The Molecular Basis of Leucine Auxotrophy of Quinone-treated Escherichia coli

ACTIVE SITE-DIRECTED MODIFICATION OF LEUCYL-tRNA SYNTHETASE BY 6-AMINO-7-CHLORO-5,8-DIOXOQUINOLINE*

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Leucyl-tRNA synthetase from Escherichia coli is rapidly inactivated by 6-amino-7-chloro-5,8-dioxoquinoline (quinone), a model substance for cytostatic quinones. Loss of activity follows pseudo-first order kinetics. The quinone masks essential —SH groups that are reactive with N-ethylmaleimide.

Specific protection of the enzyme by leucine provides evidence for active site-directed modification. Half-maximal protection is found at a concentration of 150 µM which is identical with the dissociation constant of the enzyme-substrate complex.

The competitive inhibitor leucinol also protects the enzyme from inactivation by the quinone. MgATP enhances the protective effect of leucinol about 250-fold, thus substantiating recently published findings on synergistic coupling of ligands to aminoacyl-tRNA synthetases.

The results support the assumption that the bacteriostatic quinone directly interferes with leucyl-tRNA synthetase in growing cells. Active-site-directed inhibition of the enzyme could adequately explain the phenotypically observed auxotrophy for leucine of quinone-treated E. coli.

Escherichia coli cells when treated with the bacteriostatic 6-amino-7-chloro-5,8-dioxoquinoline are characterized by a specific auxotrophy for leucine. The intracellular level of leucyl-tRNA is drastically decreased in quinone-treated cells. The concentration of free leucine, however, is not reduced. Leucyl-tRNA synthetase has been assumed to be the primary target of the quinone in growing cells (1, 2).

In order to understand the phenotypic hyperauxotrophy for leucine at the molecular level, we investigated the interaction of the quinone with purified leucyl-tRNA synthetase. Particular emphasis was laid on elucidating whether the quinone behaves as a competitive inhibitor or as an affinity label of the active site of leucyl-tRNA synthetase. Evidence was obtained that the quinone reacts with essential —SH groups of the enzyme.

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The abbreviation used is: quinone, 6-amino-7-chloro-5,8-dioxoquinoline.

** Chemically t-[4.5-3H]leucine (50 to 60 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, England. L-Leucinol was purchased from Sigma. Unfractionated transfer RNA of E. coli was purchased from Boehringer Mannheim. 6-Amino-7-chloro-5,8-dioxoquinoline was synthesized by Farbwerte Bayer, Leverkusen. For the experiments a saturated aqueous solution was used, corresponding to approximately 250 µM as determined photometrically. When stored in the dark, the solution was stable for several days as tested by the inhibitory activity. In the presence of basic organic buffers, the inhibitor was less stable.

Enzymes—For kinetic analysis, leucyl-tRNA synthetase from different sources and different purities gave comparable results. Crude enzyme was prepared from E. coli MRE 600 grown in minimal medium (3) lacking glutamic acid. The washed cells were suspended in phosphate buffer (25 mM KH₂PO₄ (pH 7.5), 0.1 mM EDTA, 1% glycerol) and disrupted by ultrasonic treatment. After centrifugation at 30,000 x g, the supernatant was diluted with glycerol (final concentration, 50%) and stored at -20°C. Partially purified enzyme was prepared from E. coli MRE 600 or E. coli K10 following the procedure described by Rouget and Chapeville (4) including separation on hydroxyapatite. Pure leucyl-tRNA synthetase from E. coli B was a gift of Dr. A. Mehler (5).

Leucyl-tRNA Synthetase Assay—The activity of enzyme was assayed by measuring the formation of [3H]leucyl-tRNA at 28°C. The standard reaction mixture (250 µl) contained 40 mM KH₂PO₄ (pH 7.5), 10 mM ATP, unfractionated tRNA (4 A₂₆₀ units), 20 µM [3H]leucine (1 Ci/mmol), 20 mM magnesium acetate, and 2.5 mM KCl. The reaction was started by the addition of enzyme. At time intervals samples of 50 µl were removed and pipetted on paper filter discs.

