JOURNAL OF BACTERIOLOGY, May 1995, p. 2798–2803 Vol. 177, No. 10
0021-9193/95/$04.00+0

Effector-Mediated Stimulation of ATPase Activity by the σ^{54}-Dependent Transcriptional Activator FHLA from *Escherichia coli*

SYLVIA HOPPER AND AUGUST BÖCK*

Lehrstuhl für Mikrobiologie der Universität München, D-80638 Munich, Germany

Received 21 November 1994/Accepted 13 March 1995

The FHLA protein is the transcriptional regulator of the genes of the formate regulon from *Escherichia coli*. The protein shares homology with the σ^{54}-dependent regulators of the NTRC family in the central and C-terminal domains but differs in possessing an extended N terminus lacking the aspartate residue which is the site of phosphorylation. Purified FHLA displays intrinsic ATPase activity which is stimulated weakly by formate and DNA. The presence of both formate and DNA carrying the upstream regulatory sequence to which FHLA binds leads to a large increase in the rate of ATP hydrolysis. Hypophosphite, a structural analog of formate, and azide, a transition state analog of formate, also stimulate ATPase activity, supporting the conclusion that formate is a direct ligand of FHLA. Half-maximal saturation of FHLA with formate took place at around 5 mM, and half-maximal saturation with target DNA took place at around 50 nM. The stimulation of ATPase activity by formate was conferred by a decrease in the apparent K_m for ATP, whereas the effect of the DNA binding site also affected the K_{cat} of the reaction. The other nucleoside triphosphates, GTP, UTP, and CTP, competed with ATP cleavage by FHLA, suggesting at least their binding to FHLA. The specific ATPase activity of FHLA was dependent on the concentration of FHLA in the assay, especially in the presence of DNA and formate. Direct liganding of the effector, therefore, leads to the same consequence as phosphorylation for the NTRC-type regulators, namely, stimulation of ATPase activity.

The formate regulon of *Escherichia coli* includes the genes for formate dehydrogenase H and hydrogenase 3, several genes (*hyp*) whose products are involved in maturation of the hydrogenases of this organism, and two genes, *fhlA* and *hycA*, whose products function in the regulation of expression of the regulon (7, 16, 18). FHLA is a transcriptional activator which binds to upstream activating sequences (UAS) located 100 to 120 bp 5′ to the start sites of transcription of the transcriptional units and activates transcription from σ^{54}-dependent promoters (3, 4, 15, 21, 22). Activation of the promoter occurs only in the presence of formate, but it has not been rigorously proven that formate per se is the effector (5, 11, 15). The HYCA protein counteracts the function of FHLA in some unknown manner. FHLA has been purified recently and demonstrated to promote the expression of genes coding for the formate hydrogenlyase components of *E. coli* in vitro (11, 22).

FHLA belongs to the large NTRC family of transcriptional regulators. These regulators consist of an N-terminal regulatory domain (domain A), a highly conserved central domain (C) which is thought to interact with RNA polymerase holoenzyme, and a domain (D) which is characterized by a helix-turn-helix structure involved in interaction with the UAS motif (9, 17, 21). These three major domains are usually separated by relatively short linker segments. The function of most of the response regulators requires the phosphorylation of an aspartic acid residue situated in a conserved position of the A domain (25). The C domain sequence includes a nucleoside phosphate binding motif, and it has been shown that phosphorylation of the aspartyl group in the A domain stimulates a nucleoside triphosphate hydrolysis reaction which is required for the conversion of a closed complex of RNA polymerase with the DNA into the open form necessary for initiation of transcription (1, 27).

FHLA belongs to a subclass of these regulators which share with the NTRC prototypes only the structural characteristics at the C and D domains (21). This subclass, which also includes the NIFA, DMPR, and XYLR proteins (9, 12, 23), lacks the conserved aspartyl residue at the regulatory domain. Regulators of this class do not therefore appear to require phosphorylation by a sensor kinase; experiments involving domain exchange between XYLR and DMPR (24) and in vitro transcription-translation analysis of the activity of FHLA (11) provided evidence that these regulators may be activated by direct interaction with their effector molecules. In this communication we prove that FHLA directly interacts with the effector formate and that binding triggers ATPase activity of FHLA. The presence of the effector also appears to cause FHLA to take on a conformation in which it is able to discriminate its specific DNA binding sequence.

