New Roles for Conserved Regions within a $\sigma^{54}$-dependent Enhancer-binding Protein*

Chih M. Lew and Jay D. Gralla†

From the Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095-1569

23 amino acid substitutions were made in the C7 and C3 regions of pspF AHTH, a protein required to convert $\sigma^{54}$ closed promoter complexes to open complexes. These mutants were assayed for transcriptional competence, for the ability to hydrolyze ATP, for their multimerization state, and for their ability to interact with $\sigma^{54}$ and its holoenzyme. C7 region mutants caused the protein to assume a compact form. This property could be mimicked by the addition of ATP, implying that compaction via C7 and ATP is part of the activation process. A number of C3 mutants were important for energy coupling, as indicated previously for several members of this activator family (1, 2). However, a patch within C3 influenced oligomerization. The C3 region was especially important in interacting with $\sigma^{54}$ during the transition from the dimer to a higher oligomer form as part of the activation process. Regions C1 and C4 contain the Walker A and B motifs that interact with ATP (13). ATPase of activator to trigger opening of the DNA by holoenzyme requires enhancer-binding activator proteins (4). Transcription occurs when a DNA loop is formed, allowing activator and holoenzyme to touch and use the ATPase of activator to trigger opening of the DNA by holoenzyme (5–7).

These enhancer-binding activator proteins often have three domains, as exemplified by the Salmonella typhimurium NtrC protein (8). The C-terminal domain is needed for protein binding to the enhancer. The N-terminal domain receives the metabolic activation signal and begins its processing. The central domain is required for activation, since it contains the information that allows the coupling of ATP hydrolysis to the melting of the DNA by holoenzyme. How ATP hydrolysis is coupled to DNA melting by the activation domain is largely unknown. The central domain contains seven conserved regions termed C1 to C7. The C3 region is believed to be critical for several reasons. First, mutants have been found in this region that have significant levels of ATPase activity and bind DNA normally but fail in activating transcription (1). Second, some mutants in this region appear to be defective in interacting with $\sigma^{54}$ (2, 9, 10). Third, there exists a distinct class of proteins that contain domains homologous to the central domain of $\sigma^{54}$ activators but activate forms of holoenzyme that lack $\sigma^{54}$; these have sequences that differ primarily in the C3 region (e.g. Rhodobacter capsulatus NtrC) (11, 12). Mutations within C3 generally cause a lack of energy coupling; however, the proposed roles of individual conserved amino acids have varied when different activators were studied (1, 2). One focus of the current work will be to study the in vitro properties of mutants within this C3 region in a common context.

Roles have been proposed for three of the other regions within this central activation domain. Regions C1 and C4 contain the Walker A and B motifs that interact with ATP (13). Region C7 is the site of a number of nonfunctional mutations, but its role is still unknown. Some studies suggest that it is involved in nucleotide binding (13, 14), and others suggest a role in activator oligomerization (15). This is an important consideration, because this class of proteins is converted from the dimer to a higher oligomer form as part of the activation process (16). For this reason, we have also studied the properties of mutations in the C7 region.

This study differs from prior ones in a number of significant ways. Prior mutations were studied in a number of different activator proteins and will now be studied in the context of a single protein. The protein chosen, pspFAHTH, is essentially a pure activation domain; the virally induced protein contains no N-terminal signaling domain, and the natural C-terminal helix-turn-helix DNA binding region has been deleted, but the dimerization region is still intact (17, 18). This should allow all activation mutants to be studied in the same context in vitro, one that bypasses potential differences due to the differing influences of the unique signaling pathways of the various activators that have been studied previously (19, 20). In addition, several recently developed assays related to activation (see below) can now be applied to this series of mutant proteins.

To do this, we collected data on genetically screened C3 and C7 mutants from several proteins and remade them in pspFAHTH. A few site-directed C3 mutants were added to extend coverage to every position within this critical region. The proteins were purified and assayed for transcriptional competence, for the ability to hydrolyze ATP, for their multimerization state, and for their ability to interact with $\sigma^{54}$ and its holoenzyme. The resulting data yield proposals for the roles of the C3 and C7 regions in the context of a simple protein that acts as a pure activation domain.

