Regulation of Histidine Catabolism by Succinate in *Pseudomonas putida*

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Received for publication 13 May 1968

The regulation of the histidine-degrading pathway is known to involve induction and repression. Our studies have shown that succinate may control the histidine-degrading pathway by sequential negative feedback inhibition. Succinate inhibited urocanase, and urocanate in turn inhibited histidase. Crude preparations of the two enzymes were made from *Pseudomonas putida* grown on L-histidine. Succinate was a competitive inhibitor of urocanase (K_i, 1.8 mM). Lactate, pyruvate, α-ketoglutarate, and glutamate did not inhibit urocanase. Urocanate inhibited histidase competitively (K_i, 0.13 mM). A multienzyme system (histidine to glutamate), when incubated with histidine and succinate, exhibited the combined effect. Succinate caused the level of accumulated urocanate to increase and indirectly blocked histidine disappearance. Growth of cells on urocanate as a nitrogen source was inhibited by 1% succinate. Succinate may play a physiological role in the biological regulation of histidine metabolism.

Modulation of enzyme activity by metabolites has been described for numerous anabolic pathways (end product inhibition) and for control of energy needs with adenylates as effectors (2, 3). Feedback inhibition of a catabolic enzyme, glycerol kinase, has been reported (19). Induction and catabolite repression have frequently been demonstrated for control of catabolic pathways. Histidine degradation (17) has been shown to be regulated by induction and catabolite repression in *Pseudomonas* (9) and in other genera (7, 11, 12, 16). Induction and repression, however, may not be adequate to provide instantaneous regulation or regulation in response to small changes in concentration. The cell would thus benefit from the ability to control, with effectors, the catabolism of a metabolite whether it is exogenous or endogenous. These concepts were discussed in relation to anabolic pathways by Davis (4).

To investigate this problem, we have studied possible inhibitors of histidase in *Pseudomonas*. We were not able to demonstrate direct "feedback" inhibition of histidase by metabolites formed as a result of the operation of the pathway. Lessie and Neidhardt (9) tested 16 potential feedback inhibitors of histidase, such as succinate, citrate, glutamate, formiminoglutamate, and adenosine triphosphate. None of these was effective. They reported that when cultures were grown in histidine and succinate, the rate of histidine degradation in vivo was 55% of the rate that was expected from the activity of histidase measured in vitro. Lessie and Neidhardt concluded that succinate inhibits histidase activity in vivo.

The purpose of our investigation was to find metabolite effectors for the control of histidine degradation. The enzymatic studies were carried out with cell extracts of *P. putida*. Enzyme activity was measured spectrophotometrically. Previously, we found "cross inhibition" of histidase from the tyrosine and phenylalanine pathways (unpublished data; 8), and in this report we present evidence that succinate may control histidine catabolism by negative sequential feedback inhibition. Succinate inhibits the second enzyme of the pathway, urocanase. This inhibition results in an accumulation of the first intermediate which, in turn, inhibits the first enzyme of the pathway, histidase. Sequential feedback inhibition of aromatic acid biosynthesis has been described previously (14).

**Materials and Methods**

*Organism.* *P. putida* A 3-12 was maintained on AC Medium (Difco) slants.

*Growth conditions.* The bacteria were grown at room temperature in the medium of Tabor and Mehler (18), according to their method. The medium contained 0.2% L-histidine·HCl·H_2O and 0.1% yeast extract. The cultures, 500 ml in 2-liter Erlenmeyer flasks, were aerated on a rotary shaker. Harvested cells were washed three times in 0.01 M potassium...
phosphate buffer (pH 7.0) and were stored at -30°C in 1-g pellets.

Preparation of cell extracts for enzyme assays. Cells were disrupted in a Hughes press and were diluted to 10 volumes in 0.01 M potassium phosphate buffer (pH 7.0). To reduce viscosity, the extract was treated with deoxyribonuclease (approximately 2 to 10 μg/ml) for 15 min at 25°C. The material was centrifuged for 20 min at 12,000 × g at 4°C. The supernatant fluid was decanted and was hereafter referred to as crude extract. The crude extract contained all of the enzymes of the histidine pathway needed to form glutamate from histidine. Urocanase and other enzymes were inactivated by heating the crude extract under nitrogen for 15 min at 78 to 83°C. Denatured protein was removed by centrifugation, and the supernatant liquid contained histidase. It was stored in crushed ice under nitrogen.

