Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 11274–11276, December 1992
Biochemistry

NAD^+ and NADH regulate an ATP-dependent kinase that phosphorylates enzyme I of the *Escherichia coli* phosphotransferase system

(protein phosphorylation/protein kinase/regulation by cell redox and electron transport chain/$NADP^+$/NADPH)

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Contributed by Saul Roseman, August 27, 1992

ABSTRACT **Crude extracts of *Escherichia coli* contain a protein kinase, EI-K, that phosphorylates enzyme I (EI) of the phospho*enol*pyruvate:glycose phosphotransferase system (PTS). Phosphorylation occurs at the active site histidine residue. The activity of EI-K was lost during purification. However, kinase activity was restored by adding NAD^+ or $NADP^+$. NADH reversed NAD^+ activation of the kinase, and the level of EI-K activity was dependent on the NAD^+/NADH ratio. Although crude preparations of EI-K showed no NAD^+ requirement, they were completely inhibited by NADH, either in the assay mixture or when the enzyme was pretreated and the NADH was removed prior to the assay. NAD^+ restored full activity to the NADH-pretreated inactive fractions. The results suggest that EI-K contains a bound cofactor that is lost during purification and that may be analogous to NAD^+. EI-K activity may serve to link some of the diverse functions of the PTS, such as sugar transport, to the metabolic state of the cell.**

The phospho*enol*pyruvate:glycose phosphotransferase system (PTS) catalyzes diverse functions in the bacterial cell, including concomitant uptake and phosphorylation of its sugar substrates, the regulation of gene expression of other catabolic operons, chemotaxis toward its substrates, etc. (for reviews, see refs. 1 and 2). In sugar uptake via the PTS, the phosphate group of phospho*enol*pyruvate (PEP) is transferred to the sugar through a chain of PTS proteins; the active sites in these proteins are generally histidine residues. The ratios of phosphorylated to unphosphorylated PTS proteins appear to regulate the activities of non-PTS proteins such as adenylate cyclase (1–3).

Enzyme I (EI) is the first protein in the PTS sequence. EI accepts the phosphate group from PEP, and we believe that it may control many of the subsequent steps in the pathway, such as the rate of sugar transport. EI can also be phosphorylated at its active site histidine residue (4) by acetate kinase and either ATP or GTP. The phosphate group in this case is transferred from the nucleotide to an acyl group in acetate kinase, and then reversibly to EI. This interaction may link the Krebs cycle to the PTS.

In a preliminary communication, we reported* that a previously undescribed ATP-requiring kinase, designated EI-K, phosphorylates EI at its active site. Crude extracts of *Escherichia coli* contain acetate kinase, but this contaminant is removed at one of the early stages of purification of EI-K.

We report here that EI-K is regulated by NAD^+ and NADH.

MATERIALS AND METHODS

Materials. EI was purified from an overproducing transformant of *E. coli* and dephosphorylated as described (5, 6). EI-K was prepared from wild-type *E. coli* K-12 (American Type Culture Collection 10798) grown to late exponential phase in a fermenter (200 liters) in minimal salts medium plus 0.01% thiamin with 0.15% glucose as the sole carbon source. Cells were harvested by centrifugation, washed with the salts medium, and stored at −20°C.

Affi-Gel blue affinity agarose was purchased from Bio-Rad. DEAE-Sepharose CL-6B and Sephadex G-50 fine were from Pharmacia LKB. HPLC anion-exchange chromatography was performed with a Zorbax Bio Series SAX column from MAC-MOD Analytical (Chadds Ford, PA).

NAD^+ (grade I), NADH (grade I), $NADP^+$ (NAD^+-free), and NADPH were from Boehringer Mannheim (or from Sigma) and solutions were prepared immediately prior to use. Phenylmethanesulfonyl fluoride, DNase I, RNase A, ATP, 3-(*N*-morpholino)propanesulfonic acid (Mops), 2-(*N*-morpholino)propanesulfonic acid (Mes), and the other coenzymes and analogs used were purchased from Sigma. Dithiothreitol was obtained from Research Organics. Enzyme-grade ammonium sulfate was from Bethesda Research Laboratories. Adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol in aqueous solution; 1 Ci = 37 GBq) was purchased from Amersham. All other chemicals were analytical reagent grade or the highest purity available.

