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Stimulation by Glutamine of the Formation of N^6-Hydroxylysine in a Cell-Free Extract From Aerobacter aerogenes 62-1

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Glutamine may serve as an activator and/or regulator of the N^6-hydroxylase (E.C. 1.14.99) of Aerobacter aerogenes 62-1. Activation and stabilization of N^6-hydroxylase activity was observed both in vivo and in vitro. Growth in a glutamine-supplemented medium resulted in (1) maximum N^6-hydroxylase activity at an earlier stage of growth and (2) higher N^6-hydroxylase activity and continued aerobactin synthesis into stationary phase. Storage of P2 in the presence of L-glutamine (1 mM) significantly increased the lifetime of the labile N^6-hydroxylase activity. Inclusion of L-glutamine in the incubation mixture typically resulted in a 2–3-fold activation of the hydroxylase activity. The stimulatory effect of glutamine was independent of and additive to the enhancement of N^6-hydroxylation by the active component(s) in the supernatant, S2 fraction. Glutamic acid-γ-semihydrazide activated slightly in the absence of glutamine but activation of the system by glutamine was decreased by this compound. Azaserine was shown to be an uncompetitive inhibitor with respect to lysine and this inhibition was not reversed by glutamine.

Key words: lysine N^6-hydroxylase, Aerobacter aerogenes 62-1, hydroxamate, siderophore, glutamine stimulation

During the past 30 yr, a large number of iron-chelating compounds of microbial origin have been discovered. These siderophores are composed of two major types: hydroxamates and phenol catecholates [1]. The primary function of these compounds is one of microbial iron assimilation, a process facilitated by their high affinity for

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ferric ions [1–4]. It is now becoming apparent that most aerobic, facultative anaerobic, and eukaryotic microorganisms possess the capacity to synthesize siderophore(s) under conditions of iron deprivation. The diversity in the biological function of siderophores has been illustrated by their abilities to promote growth [5–6] or to serve as antibiotics [7] depending on the microorganism tested. Some siderophores have been shown to function as antitumour agents [8], while others are actually carcinogenic [9]. Both naturally occurring and synthetic hydroxamates have attracted considerable attention in view of their application in chemotherapy. Thus, hydroxamates are being used in the treatment of (1) renal infections [10–12], (2) patients suffering from transfusion-induced siderosis as in Cooley anemia [13,14], (3) dermatoses [15], and (4) parasitemia in animals [16]. The potential applications of hydroxamates in agriculture [17] and industry [18] have been documented. Finally, a correlation between virulence and the ability to transport hydroxamates has been observed in the case of some enteric bacteria [19,20].

Despite the above-mentioned functions of hydroxamates, very little information is available concerning the enzyme systems responsible for their production. The biosynthesis of hydroxamates involves (1) generation of the N-hydroxylamino function, (2) acylation, and (3) in some instances, condensation of monohydroxamates to yield the desired di- or tri-hydroxamate. The difficulties experienced in the achievement of a cell-free system capable of catalysing all of the above-mentioned reactions restricted the early investigations to the identification of precursors and the determination of the sequence of events in the biosynthesis of hydroxamates [21,22].

This laboratory was successful in the development of such a cell-free system from Aerobacter aerogenes 62-1, an organism which produces the hydroxamic acid aerobactin. This cell-free system was able to catalyse the hydroxylation at the N^6 position of L-lysine, and was found to be specific for L-lysine, with neither the D-isomer of the amino acid nor its N^2- or N^6-substituted derivatives able to serve as substrates [23,24]. While none of the other common amino acids were able to undergo N-hydroxylation in the cell-free system, certain amino acids, notably glutamine and glutamic acid, were found to stimulate the N-hydroxylation of L-lysine [24]. In addition, substances that could serve as precursors of the components of aerobactin exerted a stimulatory effect on the conversion of lysine to its N^6-hydroxy derivative by the cell-free enzyme system. These observations suggested that the lysine N^6-hydroxylase system might be under metabolite control. A more extensive study of the influence of certain of these effectors was considered necessary in order to further characterize the enzyme system responsible for the conversion of lysine to its N^6-hydroxylated derivative.