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† To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, P.O. Box 951569, Los Angeles, CA 90095-1569. Tel.: 310-825-1620; Fax: 310-267-2302; E-mail: gralla@chem.ucla.edu.

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FIG. 1. Alignment of regions C3 and C7 and its mutants. Regions C3 and C7 of various family members are aligned (top). Nonconserved residues are shaded. The mutants used in this study of pspF-HTH were based on mutations found in other activators, and related proteins are shown at the bottom. They include E. coli PspF, S. typhimurium NtrC, R. melioti DctD, Pseudomonas putida XylR, B. japonicum NiaA, and E. coli DnaA. The four serine substitutions have no prior counterparts. The numbers indicate the position of the amino acid in pspF-HTH. The end of C7 is indicated by the line.

EXPERIMENTAL PROCEDURES

Plasmids and Proteins—The plasmid pMJ15 contains His6- pspF-HTH (18). The QuickChange mutagenesis kit (Stratagene) was used for site-directed mutagenesis. Escherichia coli core enzyme was from Epicentre. E. coli $\sigma^{34}$ was purified as reported (21). Plasmid pHMK3 contains $\sigma^{34}$ with a heart muscle kinase tag attached to its 3′-end (22) and was purified as described previously for $\sigma^{34}$ (21), except that induction was with 1 mM isopropyl-1-thio-β-D-galactopyranoside and growth was at 37 °C. pepF-HTH and its mutants are purified as described (16), except that a second batch purification was done. This involved elution with 300 mM imidazole and dialysis in the stated buffer without EDTA, followed by a second round of purification using the Ni²⁺-nitrilotriacetic acid beads. Unless otherwise indicated, proteins were stored in elution buffer.

In Vitro Transcription—In vitro activated transcription was conducted as described (23) with minor changes. Briefly, 75 mM $\sigma^{34}$ RNA polymerase was incubated with 0.5 mM GTP, 0.5 mM ATP, and 0.5 mM UTP in 1× buffer B (50 mM Tris-HCl at pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM β-mercaptoethanol) for 20 min at 37 °C before 1 µl of CTP mixture (50 mM CTP and 0.2 µCi/µl [α-32P]CTP) was added. This mixture was incubated for an additional 10 min at 37 °C before the sample was prepared for loading on 5% PAGE with urea. Radiolabeled RNA was analyzed with a PhosphorImager.

ATP Binding—The filter binding assay was done as described (13) with minor changes. Briefly, 5 µM pepF-HTH and its mutants were incubated in 1× ATPEase buffer (25 mM Hepes at pH 7.5, 20 mM MgCl₂, 30 mM KCl, 2 mM β-mercaptoethanol) before ATP$^{32}$P mix (0-6 mM ATP$^{32}$P, 69 µM [γ-32P]ATP) was added to the 20-µl reaction. This was incubated at 37 °C for 4 min. The reaction was applied to a polyvinylidene difluoride membrane (0.45-µm Immobilon P, prepared according to the manufacturer’s instructions; Millipore Corp.), which sat on top of a sintered glass filter, and vacuum was quickly applied to remove liquid. 1 ml of wash buffer (20 mM Hepes at pH 7.5, 10 mM MgCl₂) was immediately applied to the membrane followed by vacuum. The membrane was dried, and radiactivity was determined by scintillation counting.

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ATPase—In a standard P, release assay (13), 200 mM pepF-HTH and its mutants were incubated in 1× ATPEase buffer (25 mM Hepes at pH 7.5, 20 mM MgCl₂, 30 mM KCl, 2 mM β-mercaptoethanol, 18, 0.3 mM ATP, 20 µg/ml acetylated bovine serum albumin, 0.3 µCi/µl [γ-32P]ATP) for 15 min at 37 °C. A 1-µl aliquot was spotted onto a prerun polyethylenimine TLC plate, and electrophoresis was with 0.75 x phosphate buffer at pH 4.1. Afterward, the plate was dried and analyzed using a PhosphorImager.

Native Protein Gel—Sample dye was added to 2.5 µg of protein, sample was loaded onto a 4% stacking, 12% resolving gel, and electrophoresis was performed as described (25). In samples that contained ATP, 4 mM ATP was incubated with the protein for 5 min on ice prior to electrophoresis in a system with 4 mM ATP in the gel and electrophoresis buffer.

