Concerted Feedback Inhibition

MODIFIER-INDUCED OLIGOMERIZATION OF THE PSEUDOMONAS FLUORESCENS ASPARTOKINASE*
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SUMMARY
Concerted feedback inhibition of aspartokinase activity from *Pseudomonas fluorescens* by the combination of L-threonine and L-lysine, or by L-threonine and L-methionine was freely and rapidly reversible. Inhibition by L-threonine, or by the concerted pairs of amino acids was kinetically “mixed” with respect to L-aspartate and noncompetitive with regard to ATP.

In the presence of L-threonine the Stokes radius of the enzyme increased from 44 to 49 Å; a much larger value of about 56 Å was estimated when both L-threonine and L-lysine were added to the enzyme. The same amino acids increased the Stokes radius of the enzyme at 15° in buffer solutions containing ATP, L-aspartate, and Mg++, the substrates of the phosphorylation reaction, indicating that the larger molecular species were catalytically inactive. Although in phosphate buffer at 25° the combination of L-threonine plus L-methionine did not show an additional increment in the molecular radius over that seen in L-threonine alone, the same combination induced a large change (from 49 to 54 Å) in the buffer containing the substrates at 15°.

L-Threonine, and a combination of L-threonine plus L-lysine also increased the Svalue of the aspartokinase by about 50%; whereas, a 10% increase in the S value was noted in L-lysine alone.

The cumulative results strongly suggest that the inhibitory amino acids caused oligomerization of the enzyme. Since the inhibition of enzyme activity accompanied the structural alterations of the protein and a good correlation was seen between the degree of inhibition and the extent of enzyme oligomerization under similar experimental conditions, it is proposed that the mechanism of inhibition by L-threonine and by the concerted pairs of amino acid end products may involve modifier-induced formation of inactive enzyme oligomers.

It is generally accepted that the regulatory interactions between substrates of a given enzyme reaction and the allosteric modifiers that modulate the catalytic activity of that enzyme are indirect. Thus, binding of these modifiers on the enzyme molecule is believed to cause conformational transitions in the protein structure influencing the rate of conversion of the substrate to the product. In the previous paper (1) we have described the basic regulatory properties of the aspartokinase (ATP: L-aspartate 4-phosphotransferase, EC 2.7.2.4) from *Pseudomonas fluorescens*. The activity of this enzyme was inhibited by the concerted action of L-lysine plus L-threonine, and by L-threonine plus L-methionine; individually, L-threonine was a weak inhibitor, and L-lysine and L-methionine were slightly stimulatory. This paper reports the structural changes accompanying the binding of these modifiers to the enzyme molecule. The data suggest that the mechanism of inhibition by L-threonine, and by the concerted pairs of amino acid end products may involve modifier-induced formation of inactive enzyme oligomers. (See Ref. 2 for a preliminary report on this work.)

METHODS

Enzyme Preparation and Assay—Aspartokinase was purified from *P. fluorescens* strain P-14 according to the method described (1). The enzyme activity was measured either by the standard hydroxamate assay or by the coupled pyruvate kinase-lactate dehydrogenase system (1).

For assay of enzyme activity at high protein concentrations, a Gibson-Milnes stopped flow spectrophotometer (3) modified to produce linear absorbance was used. The standard reaction mixture contained 50 mM imidazole-HCl buffer, pH 7.6, 200 mM KCl, 10 mM MgCl2, 2 mM phosphoenolpyruvate, 0.3 mM NADH, 2 mM ATP, 2 mg per ml of rabbit muscle pyruvate kinase (a gift from Dr. M. Flashner, 320 i.u. per mg), 0.25 mg per ml of lactate dehydrogenase (Calbiochem, 6550 i.u. per mg), and 432 µg per ml of aspartokinase previously dialyzed in 20 mM histidine-KOH buffer, pH 7.6 (specific activity 200 µmoles of aspartohydroxamate formed per min per mg of protein), and the amino acid concentrations as described. Control experiments with 2-fold higher concentrations of the coupling enzymes and the substrates revealed no change in the initial rate of NADH oxidation. The amino acids also did not influence the activities of the coupling enzymes.

Other Methods—Protein was measured by the method of Lowry

et al. (4). The methods for Stokes radius determination and sedimentation velocity experiments have been reported (1).

Reagents—The sources of amino acids, marker enzymes, and other reagents were the same as those given in the previous article (1).