MATERIALS AND METHODS

Purification of FHLA and preparation of the oligodesoxyribonucleotides. Purification of FHLA was carried out as previously described (22). Two double-stranded oligodesoxyribonucleotides were used in the experiments (Fig. 1). SL1-2 is identical to the UAS located between the *hyp* and *hyc* operons, as it was protected by FHLA in DNase I footprinting experiments. SL1 has the sequence 5′ GAA TAA TGT CGA TGA TGT CGA AAT GAC ACG TCG ACA CGG CGA CGA 3′, and the sequence of SL2 is complementary to that of SL1. Oligodesoxyribonucleotide SH8-9 is identical to the sequence bordering the UAS at the 3′ side of the *fdhF* upstream region; it was used as a control. The sequence of SH8 is 5′ GAG TTT TGA ATA AAT AGT GCC CGT AAT ATC AGG GAA TGA CCC CAC 3′, and SH9 has the complementary sequence. Both double-stranded oligodesoxyribonucleotides were custom synthesized as the 45-mers SL1 plus SL2 and SH8 plus SH9 and annealed by pairwise incubation using the following temperature program: 2 min at 88°C, 10 min at 65°C, 10 min at 37°C, and 5 min at room temperature.

Assay of ATPase activity. The hydrolysis of ATP was monitored by using 10-μl reaction mixtures composed of 35 mM Tris-acetate (pH 7.5), 70 mM potassium acetate, 5 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM dithio-

* Corresponding author. Mailing address: Institute of Genetics and Microbiology, University of Munich, Maria-Ward-Straße 1a, D-80638 München, Germany. Phone: 089-17919856. Fax: 089-17919862.

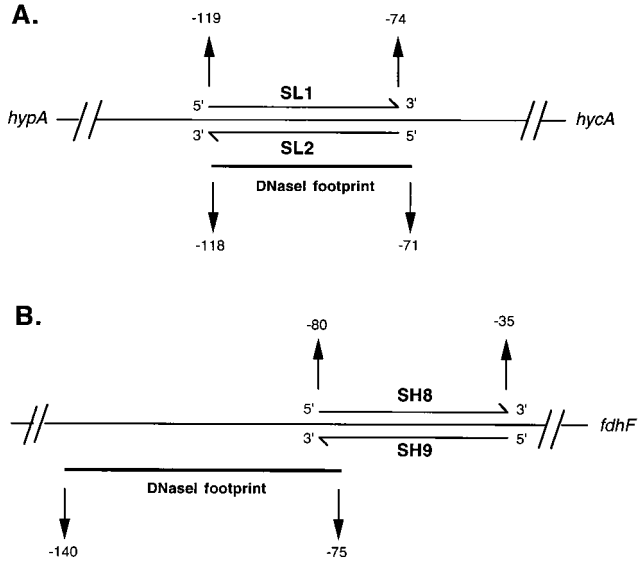

FIG. 1. Characteristics of the oligodesoxyribonucleotides used. (A) SL1-2 is a double-stranded 45-mer which comprises the region between the *hyp* and *hyc* operons, which is protected by FHLA against DNase I digestion (22). (B) SH8-9 carries DNA (double stranded, 45-mer) which borders the upstream regulatory region of the *fdhF* gene at the 3′ side (22). Numbers indicate distances from the start site of transcription.

threitol, and 5% glycerol. The buffer composition, therefore, resembled that used previously (1). Potassium formate (adjusted to the pH of the reaction mixture), FHLA protein, and oligodesoxyribonucleotides were added at the final concentrations indicated in the figure legends and tables. If not specified otherwise, [γ-^{32}P]ATP was added at 1 mM with a specific radioactivity of 0.5 mCi/μmol. Product formation was monitored either by thin-layer chromatography or by liquid scintillation counting. To this end, the reaction was stopped by the addition of 1 μl of 5% sodium dodecyl sulfate containing 20 mM EDTA. Samples (1 μl) were spotted onto polyethyleneimine cellulose plates with a fluorescence indicator (Cel 300/PEI/UV254 plates; Macherey and Nagel) and developed by chromatography in 0.75 M potassium phosphate, pH 3.5. Portions (2 μl) of 2-mg/ml solutions of ATP, ADP, and AMP were cochromatographed as a reference. The plates were dried, and the radioactivity was visualized by autoradiography at −80°C.