The reaction was stopped by immersing the filters into ice cold trichloroacetic acid (10%). After several washings with trichloroacetic acid (2.5%), once with a mixture of ether:ethanol (1:1) and finally with ether, the dried discs were counted in 6 ml of toluene containing 5 g/liter of 2,5-diphenyloxazole and 0.3 g/liter of 1,4-bis(2-(4-methyl-5-phenyloxazolyl))benzene in a Packard liquid scintillation spectrometer.

Inactivation Kinetics—Leucyl-tRNA synthetase was freshly dialyzed twice for 2 h under slight pressure of nitrogen against phosphate buffer (40 mM KH₂PO₄, pH 7.5). The enzyme thus freed of protecting mercaptoethanol was extremely labile. Extensive mixing or agitating was avoided. For inactivation studies, leucyl-tRNA synthetase was usually incubated at 28°C in 300 µl of phosphate buffer with quinone. The concentrations of enzyme and inhibitor are given in the legends to the figures. After 1, 2, 3, 4, and 5 min, samples of 50 µl were removed and immediately mixed with 12.5 µl of the assay mixture, starting the reaction of [3H]leucyl-tRNA formation. The conditions were identical with those of the standard aminocacylation assay. After 30 or 60 s, aliquots of 50 µl were pipetted on paper filter discs and treated as described. Incorporation of [3H]leucine was linear for at least 2 min under all conditions employed including different concentrations of quinone. The addition of substrates effectively stopped inactivation of the enzyme by quinone. Dilution of enzyme-inhibitor solution prior to activity measurements could therefore be avoided, preventing spontaneous loss of activity. Enzyme activity was defined as counts per min per 30 s.
RESULTS

Inactivation of Leucyl-tRNA Synthetase—When formation of leucyl-tRNA by leucyl-tRNA synthetase was measured in the presence of the quinone under the conditions of Fig. 1, the time course showed a progressive deviation from the linear control indicating time-dependent reaction of the quinone with the target. Preincubation of the quinone with each of the possible reactants revealed leucyl-tRNA synthetase to be the only target molecule under the experimental conditions employed. Incubation of leucyl-tRNA synthetase with excess of the quinone at pH 7.5 and 28°C led to a rapid loss of enzyme activity following pseudo-first order kinetics, resulting in less than 1% of initial activity (Fig. 2).

Semilogarithmic plots of the inactivation kinetics usually intersected with the ordinate at 85 to 95% of initial activity, indicating a very rapid partial loss of enzyme activity in the presence of the quinone prior to the observed time-dependent inactivation (Fig. 2). This "spontaneous" initial loss of activity was reproducible and independent of both enzyme and quinone concentrations. It was caused by the effect of mixing the synthetase and the reagent in the absence of protecting mercaptoethanol. The activity indicated by the intercept is referred to as 100% in the following experiments.

Order of Reaction—Progressive inhibition of enzyme activity in the complete assay could only be observed under non-saturating conditions with respect to the substrates leucine and ATP (Fig. 1), indicating a protective influence of substrates. The most plausible explanation for protection by substrates would be an active site-directed modification of the enzyme by the quinone.

We tried to determine whether the quinone reacts in a simple bimolecular reaction or as a so-called "affinity label" where the modification of the enzyme is preceded by a reversible binding of the inhibitor to the enzyme (6, 7).

The dependence of the inactivation kinetics on the concentration of the quinone was studied (Fig. 3A). Any of the presence of the quinone prior to the observed time-dependent inactivation (Fig. 2). This "spontaneous" initial loss of activity was reproducible and independent of both enzyme and quinone concentrations. It was caused by the effect of mixing the synthetase and the reagent in the absence of protecting mercaptoethanol. The activity indicated by the intercept is referred to as 100% in the following experiments.

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The dependence of the inactivation kinetics on the concentration of the quinone was studied (Fig. 3A). Any of the
concentrations of the quinone used was in large excess of the enzyme. The plot of the apparent first order rate constants of inactivation versus quinone concentrations proved to be linear (Fig. 3B), indicating that the reaction was first order with respect to the quinone.

