Antipain inhibits thyroxine-induced synthesis of carbamyl phosphate synthetase I in tadpole liver

[protease inhibitors/urea cycle enzymes/enzyme induction/liver protein synthesis/carbamoyl-phosphate synthase (ammonia), EC 2.7.2.5]

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ABSTRACT The increased activity of carbamyl phosphate synthetase I [carbamoyl-phosphate synthase (ammonia); ATP:carbamate phosphoryltransferase (diphosphorylating), EC 2.7.2.5] in tadpole liver observed during thyroxine-induced metamorphosis was markedly inhibited by intraperitoneal injection of the microbial protease inhibitor antipain (0.1 μmol/g of body weight, twice daily). A somewhat less than maximal inhibition was seen when antipain was given only during the first 2 days of thyroxine treatment. On the other hand, little inhibition was observed when the inhibitor was given after the third or fourth day of thyroxine treatment. Antipain also inhibited thyroxine-induced increases of ornithine transcarbamylase (EC 2.1.3.3), arginase (EC 3.5.3.1), and succinate-cytochrome c reductase (EC 1.3.99.1) activities. Among other microbial protease inhibitors tested, chymostatin was nearly as effective as antipain, leupeptin was less effective, and pepstatin was ineffective. Analysis of the total liver protein and of the immunoprecipitate by sodium dodecyl sulfate/polyacrylamide gel electrophoresis showed that the inhibition was due to decreased amounts of the enzyme protein. Antipain had no significant effect on leucine incorporation into total protein of tadpole liver. These results indicate the involvement of a proteolytic step in the pretranscriptional events in thyroxine-stimulated enzyme induction.

The activity of carbamyl phosphate synthetase (CPS-I; [carbamoyl-phosphate synthase (ammonia); ATP:carbamate phosphoryltransferase (diphosphorylating), EC 2.7.2.5]), the enzyme involved in the initial step of urea synthesis, markedly increases in tadpole liver during the early stages of thyroxine (T4)-induced metamorphosis (1). Experiments using whole animals (2, 3), liver slices (4), and liver cubes (5) established that the increase was the result of de novo net synthesis of the enzyme. CPS-I is located in the mitochondrial matrix and constitutes about 20% of the total matrix protein of adult frog liver (6). During studies on the mechanism of T4-induced synthesis of CPS-I, we found that antipain, one of the low molecular weight protease inhibitors isolated from actinomycetes (7), strongly inhibited the enzyme induction. Furthermore, antipain inhibited other biochemical changes associated with T4-induced metamorphosis.

In this paper we report the effect of antipain and other microbial protease inhibitors on T4-induced synthesis of CPS-I and related enzymes.

MATERIALS AND METHODS

Chemicals. Antipain, leupeptin, chymostatin, and pepstatin, which were originally obtained from the U.S.-Japan Cooperative Cancer Research Program, were provided by W. Troll (New York University). T4 was obtained from Calbiochem. Bovine liver ornithine transcarbamylase (EC 2.1.3.3) was a gift from M. Marshall of this laboratory.

Treatment of Animals. Premetamorphic Rana catesbeiana tadpoles, weighing 6–10 g, were obtained from Mogul-Ed (Oshkosh, WI) and fasted for a few days in dechlorinated water at 15°C before use. T4 solution (0.12 mM in 1 mM NaOH) was injected intraperitoneally (0.6 nmol/g of body weight) and the tadpoles were kept in 26 mM T4 at 22–23°C. Control animals were injected with 1 mM NaOH and kept in water. Immersion fluid was changed daily. Protease inhibitors were injected intraperitoneally twice daily (at 0800 and 2000). Livers were homogenized in 9 vol of 10 mM potassium Hepes (pH 7.4 at 4°C)/1 mM dithiothreitol. Prior to assays of CPS-I, ornithine transcarbamylase, and succinate-cytochrome c reductase (EC 1.3.99.1) activities, liver homogenates were treated with 0.5 mg of polyoxyethylene ether W-1 (Sigma Chemical Co.) per mg of homogenate protein to ensure rupture of the mitochondria.

Enzyme Assays. CPS-I was assayed with [14C]bicarbonate as a substrate by following the formation of [14C]citrulline in the presence of excess L-ornithine and ornithine transcarbamylase. The reaction mixture (0.25 ml, pH 7.4) contained 50 mM potassium N-tris(hydroxymethyl)methyl-2-aminoethanesulfonate, 5 mM ATP, 15 mM Mg(OAc)2, 10 mM N-acetylimidazole, 25 mM KHCO3 (~400 cpn/mmol), 50 mM KCl, 5 mM L-ornithine, 4 units (μmol/min) of ornithine transcarbamylase, and enzyme. Incubation was for 10 min at 37°C. [14C]Citrulline formed was determined in a liquid scintillation spectrometer (8) using Scintisol (Isolab). Ornithine transcarbamylase (9) and arginase (EC 3.5.3.1) (10) were assayed colorimetrically, and succinate-cytochrome c reductase was analyzed spectrophotometrically (11).