MATERIALS AND METHODS

Azaserine was obtained from Calbiochem and L-lysine from Aldrich. All other biochemicals were obtained from Sigma.

Preparation of Enzyme

A aerogenes 62-1 was grown and harvested as previously described [23]. The washed cells were lysed by cold osmotic shock after treatment with lysozyme-EDTA and incubated at 37°C for 1 hr [24]. The crude lysate was fractionated into the particular enzyme, P2, and supernatant cofactor, S2, as described [24].

For the inhibition studies, P2 was exhaustively dialysed against 50 mM phosphate buffer, pH 7.0, to remove endogenous lysine and glutamine.

The active component(s) in the supernatant, S2, was partially purified. Lyophilized S2 was dissolved in 70% ethanol (50 ml) and stored at −20°C for 18–24 hr. The heavy precipitate was removed by centrifugation (15 min, 12,000 g) and the supernatant containing the active component(s) was taken to dryness under reduced pressure. This material was chromatographed on Sephadex G-10 (2 × 90 cm), and the fractions effecting stimulation of P2 N^6-hydroxylase activity were rechromatographed on Bio-gel P2 (2 × 90 cm).

Assay Procedure

The typical assay consisted of the following in a 10-ml volume: 50 mM phosphate buffer (pH 7.0), 1 mM potassium pyruvate, P2 (1 ml), and S2 (2 ml). The mixture was incubated at 37°C with vigorous shaking for 2 hr. The reaction was terminated by the addition of 5 ml of a thick slurry of Dowex 50 W-X8 (H^+, 200–400 mesh).

The resin-assay mixture was heated in a water bath at 60–70°C for 1 hr, cooled to room temperature, and poured into a 1.2 × 25-cm glass column. The resin was washed with 40 ml 0.2 M HCl and the N^6-hydroxylysine was eluted with 20 ml of 6 M HCl. The 6 M HCl eluate was evaporated under reduced pressure, and the residue was dissolved in 5 ml H_2O. Aliquots of the aqueous solution were subjected to the iodine oxidation procedure to determine the N^6-hydroxylysine content [25].

Treatment of Data

The rate refers to the amount of N^6-hydroxylysine synthesized per 2-hr incubation period and was determined by iodine oxidation. Since the extent of N^6-hydroxylysine formation was linear over a 3-hr incubation period, the data reflect true initial rates of N^6-hydroxylation. For Figures 2 and 3, Ancovar covariance analysis indicated that the lines could be plotted as straight lines with common slopes [26]. Individual linear regressions were used to plot the lines for Figures 4.

RESULTS AND DISCUSSION

Previous investigations in this laboratory have established optimal conditions for the N^6-hydroxylation of L-lysine by a cell-free system of Aerobacter aerogenes 62-1. The system was resolved into particulate, P2, and supernatant, S2, components. The N^6-hydroxylase activity resided in the P2 fraction. The identity of the active component(s) or cofactor(s) in S2 has not been established. However, partial purification by 70% ethanol precipitation and gel filtration chromatographies indicate that the component(s) is small ($\leqslant$ 1,000 mol wt) and nonprotein in nature.

L-glutamine and L-glutamic acid and, to a lesser extent, L-asparagine and L-aspartic acid, had previously been found to stimulate the N^6-hydroxylation of lysine by a cell-free system prepared from A aerogenes 62-1 [24]. Glutamine was routinely used in subsequent experiments. When A aerogenes 62-1 was grown in medium supplemented with 1 mM glutamine, lysine N^6-hydroxylase activity was noted at an earlier stage of growth than that observed in cultures grown in the absence of this amino acid (Fig. 1). In the presence of glutamine, both the N^6-hydroxylase activity and aerobactin production remained high into the stationary phase of growth of the