Enzyme assays. Histidine ammonia lyase (EC 4.3.1.3), usually called histidase, was assayed by a modification of the method of Tabor and Mehler (18). This method measures the formation of urocanate from histidine by monitoring the increase in absorbance of the reaction mixtures at 277 nm. The assay was carried out at 30°C in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) connected to a recorder (E. H. Sargent and Co., Chicago, Ill.). The results were expressed as the change in absorbancy at 277 nm per min. The reaction mixtures (3 ml) contained 100 μmoles of potassium phosphate buffer (pH 7.0, unless otherwise indicated), 0.05 ml of heated extract (0.2 mg of protein), and 2 μmoles of L-histidine. The reaction was started by the addition of L-histidine.

Non-saturating levels of histidine were employed. Urocanate, which acted like a competitive inhibitor and is the product, has a high absorbance at 277 nm. The amount of urocanate which could be added to the reaction mixture was restricted because of the limited sensitivity of the spectrophotometer. When there is a high ratio of substrate to its competitive inhibitor, inhibition may not be demonstrated.

Urocanase was assayed by a modification of the method of Tabor and Mehler (18), by measuring the decrease in absorbance at 277 nm. The results were expressed in change in absorbancy at 277 nm per min. The reaction mixtures (3 ml) contained 100 μmoles of potassium phosphate buffer (pH 7.0, unless otherwise indicated), 0.2 ml of crude extract (0.8 mg of protein), and 0.1 μmole of urocanate. The reaction was started by the addition of urocanate. Other conditions and procedures were the same as those given for the histidase assay.

Enzyme reactions analyzed by chromatography. The indirect effect of succinate on histidine degradation was demonstrated in an experiment with crude extract. The 25-ml Erlenmeyer reaction flasks contained, in μmoles: L-histidine or urocanate, 4; succinate, 20; potassium phosphate buffer (pH 7.0), 50; total volume, 1.0 ml; each of one of the reaction flasks contained 0.4 ml of crude extract (1.6 mg of protein). The flasks were covered with aluminum foil, flushed continuously with nitrogen, and incubated at 30°C in a Dubnoff water-bath shaker (Precision Scientific Co., Chicago, Ill.) at 100 cycles per min. At 10-min intervals, 5-μliter samples were pipetted onto paper for chromatography. Data obtained with this procedure are given in Fig. 5.

Growth experiments. Growth studies were carried out in test tubes (18 × 150 mm) containing 10 ml of medium on a rotary shaker at 25 to 27°C. Turbidity was measured at 660 nm with a Coleman Universal spectrophotometer and was expressed as optical density (OD). The medium consisted of 1.5 g of K2HPO4, 0.5 g of KH2PO4, 1.7 mg of Fe(NH4)2- (SO4)2·6H2O, 0.2 g of MgSO4, neutralized succinic acid as indicated, and 1.4 g of urocanic acid per liter. The MgSO4 solution was autoclaved separately from the medium and was added with the peptone. Each tube was inoculated with a drop of a 24-hr culture grown in the same medium.

Paper chromatography. Chromatograms were developed in n-propanol-1 N acetic acid (3:1) in an Eastman chromagram (Distillation Products Industries, Rochester, N. Y.) apparatus for 105 min. Chromatography was carried out on Whatman no. 40 paper (15.5 × 19 cm) at room temperature. Compounds were located by spraying with ninhydrin or diazotized sulfanilic acid. Semiquantitative determination of histidine and urocanate was accomplished by visual comparison of 20 standard concentrations chromatographed under the same conditions as the unknowns, including the presence of cell extract and buffer.

Chemicals. Urocanic acid, L-histidine, imidazoleacetic acid, and imidazolopropionic acid were purchased from Calbiochem (Los Angeles, Calif.). Imidazoleactic acid was obtained from the Pierce Chemical Co. (Rockford, Ill.). L-Glutamic acid and α-ketoglutaric acid, potassium dihydrogen phosphate, and n-histidine were purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio). Succinic acid was obtained from the Fisher Scientific Co. (Fair Lawn, N. J.) and the H. M. Chemical Co., Ltd. (Santa Monica, Calif.). Lithium lactate was recrystallized from the lithium salt purchased from the Sigma Chemical Co. (St. Louis, Mo). All other chemicals were of reagent grade. Protein was determined by the method of Lowry et al. (10). Crystallized bovine plasma albumin (Armour and Co., Chicago, Ill.) was used as a standard.