Protein Determination. Protein was determined colorimetrically (12) on acid-purified samples with bovine serum albumin as a standard.

Gel Electrophoresis. Sodium dodecyl sulfate (NaDodSO4)/7.5% polyacrylamide slab gel electrophoresis was performed with a discontinuous buffer system (13). Electrophoresis was conducted for about 6 hr at a constant current of 25 mA. The gels were stained for 2 hr with 0.1% Coomassie blue/25% (vol/vol) isopropanol/10% (vol/vol) acetic acid and destained in 5% isopropanol/10% acetic acid.

Leucine Incorporation Studies. Tadpoles were injected intraperitoneally with 0.10 μCi of L-[1-14C]leucine (270 mCi/mmol, New England Nuclear) per g of body weight and each group of animals (six animals in a group) was transferred to 400 ml of 26 mM T4 solution (thyroxine-treated animals) or water (thyroxine-untreated animals) at 22–23°C. At the end of a 6-hr labeling period, each group of animals was transferred to 400 ml of ice-cold water containing Finaquel (300 mg/liter, Ayerst Laboratories Inc., New York). Livers were removed and homogenized as described above. To 100 μl of the homogenate

Abbreviations: CPS-I, carbamyl phosphate synthetase I [carbamoyl-phosphate synthase (ammonia)]; T4, L-thyroxine; NaDodSO4, sodium dodecyl sulfate.

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was added 50 µl of 50 mM leucine, and protein was precipitated by 10% trichloroacetic acid. The precipitate was dissolved in 0.5 M NaOH, unlabeled leucine was added, and protein was precipitated as above. The washing with trichloroacetic acid/ unlabeled leucine was repeated twice and the precipitate was dissolved in 0.5 M NaOH. Aliquots of the solution were assayed for radioactivity in Scintisol and for protein. Specific radioactivity of free leucine in the liver was determined essentially according to the method (14) used for the determination of lysine specific radioactivity in tadpole liver. Perchloric acid (1 M) extracts of liver homogenates were subjected to amino acid analysis; effluent fractions were collected and assayed for radioactivity.

Preparations. Frog liver CPS-I was purified to homogeneity by affinity chromatography with Affi-Gel Blue (Cibacron Blue F3GA-coupled agarose, Bio-Rad Laboratories) (15). Antibody against CPS-I was prepared in rabbits (16) and partially purified by (NH₄)₂SO₄ fractionation.

**RESULTS**

Effect of Antipain on T4-Induced Increases of CPS-I and Other Enzyme Activities. When tadpoles were injected with T4 and immersed in dilute T4 solution, CPS-I activity increased markedly (Fig. 1), as previously shown (3, 17). This increase was strongly inhibited by daily injection of antipain (twice daily). When antipain was given only for the first 2 days of T4 treatment, inhibition to almost the same extent was observed. On the other hand, antipain had little effect if administered after the third or fourth day of T4 treatment, when the enzyme induction had already started (Fig. 2). Antipain inhibited other biochemical changes associated with T4-induced metamorphosis including increases in ornithine transcarbamylase, arginase, and succinate-cytochrome c reductase activities (Table 1). The antipain action appears to be specific for T4-induced changes because the inhibitor did not significantly affect the enzyme activities of untreated animals. Recently, Brucker and Cohen showed that T4 treatment results in a marked change

<table>
<thead>
<tr>
<th>T4</th>
<th>Antipain</th>
<th>CPS-I</th>
<th>Ornithine transcarbamylase</th>
<th>Arginase</th>
<th>Succinate-cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>2.32</td>
<td>282</td>
<td>248</td>
<td>1940</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>14.8</td>
<td>514</td>
<td>1130</td>
<td>4410</td>
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<tr>
<td>+</td>
<td>+</td>
<td>8.40</td>
<td>348</td>
<td>680</td>
<td>2500</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>2.74</td>
<td>294</td>
<td>274</td>
<td>2460</td>
</tr>
</tbody>
</table>

Tadpoles were treated with T4 for 5 days. Control animals were injected with 1 mM NaOH and immersed in water. Antipain (50 mM in water, 0.1 µmol/g of body weight) or water (control) was injected twice daily (at 0800 and 2000). Livers of each group (9–12 animals) were pooled and protein and enzyme activities were measured.
Table 2. Effect of antipain, leupeptin, chymostatin, and pepstatin on T4-induced increases in enzyme activities