The results suggested a bimolecular reaction between quinone and leucyl-tRNA synthetase rather than a sequential or two-step mechanism as expected for an affinity label. The kinetic data, however, would not ultimately exclude any affinity reaction between the quinone and the enzyme since the concentration range of the quinone studied was restricted by its limited solubility.

The extrapolation of the linear plot to the point where the concentration of the quinone equals zero showed a reproducible intercept on the ordinate. This intercept did not reflect a spontaneous time-dependent denaturation of the untreated enzyme. A plausible explanation for this observation has not been found.

Resistance of Enzyme-Leucine Complex—The presence of leucine at 2 mM completely protected the enzyme against inactivation by the quinone. With leucine bound to its active center, leucyl-tRNA synthetase was assumed to be resistant to the quinone. Only the free enzyme should then react with the quinone. The ratio of free to substrate-bound enzyme depends on the dissociation constant of the enzyme-leucine complex. At a given concentration of the quinone, the rate of enzyme inactivation should then decline in the presence of increasing concentrations of leucine according to the following equation (8, 9):

\[ k_{app} = \frac{k_{app}^*}{1 + \frac{[S]}{K_S}} \]  

(1)

where \( k_{app}^* \) is the observed pseudo-first order rate constant in the absence of leucine, and \( k_{app} \) is that in the presence of leucine. \( [S] \) is the concentration of leucine and \( K_S \) is the dissociation constant of the enzyme-leucine complex.

It should be noted that the quantitative correlation described by Equation 1 only applies to the assumption of a simple bimolecular reaction between enzyme and quinone.

Protection by Leucinol—To get further support for a specific active site-directed inhibition of leucyl-tRNA synthetase by the quinone, we tested the influence of leucinol, which is competitive against L-leucine (12). Leucinol effectively protected the enzyme complex of about 4.3 times the value of the apparent Michaelis constant in the absence of ATP was arbitrarily set at 1.0. The results of two independent series of inactivation kinetics are shown (C, O).

A value of 150 \( \mu \text{M} \) was calculated for the dissociation constant from the slope of the linear plot. The value is identical with the published value of Holler et al. (11) derived by independent techniques.

Influence of MgATP and Transfer RNA—ATP in the presence of magnesium also had a protective effect on enzyme inactivation by the quinone. This protection was not complete even at saturating concentrations of MgATP. A residual rate of inactivation of about 0.3 was observed (Fig. 5). Half-maximal protection at about 2 mM MgATP accorded well with the dissociation constant of MgATP (11). In the presence of Mg\(^{2+}\) alone (up to 25 mM), there was no significant retardation of enzyme inactivation by the quinone. Transfer RNA was tested for its protective effect up to a concentration of 40 \( A_{260} \) units/\( \mu \text{L} \) of unfractionated tRNA which corresponded to 10 times the value of the apparent Michaelis constant in the aminocacylation assay. No protection was observed by tRNA, independent whether Mg\(^{2+}\) was present or absent.

Protection by Leucinol—To get further support for a specific active site-directed inhibition of leucyl-tRNA synthetase by the quinone, we tested the influence of leucinol, which is competitive against L-leucine (12). Leucinol effectively protected the enzyme against inactivation. From the concentration dependence we calculated a dissociation constant of the leucinol-enzyme complex of about 4.3 mM (Fig. 6A) again in good accordance with independent measurements of Holler et al. (11).

