes eukaryotic DNA polymerase α (Grummt *et al.*, 1979) and selectively inhibits the phosphorylation of immunoglobulin G by pp60^{src} protein kinase (Maness *et al.*, 1983).

Given the pleiotropic effects of AppppA on enzyme activity and cell physiology, we were interested in determining the number and identity of AppppA-binding proteins in *E. coli* by synthesizing and photocrosslinking (α' -³²P)8-N₃AppppA and (β -³²P)8-N₃AppppA to proteins from cells subjected to various stress conditions. We also investigated the effect of high intracellular AppppA levels on cellular responses to thermal stress. We report the results of those experiments here.

Results

Synthesis of [α' -³²P]8-N₃AppppA and [β -³²P]8-N₃AppppA

Both α' - and β -labeled [³²P]8-N₃AppppA were synthesized using [γ -³²P]ATP and [α -³²P]ATP, respectively. Using the following designation, 8-N₃A-P α -P β -P β' -P α' -A, AppppA would be labeled at the β position with γ -labeled ATP, while with α -labeled ATP, α' -labeled AppppA would be synthesized. The principal side reactions produce 8-N₃AppAN₃-8 and [³²P]AppppppA, (either γ , γ' or α , α'). The former is not radiolabeled, and the latter does not have an azido group to facilitate photocrosslinking with proteins.

CIP and SVP were added as described in Materials and methods to degrade any remaining terminal phosphates. The efficacy of this procedure was tested by FPLC analysis. Elutions of a mixed solution of ATP and AppppA showed that the former consistently eluted at 53% and the latter at

73% along a linear gradient of 50–700 mM NH_4HCO_3 , pH 8.6 (Figure 1A). Incubation with CIP abolished the peaks corresponding to ATP, ADP and AMP, while the AppppA peak remained unchanged (Figure 1B). Incubation with both SVP and CIP resulted in the eradication of all peaks (Figure 1C). Similar experiments showed that CIP incubation also did not degrade 8- N_3 AppppA (data not shown).

To confirm that the synthesized radiolabeled product was $[\beta\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$, $[\beta\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$ was incubated with extract from either wild type (AB1157) cells or *apaH*⁻ (SF436) cells, which lack AppppN hydrolase, and analyzed by 2-D TLC (Figure 2). Degradation of AppppA by the *E. coli* ApaH hydrolase occurs symmetrically (Guranowski *et al.*, 1983 and Plateau *et al.*, 1985). The results show that while $[\beta\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$ was degraded by wild type cell extract, it was not degraded by extract lacking the AppppN hydrolase. In similar experiments, the rate of degradation

of $[\alpha'\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$ was the same. The identity of the spots on 2-D TLC was confirmed by comparison with pure AppppA and ADP run under the same conditions but unexposed to cell extract (data not shown). The results show that $[\beta\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$ was successfully synthesized and represented the only detectable radiolabeled product.

Photoaffinity labeling

In all crosslinking experiments, physiologically relevant AppppA:protein ratios were used. *In vivo*, the AppppA concentration in unstressed, wild type cells is $\sim 1\text{--}3\text{ }\mu\text{M}$, and rises to around $160\text{ }\mu\text{M}$ during heat shock. Based upon Neidhardt's (1987) estimation that there are $\sim 2.35 \times 10^6$

Fig. 1. FPLC analysis of adenosine nucleotides eluted from a MonoQ anion exchange column by a 20 ml linear gradient of 0.05–0.7 M NH_4HCO_3 , pH 8.6. Vertical axis corresponds to both the elution gradient (thick line) and the absorbance at 254 nm (thin line). Horizontal axis corresponds to 1 ml fraction number. (A) Solution of 0.1 M AppppA and 0.1 M ATP. (B) Solution in (A) following incubation for 1 h with 1 U CIP at 37°C. (C) Solution in (A) following incubation for 1 h with 1 U of both CIP and SVP at 37°C.