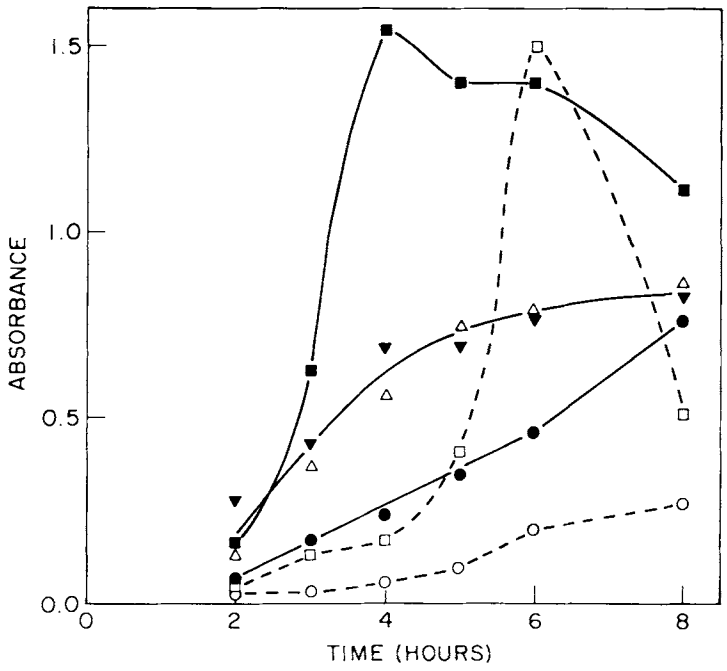

Fig. 1. Comparison of growth, aerobactin production, and N^6-hydroxylase activity in A aerogenes 62-1 in normal and glutamine-supplemented media. Growth: △—△, no gln; ▼—▼, 1 mM L-gln. Aerobactin production: ○----○, no gln; ●—●, 1 mM L-gln. N^6-hydroxylase activity: □----□, no gln; ■—■, 1 mM L-gln. Growth was followed by turbidity measurements at 450 nm (1/10 dilution). Aerobactin and N^6-hydroxylase activity were determined using iodine oxidation as described in Methods.

organism. In contrast, lysine N^6-hydroxylase activity and aerobactin synthesis decreased rapidly under similar conditions in the absence of glutamine.

Glutamine could also stimulate the activity of the lysine N^6-hydroxylase in the particulate (P2) fraction of the cell-free system, provided that the amino acid was initially present in the assay mixture. Introduction of glutamine at a later stage, for example, 1 hr after the initiation of incubation of the enzyme system with lysine, failed to stimulate the N^6-hydroxylase activity. Variation of the concentration of glutamine in the assay mixture indicated that the maximal stimulation of the N^6-hydroxylation of lysine was attained at 0.4 mM glutamine. The concentration of glutamine necessary to produce maximal stimulation of the enzyme was independent of the concentration of lysine in the assay. Glutamine did not exert its apparent stimulatory effect by serving as substrate for the N^6-hydroxylase, since amino acid analysis [27] indicated a quantitative recovery of the added glutamine in addition to a greatly increased amount of N^6-hydroxylysine. In this experiment, the amount of N^6-hydroxylysine formed was approximately three times that of a control assay to which no glutamine had been added.

The enzyme component(s) that is responsible for the N^6-hydroxylation of lysine in the cell-free system was located in the particulate P2 fraction (see Methods). The lysine N^6-hydroxylase activity in this particulate fraction was very labile [24]. How-