Glutaraldehyde Cross-linking—Glutaraldehyde cross-linking was in Buffer G (50 mM sodium phosphate at pH 7, 20 mM NaCl, and 12% glycerol). Where indicated, 4 mM ATP was incubated with 16 µM activator for 5 min at 30 °C. 160 µM glutaraldehyde was added to 16 µM protein in a 6.75-µl reaction volume. This was incubated at 30 °C for 12 min, followed by the addition of 1 µl of 1 M glycine to stop the reaction. 3 µl of 2X SDS-PAGE dye was added, heated for 2 min at 90 °C, and then loaded onto a 8% SDS-PAGE. The gel was stained with Coomassie Blue to visualize.

Electrophoretic Mobility Shift Assay—Promoter probes and electrophoretic mobility shift assay were as described (26) with minor modifications. Briefly, 1 µg annealed promoter probe was added to 7.5 mM holoenzyme in 1× STA buffer (25 mM Tris acetate at pH 8, 8 mM magnesium acetate, 10 mM KCl, 1 mM β-mercaptoethanol, 3.5% (w/v) polyethylene glycol 8000 (9)). Where indicated, 1.5 µM pepF-HTH protein or 4 mM ATP concentration was present. The 10-µl reaction was incubated at 37 °C for 10 min followed by the addition of 1 µl of 1 M glycine to stop the reaction. 3 µl of 2X SDS-PAGE dye was added, heated for 2 min at 90 °C, and then loaded onto a 8% SDS-PAGE. The gel was stained with Coomassie Blue to visualize.
RESULTS

Mutations within the C3 and C7 Regions—Fig. 1 (top) shows the alignment of the C3 and C7 regions as well as (bottom) the mutations that were made in pspFΔHTH. Most of these replicated previously identified nonfunctional mutants within Salmonella typhimurium NtrC (1), Rhizobium meliloti DctD (2), and Bradyrhizobium japonicum NifA (27). A few changes were based on other proteins (15, 28), as shown. Finally, four site-directed serine substitutions were made in the intensively studied and well conserved C3 region so that every residue would be covered by mutation. These four positions have not been studied previously in vitro. The C7 region is sparsely covered by previously identified nonfunctional mutants, and we have not attempted to saturate it. The set consisted of 23 individual substitutions in pspFΔHTH.

These 23 proteins were purified according to Jovanovic et al. (18) with some modifications. They were then tested in an in vitro transcription assay (Fig. 2 and not shown) using the σ^{54}-dependent glnAp2 promoter. All except one of the proteins, E76Q, were very defective in transcription in vitro. The lack of significant activity of all but one of these is consistent with the original mutant phenotypes (1, 2) and, in the cases studied, with in vitro transcription studies using the original proteins. The four site-directed mutants in C3 were also nonfunctional, confirming the general importance of residues within this region. Thus, the data confirms that mutations in pspFΔHTH largely reflect the same gross defects seen when the same changes were made in a variety of other proteins containing homologous domains.

Effect of Mutation on ATP Hydrolysis—The ATPase activity of this class of activator proteins is essential for function (5). The purified proteins were subjected to an ATPase assay, and the results are shown in Table I. Six of the 23 mutants, A82N, G83D, and G87K in C3, R227H and G224V in C7, and R235H near C7, lacked detectable activity. One C3 mutant, S75F, had activity that was equal to or slightly better than wild type. All of the other mutants had lowered amounts of ATPase activity, ranging as low as 6% of wild type levels.

Thus, only one of the mutants in region C3 (S75F) showed no defect in ATPase activity and yet was transcriptionally deficient. This would be a strict coupling mutant. Six other C3 mutants retained on average 50% of the wild type level of activity (plus symbols in Table I) despite showing little or no transcription. These too, are very strong candidates for coupling mutants in pspFΔHTH.