Alternatively, the reactions were ceased as described above, and 400 μl of a suspension of activated charcoal (5% in 50 mM sodium phosphate) was added. After mixing and centrifugation (3 min at 13,000 rpm in an Eppendorf centrifuge) 300 μl of the supernatant was taken and centrifuged again. A 200-μl portion of the supernatant was aspirated, and 100-μl samples of this portion were transferred into 1-ml scintillation cocktails (Ultima Gold; Packard). Radioactivity was determined in the ^{32}P channel of a Kontron MR300 liquid scintillation counter. Experiments were carried out in triplicate (except those for Fig. 2, 5, and 7, which were performed in duplicate).

Protein and nucleic acid determinations. The concentration of purified FHLA was determined spectroscopically at 280 nm by use of the FHLA-specific extinction coefficient derived from the known content of aromatic amino acids (21). The concentration of oligodesoxyribonucleotides was calculated from their A_{260} by taking the specific base composition as a basis for calculation.

RESULTS

FHLA possesses intrinsic ATPase activity. Analysis of the sequence of FHLA has revealed the existence of a potential ATP binding motif (21), and it has been speculated that all activators of σ^{54} holoenzyme probably have an ATPase activity which is required for their function (27). Therefore, we investigated whether FHLA can hydrolyze ATP. α-^{32}P- and γ-^{32}P-labelled ATP were incubated with purified FHLA protein, and the reaction products were analyzed by thin-layer chromatography and autoradiography (Fig. 2). The results show that labelled inorganic phosphate and ADP, respectively, were formed from these substrates, indicating that FHLA protein possesses an intrinsic ATP cleavage activity like that of NTRC (1, 27).

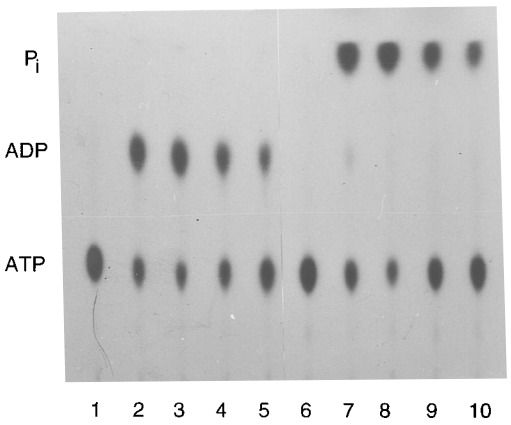

FIG. 2. ATPase activity of FHLA. Reaction mixtures with 1.88 μM FHLA (monomer) were incubated with 1 mM (lanes 2 and 7), 0.5 mM (lanes 3 and 8), 0.1 mM (lanes 4 and 9), and 0.05 mM (lanes 5 and 10) ATP. Lanes 1 and 6 were without FHLA. Assays separated in lanes 1 to 5 contained 2.3 nCi of [α-^{32}P]ATP, and those of lanes 6 to 10 contained 2.3 nCi [γ-^{32}P]ATP. Incubation took place for 90 min at 37°C, and the products were visualized by autoradiography after thin-layer chromatography.

Influence of formate and specific DNA on the ATPase activity of FHLA. By gel mobility shift assays and DNase I footprinting three DNA sites to which FHLA binds specifically have been identified: (i) the UAS of the *fdhF* gene, (ii) the UAS of the intergenic region between the divergently transcribed *hyc* and *hyp* operons, and (iii) a site located between the *hycA* and *hycB* genes (22). The sizes of the regions protected by FHLA against digestion by DNase I were between 27 and 56 bp. By comparison of the three sites different potential DNA recognition motifs were identified; however, there is no overall homology between any of the three sites (22).

NTRC (1) and DCTD (14) have an ATPase activity which can be stimulated by DNA, and we wanted to know if this is the case for FHLA as well. Therefore, we used two double-stranded 45-mer oligodesoxyribonucleotides (for a description see Materials and Methods and Fig. 1). The intrinsic ATPase activity could be increased by the addition of formate or of the SL1-2 and SH8-9 oligos (Fig. 3). Both the specific oligodesoxyribonucleotide (containing the UAS) and the unspecific oligodesoxyribonucleotide provided the same stimulation when they were added alone. However, when formate was added together with these oligodesoxyribonucleotides, the presence of the specific one dramatically increased the rate of ATP hydrolysis, whereas the presence of the control oligodesoxyribonucleotide (SH8-9) did not increase the rate above that reached by the addition of formate alone.