Mops buffer contained 50 mM Mops, 10 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 10% (vol/vol) glycerol and was adjusted to pH 7.5 at room temperature with NaOH. Mes buffer contained 50 mM Mes, 10 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 10% (vol/vol) glycerol and was adjusted to pH 6.0 at room temperature with NaOH.

Purification of EI-K. Details of the purification will be presented elsewhere. Cell pellets (20 g) were thawed in 40 ml of Mops buffer without $MgCl_2$, ruptured by three passages through a French press, and treated with 1 mM phenylmethanesulfonyl fluoride as a protease inhibitor, DNase I and RNase A (20 μg/ml each), and 10 mM $MgCl_2$. After centrifugation, the supernatant fluid was fractionated with ammonium sulfate, and EI-K activity (fraction precipitating at 50–70% saturation) was dialyzed against Mops buffer at 0–4°C. The 50–70% fraction had no polyphosphate kinase activity (which interfered with the routine assay described below). All remaining steps were conducted in the cold.

Fraction A. The protein was applied to an Affi-Gel blue column equilibrated in Mops buffer. EI-K activity was recovered by washing the gel with buffer and pooling all protein not retained by the gel. This step gives approximately a 97% yield of EI-K and only a 1.4-fold purification over the

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Abbreviations: PTS, phospho*enol*pyruvate:glycose phosphotransferase system; PEP, phospho*enol*pyruvate; EI, enzyme I of the PTS; EI-K, the kinase that utilizes ATP and phosphorylates EI.

*Dannelly, H. K., Reeves, H. C. & Roseman, S., 91st Annual Meeting of the American Society of Microbiology, May 5–9, 1991, Dallas, K-141, p. 238 (abstr.).

ammonium sulfate fraction, but the column removes all traces of acetate kinase. The unadsorbed protein is designated fraction A in the experiments that follow.

Fraction B. Fraction A was chromatographed on a DEAE-Sepharose column equilibrated in Mes buffer and eluted with a 0–500 mM KCl gradient in Mes buffer. EI-K activity eluted in a broad peak around 250 mM KCl and was concentrated to 5 mg of protein per ml by using an Amicon concentrator with a 30-kDa molecular mass cut-off membrane. At this stage, preparations showed partial activity (15–25%) in the absence of NAD^+.

An absolute NAD^+ requirement for EI-K activity was observed after further purification either by isoelectric precipitation or by fractionation on an HPLC SAX anion-exchange column at pH 7.5. The precipitation step is briefly described here.

The EI-K pool from the DEAE-Sepharose column was dialyzed against 50 mM citric acid/NaOH, pH 4.6, containing 1.0 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol for 4–12 hr at 4°C. The precipitate was removed by centrifugation at 27,000 × *g* for 15 min. EI-K activity was then precipitated by dialysis against the same citric acid buffer system adjusted to pH 4.4. The precipitate was collected by centrifugation, resuspended, and dialyzed against Mops buffer, pH 7.5. Occasionally, an insoluble precipitate was observed, which was removed by centrifugation. The final preparation is designated fraction B. It was obtained in about 13% yield and purified approximately 15-fold over fraction A.

Enzyme Assay. EI-K was assayed at 37°C by incubating the following components in final volumes of 20 μl: 7.2 μg of EI (120 pmol of EI, calculated as monomer), the Mops buffer described above, 20 mM potassium phosphate buffer, pH 7.5, 5 mM [γ-^{32}P]ATP (100 cpm/pmol), 29.2 μg of fraction A or 2.2 μg of fraction B, and NAD^+ and/or NADH as indicated. Five-microliter aliquots were removed at 0, 15, and 30 min, and the reaction was stopped by adding 20 mM EDTA. The samples were then centrifuged through Sephadex G-50 spun columns, slightly modified from the procedure given in ref. 7, to isolate ^{32}P-labeled EI (^{32}P-EI), whose radioactivity was measured in a liquid scintillation spectrometer. Incubations were performed in duplicate, and an example of the range of duplicate values is given in Fig. 1*A*. Controls consisted of samples lacking EI or the EI-K fraction. These incubations (0–30 min) gave negligible ^{32}P in the column eluates, generally <40 cpm.