The locus of coupling mutations being in the C3 region is consistent with prior studies using other proteins (1, 2). However, some differences in ATPase activity are apparent in the data. The adjacent mutants S75F and E76Q failed to show activity in NtrC (13) but do here in pspFΔHTH (Table I), as a different mutation in Ser^26 did for DctD (S75I) (2). H80R also showed activity in pspFΔHTH, whereas it did not in DctD (2). It is possible that the retention of ATPase activity in these three mutants in pspFΔHTH is related to the lack of other domains that might contribute to suppression of activity. On the other hand, G87K has ATPase activity in NtrC but none here. Overall, however, the agreement between pspFΔHTH mutants and that of other proteins is quite good.

The C7 region mutants showed varying degrees of ATPase activity, although on average they were more defective than those in the C3 region (Table I). In partial agreement, the C7 mutants studied previously in NtrC were all defective in ATP hydrolysis (13). On the whole, the evidence indicates that the C7 region is required for ATPase activity, although whether this effect is direct or indirect is not established by the existing experimental data.

ATP binding was also assayed to learn whether the lack of hydrolysis in some cases was due to a lack of binding. Since ATP binding levels in the membrane-binding assay are low, as also shown previously (13, 20), the data obtained varied somewhat for duplicates. For this reason, data from four or five experiments were needed to obtain the average ranges shown in Table I. The procedure relies on the ability of proteins to retain the nonhydrolyzable analogue ATPγS during a membrane-binding assay. Since the assay involves washing filters, the extent of ATP binding is far less than in the equilibrium experiments below. Nonetheless, the mutant data show defects relative to wild type. The results showed that of the six mutants that could not hydrolyze ATP, two bound ATPγS well (A82N and R235H; Table I), and the others bound it either with significantly reduced affinity (G83D, G87K, and G224V) or at a level below the sensitivity of the assay (R227H). We infer that at least some of these mutants cannot hydrolyze ATP because it is poorly bound. In terms of pure coupling effects, A82N and R235H are the only two mutants that can bind ATPγS but could not hydrolyze ATP (Table I).

These various ATP-related defects are very widespread in the data, despite the fact that the Walker A and B regions are consistent with the prior studies using other proteins (1, 2). However, some differences in ATPase activity are apparent in the data. The adjacent mutants S75F and E76Q failed to show activity.
still intact. This suggests that for a number of mutants the defect could be indirect. For this class of proteins, function requires the formation of higher order oligomers (4). The central activation domain has been implicated to be involved in oligomerization (29); however, it is not known which amino acids in this domain are involved. To assay for these, we next studied the multimerization states of the collection of mutants.

Effect of Mutation on the State of Multimers—PspFΔHTH has been reported to exist predominantly as a dimer (72 kDa) with higher molecular weight oligomers occurring at high concentrations of protein (18). We repeated the native gel assay to confirm this under the present conditions and to learn the multimerization status of the collection of mutants. This initial assay was conducted using normal solution concentrations of pspFΔHTH, where dimers are expected to predominate. In this assay, pspFΔHTH runs as a very broad band with an apparent molecular weight somewhat higher than that expected for a dimer (Fig. 3A, Wt). This broad band has been seen previously (18) and may represent the different conformers of pspFΔHTH (30) undergoing kinetic exchange. Although at this concentration the dimer is the predominant form in solution, it is difficult to say with certainty what forms exist within the broad band. At this protein concentration, no specific bands that would clearly represent higher order oligomers are seen.

When the mutant proteins are assayed, many show smears with mobility altered slightly from wild-type or with lesser intensity. These and other results were reproducible using two different preparations of proteins. The mobility changes may represent alterations in the equilibrium population of different oligomers undergoing kinetic exchange during the native gel electrophoresis.

Two patches of mutants, however, show bands that are more discrete than wild-type and also have altered mobility. One of these consists of the adjacent amino acids Leu^{77}, Phe^{78}, and Gly^{79} in the C3 region. In these cases, the broad band observed with wild type is replaced by a series of discrete bands beginning with an apparent dimer and including apparent higher molecular weight forms (Fig. 3A, L77S, F78S, and G79S versus wild type). These forms probably correspond to tetramers and hexamers, since the pattern resembles that seen for wild-type protein at high concentration (18). Mutation of the adjacent residue Glu^{76} shows a sharpened band but little evidence of oligomerization (Fig. 3A). No other mutations caused this pattern of band sharpening. We infer that this patch within the C3 region contains determinants that strongly affect the propensity of pspFΔHTH to form higher order multimers. The (E)LPG sequence is highly conserved within the δ^4 family of activators (8), so this phenomenon could apply to other proteins. Alanine substitutions within this sequence in DctD led to some small defects in expression in vivo, but in vitro transcription and oligomerization state were not tested (2).