ever, storage of P2 preparations in the presence of 1 mM glutamine dramatically increased the lifetime of the enzyme. The other component of the cell-free N^6-hydroxylase system from A aerogenes 62-1 was the supernatant fraction, S2. This material was found to be a heat-stable, dialysable, nonprotein compound, and served to stimulate the lysine N^6-hydroxylase activity present in the particulate P2 fraction. However, it is unlikely that the stimulatory effect of S2 is due to the presence of glutamine in this preparation, since storage of P2 with S2 at 4°C did not increase the stability of the lysine N^6-hydroxylase activity in the particulate P2 fraction. In addition, amino acid analysis [27] of purified S2 failed to detect the presence of either glutamine or glutamic acid, aspartic acid or asparagine. The stimulatory effect of glutamine in the assay was also found to be independent of, and additive to, that effected by S2. The presence of S2 was not required in order for glutamine to exert its stimulatory effect on the lysine N^6-hydroxylase in the particulate P2 fraction (Table I). These observations suggest that the ability of glutamine to stimulate the conversion of lysine to its N^6-hydroxy derivative by the cell-free system from A aerogenes 62-1 is due to the interaction of the amino acid with the enzyme component(s) present in the particulate P2 fraction. Since glutamine was apparently unable to serve as substrate, the observed glutamine-induced stabilization and activation of the N^6-hydroxylase activity, in the presence of saturating levels of lysine, could be due to the binding of glutamine by the enzyme system at some site other than the catalytic site of the enzyme. In order to further investigate this possibility, the effect of a number of analogues of glutamine on lysine N^6-hydroxylation was studied.

The effect of glutamic acid-γ-semihydrazide varied depending on the presence or absence of glutamine in the reaction mixture. In the absence of glutamine, glutamic acid-γ-semihydrazide exerted a small, but definite (20% over control), activation of lysine N^6-hydroxylation when present at 1 mM concentration in the assay. However, when glutamic acid-γ-semihydrazide was added to reaction mixtures in which the N^6-hydroxylase had been stimulated by the inclusion of glutamine, the activation regularly noted with this latter compound was abolished. In experiments in which the lysine concentration was varied, in the presence of 0.5 mM glutamine and various fixed concentrations of glutamic acid-γ-semihydrazide, the inhibition of the glutamine-stimulated N-hydroxylase activity by glutamic acid-γ-semihydrazide was ob-

TABLE I. Effect of L-Glutamine in N^6-Hydroxylase Enzyme System*

Enzyme fraction	L-Glutamine	Activity (% control)
P2	−	48
S2	−	⩽1
P2 + S2	−	100
P2	+	108
S2	+	⩽2
P2 + S2	+	189

*Assays consisted of 50 mM phosphate buffer (pH 7.0) and 1 mM L-lysine. Incubation was for 2 hr at 37°C. P2 (1 ml), S2 (2 ml), and L-glutamine (1 mM) were added as indicated. It should be noted that although L-glutamine enhanced N^6-hydroxylase activity approximately twofold, L-glutamine was neither hydroxylated nor utilized. Amino acid analyses of reaction mixtures indicated an initial glutamine concentration of 1.1 μmol/ml and a final concentration of 1.3 μmol/ml.