Synergistic Coupling of Ligands to Leucyl-tRNA Synthetase—Holler and co-workers (11) have investigated aminocetyl-tRNA synthetases with respect to synergistic binding of ligands to the active site. The affinity of leucinol to leucyl-tRNA synthetase was reported to be enhanced 10-fold in the presence of MgATP. The protection studies showed that
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Fig. 6. Protective effect of leucinol on inactivation of leucyl-tRNA synthetase by quinone. A, leucyl-tRNA synthetase, partially purified (24 μg), was incubated at 28°C in 300 μl of buffer (50 mM KH₂PO₄, pH 7.5) with quinone (170 μM) and leucinol at different concentrations (0 to 4 mM). After 1, 2, 3, 4, and 5 min, samples of 50 μl were removed and assayed for enzyme activity as described. The apparent first order rate constants (k_app) of the inactivation kinetics were plotted versus k_app x [leucinol] according to Equation 2. k_app in the absence of leucinol was arbitrarily set at 1.0. B, leucyl-tRNA synthetase, highly purified (12 μg), was incubated at 28°C in 300 μl of buffer (KH₂PO₄, 50 mM, pH 7.5) with quinone (170 μM) and leucinol at different concentrations (0 to 33 μM) in the presence of MgATP (25 mM, pH 7.5). The results of two independent series of inactivation kinetics are shown (○, ■).

Fig. 7. Protection of leucyl-tRNA synthetase by leucyl-adenylate. Leucyl-tRNA synthetase (crude extract, 14 μg), was incubated at 37°C in 300 μl of buffer (50 mM sodium cacodylate, pH 8.2), 25 mM magnesium acetate with quinone (QU, 35 μM, ○) in the presence of 5 μM leucine (■), 200 μM ATP (▲), or 200 μM ATP plus 0.5 μM leucine (■) and 5 μM (□). At the times indicated, samples of 50 μl were removed and assayed for enzyme activity. Similar results were obtained using highly purified enzyme and phosphate buffer pH 7.5.

MgATP at saturating concentration decreased the value of the dissociation constant of leucinol even more than 200-fold to about 15 μM (Fig. 6B), substantiating the existence of a synergism in the formation of the ternary enzyme-ligand complex of leucyl-tRNA synthetase.

Protection by Leucyl-adenylate—Leucyl-adenylate also exhibited effective protection of purified leucyl-tRNA synthetase against quinone inactivation. In order to somehow simulate intracellular conditions we also used a very crude enzyme preparation (30,000 X g supernatant) for protection studies. In the presence of leucine (5 μM) plus MgATP (200 μM) an almost complete protection was observed (Fig. 7). At the concentrations employed, leucine or ATP alone was without any protective effect. The experiment demonstrates that in the presence of ATP very low concentrations of leucine are sufficient to exert complete protection, obviously via formation of leucyl-adenylate.

Quinone Masks Essential —SH Groups of Leucyl-tRNA Synthetase—In the preceding sections, modification of leucyl-tRNA synthetase by the quinone was assumed to be irreversible under the conditions employed. Loss of enzyme activity followed pseudo-first order kinetics to less than 5% of initial activity. Furthermore, dilution of inactivated enzyme or filtration through Sephadex G-50 did not restore enzyme activity. Incubation with high concentrations of 2-mercaptoethanol almost completely restored enzyme activity (Fig. 8), suggesting that the quinone modifies essential —SH groups of the enzyme. In the presence of thiols, such as cysteine or glutathione, the enzyme could not be inactivated by the quinone.

