Histidine Regulation in Salmonella typhimurium

XIV. INTERACTION OF THE HISTIDYL TRANSFER RIBONUCLEIC ACID SYNTHETASE WITH HISTIDINE TRANSFER RIBONUCLEIC ACID*

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SUMMARY

Mutations in any of the six genes of Salmonella typhimurium lead to constitutivity of the histidine operon. Four of these genes, hisR, hisU, hisW, and hisT, are believed to be involved with the production of histidine tRNA. We show that the tRNAHis isolated from each of these mutants is aminoacylated normally, indicating that the constitutivity of these mutants cannot be ascribed to a reduced ability of their histidine tRNA to be charged.

HistT mutants are known to have a structurally altered tRNAHis. These mutants are apparently constitutive because of failure of the altered tRNA to interact properly with the repression mechanism. The binding of charged tRNAHis from hisT mutants to the histidyl-tRNA synthetase, one candidate for the corepressor of the histidine operon, has been tested by filter binding. Under several different assay conditions the binding of histidyl-tRNAHis from the wild type and from hisT1504 is the same within experimental error.

An assay for the enzymatic deacylation of histidyl-tRNAHis was developed to check the binding constants obtained by the filter binding method. The synthetase has surprisingly low Kd values for the substrates of the reverse reaction; 45 μM for AMP and 15 μM for pyrophosphate. The Kd for charged tRNAHis, 40 nM, is the same as the dissociation constant found for charged tRNAHis by filter binding and also the same as the Kd for uncharged tRNAHis in the aminocyclation reaction. The Kd of the enzyme for tRNAHis is shown to increase as the histidine concentration is increased, over a range of histidine concentrations that are found physiologically. It is shown that an increase in pyrophosphate concentration increases the inhibitory effect of AMP on synthetase activity, and it is suggested that this has physiological meaning.

The histidine operon of Salmonella typhimurium is composed of the nine genes which code for the enzymes of histidine biosynthesis. These genes occupy contiguous positions on the Salmonella chromosome, and their activity is controlled as a unit in response to the need of the cell for histidine biosynthesis. To unravel the mechanism of control of the activity of these genes, a large number of constitutive mutants have been isolated.

The constitutive mutations map in six different chromosomal locations (2–6). The mutations of one set map at the beginning of the operon and are cis dominant, suggesting that they are operator mutants (7, 8). The remaining loci, hisR, hisU, hisT, hisW, and hisU, are all recessive (7). Each of these remaining loci has been implicated in the production of tRNAHis or in its charging. HisT codes for the histidyl-tRNA synthetase (3, 9), hisR appears to be a structural gene for histidine tRNA (10–12), and hisT codes for an enzyme which converts uridine to pseudouridine in the anticodon loop of the histidine tRNA (13). HisU and hisW may also be involved in tRNA maturation, as both mutant types appear to have reduced levels of several species of tRNA (12).

In this paper we examine the interaction of the histidyl-tRNA synthetase with histidine tRNA isolated from the constitutive mutants. These studies are divided into two parts. In the first part, both the Kd and Vmax of tRNAHis isolated from the wild strains were determined in the aminoacylation reaction. These studies serve as a probe for possible differences in tRNA structure, and as a test of the possibility that the constitutivity of these strains might result from reduced charging of an altered histidine tRNA. The second part of the paper examines the possibility that the histidyl-tRNA synthetase, in complex with charged histidine tRNA, might be the repressor for the operon (14). In these experiments the binding to the enzyme of charged histidine tRNA from hisT mutants is compared with that from the wild type. Histidine tRNA is known to be present in normal quantities in hisT mutants, and to be charged to the normal extent (11, 12), making it likely that these mutants are derepressed because of failure of the charged tRNA to interact properly with the repression mechanism. If binding of charged histidine tRNA to the synthetase is required for repression, it might be expected that charged tRNA from hisT mutants would bind less tightly to the enzyme than the wild type tRNA. The binding studies with charged tRNAHis were done using the filter binding...
method of Yarus and Berg (15). In addition, the affinity of charged tRNA for the enzyme was determined by measuring its $K_a$ in the enzymatic deacylation of the tRNA. Besides serving as a check on the validity of the filter binding procedure, the deacylation reaction may prove useful for mechanistic studies of the aminocacylation reaction.