RESULTS

Inhibition of urocanase by succinate. Urocanase, the second enzyme in histidine catabolism, was inhibited by succinate (Fig. 1). The plot in this figure indicates that succinate is competitive with urocanate. In this experiment, we found 49% inhibition by 3.3 mM succinate under the assay conditions described in Materials and Methods. An average Ks of 1.8 mm was calculated from the data in plots of four experiments similar to that shown by Fig. 1. The average Ks was 0.06 mm (four experiments).

Effect of succinate concentration on the inhibition of urocanase. The saturation curve for inhibition of urocanase by various concentrations of succinate is shown in Fig. 2. The curve is hyperbolic up to 8 mm and levels off at approximately
75% inhibition. Apparently, succinate alone cannot completely block this reaction.

Specificity of inhibition of urocanase. Table 1 presents the data obtained when several compounds were tested for their ability to inhibit urocanase. Imidazoleacetic acid had no effect. and imidazolelactic acid showed a slight inhibitory effect in three trials. Pyruvate, α-ketoglutarate, lactate, and glutamate did not inhibit the reaction. Under the same conditions, succinate inhibited the reaction by nearly 50%. Although this experiment was not a thorough study of the analogues of the substrate and inhibitor, it did indicate that several compounds related structurally and metabolically to the substrate and inhibitor are not effectors. If succinate is converted by the cell extract to another substance, which is the actual inhibitor, then we would expect that metabolically related compounds would also act as inhibitors.

Other aspects of inhibition of urocanase by succinate. Succinate inhibited the urocanase reaction as much when added after the reaction had proceeded for 2 min as when added before the substrate. Thus, succinate had an immediate inhibitory effect. This result supports the idea that succinate is not converted to another compound which is the actual inhibitor.

An experiment was performed to examine the effect of divalent cations (0.33 mM) on succinate (3.3 mM) inhibition. These cations, Cu++, Co++, Zn++, Sr++, Mn++, Ni++, and Mg++, did not antagonize or enhance the inhibition by a significant amount. A higher concentration (1.0 mM) of cations caused precipitation in the reaction mixture.

Inhibition of histidase by urocanate. Urocanate inhibited histidase. Figure 3 shows data from an experiment plotted in the reciprocal Lineweaver-Burk form. The plot indicates competitive inhibition, which is typical of product inhibition. Urocanate (0.1 mM) inhibited histidase 33% in the routine assay. The $K_i$, 0.13 mM (average of three values), was calculated with data from curves like the one shown in Fig. 3. The $K_m$ was 2 mM. Higher concentrations of urocanate were not used because of its high absorbance. Inhibition of liver histidase by urocanate has been reported (5).

Influence of pH on the inhibition of histidase by urocanate. Histidase has a broad pH optimum from 8.5 to 9.5. The data in Fig. 4 show activity increasing from pH 6.9 to pH 8.0. In the same pH range, per cent inhibition was highest at pH 6.9 and dropped off as the pH was raised. The pH of the reaction mixture was recorded in these studies. Histidase is usually assayed at pH 9.2.

![Graph of Lineweaver-Burk plot for the inhibition of urocanase by succinate.](image)

**Table 1. Effect of metabolites related to substrate and inhibitor on urocanase**

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Conc (mM)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>2.7</td>
<td>53</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>2.7</td>
<td>94</td>
</tr>
<tr>
<td>L-Lactic acid</td>
<td>2.7</td>
<td>100</td>
</tr>
<tr>
<td>α-Ketoglutaric acid</td>
<td>2.7</td>
<td>105</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imidazoleacetic acid</td>
<td>2.7</td>
<td>101</td>
</tr>
<tr>
<td>Imidazolelactic acid</td>
<td>2.7</td>
<td>87</td>
</tr>
</tbody>
</table>

*a Compound were neutralized.
*b The conditions of the reaction are described in Materials and Methods.
histidase by Vol.