Further evidence of the site of quinone attack was provided by showing that it masked the —SH groups of leucyl-tRNA synthetase that are reactive with N-ethylmaleimide (13). In the following experiment (Fig. 9), we took advantage of the observation that inactivation of leucyl-tRNA synthetase by N-ethylmaleimide cannot be restored by 2-mercaptoethanol in contrast to the modification by quinone. At time intervals following the addition of the quinone, residual enzyme activity was destroyed by the addition of N-ethylmaleimide. Then 2-mercaptoethanol was added in excess. Only quinone-modified enzyme should be restored. It is seen in Fig. 9 that the degree of quinone inactivation before addition of N-ethylmaleimide agreed well with the amount of enzyme that could be reactivated by 2-mercaptoethanol. The results indicate that one or several enzymically essential sulphydryl groups are protected by quinone against alkylation by N-ethylmaleimide.
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FIG. 9. Protection against N-ethylmaleimide modification by quinone. Leucyl-tRNA synthetase, partially purified (20 μg), was incubated at 28°C in 300 μl of buffer (50 mM Tris-HCl, pH 8.3) with quinone (100 μM). At the times indicated, samples of 50 μl were removed and assayed for enzyme activity (∗). N-Ethylmaleimide (1.7 mM) was added to parallel samples and further incubated. N-Ethylmaleimide at 1.7 mM completely inactivated control enzyme within 10 to 20 s. After 1 min, 2-mercaptoethanol was added to a final concentration of 50 mM. Enzyme activity was assayed after further 6 min of incubation (∗∗). In intact E. coli cells a remarkable decrease of aminoacylated tRNA1leu has been found to be the reason for the bacteriostatic effect of 6-amino-7-chloro-5,8-dioxoquinoline (1, 2). Since the intracellular concentration of leucine was not decreased in quinone-treated cells, leucyl-tRNA synthetase has been assumed to be the target of quinone attack. Nonetheless, addition of leucine to the medium prevented the growth inhibition by quinone. The results presented here provide evidence that the interaction of the medium with the enzyme is the cause of the phenotypically observed hyperauxotrophy for leucine of quinone-treated cells.

Quinone probably inactivates leucyl-tRNA synthetase from E. coli covalently in a simple bimolecular mechanism, excluding a competitive inhibition with respect to leucine that could have been expected from the in vivo studies. Competitive protection of the enzyme by leucine or leucyl-adenylate against quinone attack, however, would sufficiently explain the observed hyperauxotrophy for leucine, provided that leucyl-tRNA synthetase is not saturated with leucyl-adenylate in cells growing in minimal medium. The stimulation of protein synthesis in untreated cells by the addition of amino acids to the minimal medium may indeed reflect the possibility of increasing the degree of saturation of aminoacyl-tRNA synthetases with their corresponding aminoacyl-adenylates (14).

From this model we had to postulate that the effect of leucine in overcoming the bacteriostasis should only be observed when leucine was added to the culture prior to the addition of quinone. The results shown in Fig. 10 are in line with this prediction. The faint stimulation by leucine of cells which had already been inhibited by the quinone might reflect the "kinetic reserve" of still unmodified leucyl-tRNA synthetase.

Other in vitro aminoacyl-tRNA synthetases from E. coli could also be inhibited by the quinone at comparable rates. The selective effect of the quinone on leucyl-tRNA synthetase in the intact cell might be explained by a particularly low degree of saturation of the aminoacyl-tRNA synthetase with leucyl-adenylate.

Studies with N-ethylmaleimide revealed that it is the masking of essential -SH groups that leads to the inactivation of the enzyme. Complete reactivation of enzyme activity could be achieved by the presence of excess mercaptoethanol. The quinone might thus be a useful reagent for the temporary blocking of essential -SH groups. The chemical nature of the reaction between quinone and cysteine residues remains to be clarified (15, 16).

The importance of free -SH groups for the enzymatic activity of leucyl-tRNA synthetase of E. coli was studied by Rouget and Chapeville (13). Titration with p-chloromercuribenzoate revealed two rapidly reacting sulfhydryl groups which are necessary for enzyme activity.

Our protection studies with substrates and the competitive inhibitor leucinol indicate that the quinone-reactive groups are within the active center, although an indirect protective influence of bound substrates cannot be excluded. The high selectivity of protection with respect to the different substrates seems to favor the assumption of active site-located modification by the quinone. Furthermore, one intrinsic feature of the active center, as is the synergistic coupling between the bindings of ligands, was reflected by the protection of the enzyme against quinone inactivation by leucinol in the presence of MgATP. At low concentrations of leucinol, the addition of small amounts of MgATP drastically enhanced the protective effect of leucinol. Our results are in agreement with those of Holler et al. (11) in that MgATP lowers the dissociation constant of the enzyme-leucinol complex.

The role of the reactive -SH groups of leucyl-tRNA synthetase in the binding of substrates and for the catalytic reaction is under investigation.

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Unpublished results.
Inactivation of Leucyl-tRNA Synthetase by Quinone

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