Results are presented as pmol of ^{32}P-EI formed per 20-μl incubation mixture.

As will be described elsewhere, the incubation conditions are optimal for EI-K activity, and specific for γ-labeled ATP (GTP is inactive). Activity was proportional to the quantity of EI-K and time of incubation. $NADP^+$ and NADPH could substitute for NAD^+ and NADH, respectively, in all cases. Fraction B contained no detectable NAD^+ kinase activity.

RESULTS

Fraction B. We have reported* that EI-K transfers the γ-phosphate of ATP to the active-site histidine residue of EI. The activity was, however, not retained upon further purification, and it appeared possible that EI-K contained a bound cofactor which was lost during the fractionation steps. A number of potential cofactors were therefore tested with the inactive enzyme, and two of these, NAD^+ and $NADP^+$, restored enzymatic activity.

Typical results are shown in Fig. 1*A*. Fraction B was virtually inactive in the absence of NAD^+ and was highly active in the presence of 1 mM NAD^+. Under these conditions, one-third of the EI was phosphorylated in 30 min. The rate remained approximately constant until about 90% of the EI was phosphorylated, after which the rate decreased substantially. The initial rate of EI phosphorylation was proportional to the quantity of EI-K.

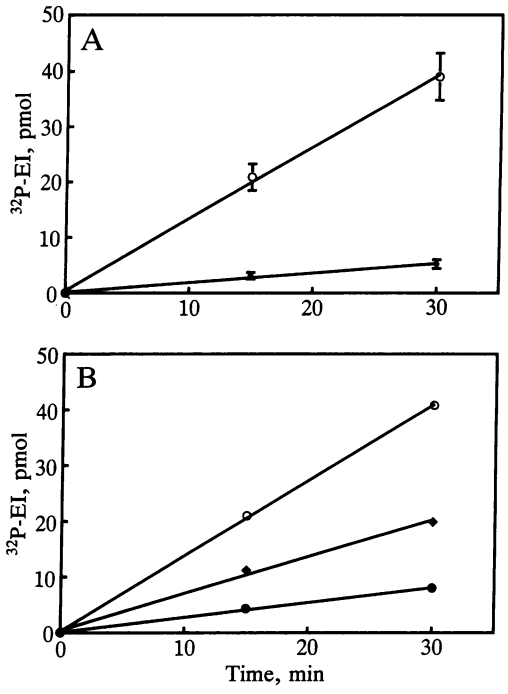

FIG. 1. EI-K activity of fraction B. (*A*) Incubation mixtures supplemented with fraction B were prepared as described in the text (*Enzyme Assay*) with (○) or without (●) 1 mM NAD^+. The range of values obtained with duplicate samples is given by the bars. (*B*) The mixtures contained 1 mM NAD^+ (○), 1 mM each NAD^+ and NADH (◆), or 1 mM NAD^+ and 5 mM NADH (●).

NADH was unable to replace NAD^+ even when used at 10 mM (data not shown). In fact, NADH was a potent inhibitor of the kinase, reversing the NAD^+ stimulatory effect (Fig. 1*B*). The degree of inhibition depended on the ratio of NAD^+ to NADH.

One explanation for these results was nonspecific oxidation and reduction (for example of SH groups in the kinase). The specificity of the NAD^+/NADH effect was therefore examined. As will be reported elsewhere in detail: (*i*) Pretreatment of fraction B with NAD^+ was ineffective—i.e., NAD^+ had to be present during the assay. Thus, it appears unlikely that the NAD^+ effect resulted from activation of the kinase by ADP-ribosylation (8). (*ii*) The following compounds did not replace β-NAD^+: FAD, α-NAD^+, NMN^+, pyridine aldehyde adenine dinucleotide, or ADP-ribose. (*iii*) Dithiothreitol (10 mM) did not replace NADH as an inhibitor. Furthermore, the assay was routinely performed in the presence of 1–2 mM dithiothreitol, and the NAD^+ activation was observed with concentrations of the latter compound as low as 0.1 mM.