Other mutants that differed in mobility from wild type were restricted to the C7 region. In fact, all C7 mutants tested had altered mobility on native gels (Fig. 3B). The broad band characteristic of wild type protein was converted into more discrete bands by the C7 mutations. In four of the five mutants, this band ran significantly lower on the gel (Fig. 3B, e.g. G224D versus Wt), reducing the apparent molecular mass from 90 to 66 kDa (the theoretical dimer molecular mass is 72 kDa). Thus, the C7 region plays a role in influencing the conformational state of the pspFΔHTH dimer. In most cases, mutations convert a conformational diverse collection of states (a broad band with low mobility, Wt in Fig. 3B) to unique species with apparently compacted conformations (narrow bands with high mobility; e.g. G224D in Fig. 3B). Although the concentration used in this assay may be higher than found in vivo, the result shows that certain mutants alter the propensity to assume these various states. Most of the mutants show some evidence of discrete bands of differing mobility, particularly in the case of R227C. Moreover, not all the bands have precisely identical mobility. Thus, it appears that the C7 mutations create predominantly more compact forms of the protein, but many conformational states may still be accessible.

Effect of ATP on the State of Multimers—We hypothesized that the C7 region mutations might be partially mimicking the effect of ATP, and so the experiments were repeated in the presence of ATP. The sample, gel, and buffer reservoir contained ATP to maximize its occupancy of the proteins during electrophoresis. The results for selected mutants of interest are shown in Fig. 3C.

The effect of ATP on the native gel mobility of the wild-type protein was dramatic. The broad band was converted to a
narrow band with much greater mobility (Fig. 3, compare Wt in A with Wt in C). In fact, the effect of ATP on wild-type closely mimicked the effects of C7 mutants (Fig. 3, compare Wt in C and G224D in B). It appears that ATP converts a mixture of conformers to a unique compact form. That the C7 mutants mimic this effect is supported by the observation that ATP has little effect on their altered mobility on native gels (Fig. 3, B and C).

Mutations in the LFG “oligomerization” sequence of region C3 responded differently to ATP. The effect that is seen is similar to wild-type protein only in the sense that the bands are shifted to faster migrating species. However, only a small proportion of the mutant band population has the same mobility as the wild type band (Fig. 3C, L77S, F78S, G78S, and wild type). Most of the bands simply appear to broaden when ATP is added. In this sense, the effect is the reverse of that seen with wild type; ATP causes the LFG mutant population to be somewhat more heterogeneous rather than less. The observation that these mutants can respond to ATP, despite poor retention in the filter-binding assay, is probably due to the use of an “ATP-saturated” electrophoresis assay, where ATP is included in both the gel and buffer.

Glutaraldehyde Cross-linking Shows a Dimer Multimerization Pattern—We wished to confirm that the species seen consist mostly of dimers and multimers thereof. Under the conditions of these experiments, wild type pspFΔHTH has been reported to exist predominantly as a dimer in solution, which in a native gel appears as a smear (18). A population of more discrete higher order oligomers can be seen when higher concentrations of protein are used (18). Since native gel electrophoresis could dissociate higher oligomers to dimers, we used solution cross-linking to assess the multimerization state. Glutaraldehyde can covalently cross-link lysines between subunits that are transiently associated together, even if they are in equilibrium with lower order species. Because lysines are distributed throughout the surface of the protein (31), cross-links could be made between various positions, and each cross-linked complex could have a unique mobility on a denaturing gel, mostly resulting in the very broad bands typical of such experiments. Since not all subunits will be cross-linked, one expects to see a distribution of mobilities of cross-linked species, perhaps ranging downward from the highest order multimer. The products are run on denaturing gels, and the appearance of bands higher than monomer can indicate multimer formation.