Fig. 2. Analysis by 2-D TLC of the degradation products of $[\beta\text{-}^{32}\text{P}]8\text{-N}_3\text{AppppA}$ after incubation with: (A) wild type extract for 2 min, (B) wild type extract for 10 min, (C) *apaH* mutant extract for 2 min and (D) *apaH* mutant extract for 10 min. Migration proceeded left to right in the first dimension, then bottom to top.

protein molecules in a typical cell, and given an average cell volume of $\sim 10^{-15}$ l, the calculated protein concentration is $\sim 3.9 \times 10^{-3}$ M. Thus, *in vivo*, the ratio of protein to AppppA ranges from a $\sim 10^3$ -fold excess in the naive state to ~ 25 -fold excess during heat shock. The concentrations of protein and AppppA chosen for all of our *in vitro* assays (2.56×10^{-5} M protein and 1.8×10^{-7} M [32 P]8-N₃AppppA) gave a ~ 140 -fold excess of protein, a compromise between the naive and heat shocked ratios.

Fig. 3. Time course of photocrosslinking with proteins from crude extracts of *apaH* mutants by [α' - 32 P]8-N₃AppppA, as described in Materials and methods. At the times indicated, UV illumination was stopped and an aliquot was removed. Crosslinked proteins in each aliquot were separated on 10% SDS–polyacrylamide gels.

Fig. 4. Proteins from crude extracts of *apaH* mutants were photocrosslinked with [α' - 32 P]8-N₃AppppA for 2 min, then separated on 10% SDS–polyacrylamide gels. (A) Silver stained gel. (B) Autoradiograph of the same gel. Note that the only band visible in the absence of UV illumination is not visible under normal photocrosslinking conditions.

In order to determine the shortest amount of time that gave detectable crosslinking, a time course of protein labeling with [α' - 32 P]8-N₃AppppA was done. The results show that 120 s was the shortest period of UV irradiation that gave strong labeling (Figure 3). In all subsequent experiments, photocrosslinking was for 120 s.

Photocrosslinked proteins from *apaH* mutants were separated on a 10% SDS–polyacrylamide gel, silver stained and used to expose autoradiograph film. Comparison of band intensities on the autoradiogram with protein bands stained by silver staining show no correlation between protein abundance and AppppA binding (Figure 4). None of the labeling can be attributed to degradation products because, of the two equivalents of ADP formed by symmetrical cleavage of [α' - 32 P]8-N₃AppppA, one would be 8-N₃ADP, which cannot be seen by autoradiography, and one would be [α' - 32 P]ADP, which cannot effectively photocrosslink proteins at the fluence used, as shown by the lack of labeling in the absence of UV illumination (Figure 4). These results show that all of the labeling was due to [α' - 32 P]8-N₃AppppA, since it alone possesses both a radiolabeled phosphate and an azido-modified adenine.

When the salinity of the photocrosslinking buffer was increased from 10 mM to 200 mM, a few bands, primarily in the 20–40 kDa range, disappeared, indicating that AppppA was most likely binding to these proteins on the basis of non-specific charge–charge interactions (Figure 5). However, the labeling of most proteins remained undiminished, a further indication of the specificity of binding, and a few bands became brighter. Most likely, the enhanced labeling of some proteins was due either to the elimination of charge–charge repulsions at the binding site, or to an increased availability of [α' - 32 P]8-N₃AppppA when

Fig. 5. Autoradiograph of photocrosslinked proteins from wild type and *apaH* mutants under conditions of low and high salt. Arrows denote proteins whose photocrosslinking was appreciably affected by high salt.

Fig. 6. Competition of photocrosslinking with proteins from crude extract of *apaH* mutants by [α '- 32 P]8-N₃AppppA. Immediately before the addition of [α '- 32 P]8-N₃AppppA to high salt photocrosslinking reactions, either non-radiolabeled 8-N₃AppppA or 8-N₃ATP was added at the indicated concentrations.