Fraction A. The cruder EI-K preparation, fraction A, showed no requirement for NAD^+ (Fig. 2*A*). These results suggested that EI-K in fraction A contained a bound cofactor, possibly related to NAD^+, which was lost upon further purification. To test this idea, fraction A was pretreated with 10 mM NADH for 30 min at 37°C, the NADH was removed by gel filtration, and the sample was assayed in the presence and absence of NAD^+. It is evident from Fig. 2*B* that the pretreatment resulted in complete loss of EI-K activity. When NAD^+ was present in the assay mixture, however, EI-K activity was fully restored.

DISCUSSION

We have emphasized in earlier papers (2–6, *) that EI is likely to play a key role in regulating the PTS. Thus, the phosphor-

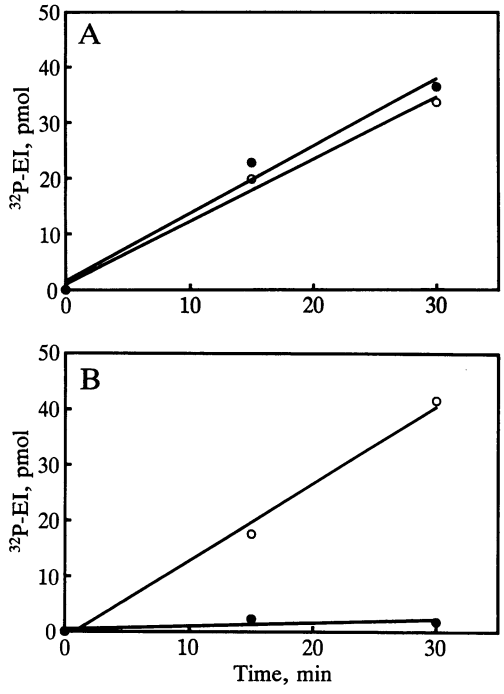

FIG. 2. EI-K activity of fraction A. (*A*) Incubation mixtures contained fraction A (*Enzyme Assay*) with (○) and without (●) 1 mM NAD^+. (*B*) Fraction A was incubated with 10 mM NADH in Mops buffer at 37°C. After 30 min, the protein was separated from the NADH by centrifuging through a Sephadex G-50 spun column equilibrated with the Mops buffer. The standard EI-K activity assay of the protein fraction was conducted with (○) or without (●) 1 mM NAD^+.

ylation of EI may be the crucial step in regulating diverse physiological functions in the bacterial cell.

We now know three different mechanisms for phosphorylating EI at its active site histidine: (*i*) autophosphorylation by PEP; (*ii*) phosphorylation by acetate kinase and ATP (or GTP), which may link the PTS to the Krebs cycle (4); and (*iii*) the ATP-requiring kinase, EI-K, described here.

EI-K appears to be a unique protein kinase. While NAD^+ and NADH are allosteric regulators of many enzymes, we are aware of only two protein kinases in which the pyridine nucleotides act as effectors. Isocitrate dehydrogenase kinase/phosphatase activities (9) are reversibly inhibited by NADH (or NADPH), and mammalian pyruvate dehydrogenase kinase and phosphatase have been shown to be sensitive to the NAD^+/NADH molar ratios in the cell (10). However, no other protein kinase, when purified free of an apparent cofactor, shows an absolute requirement for NAD^+ for activity. The identity of the cofactor remains to be determined, but whatever it may be, it appears that EI-K provides a direct link between the PTS and the redox state of the cell, and therefore to the electron transport chain.

We thank Dr. Bruce Anderson for helpful discussions, Pat Coyle and Dr. Harry Price for providing purified EI, and Derek Nielson for technical assistance in EI-K preparation. We also thank Pat Coyle for his expertise in operating the fermenter. This work was supported by Grant GM38759 from the National Institutes of Health.

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