The inhibition by urocanate is probably not as effective at this pH as it is at about pH 7.0.

Specificity of inhibition of histidase. In crude extracts, histidase was inhibited by urocanate and by imidazolelactate at a much higher concentration, but it was not inhibited by three other imidazoles (Table 2). Succinate and other metabolites were not inhibitors. Edlbacher et al. (6) found that pyruvate (0.5 mM) inhibited rat liver histidase by 32% at pH 8.0. We did not find inhibition of the bacterial histidase by 1.67 mM pyruvate. Many compounds have been investigated for inhibition of histidase (6, 8, 9, 15).

Other aspects of the inhibition of histidase. The addition of reduced glutathione (1.67 mM), which is usually included in the histidase assay (18), had no effect on the inhibition by urocanate. When the experiment illustrated in Fig. 4 was repeated with the addition of reduced glutathione, the curves were superimposable. This result markedly contrasts with our previous finding that inhibition of histidase by tyrosine and p-hydroxyphenylpyruvate is very significantly decreased by 1.67 mM reduced glutathione (8).

Eight cations were tested for their ability to antagonize the inhibition of histidase by urocanate. The assay was performed as described in Materials and Methods, with 0.1 mM urocanate as inhibitor and with 0.1 mM cations. Controls without urocanate were run. No significant change in the inhibition by urocanate resulted from the addition of Co++, Zn++, Ba++, Sr++, Mn++, Ni++, and Mg++. Histidase was inhibited 66% by 0.1 mM Cu++ (in absence of urocanate). It has been shown that ethylenediaminetetraacetate and cysteine inhibit histidase and that added zinc protects the enzyme from inhibition (15). Apparently, inhibition of histidase by urocanate is not closely related to metal binding.

Sequential feedback inhibition of urocanase and histidase by succinate. Experiments using paper chromatography, instead of spectrophotometry, for analysis were carried out. With this technique, it was possible, in the presence of all of the enzymes of the pathway, to demonstrate the buildup of urocanate because of succinate inhibition and the simultaneous inhibition of histidase by the "elevated" level of urocanate. Although
these experiments were semiquantitative, they confirmed previous results. Crude extract converts histidine to glutamate rapidly. When succinate was added and the metabolism of histidine was measured, histidine disappearance was retarded. In Fig. 5A, an experiment in which both histidine and urocanate were determined is shown. These data (Fig. 5A) represent two flasks, one containing histidine, the other histidine and succinate. The decreased rate of histidine utilization and the increased urocanate level with added succinate were reproduced four times. When four levels of succinate were used, the inhibition of histidine degradation was greater for each higher level (results not shown). In addition, the urocanate concentration increased with increases in the inhibitor; i.e., the more succinate that was added, the more urocanate that accumulated.

The inhibitory effect of succinate on the metabolism of urocanate is shown in Fig. 5B. The same experimental technique was used to demonstrate the inhibition of histidine disappearance by added urocanate (results not shown). Although urocanate itself disappeared during the experiment, even cursory inspection of the chromatograms revealed that the rate of histidine metabolism was retarded by added urocanate.

Urocanate was identified in these experiments by its RF value in two solvents, by its reaction with diazotized sulfanilic acid (it gives a spot of characteristic orange color), and by its typical dark blue color when viewed under ultraviolet light (253.7 mλ), owing to the quenching of background fluorescence.

_Growth experiments._ We reasoned that cells depending on L-histidine or urocanase as the only sources of nitrogen would not be able to grow as well in the presence of succinate, the proposed feedback inhibitor of the pathway, as in its absence. When urocanate was the nitrogen source, 0.026 M succinate stimulated growth, 0.043 M succinate had no influence on the growth rate, and the highest level of succinate used (0.085 M or 1%) completely stopped growth (results not given). This inhibitory effect may be the result of catabolite repression of urocanase or of feedback inhibition.

When L-histidine was used as a nitrogen source in a similar experiment, succinate had virtually no effect on growth rates (results not given). These growth experiments do not provide conclusive evidence of a physiological role for succinate as a feedback inhibitor.

