Volume 1 number 7 July 1974 Nucleic Acids Research

Defective in vitro binding of histidyl-transfer ribonucleic acid to feedback resistant phosphoribosyl transferase of *Salmonella typhimurium*

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Received 4 June, 1974

ABSTRACT

We previously proposed that the first enzyme for histidine biosynthesis in *Salmonella typhimurium* plays a role in regulating expression of the histidine operon and that in order to play this role the enzyme must form a complex with histidyl-tRNA. Among the many observations that led to these conclusions were 1) that regulation of the histidine operon is defective in strains carrying a mutation in the gene for the first enzyme that renders the enzyme resistant to inhibition by histidine; and 2) that the enzyme purified from the wild type strain interacts specifically, and with high affinity, with histidyl-tRNA. The present study was carried out to test the prediction that the enzyme purified from the mutant strain described above would display a defect in its interaction with histidyl-tRNA. This prediction was fulfilled by the finding that purified histidine-insensitive enzyme does not bind histidyl-tRNA. Our results therefore suggest that the capacity of the enzyme to bind histidyl-tRNA *in vitro* is a reflection of its regulatory function *in vivo*.

INTRODUCTION

The full mechanism of repression of the histidine operon of *Salmonella typhimurium* has not yet been elucidated. Aminoacylated histidine transfer ribonucleic acid (histidyl-tRNA) has been shown to be required for repression by biochemical studies[1] and by studies on regulatory mutants that display defective synthesis or aminoacylation of histidine tRNA[2-8]. It has been assumed that histidyl-tRNA fulfills its regulatory function as a complex formed by interaction with a regulatory protein[9,10], but intensive efforts to identify such a protein by genetic means have not yet been successful. We have considered the possibility that a regulatory protein for the histidine system has a dual function, acting not only in regulation of the histidine operon but also in some capacity vital for growth of the organism on minimal medium[10]. An obvious candidate would be one of the enzymes for histidine biosynthesis.

Previous studies from this laboratory suggested that the allosteric enzyme, phosphoribosyltransferase [N-1-(5'-phosphoribosyl)adenosine triphos-

phate: pyrophosphate phosphoribosyltransferase, EC 4.2.1c], the enzyme that catalyzes the first step of the pathway for histidine biosynthesis in Salmonella typhimurium and that is sensitive to inhibition by histidine, plays a role in the repression process[11-16]. While studying the kinetics of repression of the histidine enzymes in various mutants of the histidine operon, we noted that when the function of the feedback sensitive site of phosphoribosyltransferase was altered, either chemically or by mutation, the kinetic pattern of repression by histidine was altered and repression by the histidine analogue, 1,2,4-triazolalanine, was prevented althogether[12,15]. Since 1,2,4-triazolalanine is thought[17] to cause repression of the histidine operon by becoming aminoacylated to $tRNA^{His}$ we suggested that phosphoribosyltransferase fulfills its regulatory function in vivo by interacting with histidyl-tRNA. Basic to this idea is the prediction that it should be possible to demonstrate an interaction between phosphoribosyltransferase and histidyl-tRNA in vitro. This prediction was verified by the findings that phosphoribosyltransferase binds histidyl-tRNA in preference to other species of aminoacylated tRNA[13,15] and binds histidyl-tRNA in preference[15] to $tRNA^{His}$. The present study was undertaken to determine whether the regulatory alterations noted in feedback resistant mutants in vivo are reflected in an alteration of the binding of purified feedback resistant phosphoribosyltransferase to histidyl-tRNA in vitro.

MATERIALS AND METHODS

Purification, storage, and assay of phosphoribosyltransferase.