RESULTS

Nature of Inhibition

Since L-threonine, and the combination of L-threonine plus L-lysine, and L-threonine plus L-methionine inhibited enzyme activity, the effects of these amino acids on the substrate saturation curves were examined. Fig. 1 shows that in the standard hydroxamate assay, inhibition by 0.5 mM each of L-threonine and L-lysine, or by 5 mM each of L-threonine plus L-methionine was not overcome by increasing the aspartate and ATP concentrations up to 50 and 60 mM, respectively. The same results were obtained when 20 mM L-threonine alone was used to inhibit the enzyme activity. The double reciprocal plots of the reaction rates versus substrate concentrations (Fig. 1, B and D) confirmed the “mixed” inhibition kinetics with respect to aspartate and the noncompetitive nature of inhibition with relation to ATP. In the coupled assay, inhibition of enzyme activity by L-threonine also appeared to be “mixed” with respect to aspartate and noncompetitive with regard to ATP. These data suggest that the binding sites for the inhibitors, ATP and aspartate, are separate, and that indirect interactions are to be expected between these ligands when they occupy their respective sites on the enzyme molecule.

Reversibility and Rate of Inhibition

Concerted feedback inhibition by L-threonine and L-lysine was freely and rapidly reversible. An enzyme solution incubated in the presence of 5 mM each of these amino acids at 25°C up to a period of 2 hours showed full activity within 30 s when diluted and assayed in the absence of these modifiers as compared to a control sample not incubated with the amino acids. A reciprocal experiment in which an enzyme solution was freed of the inhibitors by exhaustive dialysis and subsequently assayed in the presence of the inhibitors revealed the expected reduction of activity in less than 15 s. These data show that both the onset of inhibition as well as the reversibility of inhibition was as rapid as could be measured by the conventional spectrophotometric assay (also, see below).

Structural Alterations in Protein

Changes in Stokes Radius—Gel filtration of aspartokinase on calibrated Sephadex G-200 columns revealed that the elution profile of the enzyme was drastically altered in the presence of the amino acid modifiers (Fig. 2). At 25°C, the enzyme in 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl eluted slightly after yeast alcohol dehydrogenase (Fig. 2A); a Stokes radius of about 44 A was calculated from the equations described (6, 7). When the gel filtration buffer was supplemented with 5 mM L-threonine (Fig. 2B), the peak of enzyme activity shifted to an earlier fraction to correspond with a molecule having a Stokes radius of about 46 A as evidenced by its elution profile coinciding with that of pyruvate kinase (Fig. 2C). From a series of similar Sephadex G-200 experiments in 50 mM phosphate buffer, pH 7.5, containing 100 mM KCl, the Stokes radius of the enzyme was determined in the presence of several amino acids either added individually or in certain combinations; the results are summarized under Experiment I in Table I. It is clear that L-methionine, L-isoleucine, or L-lysine did not alter the Stokes radius significantly; whereas, L-threonine alone, and the combination of L-threonine plus L-lysine increased the molecular radius of the enzyme. Although inclusion of L-methionine in the phosphate buffer already supplemented with L-threonine did not cause additional change in the Stokes radius at 25°C, a synergistic effect of these amino acids on the change in the Stokes radius was observed at 15°C in histidine buffer in the presence of the substrates (see Experiment II, Table I).

The increases in Stokes radii by these modifiers were also ob-
An article discussing the effects of various amino acids on the molecular structure of aspartokinase. The text describes experiments conducted to determine the Stokes radii of the enzyme in the presence and absence of amino acids. The results indicate that certain amino acids, such as L-lysine and L-methionine, can alter the protein structure in a way that is not detected by gel filtration.

The article also presents tables and graphs to illustrate the data. One table, Table I, lists the Stokes radii of aspartokinase in the presence and absence of various amino acids. Another table shows the relationship between inhibition of activity and structural alterations in the enzyme molecule. The text concludes with a discussion of the results of gel filtration experiments and sedimentation velocity centrifugation studies, indicating that the amino acid modifiers inhibit enzyme activity.
FIG. 3. Sedimentation velocity patterns of aspartokinase in the presence and absence of various amino acids. Purified enzyme was dialyzed extensively against 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 10 mM β-mercaptoethanol and the amino acids as indicated: \( A \), no amino acid; \( B \), 5 mM L-lysine; \( C \), 5 mM L-threonine; \( D \), 5 mM L-threonine plus 5 mM L-lysine. Centrifugations were carried out in single sector cells at 20°; photographs were taken at 8-min intervals at a bar angle of 60° after the rotor had reached two-thirds of the top speed of 52,000 rpm. Sedimentation was from left to right. In all cases, the first picture shown was taken at 48 min. The following protein concentrations were used: \( A \), 3.33 mg per ml; \( B \), 3.88 mg per ml; \( C \) and \( D \), 3.96 mg per ml.

TABLE II

Influence of amino acids on sedimentation coefficient and molecular weight of aspartokinase

Sedimentation values were determined from velocity centrifugation in model E ultracentrifuge in 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 10 mM β-mercaptoethanol, and the amino acids as indicated. For other details, see legend of Fig. 3. Molecular weights were estimated from the appropriate Stokes radii (as determined by gel filtration on Sephadex G-200), and from the sedimentation coefficient values obtained as above using the equation described by Siegel and Monty (7).