Next, the kinetic parameters of FHLA in the ATP hydrolysis reaction were determined in order to assess whether the effects of formate and/or of the DNA binding sites are produced via a change of the affinity for ATP or of the maximal velocity of the reaction. Table 1 shows that with ATP as the sole reaction component FHLA was not saturated with the substrate at the concentration of 1 mM used in the assays. When added alone, both formate and the DNA increased the affinity for ATP significantly. The V_{max} was slightly increased in the presence of formate, but it was reduced in the presence of SL1-2 DNA. However, a considerable increase in V_{max} was experienced when both formate and SL1-2 DNA were present. Thus, in contrast to regulators of the NTRC or DCTD type, in which phosphorylation as well as DNA triggers an increase of the

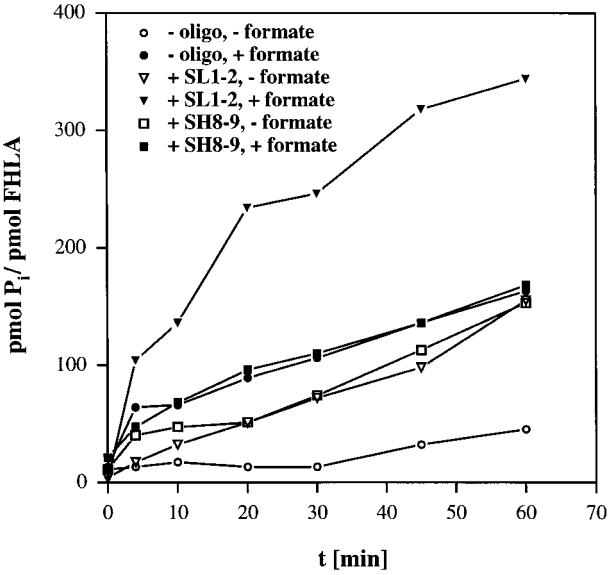

FIG. 3. Rate of ATP hydrolysis by FHLA in the absence and presence of formate and of the oligodesoxyribonucleotides (oligo) SL1-2 and SH8-9. Formate was present at 10 mM, the oligodesoxyribonucleotides were present at a 1 μM final concentration, and FHLA was present at 471 nM (monomer).

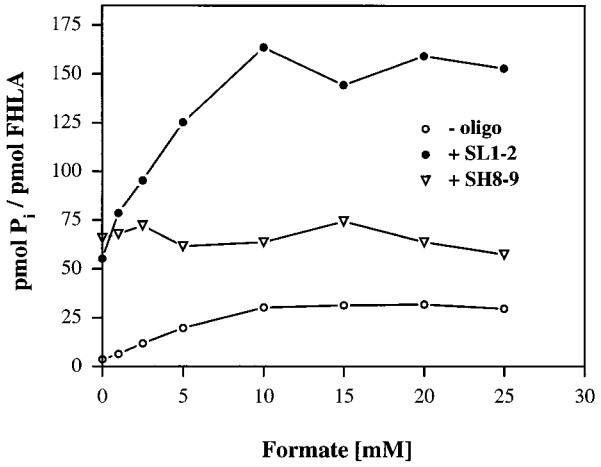

FIG. 4. Formate concentration dependence of the rate of ATP hydrolysis by FHLA (at a 471 nM final concentration) in the absence and presence of the oligodesoxyribonucleotides (oligo) SL1-2 (1 μM) and SH8-9 (1 μM). The incubation time was 60 min at 37°C.

V_{max} of the ATPase activity (14, 27), FHLA displays a more complex kinetic behavior: both the K_m for ATP and the V_{max} are affected by binding of the ligands. In all further experiments the ATP concentration was kept at 1 mM, a value which still allows the discrimination of the influence of the binding of the ligands on the affinity for ATP.

Concentration dependence of the stimulation of ATPase activity by formate and oligodesoxyribonucleotides. Figure 4 presents the dependence of ATPase activity on the concentration of formate. Maximal rates were observed at about 10 mM formate, irrespective of whether the specific oligodesoxyribonucleotide was present or not. The formate concentration resulting in optimal ATPase activity is in the same range as that for optimal promoter activity in the coupled in vitro transcription-translation system (11). In the presence of the control oligodesoxyribonucleotide SH8-9 there was no statistically significant increase of ATP hydrolysis by formate.