Wild type phosphoribosyltransferase was purified from a constitutive mutant by the method of Parsons and Koshland[18]. Frozen cells (200 grams) of S. typhimurium, hisT1504, were suspended in 0.1 M Tris-HCl, pH 7.5, containing NaCl (0.1 M), L-histidine (0.4 mM), EDTA (0.5 mM), and dithiothreitol (1.0 mM)[19]. Cells were ruptured by sonication of 50 ml portions and centrifuged at 20,000 x g for 40 min. The method of Parsons and Koshland[18] involves the following purification steps: heat treatment at 53° for 6-7 min, precipitation with ammonium sulfate between 30 and 45% saturation, precipitation at pH 4.8 to remove histidine, and a final purification step that depends on the differential solubility of phosphoribosyltransferase in 50% ammonium sulfate in the absence and presence of 1 mM histidine. Thus, the enzyme collected as a precipitate at 50% saturation with ammonium sulfate in the absence of histidine, all remains soluble at 50% saturation in the presence of 1 mM histidine. These treatments resulted in an approximately 100-fold purifica-

tion of the enzyme, with an 18% overall recovery. The specific activity of the final preparation, assayed by the method of Ames et al[20], as described in reference 14, was about 8,000 units/mg. Electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulfate showed one major band, comprising about 95% of the protein, and two very faint bands. The enzyme was stored in 10 mM Tris-HCl, pH 7.5, containing EDTA (1 mM) and glycerol (50% by volume).

Feedback resistant phosphoribosyltransferase was purified from the double mutant, hisT1504hisG1102, constructed by Dr. B. Ely (unpublished data). This strain contains the constitutive mutation, hisT1504, and a mutation, hisG1102, that leads to the production of a phosphoribosyltransferase that is catalytically active but is resistant to inhibition by histidine. During purification by the method of Parsons and Koshland[18], it was noted that the feedback resistant enzyme failed to display the same differential solubility as that displayed by the wild type enzyme in ammonium sulfate solutions in the presence and absence of 1 mM histidine. When the histidine concentration in the buffer was increased to 10 mM about 30% of the feedback resistant enzyme remained soluble in 50% ammonium sulfate. This fraction had a specific activity of 4,123 units/mg. Electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulfate showed one major band, comprising at least 70% of the protein, and several faint bands representing proteins with lower migration rates. The specific activity of the feedback resistant phosphoribosyltransferase was increased by approximately 40% by a modification[12] of the reactivation method of Martin[21]. It was stored and assayed in the same manner as was the wild type enzyme. It was fully active in the presence of histidine at a concentration (2.0 mM) at which the wild type enzyme was almost fully inhibited.

Aminoacylation of transfer-RNA.

Partially purified E. coli B $tRNA^{His}$ (41% pure), was a generous gift from Donald A. Kelmers of Oak Ridge National Laboratory. It was dialyzed against water and stored lyophilized at -20°. Samples of $tRNA^{His}$ were aminoacylated with tritiated L-histidine (specific activity, 11.4 Ci/mmole, New England Nuclear Corp.) by the method of Muench and Berg[22] with the use of aminoacyl-tRNA synthetase prepared by the method of Zubay[23]. Between 20% and 23% of the total tRNA was aminoacylated.

Binding Studies

Binding of phosphoribosyltransferase to tRNA was studied by a modifica-

tion[15] of the nitrocellulose filter binding technique of Yarus and Berg[24] (see legend for Fig. 1 for details).

RESULTS AND DISCUSSION

Purification of wild type phosphoribosyltransferase from hisT1504 was carried out by the method of Parsons and Koshland[18] without difficulty. This method depends primarily upon the fact that the enzyme is more soluble in ammonium sulfate when in the presence of histidine than it is when in the absence of histidine, presumably because of the confromational change histidine is known to produce in the enzyme[25]. Final preparations had specific activities between 7,000 and 8,000 units per mg. However, when the same method was applied to extracts of hisT1504hisG1102, the results were poor. The point in the procedure at which the feedback resistant enzyme failed to undergo the same degree of purification as did the wild type enzyme was the one that depended upon its differential solubility in ammonium sulfate in the presence and absence of histidine. It is tempting to speculate that the reason for this failure of the feedback resistant enzyme to show the differential solubility displayed by the wild type enzyme is related to its failure to undergo the confromational change that histidine produces in the wild type enzyme[25]. For this reason, the concentration of histidine in the buffer was increased from 1 mM to 10 mM during the differential ammonium sulfate precipitation. Although some shift in solubility did occur under these conditons, the shift was not so large as in the case of the wild type enzyme. Therefore, the final specific activity achieved was only about half of that achieved with the wild type enzyme.