**MATERIALS AND METHODS**

The origin of the bacterial strains, growth of the bacteria, and preparation of the tRNA are described elsewhere (11). L-[3H]Histidine (250 mCi per mm) and L-[3H]histidine (2.5 Ci per mm), both in 50% ethanol, were obtained from New England Nuclear. The radioactive histidine was stored at $-20^\circ$C, and before use an aliquot was evaporated to dryness and dissolved in an equal volume of water. The crystalline sodium salt of ATP, unlabeled L-histidine, and bovine serum albumin, type V, were obtained from Sigma. AMP was a product of Schwartz BioResearch, and sodium pyrophosphate was from Mallinckrodt. Homogeneously pure histidyl-tRNA synthetase (specific activity 15000 units per mg) was prepared as described previously (16). Glass fiber filters (type A, 1 inch in diameter) were purchased from Gelman Instrument Company.

**Determination of $K_a$ and $V_{max}$ for tRNA$^{His}$ in Aminocacylation Reaction**—In a final volume of 0.25 ml the reaction mixture contained 8 mm MgCl₂, 4 mm ATP, 15 μM [3H]histidine, and 0.1 M sodium cacodylate at pH 7.5. The concentration of tRNA$^{His}$ was varied from about 20 nM to 200 nM. Tubes were incubated previously for 2 min and enzyme added to start the reaction. When assays were run at 37°C, histidyl-tRNA synthetase was usually used at a final concentration of 6.1 μg per ml; when assays were run at 20°C the enzyme concentration was usually 15 ng per ml. Samples of 75 μl were taken at 1, 2, and 3 min, the reaction stopped by the addition of 3 ml of cold 10% trichloroacetic acid, and the resulting suspension chilled in ice. The precipitate was collected on a glass fiber disc and washed four times with 5 ml of 10% trichloroacetic acid, four times with 5 ml of 95% ethanol, and twice with 5 ml of ether. The air-dried filters were counted in Bray's scintillation fluid (18). In other experiments 0.2 ml of the filtrate was pipetted onto glass fiber filters, the filters dried at 120°C, and then counted in the 2,5-diphenylloxazole-1,4-bis-[2-(5-phenyloxazolyl)] benzene-toluene solution described above. Counting was done in a Nuclear Chicago Mark I liquid scintillation counter at an efficiency of 30%.

**Preparation of Histidyl-tRNA$^{His}$**—The tRNA$^{His}$ is aminocylated using the reaction conditions described above, except that a large excess of enzyme is used to insure that the reaction goes rapidly to completion. The reaction is stopped by the addition of 13 volumes of 67% ethanol at $-20^\circ$C, which contains 10 mm MgCl₂ and 10 mm sodium acetate, pH 4.5 (the large volume of ethanol is necessary to keep the ATP in solution). The precipitate is collected by filtration through Millipore filters, type HA, 0.45 μ (Millipore Filter Corporation, New Bedford, Mass.), and washed with 20 ml of the buffered alcohol. Up to 50 A$_{260}$ units of tRNA are collected on a single Millipore filter. The filters are air-dried, and the tRNA eluted into a buffer containing 1 mm MgCl₂ and 1 mm sodium acetate, pH 4.5, by shaking in the cold for 15 min. It was found convenient to perform the elution in a glass scintillation vial, usually with 1 ml of buffer.

A slight variation of this procedure was used to prepare charged tRNA$^{His}$ for the binding reaction. The tRNA was charged to completion as described above, then the reaction was quenched by the addition of 0.25 volume of ice-cold 1 M acetic acid followed by 2.5 volumes of ice-cold water. The resulting solution was applied to a column (1 × 11 cm) of DEAE-cellulose (Selecta-cel 70, Schleicher and Schuell) equilibrated at 4°C with 10 mm sodium acetate, pH 4.5, 10 mm MgCl₂, and 0.1 M NaCl. After the column was washed with 45 ml of the equilibration buffer, the column was eluted with the same buffer made 1 M in NaCl and 3 ml fractions were collected. High salt fractions containing radioactivity were pooled and mixed with 2 volumes of ethanol at $-18^\circ$C. After 1 hour at $-18^\circ$C, the precipitated nucleic acid was collected by vacuum filtration on several Millipore filters according to the method described above.