Amounts of pspFΔHTH and its mutants slightly higher than those used for the native gel analysis were cross-linked with glutaraldehyde and then run on denaturing gels. Fig. 4 shows that glutaraldehyde treatment of pspFΔHTH (Wt) is required to induce formation of broad bands with apparent molecular mass centers near 36, 78, and 135 kDa with a small amount of a higher molecular mass species. These correspond roughly to the molecular masses expected for monomers, dimers, and tetramers. Glutaraldehyde also induces a fast migrating compact band (Fig. 4, dot) that was proposed to be an internally cross-linked monomer (15). The lack of a broad band in the trimer position (108 kDa) supports the view that the protein exists as a dimer and its multimers, as proposed previously (18). The population of multimers seen probably underrepresents the abundance of higher order species, since multiple cross-links are required for a multimer to be detected in this assay.

The cross-linking pattern was also not altered by mutation of pspFΔHTH (data not shown). However, the intensity of the bands increased slightly with ATP for both wild-type and mutant (Fig. 4, lanes with ATP versus without; data not shown). Since ATP also compacts the dimer (Fig. 3C), this form may be preferred for multimer formation.

Assay for the Ability to Engage the DNA Nontemplate Strand during Activation—During activation, pspFΔHTH and related proteins trigger events that allow σ^44 holoenzyme to engage the nontemplate DNA strand (7). This interaction is required for transcription initiation and precedes it. It is stimulated by the presence of ATP or ADP in the case tested, that of NtrC (23). Nontemplate strand engagement may be assayed by adding activator protein to systems containing σ^44 holoenzyme and fork junction probes in which the nontemplate strand from −11 to −7 is in single-stranded form, whereas the upstream DNA remains duplex. When pspFΔHTH or NtrC is present, a conformational change is induced that confers altered mobility on the holoenzyme-DNA complex. This mobility shift requires that the DNA probe contain the exposed nontemplate single strand and is thus considered an assay for the engagement of this single-stranded segment (23).

ATP stimulated this process for NtrC (23), and the data show that this is also true for pspFΔHTH (Fig. 5, Wt). ADP and the poorly hydrolyzed analogues ATP-βS and AMPPNP also stimulate this conformational change for pspFΔHTH (not shown). With the exception of ATP-γS, this pattern was also observed for NtrC (23).

When the mutants were tested in this assay, all were able to induce formation of the shifted band (Fig. 5, top). Thus, it appears that none of the mutants block the ability of pspFΔHTH to interact with σ^44 holoenzyme in a way that leads to nontemplate strand engagement. There were some potentially interesting differences in how the engagement was stimulated by ATP. The (E)LFG oligomerization patch shows a slightly elevated response to ATP, as seen by comparing the intensity of the upper and lower bands with the ratio associated with wild type (Fig. 5, bottom). This may imply that mutants with an increased propensity to oligomerize (Fig. 3A, L77S) are more responsive to ATP in directing nontemplate strand engagement. There also appears to be a small effect with the C7 region mutants at G224V and R227H. But overall,
these effects are small, and the main point is that all mutants retain the ability to induce DNA strand switching. Apparently, neither the C3 nor the C7 region is essential for this interaction.

Transition State Binding by the Mutants—A different gel electrophoresis assay has been developed that assesses the ability of pspFΔHTH to bind directly to isolated σ^{54} (Fig. 6). A band with shifted mobility is seen only when the ATP transition state analog ADPAlF\_x (where x = 3 or 4) is present along with pspFΔHTH and \(^{32}\)P-labeled heart muscle kinase-tagged σ^{54} (Fig. 6, Wt versus Wt + ADPAlF\_x). This indicates that the assay probably measures the ability of the activator to correctly participate in the transition state for ATP hydrolysis. Because the strand engagement assay is stimulated by ATP binding but not hydrolysis, engagement may precede hydrolysis in activation of transcription initiation. We tested the collection of pspFΔHTH mutants in the transition state binding assay. A mutation in the C3 amino acid Thr^{66} was shown previously to fail to bind in this assay (9).