Previous studies demonstrated that the capacity of phosphoribosyltransferase to bind histidyl-tRNA diminishes pari passu with the loss of sensitivity to inhibition by histidine that occurs upon aging of the enzyme[14]. For this reason we expected that enzyme that was insensitive to histidine inhibition by virtue of a mutation-induced structural defect might also display some loss of the ability to bind histidyl-tRNA, even when freshly prepared. However, there remained the possibility that by virtue of the length of the previously employed purification procedure[19], the binding characteristics of the enzyme were altered during its isolation, since wild type enzyme is known to lose its capacity to bind tRNA upon aging[14]. The recently reported rapid purification procedure[18] circumvents this problem, and the use of partially purified histidyl-tRNA has allowed a more rigorous test of the binding characteristics of the enzyme. Fig. 1 Shows the results of experiments in

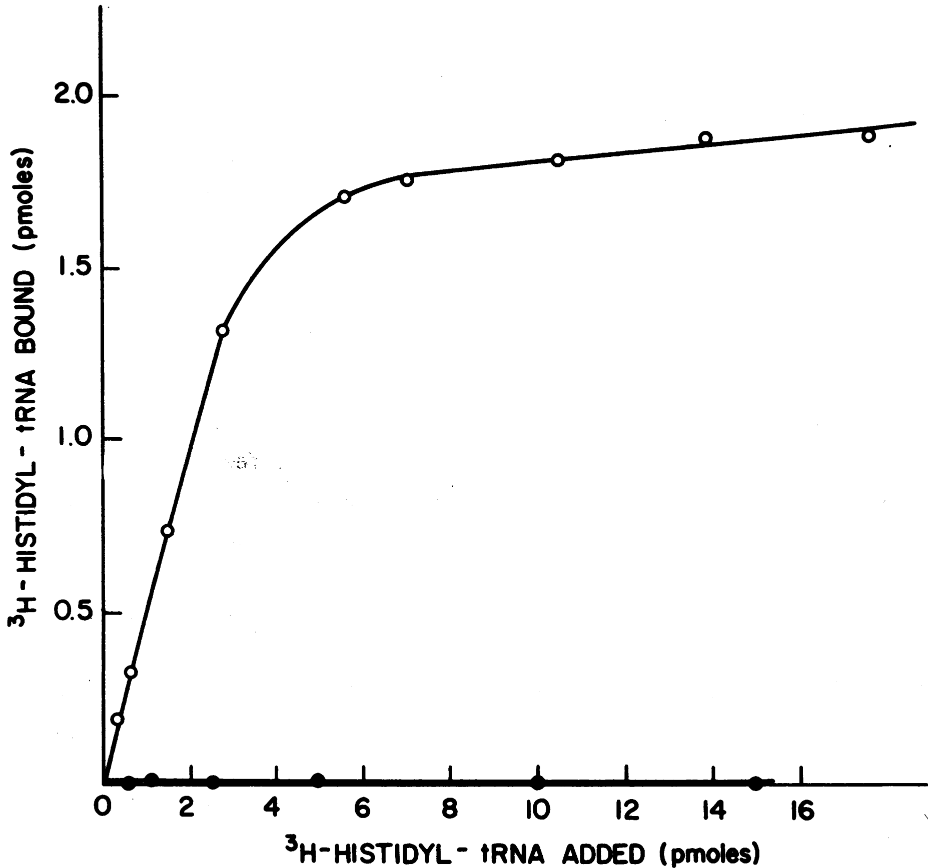

Fig. 1. Binding of ^{3}H-histidyl-tRNA to phosphoribosyltransferase. Samples (7.0 μg) of enzyme were incubated with various amounts of partially purified ^{3}H-histidyl-tRNA in 0.033 M potassium cacodylate buffer, pH 6.5, containing $MgCl_2$ (5×10^{-3}M), in a total volume of 0.2 ml. In all cases, the reaction was started by the addition of enzyme. After incubation, the reaction mixture was diluted with 3 ml of ice-cold cacodylate buffer and filtered through nitrocellulose filters (Schleicher and Schuell Co., B6). Filters had been prewashed and presoaked in the same buffer. To ensure complete transfer of the reaction mixture onto the filter, each tube was rinsed four times with cacodylate buffer (3 ml in each wash). The filters were then dissolved in Bray's solution[26] and counted in a scintillation spectrometer (Nuclear-Chicago Corp., Mark II) with greater than 50% efficiency. The preparations of phosphoribosyltransferase were wild type (from hisT1504; O——O) and feedback resistant (from hisT1504hisG1102; ●——●).

which the binding of histidyl-tRNA to feedback resistant enzyme is compared with the binding to wild type enzyme. The wild type enzyme gave the response previously reported[15]. Increasing concentrations of histidyl-tRNA gave increasing amounts of binding, until saturation. Using the same amount of protein from the preparation of feedback resistant enzyme, no binding was observed even with histidyl-tRNA at a concentration three times higher than that necessary to saturate the wild type enzyme. In other experiments, in which the amount of enzyme was increased 4-fold we found that the feedback resistant enzyme still did not bind histidyl-tRNA. These results were routinely reproducible.