Stimulation of ATPase activity by the specific oligodesoxyribonucleotide (SL1-2) in the presence of formate occurred at concentrations in the nanomolar range (Fig. 5). Maximal rates were observed at about 100 nM, which amounts to a ratio between FHLA monomers and SL1-2 oligodesoxyribonucleotide of 4:1 to 5:1.

Substrate specificity of FHLA. To verify that formate directly interacts with FHLA, we tested the activities of two analogs of formate, namely, hypophosphite and azide. Hypophosphite is a competitive inhibitor of formate for several enzymes (13), and it has been shown that hypophosphite can substitute for formate in activating *hycA* gene expression in vivo (26) as well as in vitro (11), whereas azide is a transition state analog (6). Figure 6 illustrates that azide stimulates ATP hydrolysis by FHLA more than the cognate substrate, formate. Hypophosphite, in contrast, increases the rate only at lower concentrations and appears to be inhibitory at higher levels. Formamide and formaldehyde were not stimulatory at all (results not shown), which is in agreement with the data obtained by Blanchard and Cleland (6), who demonstrated that uncharged formate analogs like formamide and formaldehyde are not oxidized by certain formate dehydrogenases and that they do not bind to the enzyme.

The nucleotide specificity of the ATP hydrolysis reaction was analyzed by performing competition experiments in which a 20-fold excess of each of the four nucleoside triphosphates was added to a reaction mixture containing 1 mM [γ-^{32}P]ATP

TABLE 1. Kinetics of ATP hydrolysis by FHLA[a]

Component(s) added	Apparent K_m (mM ATP)	V_{max} (pmol of $P_i \cdot min^{-1} \cdot$ pmol of FHLA monomer^{-1})
None	2.9	14.8
Formate (10 mM)	0.5	20.5
SL1-2[b]	0.8	8.6
Formate + SL1-2[b]	1.0	47.6

[a] FHLA was present at a final concentration of 471 nM. Values are the averages of those from two independent determinations.
[b] SL1-2 DNA was added at a final concentration of 0.5 μM.

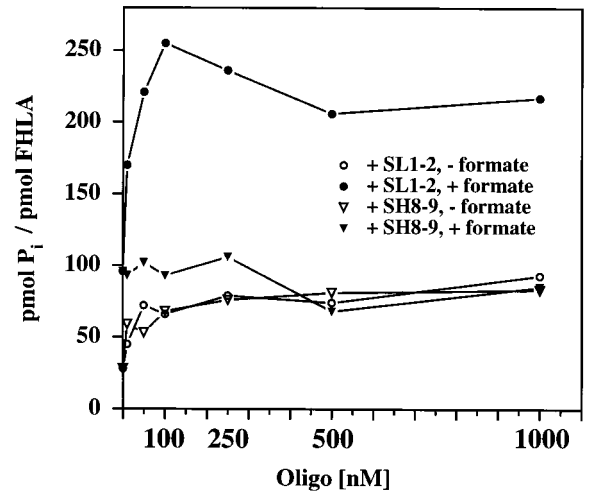

FIG. 5. DNA dependence of ATP hydrolysis by FHLA (471 nM monomer) in the absence or the presence of 10 mM formate. Incubation took place for 30 min at 37°C. Oligo, oligodesoxyribonucleotide.

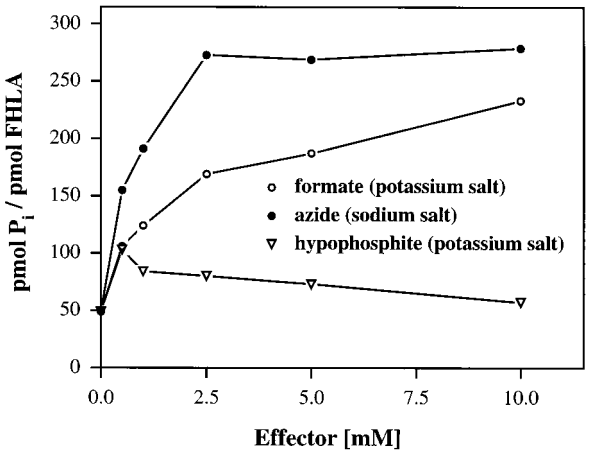

FIG. 6. Dependence of ATP hydrolysis by FHLA (471 nM monomer) on the presence of different concentrations of potassium formate, sodium azide, and potassium hypophosphite. The incubation time was 60 min at 37°C.