Collection of tRNA on membrane filters has proved to be a convenient method for dealing with small quantities of tRNA. This method has been used with total amounts as small as 0.1 A$_{260}$ unit (5 μg) and concentrations as low as 0.01 A$_{260}$ unit per ml. A number of different filter types have been screened to select that most suited for collection of tRNA samples. The Millipore filter type HA, 0.45 μ, proved to be superior to eight other types tested for tRNA collection (17). In using these filters, however, one must not exceed an alcohol concentration of about 75%, as higher concentrations (95%) partially dissolve the filters. Elution of tRNA from the filters is rapid, and does not appear to be sensitive to the buffer used. The elution is about 95% complete in 5 min, and 100% complete by 15 min. Recoveries of A$_{260}$ units range from 80 to 100%.

**Deacylation Assay**—In a final volume of 0.25 ml the reaction mixture contained 0.1 mm sodium cacodylate at pH 7.5, 8 mm MgCl₂, 1 mm AMP, 1 mm PP₃, and enzyme and [3H]- or [3H]histidyl-tRNA$^{His}$ as indicated in the table and figure legends. Tubes were previously incubated for 2 min at 37°C, and histidyl-tRNA$^{His}$ added to initiate the reaction. At intervals of 1, 2, and 3 min, 75-μl samples were withdrawn and the reaction quenched by the addition of 1 ml of 5% trichloroacetic acid. The chilled precipitate was then filtered through a glass fiber filter. In some experiments 0.5 ml of the filtrate was neutralized with 0.1 ml of a NaOH solution 0.1 mm sodium cacodylate and counted in Bray's scintillation fluid (18). In other experiments 0.2 ml of the filtrate was pipetted onto glass fiber filters, the filters dried at 120°C, and then counted in the 2,5-diphenyl-oxazole-1,4-bis-[2-(5-phenyloxazolyl)] benzene-toluene solution described above. Counting was done in a Nuclear Chicago Mark I liquid scintillation counter. In Bray's solution efficiencies of 15% for 3H and 80% for 14C were obtained. In the 2,5-diphenyl-oxazole-1,4-bis-[2-(5-phenyloxazolyl)] benzene-toluene solution efficiencies were 30% for 3H and 90% for 14C.

**Binding Assay**—Histidyl-tRNA synthetase and tRNA$^{His}$ charged with histidine were incubated together in 120 μl of reaction mix of composition described in the figures and tables. At the end of the incubation period, a 100 μl sample was withdrawn and filtered through a Schleicher and Schuell B-6 nitrocellulose filter, previously moistened in water. The filter was then washed with 0.6 ml of the buffer used in the assay, dried for 10 min at 110°C, and radioactivity determined using the 2,5-diphenyl-oxazole-1,4-bis-[2-(5-phenyloxazolyl)] benzene-toluene solution described above. Blank reactions were performed by substituting an identical amount of bovine serum albumin for the synthetase.

**Analysis of Binding Data**—The straight line best fitting a double reciprocal plot of histidyl-tRNA$^{His}$ bound versus histidyl-tRNA$^{His}$ free in solution was obtained by a least squares approximation as described by Yarus and Berg (15).
RESULTS

Kinetic Analysis of tRNA in Aminoclaylation Reaction—Both the Km and Vmax were determined for histidine tRNA isolated from the wild type and from the regulatory mutants, hisR, hisU, hisW, hisT, and hisO. The tRNA from hisO served as a derepressed control, since hisO is known to be an operator region (7, 8, 19), and therefore not involved in tRNA production. Typical double reciprocal plots of the kinetic data are presented in Fig. 1. Table I presents the Km and Vmax values determined. As can be seen, the parameters determined for tRNA isolated from the mutant strains do not differ significantly from those determined for tRNA isolated from the wild type.

The sensitivity of this probe of tRNA structure is limited by the variation found between different preparations of the wild type tRNA. This is particularly noticeable for the first preparation listed. This batch of tRNA was that used to determine the published value of 110 nM for the tRNA\textsuperscript{His} Km (16), and therefore not involved with tRNA production. The “Silbert” method is essentially the same, except LiCl replaces NaCl in the buffers (10). The “thiosulfate” preparations are the same as the “standard,” except 2 mM sodium thiosulfate was present in all buffers to protect sulfhydryl groups in the tRNA (11).