It has been known for some time that although wild type phosphoribosyltransferase does bind histidyl-tRNA specifically[15], it also binds all other species of tRNA[14,15]. In order to assess the ability of feedback resistant enzyme to bind all species of tRNA, we investigated the binding of the enzyme to total ^{3}H-tRNA of E. coli. Once again, we found that, whereas the wild type enzymes bound well, the feedback resistant enzyme did not. At a given concentration of total tRNA, more than ten times as much wild type enzyme became bound than feedback resistant enzyme. The small amount of binding of the feedback resistant enzyme to total tRNA was no greater than that found with bovine serum albumin, and was therefore considered to be entirely non-specific.

The finding that feedback resistant phosphoribosyltransferase does not bind histidyl-tRNA was strongly suggested by a previous study[14] in which we did not have purified histidyl-tRNA and in which we did not have a sufficient amount of the mutant enzyme to do extensive studies of binding. We can now state that two preparations of feedback resistant enzyme, purified by entirely different methods, when tested with several entirely different preparations of transfer RNA, both lack the binding characteristic of wild type enzyme.

Because the feedback resistant phosphoribosyltransferase was catalytically active, displaying the same K_m for ATP and the same K_m for phosphoribosyl pyrophosphate (PRPP) as that displayed by the wild type enzyme[19], we conclude that its loss of the capacity to bind tRNA was not due to an alteration of the active site of the enzyme. This is in agreement with previous evidence that tRNA binds to the enzyme at a site distinct from the catalytic site[14,15]. Because histidine does not alter the binding of histidyl-tRNA to wild type enzyme, even when present at a concentration five orders of magnitude higher than that of the tRNA and three orders of magnitude higher than that of the K_I[14], we conclude that the enzyme binds histidyl-tRNA at a site distinct

from the feedback sensitive site. Thus, it appears that phosphoribosyltransferase has different sites specific for binding ATP, PRPP, histidine, and tRNA[27].

Although we have in the past considered it likely that the highly specific binding of phosphoribosyltransferase to histidyl-tRNA is related to a regulatory activity of the enzyme in vivo, the evidence for such a relationship was circumstantial. The studies reported here provide more direct evidence, since they demonstrate that a defect in the repression process in vivo is reflected in a defect in the binding in vitro. That is, purified feedback resistant phosphoribosyltransferase from a strain that has a defect in its pattern of repression does not bind histidyl-tRNA. Although these findings allow us to further implicate phosphoribosyltransferase in the autogenous regulation of the histidine operon, they do not allow us to specify the mechanism of that regulation in greater detail.

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27. We have recently found (M. Meyers, T. Vogel, M. Levinthal, C.B. Bruni, J.S. Kovach, R.G. Deeley, and R.F. Goldberger, unpublished data) that phosphoribosyltransferase also binds specifically to some region in the bacterial DNA of a defective ϕ80 transducing phage carrying the histidine operon.

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