Fig. 7. Competition of *in vitro* (γ - 32 P)ATP-dependent phosphorylation of proteins from wild type extract with increasing concentrations of AppppA. (γ - 32 P) ATP was present at a concentration of 0.1 mM.

non-specific interactions were eliminated. Subsequent experiments designed to purify several AppppA-binding proteins have shown that 500 mM salt has no effect on binding (Fuge, W. and Farr, S.B., manuscript in preparation).

To determine the specificity of binding, competition assays were performed with cold 8-N₃AppppA and 8-N₃ATP. The results show that with a 10³-fold excess of competitor, 8-N₃AppppA reduced protein labeling more effectively than 8-N₃ATP (Figure 6). With a 10⁴-fold excess of competitor, the difference was more dramatic; 8-N₃AppppA competed away almost all protein labeling, while 8-N₃ATP had little effect. Co-operative binding of AppppA to many proteins could explain the non-linear nature of this competition.

To confirm the specificity of binding further, competition assays were performed by adding increasing concentrations of AppppA into an *in vitro* phosphorylation reaction. The results show that AppppA does not effectively compete with ATP for binding to *E. coli* kinases in general (Figure 7). Given the similarity of structures between AppppA and ATP, it is likely that at sufficiently high concentrations, some ATP-binding proteins will bind AppppA, and *vice versa*. The above results, however, suggest a high degree of specificity of most AppppA-binding proteins at physiologically relevant concentrations.

Two-dimensional gel analysis of AppppA-binding proteins

In order to identify the AppppA-binding proteins, two-dimensional PAGE was performed as described in Materials and methods.

The results confirm that several proteins were labeled by 8-N₃AppppA at physiologically relevant concentrations (Figure 8A and B). Apparently, many of these AppppA-binding proteins are present in low abundance, since they were not detected as ³⁵S-labeled proteins. Comparison of AppppA-binding proteins with published gels suggested that several of these proteins are induced by both heat and oxidative stress, including DnaK, GroEL, E89, C45 and C40 (Neidhardt *et al.*, 1981; Tilly *et al.*, 1983a and b; Morgan *et al.*, 1986; Walkup and Kogoma, 1989).

To confirm the identification of these proteins, the above experiment was repeated using extract from cells exposed to heat shock or oxidative stress. The results (Figure 8C and D) show that these five AppppA-binding proteins are induced under both stress conditions. The remaining AppppA-binding proteins did not appear to be induced by either heat shock or oxidative stress, and could not be confidently identified.

To confirm these results further, extracts from *apaH* mutants carrying either *dnaK* or *groEL* on high copy plasmids and exposed to heat shock were analyzed in photocrosslinking experiments. Comparison of heat shocked versus control cells confirms that DnaK, E89 and GroEL bind AppppA (Figure 9). Both C45 and C40 are the same size as non-heat shock AppppA-binding proteins, so their induction by heat was less obvious on 1D gels. Overproduction of GroEL on a plasmid further enhanced the strength of the corresponding radiolabeled band. Overproduction of DnaK on a plasmid did not, suggesting the possibility that binding of AppppA to DnaK is facilitated by another protein that is rate limiting. Finally, these results confirm that the heat shock protein E89 strongly binds AppppA.

Heat sensitivity of *apaH* mutants

Since AppppA was found to bind several heat shock and oxidative stress proteins, we tested whether *apaH* mutants showed altered sensitivity to heat. Compared with isogenic wild type, *apaH* mutants show dramatically increased sensitivity to killing by heat at 55°C (Figure 10). This sensitivity is abolished when *apaH* mutants carry *apaH*⁺ on a plasmid. Although *apaH* mutants did develop thermo-tolerance if pre-exposed to 43°C, they remained significantly more sensitive than pre-exposed wild type cells. Furthermore, unlike wild type cells, *apaH* mutants do not grow at 43°C (Farr *et al.*, 1989). These results suggested the possibility that AppppA binding to heat shock proteins