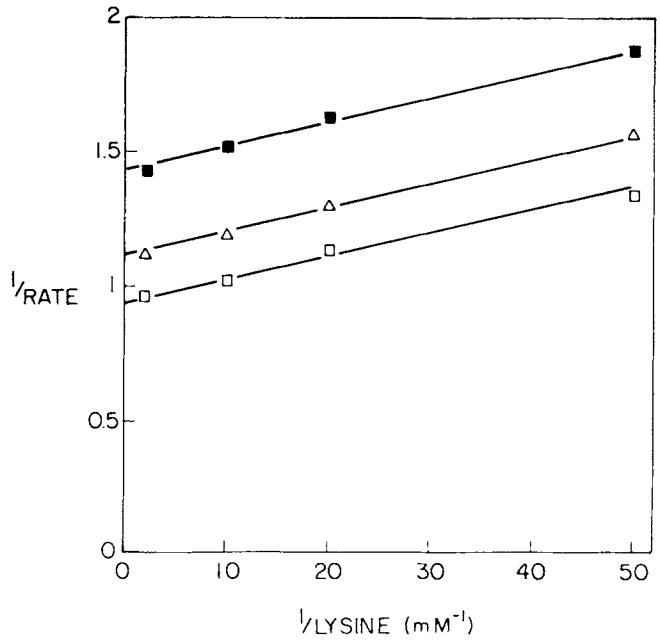

Fig. 2. Effect of glutamic acid-γ-semi-hydrazide on N-hydroxylation: The double-reciprocal plot of 1/rate vs 1/(lysine). All samples contained 50 mM phosphate (pH 7), 1 mM pyruvate, 0.5 mM glutamine, P2 + S2 plus lysine to the appropriate concentration. □—□, no glutamic acid γ-semihydrazide; △—△, 0.5 mM: ■—■, 1 mM.

served to be uncompetitive (Fig. 2), with an apparent inhibition constant of 1.4 mM for glutamic acid-γ-semihydrazide in the presence of 0.5 mM glutamine. These observations suggest that both glutamine and glutamic acid-γ-semihydrazide bind at the same site on the enzyme, and that this site might be distinct from the catalytic site to which lysine binds, since both glutamine and glutamic acid-γ-semihydrazide are able to stimulate the formation of N^6-hydroxylysine in the presence of saturating amounts of lysine. However, the maximal stimulation of the N^6-hydroxylase by glutamic acid-γ-semihydrazide was approximately 20% over control values, in contrast to the 2–3-fold increases in lysine N^6-hydroxylation in the presence of glutamine. Thus, when both glutamine and glutamic acid γ-semihydrazide are present in reaction assays, competition between these two effectors for the same binding site would result in a decrease in enzyme activity relative to that normally observed in experiments performed in the presence of glutamine alone. Despite the lower N^6-hydroxylase activity due to the competition between these two effectors, the extent of N^6-hydroxylysine formation observed in their presence was still greater than that noted when neither compound was present, possibly since neither compound appears capable of binding to the catalytic site in order to compete with, and inhibit, the N^6-hydroxylation of lysine.

Azaserine is an analogue of glutamine that has been observed to inhibit a number of enzymes which are involved in glutamine metabolism [28], and thus was considered of use in the study of the glutamine stimulation of lysine N^6-hydroxylation. In

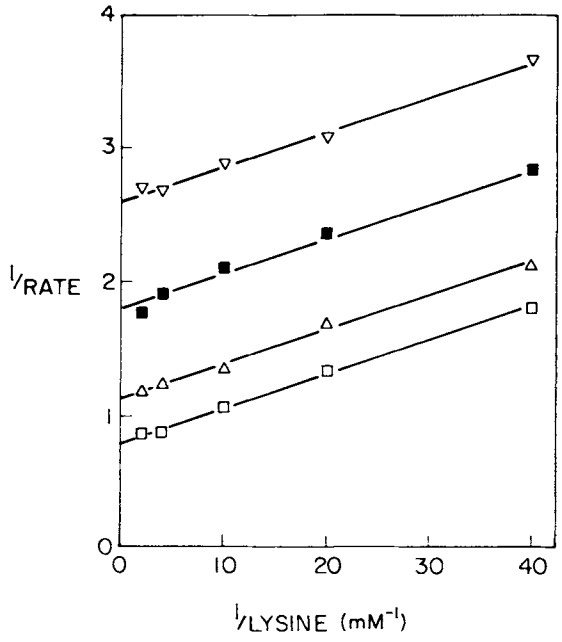

Fig. 3. Effect of azaserine on N^6-hydroxylase activity: The double reciprocal plot of 1/rate vs 1/(lysine). Assays were performed as described (see Methods) with addition of L-lysine to the appropriate concentration. □—□, no azaserine; △—△, 0.03 mM: ■—■, 0.05 mM; ▽—▽, 0.10 mM.