<table>
<thead>
<tr>
<th>Additions</th>
<th>Sedimentation coefficient ( \times 10^3 ) s(^{-1} )</th>
<th>Molecular weight</th>
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<tr>
<td>None</td>
<td>6.50</td>
<td>137,000</td>
</tr>
<tr>
<td>L-Lysine (5 mM)</td>
<td>7.15</td>
<td>146,000</td>
</tr>
<tr>
<td>L-Threonine (5 mM)</td>
<td>9.58</td>
<td>225,000</td>
</tr>
<tr>
<td>L-Lysine + L-threonine (5 mM each)</td>
<td>9.76</td>
<td>288,000</td>
</tr>
</tbody>
</table>

Fig. 4. Inhibition of enzyme activity by L-threonine plus L-lysine at high protein concentration. Activity of 432 \( \mu \)g per ml of aspartokinase purified through Sephadex G-200 II step (1) was measured in the Gibson-Milnes stopped flow spectrophotometer. The concentrations of the inhibitors were as follows: Curve \( A \), no inhibitor; Curve \( B \), 0.1 mM each of L-threonine and L-lysine; Curve \( C \), 0.2 mM each of L-threonine and L-lysine; Curve \( D \), 2.5 mM each of L-threonine and L-lysine. For other details, see "Methods." The inset shows the activity of 2 \( \mu \)g per ml of the enzyme as measured with the stopped flow apparatus in an otherwise identical reaction mixture except that no inhibitor was present; in this case, the decrease in \( A_{340} \) was recorded directly on a strip-chart recorder.

under assay conditions (2 to 8 \( \mu \)g per ml) also caused drastic structural changes in the molecule at protein concentrations ranging from 400 \( \mu \)g per ml to 4 mg per ml. In view of this, it was necessary to show that the activity of aspartokinase was also subject to concerted feedback inhibition at higher enzyme concentrations. The data of stopped flow experiments given in Fig. 4 clearly reveal that the aspartokinase activity was inhibited by the combination of L-threonine and L-lysine at a protein concentration of 432 \( \mu \)g per ml. Since the experimental conditions for

\footnote{The stopped flow experiments revealed an interesting aspect of the phosphorylation reaction that could be easily missed in the coupled pyruvate kinase-lactate dehydrogenase assay with a conventional spectrophotometer. A significant lag in NADH oxidation (about 100 ms) preceded the linear rate; with increasing concentrations of the amino acid inhibitors, this lag was almost...}
Fig. 5. Relationship between the changes in the Stokes radius and per cent inhibition of enzyme activity as a function of equimolar concentrations of L-threonine plus L-lysine. ○, Stokes radius values taken from Experiment II, Table I; Δ, per cent inhibition of activity at 2.1 μg per ml of enzyme; ⋄, per cent inhibition of activity at 432 μg per ml of enzyme (calculated from the data given in Fig. 4). See text for other details.

The stopped flow measurements were almost identical with those of the gel filtration studies shown under Experiment II in Table I (with the exceptions that the enzyme activity was assayed at 25° in the imidazole-HCl buffer, at pH 7.8, and the assay mixtures also contained the coupling enzymes and their substrates), we conclude therefore that the enzyme species with larger molecular radii were catalytically inactive.

Fig. 5 summarizes the data of the per cent Stokes radius change and per cent inhibition of enzyme activity as a function of increasing equimolar concentrations of L-lysine plus L-threonine. The results show a sharp transition from a smaller active to a larger inactive form of the enzyme; the change is about 90% complete within a narrow range of inhibitor concentrations from zero to 0.5 mM each. A good correlation can be seen between the degree of inhibition (at enzyme concentrations of 2 and 432 μg per ml) and the extent of enzyme oligomerization (at protein concentration of 420 μg per ml) as evidenced by the increase in the Stokes radius.

DISCUSSION

A wide variety of regulatory enzymes is known to undergo drastic structural changes leading to enzyme aggregation in the presence of feedback modifiers. Some examples are: homoserine dehydrogenase of Rhodospirillum rubrum (8), chorismate mutase-prephenate dehydratase of Salmonella typhimurium (9), and cytosine triphosphate synthetase (10), ribonucleotide reductase (11), lysine-sensitive aspartokinase (12), and threonine-sensitive aspartokinase I-homoserine dehydrogenase I (13, 14) of Escherichia coli. Nevertheless, it has been clearly established that, in many cases, the aggregation phenomenon may not be directly implicated as the physical basis for the regulation of enzyme activity (9, 14, 15).