**DISCUSSION**

This investigation revealed a mechanism whereby succinate can inhibit histidase indirectly by inhibiting urocanase. We propose that succinate contributes to the regulation of histidine catabolism by feedback inhibition, and thus couples the histidine pathway to the energy requirements of the cell (Fig. 6). The concentration of succinate, a member of the tricarboxylic acid cycle, may fluctuate with the energy demands of the cell and with the supply of intermediates that enter the cycle. For example, in the presence of exogenous histidine and glucose, it is advantageous to the cell to spare histidine for protein synthesis (and for amino acid synthesis via transaminases) and to utilize glucose for energy requirements. Induction and catabolite repression

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**Fig. 5.** Sequential inhibition of histidine catabolism by added succinate in a cell extract. (A) Effect of succinate on histidine degradation. Flask with succinate: μmoles of histidine (△); μmoles of urocanate (▲). Control flask: μmoles of histidine (○); μmoles of urocanate (●). (B) Effect of succinate on urocanate degradation. Flask with succinate: μmoles of urocanate (▲). Control flask: μmoles of urocanate (●). Conditions and experimental details are given in Materials and Methods.

**Fig. 6.** Regulation of histidine degradation by repression, induction, and negative sequential feedback inhibition. Broken lines join negative effectors to the reactions which are inhibited (X) in the histidine degradation pathway (17). The induction and repression of histidase and urocanase has been described for _P. aeruginosa_ (9).
serve this control function. Succinate as an indirect negative effector of histidase may act as a “fine adjustment” of this control system. This would enable the cell to respond quickly and favorably to small or abrupt changes in energy requirements or to exogenous metabolites.

Lessie and Neidhardt (9) have suggested that pyrophosphate may act as a signal to link energy demands of the cell to histidine degradation. They found that pyrophosphate inhibits histidase, and proposed that pyrophosphate concentration depends on adenosine triphosphate concentration which reflects energy requirements. However, as these investigators pointed out, the level of pyrophosphate required for inhibition in vitro may not be reached in vivo. Their experiments also clearly showed that histidase is repressed and inhibited by succinate in vivo. Our results indicate a direct interaction of succinate with histidine-degrading enzymes without involving pyrophosphate. We conclude that our in vitro experiments combined with the in vivo experiments of Lessie and Neidhardt demonstrate a possible physiological role of succinate as a modulator of the histidine degradative pathway and thus as a link to energy needs.

It is interesting to speculate on the purpose of a negative feedback control by succinate of the second enzyme of the pathway (Fig. 6). Presumably, this system would tend to keep the urocanate pool larger than it would be if feedback control operated directly on histidase. There is evidence that urocanate is the inducer of the histidine-degrading pathway in Aerobacter (12) and Pseudomonas (9). Furthermore, the studies with Aerobacter and Pseudomonas indicated that a low level of histidase is always present to form the inducer, urocanate, from histidine.

This low histidase concentration probably results from induction by the urocanate pool. This control system permits inducer formation and operation of the pathway when either externally supplied histidine or sudden energy demands (signalled by low endogenous succinate) requires operation or formation of the histidine-degrading machinery. Therefore, the succinate inhibition that we observed in this study probably helps to maintain the inducer concentration and thus a low level of the enzymes of the pathway. In other words, succinate acts as a negative effector for the pathway, while allowing for formation of the inducer of the pathway. Lessie and Neidhardt found that succinate was the best “repressor” of seven compounds tested (9). We believe these multiple roles would tend to delicately balance and integrate the total regulation of the pathway: (i) urocanate is an inducer and a negative effector; (ii) succinate is a “repressor” and a negative effector, and acts to maintain the inducer concentration.

Finally, one may speculate that this regulatory system is linked to the biosynthetic pathway of histidine by repression and inhibition. In Escherichia coli (13) and in Salmonella typhimurium (1), histidine is a negative feedback inhibitor of the first enzyme of histidine biosynthesis, phosphoribosyl-adenosine triphosphate pyrophosphorylase. The biological control mechanism which we have outlined in this paper harmonizes many aspects of histidine metabolism of the cell. It allows cells to economize on protein synthesis, to respond to energy demands, to regulate endogenous synthesis of histidine, to conserve endogenous histidine, and to utilize exogenous histidine.

ACKNOWLEDGMENT

This investigation was supported by the Veterans Administration, Department of Medicine and Surgery, Research Service, Project M18-58.

LITERATURE CITED