Fig. 8. Two-dimensional protein gel electrophoresis of extract from wild type *E.coli* photocrosslinked by [α' - 32 P]8-N₃AppppA, spiked with isogenic extract labeled with [35 S]L-methionine. Two pieces of film were exposed by the gels simultaneously, as described in Materials and methods. (A) Film exposed by [35 S]L-methionine labeled proteins from cells grown at 30°C. (B) Film exposed by [α' - 32 P]8-N₃AppppA photocrosslinked proteins. Circled proteins on (A) are those that are photocrosslinked by [α' - 32 P]8-N₃AppppA, as determined by overlaying (A) with (B). (C) Extract from isogenic cells exposed to heat stress at 43°C during labeling with [35 S]L-methionine, then added to extract photocrosslinked with [α' - 32 P]8-N₃AppppA as above. Arrows indicate proteins that are induced during heat stress, as compared to the control. Proteins with both arrows and circles are heat shock proteins that are also photocrosslinked by [α' - 32 P]8-N₃AppppA, as determined by overlaying 35 S- and 32 P-exposed films (32 P-labeled radiograph not shown). (D) Extract from oxidatively (H₂O₂)-stressed cells during labeling by [35 S]L-methionine, then added to extract photocrosslinked with [α' - 32 P]8-N₃AppppA as above. Arrows indicate proteins induced by H₂O₂ stress, and those that are also circled are photocrosslinked by [α' - 32 P]8-N₃AppppA (32 P-labeled radiograph not shown). Letters to the following proteins; A = E89, B = DnaK, C = GroEL, D = C45, E = C40.

reduced their effectiveness in protecting cells from heat shock injury.

Discussion

We have shown that AppppA specifically binds to several proteins, including the stress proteins DnaK, GroEL, E89, C45 and C40. While photocrosslinking appeared to label a large portion of proteins E89, C45 and C40, a much smaller stoichiometric amount of DnaK and GroEL was labeled. Although it is unknown why AppppA would bind to only a portion of DnaK and GroEL, previous investigators similarly have observed that only a fraction of DnaK is phosphorylated (Zylicz *et al.*, 1983). Perhaps there are subpopulations of both proteins with modifications required for binding to AppppA or for phosphorylation. Moreover, there may be an interaction between these two activities.

Preliminary results suggest that AppppA influences the phosphorylation of DnaK; in wild type cells, the level of phosphorylated DnaK increases upon a shift from 30°C to 43°C, while in *apaH* mutants, the level of phosphorylated DnaK is already constitutively high at 30°C and does not increase further upon temperature shift (S.B.Farr and D.B.Johnstone, unpublished observations).

Our results also offer a possible physiological role for the binding of AppppA to DnaK and GroEL. Previous investigators have shown that *dnaK* and *groEL* mutants are hypersensitive to killing by heat, and are unable to grow at 43°C (Paek and Walker, 1987; Fayet *et al.*, 1989). We have shown that AppppA overproduction (under-degradation) causes cells to become hypersensitive to killing by heat, and renders cells unable to grow at 43°C (Farr *et al.*, 1989). It is possible, therefore, that AppppA binding may inhibit or alter DnaK and/or GroEL functions that are required for

Fig. 9. Proteins from *apaH* mutants carrying no plasmid, pOF12 (*groEL*, *groES*), or pMOB45 (*dnaK*) and grown either at 30°C or exposed to 43°C for 10 min, were photocrosslinked with [α - 32 P]8-N $_3$ AppppA and separated on 10% SDS–polyacrylamide gels. Arrows mark the proteins GroEL, DnaK and E89. Panel (A) is a silver stained gel, and panel (B) is the corresponding autoradiograph.

the survival of thermal stress. Consistent with the possibility that AppppA alters or inhibits correct functioning of DnaK and or GroEL, high levels of AppppA in *apaH* mutants cause delayed λ phage morphogenesis in a λ cl^{ts} lysogen (S.B.Farr, unpublished data), a process that requires both DnaK and GroEL (reviewed by Friedman *et al.*, 1984). Additionally, plasmid encoded DnaK rescues *apaH*, *ksgA* mutants from filamentation (Leveque *et al.*, 1990), which suggests that enhanced production of DnaK can compensate for the deleterious effects of AppppA overproduction.