Among the tRNAs analyzed was that from the cold-sensitive hisW mutant, JL250. To determine if the immediate cessation of growth displayed by this mutant when shifted to 20° was due to a cold-sensitive tRNA\textsuperscript{His}, the Km for the JL250 tRNA\textsuperscript{His} was also determined at this lower temperature (Table I, values in brackets). No dramatic difference in Km or Vmax was found for the tRNA\textsuperscript{His} of the mutant relative to that from a number of other Salmonella strains which were assayed as controls.

Measurements of the Km for tRNA\textsuperscript{His} discussed above were made at a histidine concentration of 15 μM, the concentration of the internal histidine pool of Salmonella growing on minimal salts-glucose medium (20). As shown in Fig. 2, however, the
FIG. 2. Effect of histidine concentration on the $K_m$ for tRNA$\text{His}$ in the aminoaacylation reaction. Except for the variation in histidine concentration, the assays were performed as described under "Materials and Methods." Enzyme was used at a final concentration of 14.4, 7.2, and 3.6 ng per ml for the assays at 5, 15, and 50 $\mu$M histidine, respectively.

$K_m$ for tRNA$\text{His}$ depends upon the concentration of histidine, with higher histidine concentrations giving higher values for the $K_m$. We are not sure whether this variation of tRNA $K_m$ with histidine concentration has any physiological significance, since the in vivo concentrations of the synthetase and uncharged tRNA$\text{His}$ (1.5 $\mu$M and 0.23 $\text{mM}$, respectively (12, 16, 17)) are much higher than the $K_m$ of the enzyme for the tRNA (0.04 $\mu$M).

Binding of Charged Histidine tRNA to Histidyl-tRNA Synthetase—HisT mutants produce a structurally altered histidine tRNA (11, 13). However, the mutant tRNA is present in the cell in wild type quantity (11, 12) and is charged normally (12, Table I). Apparently hisT mutants are derepressed through failure of the charged histidine tRNA of hisT mutants to interact properly with the repression mechanism. If the histidyl-tRNA synthetase is the corepressor for the histidine operon (14), one might expect it to bind charged histidine tRNA from hisT mutants less strongly than the charged tRNA from the wild type. This prediction was tested using the filter binding method for measuring the affinity of tRNA for the enzyme. The method used was essentially that of Yarus and Berg (15), except that the reaction conditions were modified to more closely approximate the physiological conditions under which repression of the histidine operon has been studied in S. typhimurium. Accordingly, the incubations were done at pH 7.0, rather than pH 5.5, and at temperatures of 25° and 37°, rather than 17°. In addition, the data of Yarus and Berg (21) indicate that formation of synthetase-isoleucyl-AMP complex occurs faster than dissociation of charged tRNA$\text{His}$ from the enzyme. If the same is true for the histidyl-tRNA synthetase, the form of the synthetase expected to predominate in vivo during conditions of repression is the synthetase-histidyl-AMP complex. Thus, we also studied the binding of charged tRNA$\text{His}$ from the wild type and from hisT1504 to the synthetase-histidyl-AMP complex as well as their binding to the synthetase alone. When required, the histidyl-AMP-synthetase complex was generated by preliminary incubation of the enzyme with 15 $\mu$M histidine and 4 $\text{mM}$ ATP. The apparent lesser binding of charged tRNA$\text{His}$ to the histidyl-AMP-synthetase complex below pH 6 is probably due to denaturation of the synthetase at low pH during this preliminary incubation. After preincubation tRNA was added to give a final reaction volume of 120 $\mu$l. All assays were at room temperature and were as described under "Materials and Methods."