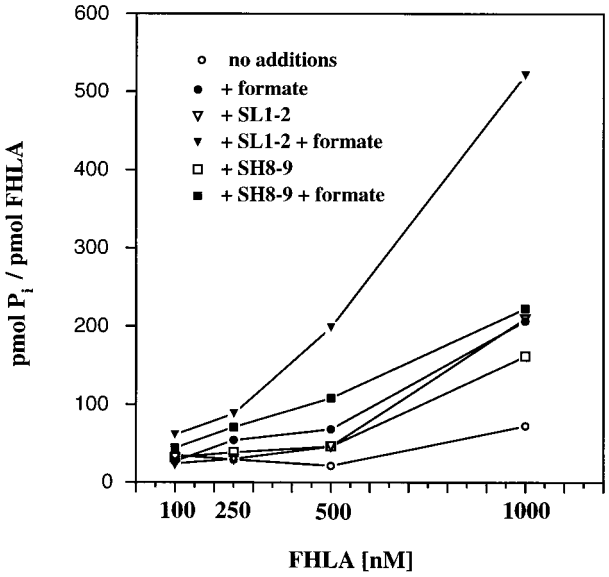

FIG. 7. Dependence of the specific ATPase activity of FHLA on the concentration of FHLA in the reaction mixture. If present, formate was added at 10 mM and DNA was added at 0.5 μM. Incubation took place for 60 min at 37°C.

(Table 2). The three other nucleoside triphosphates inhibited the reaction in the decreasing order GTP > UTP > CTP. The idea that GTP indeed is hydrolyzed was tested in reactions in which ATP was replaced by [γ-^{32}P]GTP. It was found that GTP is hydrolyzed at almost the same rate as ATP, and the GTPase activity showed the same regulatory pattern as the ATPase activity, i.e., stimulation by formate and DNA (data not shown). Thus, FHLA is a σ^{54}-dependent transcriptional activator which hydrolyzes ATP and GTP and at least binds UTP and CTP. NTRC-phosphate can hydrolyze ATP and GTP but not CTP or UTP (27); the same nucleotide specificity has been shown for $DCTD_{L143}$, a constitutively active, truncated form of *Rhizobium meliloti* DCTD (14). In comparison, the central domain of NIFA is able to hydrolyze GTP, ATP, and UTP, but not CTP (2).

Dependence of the specific ATPase activity of FHLA on the FHLA concentration. In the in vitro transcription-translation experiments it was observed that the FHLA concentration must exceed a certain threshold level for activation of the promoters of the formate regulon. Beyond this threshold level, which is lowered in the presence of formate, there is a steep increase of activity in a very narrow concentration range (11). The specific activity of FHLA as a function of its concentration is reflected in the ATP hydrolysis reaction. Figure 7 shows that the specific activity indeed increased with increasing FHLA concentrations and that this effect was most pronounced in the presence of formate and the specific oligodesoxyribonucleotide.

TABLE 2. Nucleotide specificity of ATPase activity of FHLA[a]

NTP added (20 mM)[b]	Formate (10 mM)[c]	SL1-2 (0.25 μM)[c]	pmol of P_i/pmol of FHLA
None	−	−	45
ATP	−	−	13
GTP	−	−	11
CTP	−	−	19
UTP	−	−	11
None	+	−	261
ATP	+	−	8
GTP	+	−	21
CTP	+	−	51
UTP	+	−	25
None	+	+	580
ATP	+	+	25
GTP	+	+	68
CTP	+	+	142
UTP	+	+	89

[a] All assays contained [γ-^{32}P]ATP at 1 mM and a specific radioactivity of 0.5 mCi/μmol.
[b] NTP, nucleoside triphosphate.
[c] −, absent; +, present.