However, the basis for heat sensitivity of *apaH* mutants is probably not due solely to the effects of AppppA-binding on DnaK, as DnaK overproduction by a high copy plasmid does not rescue *apaH* mutants from heat sensitivity (data not shown). We do not yet know the effect(s) of AppppA binding on GroEL function(s), but since only a fraction of the total GroEL protein is bound, and since GroEL functions, unlike DnaK, do not include the regulation of other heat shock proteins, the effects are not likely to cause severe heat sensitivity. Of the five stress proteins, E89 has the highest apparent affinity for AppppA, and it is possible that a significant fraction of E89 is AppppA-bound. The roles of E89, C45 and C40 in protecting the cell from thermal killing are unknown, but in a separate series of experiments we have found that *ksgA* mutants show significantly reduced

induction of E89 when heat shocked and are hypersensitive to killing by heat (D.B.Johnstone and S.B.Farr, submitted).

It is interesting to note that AppppA is synthesized when the cell is exposed to heat stress, starvation and oxidative stress (Lee *et al.*, 1983; Bochner *et al.*, 1984; VanBogelen *et al.*, 1987; Farr *et al.*, 1989; J.Kitzler, personal communication). These same conditions induce DnaK, GroEL and E89; the effect of starvation on C45 and C40 could not be unambiguously determined from the literature (Morgan *et al.*, 1986; Spence *et al.*, 1990; VanBogelen *et al.*, 1987; Walkup and Kogoma, 1989; Matin, 1991; this work). Moreover, *apaH* mutants are sensitive to killing by heat, starvation and oxidative stress (this work; A.Eisenstark, personal communication). If the timing of production and down-regulation of stress proteins is crucial for optimal function, and if AppppA alters or inhibits that function, this may explain why *apaH* mutants are sensitive to the same stresses that normally lead to its production in wild type cells. Constitutively high AppppA may bind to, and alter or inhibit the function(s) of stress proteins before they have fulfilled their functions under that stress.

In addition to stress-inducible proteins, AppppA binds to other *E. coli* proteins, most of which have not yet been identified. AppppA has been reported to affect several physiological processes in many cell types, both *in vivo* and

Fig. 10. Cell killing by exposure to 55°C, with and without a preincubation period of 10 min at 43°C. Horizontal axis shows the times at which aliquots were removed from the 55°C bath. Vertical axis represents cell survival. Wild type (□), *apaH*⁻ (○), *apaH*⁻ + pAQ14 (△). Open figures indicate untreated cells, closed figures indicate cells exposed to 43°C for 10 min prior to shift to 55°C.

in vitro (for reviews, see Varshavsky, 1983; Leveque *et al.*, 1990a). Further identification of AppppA-binding proteins may help explain the numerous effects attributed to AppppA.

Materials and methods

Strains and plasmids

Strains of *E. coli* used were AB1157 (*F*⁻, *thr*-1, *proA*2, *his*-4, *thi*-1, *argE*2, *lacY*1, *galK*2, *rpsL*, *supE*44, *ara*-14, *xyl*-15, *mtl*-1, *tsx*-33; Bachman, 1972), SF436 (AB1157 *apaH*::Tn::mini-kan, Farr *et al.*, 1989) and SF790 (SF436 transformed with pAQ14). Plasmid pAQ14 carries a 3.5 Kb *EcoRI*–*Bam*HI fragment from pUC1247 that contains the *ksgA*, *apaG* and *apaH* genes (Mechulam *et al.*, 1985). Plasmid pOF12 carries *groES* and *groEL* (Fayet *et al.*, 1989) and pMOB45 carries *dnaK* (Zylicz *et al.*, 1983).