Table II

| Buffer                     | Radioactivity retained on filter (cpm) |
|---------------------------|----------------------------------------|
| Sodium phosphate          | 116 28                                  |
| Sodium cacodylate          | 719 28                                  |
| Imidazole-HCl              | 1138 168                                |
| Triethanolamine-HCl        | 778 176                                 |

FIG. 3. Effect of buffer pH on the binding assay. Sixty-six picomoles of histidyl-tRNA synthetase and 10 pmols of [3H]histidyl-tRNA were incubated for 15 s at room temperature in 100 mM MgCl$\text{2}$ and 50 mM buffer as indicated, pH 7.0. The assays were otherwise performed as described under "Materials and Methods."
Summary of results obtained in binding experiments using charged tRNA<sup>His</sup> from LT-2 and hisT1504

In Experiment A, varying quantities of charged tRNA<sup>His</sup> were added to 110 pmol of synthetase in 10 mM MgCl<sub>2</sub> and 50 mM sodium cacodylate, pH 7.0, at room temperature (24°). Fifteen seconds after the addition of tRNA, samples were filtered and counted as described in “Materials and Methods.”

In Experiment B, conditions were as in Experiment A, except before the addition of charged tRNA<sup>His</sup> the synthetase was incubated previously at 24° for 10 to 25 min in 10 mM MgCl<sub>2</sub>, 15 μM histidine, 4 mM ATP, and 50 mM sodium cacodylate, pH 7.0.

In Experiment C, conditions were as in Experiment B, except that before the addition of charged tRNA<sup>His</sup> the temperature was shifted to 37°.

| Strain        | Temperature | Other substrates | K<sub>d</sub> (nM) |
|---------------|-------------|------------------|-------------------|
| Experiment A  |             |                  |                   |
| LT-2          | 24°         |                  | 12, 19, 52        |
| hisT1504      | 24°         |                  | 13, 22            |
| Experiment B  |             | 4 mM ATP and 15 μM histidine | 33, 83          |
| LT-2          | 24°         |                  | 33, 29            |
| hisT1504      | 24°         |                  | 33, 29            |
| Experiment C  |             | 4 mM ATP and 15 μM histidine | 44              |
| LT-2          | 37°         |                  | 44                |
| hisT1504      | 37°         |                  | 46, 45            |

The dissociation constants obtained are presented in Table III, and a typical experiment is shown in Fig. 4. Within the range of variation the results for wild type and hisT1504 tRNA are the same. The results also indicate that the binding of charged tRNA<sup>His</sup> from both strains to the synthetase-histidyl-AMP complex is less tight than binding to the free enzyme. This effect parallels the increase in tRNA<sup>His</sup> with increasing histidine concentration noted in the deacylation reaction (Fig. 2).

From our results we can determine the number of histidyl-tRNA<sup>His</sup> molecules bound per molecule of histidyl-tRNA synthetase when saturating amounts of tRNA are present. We find 0.07 and 0.25 tRNA molecules per molecule of synthetase are bound without the addition and with the addition of ATP and histidine, respectively. Our synthetase preparation is clearly not fully active. Presumably the increased binding in the presence of ATP and histidine is due to the activation of some of the enzyme molecules by these substrates, although this has not been tested. For these experiments we employed the highly purified, homogeneous material described by De Lorenzo and Ames (16). Our enzyme has a specific activity similar to the activity of freshly purified enzyme as judged by its ability to charge tRNA<sup>His</sup>. The reasons for the low binding activity and the variability in the determination of dissociation constants are currently under investigation.

Interaction of Charged Histidine tRNA with Histidyl-tRNA Synthetase as Measured by Deacylation Assay—The precise mecha-
Interaction of Histidyl-tRNA Synthetase with Histidine tRNA

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FIG. 5. Rate of enzymatic deacylation of histidyl-tRNAHis as a function of pyrophosphate concentration. The reaction was performed as described under "Materials and Methods," except the pyrophosphate concentration was varied as indicated. The reaction mixture contained histidyl-tRNA synthetase at a concentration of 27 ng per ml and [3H]histidyl-tRNAHis at a concentration of 26 A 160 units per ml (380 nM).

FIG. 6. Rate of enzymatic deacylation of histidyl-tRNAHis as a function of AMP concentration. The reaction was performed as described under "Materials and Methods," except the AMP concentration was varied as indicated. The reaction mixture contained histidyl-tRNA synthetase at a concentration of 22 ng per ml, and [3H]histidyl-tRNAHis at a concentration of 26 A 160 units per ml (380 nM).

FIG. 7. Rate of deacylation reaction as a function of histidyl-tRNAHis concentration. The reaction was conducted using [3H]histidyl-tRNAHis as described under "Materials and Methods," with enzyme at a final concentration of 11.2 ng per ml.