The data presented here on the concerted feedback inhibition of aspartokinase from P. fluorescens indicate that, in this instance, aggregation of the enzyme by the amino acids may be an integral part of the mechanism of feedback control. The following observations are relevant in this regard. (a) In the absence of feedback modifiers, the Stokes radius of the enzyme at a protein concentration of 420 μg per ml in buffer without the substrates of the phosphorylation reaction was about 44 A. At the same protein concentration but in buffer supplemented with substrates, the Stokes radius was about 41 A (Table II) indicating that the substrates did not alter the protein structure to a significant extent. (b) In the presence of the inhibitory ligands, the molecular radius of the enzyme increased drastically; in the presence of the substrates and cofactors at a protein concentration of 420 μg per ml (Table II). The change in the molecular radius was proportional to the concentrations of the ligands present during gel filtration, and almost 90% of the maximum change was complete at inhibitor concentrations of 0.5 mM each (Fig. 5, open circles). (c) Assay of enzyme activities at 432 μg per ml using stopped flow technique revealed that inhibition of aspartokinase activity by the combination of L-threonine plus L-lysine increased with increasing concentrations of these ligands reaching 90% of the maximum inhibition at 0.5 mM each of the amino acids (cf. Figs. 4 and 5, closed circles). (d) Upon removal of the inhibitory ligands either by dialysis or by gel filtration, full enzyme activity was restored; under these conditions a return from the oligomeric form of the enzyme to the "native" molecular size was shown by a decrease in Stokes radius and a reduction in the sedimentation coefficient (see enzyme purification scheme, Ref. 1).

The above findings clearly show that, at least in one instance, i.e. at a protein concentration of about 420 μg per ml in the ligand concentration range from 0 to 0.5 mM each, a good correlation exists between the inhibition of enzyme activity and ligand-mediated oligomerization of the protein.

It is possible to argue that the changes in the elution profile on gel filtration in the presence of the amino acid modifiers might have been the result of some conformational changes leading to unfolded forms of the enzyme; alternatively, the large molecular radius could have reflected protein aggregation. A 50% increase in the sedimentation coefficient of the enzyme (as well as a large increase in molecular radius) in the presence of the modifiers (Table II) appears to rule out intramolecular conformational changes in the protein molecule; an unfolding of the protein would accompany a decrease in the S value, since a larger molecule of the same mass would sediment more slowly. In addition, an approximately 30% increase in the Stokes radius is compatible with a doubling of the molecular weight (17).

The approximate molecular weight of 137,000 of the enzyme in the absence of the modifiers (also, cf. 1) and a molecular weight completely abolished. The inset given in Fig. 4 shows that even at an enzyme concentration of 2 μg per ml but in an otherwise identical assay mixture without the feedback inhibitors, the lag was clearly defined; in the presence of the inhibitors this lag was eliminated. These observations ruled out the possibility that the coupling enzymes were rate-limiting. Since the enzyme solution for these experiments was freed of the inhibitory amino acids by exhaustive dialysis, one possible explanation for the observed lag might be that the aspartokinase molecules assume a different conformation in the presence of the substrates and that this new conformational form is enzymatically active.

4 Although the enzyme activity is also severely inhibited by the feedback modifiers when assayed at protein concentrations varying from 2 to 8 μg per ml (see Fig. 5, open triangles, and also cf. Ref. 1), data are not yet available on the ligand-mediated oligomerization of the enzyme in this low protein concentration range. Further studies using active enzyme centrifugation technique (18) in the presence and absence of the inhibitory ligands would be necessary to establish the state of oligomerization of aspartokinase at the concentrations used for the routine activity assays.
of 268,000 in the presence of 5 mM each of L-threonine and L-lysine (Table II) also supports the notion that the feedback inhibitors caused aggregation of the enzyme. An intermediate molecular weight value of 225,000 in the presence of L-threonine with one well defined symmetrical peak (cf. Figs. 2 and 3) might reflect an average value of more than one form of the enzyme in rapid equilibrium. We may recall that the Stokes radius of aspartokinase was found to be about half-way between the fully aggregated and the unaggregated species in the presence of this ligand (Table I).

One important question remains to be answered in order to demonstrate convincingly a direct relationship between the structural change in the protein and the inhibition of enzyme activity: does the rate of formation of enzyme oligomers occur as rapidly as the regulatory event itself? We have shown that the reversible concerted feedback inhibition of aspartokinase by L-threonine plus L-lysine occurred at least as rapidly as could be measured with the conventional spectrophotometric method and by the stopped flow apparatus. However, at present we have little information on the rate of formation of enzyme oligomers. Thus, we cannot completely rule out the possibility that a rapid intramolecular conformational change may precede aggregation of the enzyme molecule. Nevertheless, the cumulative data obtained thus far strongly suggest that modifier induced formation of inactive enzyme oligomers is an integral part of the mechanism of feedback inhibition control.

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REFERENCES
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