Among the collection of proteins, only wild type and four mutants were able to induce a shifted band in this assay. These are shown in Fig. 6 along with two of the many mutants that failed to induce a mobility shift. The lack of mobility shifts by most mutants indicates that both the C3 and C7 regions have a significant influence on the ability of pspFΔHTH to bind σ^{54} in the presence of an ATP hydrolysis transition state analogue. For each of the four mutants that did induce band formation, the presence of the transition state analogue was required, as expected. Three of the four mutants are F78S, A82N, and G83D, all of which fail to transcribe in vitro (the fourth is E76Q, which does transcribe). The three inactive mutants hydrolyze ATP poorly (Table I) despite being capable of forming the transition state complex. This suggests that they form the transition state but then fail to complete the hydrolysis and release of products that probably drive the required conformational changes in holoenzyme. Other mutants that did not induce transition state band formation encompass the full range of ATP hydrolysis activity. It seems that ATP hydrolysis efficiency is not closely related to success or failure in this assay.

All but one of the mutants failed to transcribe in vitro. Thus the transition state assay is reasonably, but not perfectly, indicative of the lack of transcription. One of the four mutants that could induce band formation was E76Q (albeit with slightly altered mobility); this was the only mutant that could transcribe. The other mutants that induce band formation were also in the C3 region, F78S (in the LFG region), A82N, and G83D (Fig. 6). Since these three could not transcribe, it appears that their defects are very late in the transcription initiation pathway.

DISCUSSION

In this work, 23 individual substitutions were introduced into the central domain of pspFΔHTH (Fig. 1), which essentially acts as a constitutive activation domain for promoters that rely on σ^{54} holoenzyme. Most of these had been discovered previously in genetic screens of other members of this activator family (1, 2, 27, 28), and, in agreement, in vitro transcription failed with all but one of the mutants (Fig. 2 and data not shown). A number of prior in vitro studies, often using different members of the family, have led to proposals for lack of function that were in partial agreement (13, 15). All of the pspFΔHTH mutants were purified, and various assays were applied to uncover the range of defects in the context of a single, simple protein. The results uncovered unexpected roles for highly conserved regions that are closely related to the mechanism of activation and confirmed the importance of region C3. They also raise the possibility that there is more than one site on pspFΔHTH that interacts with holoenzyme. This issue and the properties of the C7 and C3 region mutants will now be discussed in the context of how activation occurs.

C7 Mutants and a New Role for ATP—We studied the properties of five nonfunctional mutants in two residues within region C7 and in the nearby residue Arg\(^{21}\) (Fig. 1, C7). We also saturated the C3 region with mutations. As shown above, the major distinction is the influence of C7 mutations on the protein conformational state. A lesser distinction is related to ATP hydrolysis. For each C7 residue, one mutant change blocked ATP hydrolysis (Table I, G224V, R227H, and R235H). This is in contrast to the C3 mutants, where only three of 14 residues were changed in a way that blocked hydrolysis. This result indicates a role for the C7 region in ATP hydrolysis, although it cannot be determined whether it is direct or indirect.

However, the most striking consequence of these C7 mutations is their ability to alter the conformation of pspFΔHTH so that it runs very differently on native protein gels (Fig. 3B, compare Wt with G224D and R227H). Wild-type pspFΔHTH runs as a broad band with an apparent molecular mass above 90 kDa. By contrast, the C7 mutations have two effects on mobility. First, the band is shifted to near 66 kDa. Second, the breadth of the band is reduced greatly, and it now runs as a more conformationally distinct species. This indicates that the C7 region has a role in influencing the interconversion between
heterogeneous and more homogeneous compact forms of pspFΔHTH.

We were able to mimic the mobility changes induced by mutations simply by adding ATP to wild type. This also converts the broad band into a discrete one with much greater mobility (Fig. 3, compare Wt in A with Wt in C). The cross-linking results do not show any decrease in dimer population induced by ATP (Fig. 4, arrow at 78). We speculate that one natural effect of ATP is to convert a population of dimers to a more compact and homogeneous form and that the C7 region is involved in this process. Because the mutants do not transcribe it seems likely that they are locked in forms that cannot properly use ATP. In support of this, all of the C7 mutants fail to form a stable ATP-dependent transition state complex with 6 data not shown.