DISCUSSION

In this paper we examined the interaction of the histidyl-tRNA synthetase with histidine tRNA isolated from those constitutive mutants possibly defective in tRNA production. Since several of these strains are thought to have an altered tRNAHis, it was considered that constitutivity might result from failure of these tRNA's to be charged normally. Accordingly, the Km and Vmax for the tRNA in the aminoacylation reaction were determined. Failure to find any kinetic difference between tRNAHis isolated from the mutant and wild type strains suggests the tRNAHis in these strains should be charged to the normal extent in vivo. This prediction has recently been verified (12), requiring alternate hypotheses to be considered for the cause of constitutivity. In the case of hisR, constitutivity likely arises from a decrease in the total level of histidine tRNA in the cell (10-12). A lowering of the histidine tRNA content of hisR and hisW strains may also be responsible for the constitutivity of these mutants (12). HisT mutants, however, appear to have both the normal content of histidine tRNA, and normal charging of that tRNA within the cell (11, 12). At the same time, hisT
mutants are known to produce a structurally altered histidine tRNA (11, 13). It would appear, therefore, that constitutivity of hisT mutants is caused by a failure of the charged histidine tRNA in hisT mutants to interact properly with the repression mechanism.

One possibility which we have been considering is that the histidyl-tRNA synthetase, in complex with charged histidine tRNA, is the repressor for the histidine operon (14). If such were the case, one might expect charged histidine tRNA from the wild type to bind to the enzyme, but that the tRNA from hisT mutants would not. However, the data presented here show that charged tRNA<sup>His</sup> from hisT mutants binds with the same affinity as the wild type. This finding is consonant with the results of the aminoaacylation reaction, in which uncharged tRNA<sup>His</sup> from the wild type and from hisT1504 yielded the same 

Although these results do not support the hypothesis that the synthetase is the corepressor of the histidine operon, neither do they rule it out. The altered structure of charged tRNA<sup>His</sup> from hisT mutants might cause the synthetase to function inefficiently as a corepressor while still causing no significant change in the strength of tRNA binding to the synthetase. Biochemical and genetic studies are in progress to test more conclusively the possibility that the histidyl-tRNA synthetase is the coregulator of the histidine operon.

An assay of the enzymatic deacylation of charged tRNA was developed to check the validity of the filter binding assay. The deacylation reaction may prove valuable for the analysis of enzyme-tRNA interactions in systems where filter binding does not work, particularly when physiological conditions are desired. The reaction may also be useful for kinetic analysis of the reaction mechanism. Of particular interest is the low 

The synthesis of AMP in the deacylation reaction. This 

An 8-fold lower than that for ATP in the forward reaction. It has been shown that the 

The 

has previously been shown to be subject to energy charge control (inhibition by AMP and ADP) in the absence of pyrophosphate (22). Since the presence of pyrophosphate increases the binding of AMP, energy charge control should be stronger when pyrophosphate is present, and this may well have physiological meaning. No data have been found concerning the in vivo concentration of pyrophosphate in bacteria, but pyrophosphate concentrations as high as a millimolar have been recently reported for rat and guinea pig liver (23).

The filter binding assay and the deacylation assay give the same binding constant for charged histidine tRNA as is found for uncharged tRNA<sup>His</sup> in the aminoaacylation reaction. The similarity in binding constants could not have been predicted 

a priori: although charged and uncharged tRNA are similar in gross structure, several workers have found that charging of tRNA leads to a change in its conformation (24–27). Such changes in conformation could lower the affinity of the charged tRNA for the synthetase. The strong binding which is observed between the synthetase and histidine tRNA has two interesting implications. First, even if the synthetase-charged tRNA complex is not directly involved in repression, the ability of the synthetase to complex a large portion of the charged tRNA in the cell must be considered when describing the mechanism of control of the histidine operon. Second, the strong binding of charged tRNA suggests that it is a good inhibitor of the aminoaacylation reaction. We have found this to be the case. If approximately 80% of the histidine tRNA in the cell is in the charged state (12), only a fraction of the synthetase can be expected to be active in vivo. Such product inhibition might serve to regulate the rate of the aminoaacylation reaction.

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1 Unpublished experiments.
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