Protein extracts

Protein extracts were prepared by growing cells in 200 ml of LB media with antibiotics where indicated (kanamycin, 40 µg/ml; ampicillin, 50 µg/ml, at 30°C at 200 r.p.m. to mid-log phase. Cells were then chilled, pelleted by centrifugation, and resuspended in 2 ml lysis buffer (50 mM MES–KOH, pH 8.9, 10 mM CaCl₂, 5 mM MgCl₂, 5 mM β-mercaptoethanol). While on ice, cells were lysed in a Sonifier W-350 cell disruptor, then cell debris was cleared by centrifugation. The supernatant was dialyzed twice at 4°C for 14 h in 1000 volumes of 20 mM potassium phosphate buffer, pH 7.5. Protein concentrations were determined by the Bradford Assay (Bio-Rad).

Heat sensitivity

Cultures were grown overnight from single colonies in LB with appropriate antibiotics to an OD of ~2.4. The following morning, 30 µl of overnight

culture were diluted into 3 ml LB without antibiotics. At an OD₆₀₀ of 0.3–0.4, cultures were divided into three samples of 0.5 ml each. The first sample was maintained at 30°C as a control. The second sample was placed into a 55°C shaker bath. Aliquots were removed at times indicated in the figure legend. The third sample was pre-incubated at 43°C, then transferred to the 55°C shaker bath, and treated as for the second sample. Samples were diluted in 1 × M9 salts, and plated in triplicate onto LB plates. Colonies were counted following overnight incubation at 30°C.

Synthesis of [α '-³²P]8-N₃AppppA and [β -³²P]8-N₃AppppA

[α '-³²P]8-N₃AppppA and [β -³²P]8-N₃AppppA were synthesized as described previously by Prescott and McClellan (1990), with modifications. 12 µl of 0.05 M 8-N₃AMP (Sigma), 2 µl of 300 mM MgCl₂, and 250 µCi of [³²P]ATP, labeled at either the γ position (ICN Biomedicals, specific activity, 4000 Ci/mmol) or the α position (ICN Biomedicals, specific activity, 3000 Ci/mmol) were combined under a 15 W red light. While in darkness, the mixture was dried in a Savant SpeedVac Concentrator, then redissolved in 2 µl of 1.7 M HEPES–NaOH, pH 6.8 and 1 µl of 5 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (CDI, Sigma) and sealed in aluminum foil. The solution was then incubated at 30°C for 16 h with occasional agitation. The reaction was quenched by dilution with 200 µl of 50 mM NH₄ HCO₃, pH 8.6. Afterwards, the solution was incubated at 37°C for 1 h with 2 U of calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) to degrade selectively any remaining [³²P]ATP and 8-N₃AMP.

Unlabeled 8-N₃AppppA was synthesized as above, but with equal concentrations of 8-N₃AMP and ATP (Boehringer Mannheim) and lyophilized to increase the concentration. The identity and concentration of unlabeled 8-N₃AppppA was determined by comparison with pure AppppA (Boehringer Mannheim) on TLC with 0.75 M KH₂PO₄ (pH adjusted to 3.5 with H₃PO₄) (Bochner *et al.*, 1984). 8-N₃ATP was from Sigma.

FPLC analysis of nucleotides

Solutions of 0.1 M AppppA (Boehringer Mannheim) and 0.1 M ATP (Sigma) in 50 mM NH₄HCO₃ were incubated for 1 h at 37°C without CIP or snake venom phosphodiesterase (SVP, Worthington), with 1 U of CIP or with 1 U of both CIP and SVP. Samples were then loaded onto a Pharmacia MonoQ HRS/5 anion exchange column, eluted by a 20 ml linear gradient of 0.05–0.7 M NH₄HCO₃, pH 8.6, and analyzed at 254 nm.

Two-dimensional TLC analysis of nucleotides

Two-dimensional TLC analysis was done as described previously (Bochner *et al.*, 1984), except that the assay was performed under a 15 W red light at a distance of 0.5 m. Concentrations of protein and radiolabeled 8-N₃AppppA were identical to those used in the crosslinking reactions. Samples were incubated on ice for 10 min before being spotted onto PEI–cellulose (J.T.Baker).