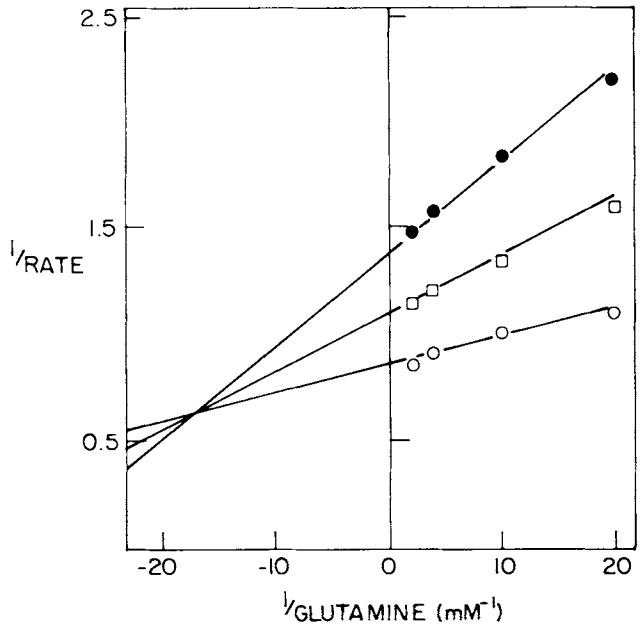

Fig. 4. Effect of glutamine on azaserine inhibition: Plot of 1/rate vs 1/(glutamine). All assays contained 50 mM phosphate (ph 7), 1 mM pyruvate, 0.5 mM L-lysine, P2 + S2 plus L-glutamine to the appropriate concentration. ○—○, no azaserine; □—□, 0.05 mM: ●—●, 0.1 mM.

contrast to the data obtained with glutamic acid-γ-semihydrazide, azaserine was found to inhibit the activity of the N^6-hydroxylase even in the absence of glutamine. This inhibition was, however, uncompetitive with respect to the substrate, lysine, as shown in Figure 3. An apparent inhibition-constant of about 0.03 mM was observed. The addition of glutamine to reaction mixtures that contained azaserine failed to alleviate the azaserine-induced inhibition of the lysine N^6-hydroxylase. However, as shown in Figure 4, glutamine was able to enhance the activity of the residual uninhibited fraction of the enzyme.

The compounds that have been discussed above, glutamine, glutamic acid-γ-semihydrazide, and azaserine, all bear structural similarities to lysine, the substrate for the N^6-hydroxylase in the cell-free system from A aerogenes 62-1. However, only one of these compounds, azaserine, was found to be an inhibitor with respect to lysine, and in this case, the inhibition was uncompetitive. Both glutamine and glutamic acid-γ-semihydrazide were observed to enhance the formation of N^6-hydroxylysine by the cell-free system, even in the presence of saturating levels of the substrate, lysine. It thus appears that these compounds exert their influence by binding to some site in the enzyme system that is distinct from the catalytic site for lysine. This site may serve an activatory or regulatory role in the N^6-hydroxylation of lysine. We are unable to state whether the site to which glutamine binds in order to stimulate the lysine N^6-hydroxylase is on the same enzyme molecule as the site that catalyzes the formation of N^6-hydroxylysine, due to the crude nature of the cell-free system. If these sites share the same enzyme molecule, then the data presented in this report might indicate that the lysine N^6-hydroxylase from A aerogenes 62-1 could be subject to allosteric control. It is also possible that glutamine could bind to a protein moiety separate from the N^6-hydroxylase. Subsequent association of this moiety with the other component(s) in the cell-free extract could result in enhanced rates of N^6-hydroxylysine synthesis. Clearly, substantial purification of the active components of the N^6-hydroxylase system must be achieved before any concrete hypothesis can be made. Attempts at purification of the active components in the particulate P2 fraction of the cell-free system have been unsuccessful, due to the labile nature of the lysine N^6-hydroxylase activity. The observation, in this report, that glutamine is able to stabilize the N^6-hydroxylase activity in the particulate P2 fraction may be of considerable use in subsequent purification schemes.

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