PspFΔHTH and other 6 activators belong to the AAA+ protein superfamily, and the analogy suggests that C7 region residue Arg827 has the potential to interact with a phosphate of ATP (14). In members of the AAA+ family, a single ATP binds between each pair of monomer subunits in the multimer (32). We found that mutation G40V, in the Walker A motif that presumably binds the alpha phosphate (13), also leads to compact dimer formation (Fig. 3B). This information and the native gel analysis suggest that pspFΔHTH alters its dimer conformation to accommodate ATP in a form suitable for hydrolysis of the beta-gamma bond; this links the monomer subunits together into a more rigid complex (Fig. 3C).

C3 Mutants and the (E)LFG Oligomerization Patch—The C3 region has been proposed to be required for interacting with 6 and for assisting the coupling of ATP hydrolysis to DNA melting by the 6 helozyme (1, 2, 9). In the 6 activator family, C3 is implicated to be the switch region, which undergoes drastic nucleotide-dependent conformational changes. Thus, the expected effects of C3 mutations might be complex. We have studied 18 C3 mutations in pspFΔHTH and see several interesting phenotypes (Fig. 1).

Only four of the mutants retain the ability to form a transition state complex with an ATP analogue, pspFΔHTH and 6 (Fig. 6). Thus, most of C3 is needed for forming this state, in agreement with its proposed involvement in energy coupling. Three of the four exceptions are F78S, A82N, and G83D, all of which fail to transcribe in vitro (the fourth is E76Q, which does transcribe). The three inactive mutants hydrolyze ATP poorly (Table I) despite being capable of forming the transition state complex. This suggests that they form the transition state but then fail to complete the hydrolysis and release of products that probably drive the required conformational changes in helozyme. Other mutants that did not induce band formation encompass the full range of ATPase activities. It seems that ATP hydrolysis efficiency is not closely related to success or failure in this assay.

Among the 14 mutants that fail in the transition state assay, three are within residues that have been suggested to interact with 6 helozyme. These are Ser75 and His80, the DctD analogues of which fail to cross-link to the helozyme (2) and Thr86, at which mutation can be suppressed by a mutation in 6 (9). Mutations in these positions retain some ability to hydrolyze ATP (Table I). However, these and all other C3 mutants preserve the ability of pspFΔHTH to trigger the beta-helozyme to engage the nontemplate strand (Fig. 5, top). Thus, there appear to be two potential interaction regions for activators, one within C3 and a different unknown region elsewhere in the central domain.

Mutations within each residue of the LFG sequence (residues 77–79) showed an enhanced ability to form higher order oligomers on native gels (Fig. 3A). The effect of ATP in this case is distinct, since it induces only a small number of compact forms, in contrast to wild type and all other C3 mutants. In addition, ATP converts discrete oligomer bands to a broad family of bands with greater mobility (Fig. 3, compare A with C, L77S, F78S, and G78S). Because the LFG substitutions were not guided by screens but were simple serine substitutions, it seems unlikely that each has created new interactions leading to oligomerization. Instead, it may be that the LFG patch is a natural deterrent to premature oligomerization for activators in which this is controlled by signaling. This region, in addition to the adjacent residue, Gly76, may also have a role in deterring premature ATP-dependent engagement of the nontemplate strand, since its mutation favors this step (Fig. 5, bottom). Both deterred properties, oligomerization and strand engagement, are required for activator function. The patch may simply interfere with these functions until signaling occurs, contributing to the tightness of regulation.

In the case of pspF, function is normally repressed by the pspA protein (24). Under activating conditions, pspA is signaled to release pspF. As the concentration of pspF rises, it begins to oligomerize. The LFG mutations reduce the concentration threshold (33) needed for oligomerization of pspFΔHTH (18) (Fig. 3A, L77S). Thus, the LFG patch helps prevent premature activation and may do so for family members with more complex signaling pathways.

Thus, the C3 region probably plays both positive and negative roles during regulation. Prior to signaling, it prevents premature activation, but after signaling it is used to help form the correct complex containing activator and ATP. It also appears to play a role in the conformational changes that apparently follow the formation of the transition state. The overall signaling pathway also should include compaction of the dimer when ATP is bound, probably using determinants in the C7 region. However, neither region seems to be needed to allow pspFΔHTH to trigger the switch of helozyme to engage the nontemplate strand, so this determinant should lie elsewhere. Among the many questions remaining are learning which interfaces between activator and helozyme catalyze these various steps.

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