Photoaffinity labeling of proteins

Crosslinking reactions were carried out in the recessed well on the top of a 1.5 ml Eppendorf tube filled with ice. All ingredients were chilled on ice and mixed at 4°C. Ingredients were added in the following order: 10 mM potassium phosphate buffer, pH 7.6, to allow for a final volume of 40 µl, 41 µg of protein extract, and 3 µl of 2.4 µM [³²P]8-N₃AppppA, which was added under a 15 W red light in a dark room. For competition experiments, cold competitor 8-N₃AppppA or 8-N₃ATP in 50 mM NH₄HCO₃ was added at the indicated concentrations. For the determination of the effects of salt on photocrosslinking, 200 mM KCl was added to 10 mM potassium phosphate buffer at a final pH of 7.6.

As quickly as possible, samples were spread into a thin film and irradiated at 265 nm and at a fluence of 3 W m⁻². Samples used in 1-D SDS–PAGE analysis were then pipetted into 1 vol of 2 × Laemmli buffer, steeped in boiling water for 5 min and frozen on dry ice–ethanol until thawed for loading. Samples used in 2-D PAGE analysis were pipetted into 40 µl of buffer A (5.71 g BRL urea, 0.5 ml mixture of ampholines pH 3.5–10, pH 4–6, pH 6–8 and pH 7–9 (Pharmacia-LKB) in the ratio 4:4:4:3, 0.5 ml β-mercaptoethanol, 2.0 ml of 10% NP40, and H₂O to 10 ml), then frozen on dry ice–ethanol and sealed in aluminum foil.

In order to determine the least amount of time that gave strong photocrosslinking, samples were UV irradiated for increasing durations (see results). The optimum UV irradiation time was 120 s. Other than the time-course experiment, all samples were thus UV irradiated for 120 s.

Two-dimensional gel analysis of AppppA-binding proteins

Cells were grown at 30°C and 200 r.p.m. in 6 ml of 1 × M9 salts supplemented with 0.4% glucose, 0.2% casamino acids and 10 µg/ml each

of thiamine and thymine to an OD₆₀₀ of 0.4–0.5. Cells were then aliquoted into three tubes of 2 ml each. The control tube was returned to 30°C and labeled for 10 min with 10 µl of [³⁵S]L-methionine (28 µCi/µl, specific activity, 715 Ci/mmol). Labeling was quenched with 20 µl of 0.1 M L-methionine, and by chilling on ice. To label proteins during heat stress, cells were placed on a 43°C water bath and incubated for 5 min before labeling. To label proteins during H₂O₂ stress, cells were incubated in 100 µM H₂O₂ for 10 min prior to labeling. After labeling, cells were chilled, pelleted, resuspended in 20 µl of buffer A and frozen. Lysis was done by six cycles of freeze–thaw in tepid water and dry-ice–EtOH. Cell debris was cleared by centrifugation.

Cell extract (10 µl) with [³⁵S]L-methionine was mixed with 20 µl of extract crosslinked with (α'-³²P)8-N₃AppppA as described above and loaded onto the first dimension. Electrophoresis was done essentially as described by O'Farrell (1975). Two pieces of Kodak X-OMAT/AR film separated by two layers of plastic wrap were simultaneously exposed to the dried gels. An intensifying screen was placed on top of the second piece of film. ³⁵S-labeled proteins exposed the bottom film, but were blocked from exposing the top film by the plastic wrap, while proteins crosslinked with (α'-³²P)8-N₃AppppA preferentially exposed the top film, due to the enhancing screen. ³²P-labeled proteins were not appreciably visible on the bottom film. Overlaying the two pieces of film allowed precise determination of which proteins bind AppppA.

In vitro phosphorylation

Cell extract was prepared as described above. At a final volume of 50 µl, 41 µg of protein from AB1157 was labeled for 20 min at 30°C with 400 µCi [^γ-³²P]ATP (7000 Ci/mmol). Cold competitor AppppA was added as indicated in the figure legend. Reactions were terminated by adding 1 vol 2× Laemmli buffer as described above.

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