DISCUSSION

The results described above have shown that the transcriptional activator FHLA exhibits intrinsic ATPase activity which is stimulated by formate or by double-stranded DNA irrespective of whether the DNA contains the specific binding site for FHLA or not. A certain unspecific stimulation of ATPase activity by DNA, which was discussed to be due to nonspecific DNA binding (1), is characteristic of NTRC (1) and $DCTD_{L143}$ (14) as well. However, when both formate and the DNA carrying the binding site are present, a dramatic increase in ATP hydrolysis takes place. It appears that FHLA loosely interacts with DNA in an unspecific manner and that the presence of formate changes its conformation such that it enters a state in which it is capable of selective and stable interaction with its binding site on the DNA. The ATPase activity was dependent on the concentration of FHLA in the assay, and this concentration dependence was most pronounced in the presence of formate and the specific oligodesoxyribonucleotide. Such behavior is characteristic of other σ^{54}-dependent activators such as $DCTD_{L143}$ (14) and NTRC-phosphate (1, 27). These proteins are dimers in solution, and it is suggested that they may form a larger protein complex either on DNA upon cooperative binding to the UAS or in solution at relatively high protein concentrations (1, 14, 27). This oligomerization is presumed to enhance ATPase activity. For NTRC it has been shown that phosphorylation induces strong cooperative binding to DNA (28) and that oligomerization at the *glnA* enhancer, which is composed of two NTRC binding sites, is required for transcriptional activation (19). For FHLA it is unknown at present

whether the increase in ATPase activity is a direct consequence of the binding of formate and/or the DNA or whether these ligands change the oligomeric state of the protein and this results in an increase of the K_{cat} of the protein. There are some arguments for and some arguments against the assumption that FHLA can change its oligomeric state. A hint at a possible oligomerization comes from the observation that binding of FHLA to the sites within the two intergenic regions seems to occur cooperatively (22). This may indicate binding of more than one activator molecule or attainment of a supramolecular structure of the complex. An argument against oligomerization is the fact that purified FHLA in the native state is a homotetramer (22) and that maximal stimulation of its ATPase activity takes place at a stoichiometric ratio of 4:1 to 5:1 (FHLA monomer/oligodesoxyribonucleotide). This suggests that one SL1-2 oligodesoxyribonucleotide is bound by one tetramer and that FHLA does not change its degree of oligomerization upon binding to DNA. However, we have no information concerning what fraction of the protein is active in the assay, nor is it clear how many binding sites for FHLA are present on SL1-2.

We now have good evidence that FHLA does not need to be phosphorylated to act as a transcriptional regulator: (i) the lack of the conserved sequences in the N terminus which are characteristic of phosphorylated response regulators and (ii) the fact that ATP hydrolysis by FHLA was induced by formate and the target DNA in the absence of any other component. Furthermore, neither acetyl-phosphate, carbamyl-phosphate, nor phosphoamidate had any effect on the activity of FHLA in ATP hydrolysis or in a coupled transcription-translation system (data not shown). In contrast to this, the ATPase activity of NRI (NTRC), which is the target of phosphorylation by NRII (NTRB), is stimulated by acetyl-phosphate, carbamyl-phosphate, and phosphoamidate (10). However, it might not be a general feature of all two-component receiver domains to be phosphorylated by these compounds in vitro even if they are phosphorylated in vivo by a sensor kinase. FHLA is converted into the active state by liganding its effector, formate. It is intriguing that the transition state analog azide is a better effector than the cognate substrate. This indicates that formic acid might be bound to FHLA in a straight conformation and not in an angular one such as formate has in solution. The affinity of FHLA to formate lies in the 5 mM range and, therefore, appears to be quite high. It is, however, well within the physiological range, since the induction of the formate hydrogenlyase system, whose main physiological purpose is pH homoestasis, occurs only at such high formate concentrations (20).

Binding of the effector formate to FHLA appears to serve the same function as phosphorylation of the NTRC-type response regulators, namely, stimulation of ATP hydrolysis. Other regulators of the FHLA class like XYLR and DMPR may function in an identical manner, since it has been demonstrated recently by domain exchange experiments that these regulators also appear to bind their effectors directly (24). Also, a σ^{70}-dependent activator, TYRR, was shown to exhibit ligand-dependent ATPase activity (8). It is, at present, premature to speculate on the functional advantage of a phosphorylation cascade relative to activation by liganding of an effector. One advantage of sensor-regulator communication via phosphorylation, however, may lie in the spatial separation between sensing and gene activation and in the higher level of versatility of constructing new systems via module exchange.

ACKNOWLEDGMENTS

We are greatly indebted to Verena Weiss for samples of phosphoamidate.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SPP Regulationsnetzwerke in Bakterien).

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