<table>
<thead>
<tr>
<th>T4 inhibitor</th>
<th>Enzyme activity, nmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPS-I</td>
</tr>
<tr>
<td>- None</td>
<td>3.47 ± 0.88</td>
</tr>
<tr>
<td>+ None</td>
<td>21.9 ± 2.6</td>
</tr>
<tr>
<td>+ Antipain</td>
<td>6.25 ± 0.65</td>
</tr>
<tr>
<td>+ Leupeptin</td>
<td>16.3 ± 1.6</td>
</tr>
<tr>
<td>+ Chymostatin</td>
<td>7.35 ± 2.00</td>
</tr>
<tr>
<td>+ Pepstatin</td>
<td>20.3 ± 4.5</td>
</tr>
</tbody>
</table>

Tadpoles were treated with T4 for 4 days. Antipain or leupeptin (50 mM in water, each), chymostatin or pepstatin (50 mM in dimethyl sulfoxide, each), or water (control) was injected twice daily. Values represent means ± SD (six animals). ND, not determined, because the injection of dimethyl sulfoxide alone increased arginase activities to some extent; dimethyl sulfoxide injection had no effect on CPS-I and ornithine transcarbamylase activities.

in distribution of mitochondria on isopycnic centrifugation (18, 19). This change was also inhibited by antipain (data not shown).

**Specificity of Protease Inhibitors.** Table 2 shows the effect of other protease inhibitors of the same class on increases of CPS-I, ornithine transcarbamylase, and arginase activities. Leupeptin inhibited the increases of the three enzyme activities to a lesser extent than did antipain. Chymostatin inhibited the increases of CPS-I and the transcarbamylase activities almost as strongly as antipain. The time course of the inhibition of CPS-I induction by chymostatin was similar to that by antipain (data not shown). On the other hand, pepstatin did not affect the increases of the two enzyme activities.

**Gel Electrophoresis.** Total liver protein of control and of T4- and protease inhibitor-treated tadpoles was analyzed by

![Image](image.png)

**Fig. 3.** NaDodSO4/polyacrylamide gel electrophoresis of total liver protein of T4- and protease inhibitor-treated tadpoles. Animals are those of Table 2. Protein samples were heated for 3 min in boiling water in 63 mM Tris-HCl, pH 6.8/10% (wt/vol) glycerol/5% (wt/vol) 2-mercaptoethanol/2.5% (wt/vol) NaDodSO4 before electrophoresis. Lanes 1–6 are liver homogenates (40 μl of 10% homogenate). Lanes: 1, control; 2, T4-treated; 3, T4 plus antipain; 4, T4 plus leupeptin; 5, T4 plus chymostatin; 6, T4 plus pepstatin. Lane 7, CPS-I (2 μg).

![Image](image.png)

**Fig. 4.** NaDodSO4/polyacrylamide gel electrophoresis of protein immunoprecipitated from T4- and protease inhibitor-treated tadpole liver extracts. Animals are those of Table 2. Liver homogenates were treated with polyoxyethylene ether W-1 (0.5 mg/mg of protein) to solubilize CPS-I and centrifuged at 105,000 × g for 60 min. Aliquots of the supernatants adjusted to contain 0.01 unit (μmol/min) of CPS-I were incubated with a sufficient amount of antibody for 15 min at 37°C and then overnight at 4°C in the presence of antipain, leupeptin, chymostatin, or pepstatin (0.1 mM, each). Immunoprecipitates were collected by centrifugation and washed twice with 1 ml of 17.5 mM Na phosphate, pH 7.4/0.15 M NaCl/1% (wt/vol) Triton X-100/1% (wt/vol) Na deoxycholate. The immunoprecipitates were heated as in Fig. 3. Lanes 1–6 are immunoprecipitates from tadpole livers. Lanes: 1, control; 2, T4-treated; 3, T4 plus antipain; 4, T4 plus leupeptin; 5, T4 plus chymostatin; 6, T4 plus pepstatin. Lane 7, CPS-I (4 μg). The lower two polypeptide bands correspond to the heavy chain and light chains of immunoglobulin.
Table 3. Effect of antipain on leucine incorporation into tadpole liver protein

<table>
<thead>
<tr>
<th>T4</th>
<th>Antipain</th>
<th>CPS-I activity*</th>
<th>Specific radioactivity†</th>
<th>Leucine incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>3.80 ± 0.71</td>
<td>14.6</td>
<td>4360 ± 610</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>3.31 ± 0.24</td>
<td>22.1</td>
<td>5540 ± 620</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>17.48 ± 3.11</td>
<td>18.2</td>
<td>3320 ± 820</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>8.13 ± 1.56</td>
<td>25.0</td>
<td>3940 ± 1000</td>
</tr>
</tbody>
</table>

Tadpoles were treated with T4 or antipain or both as described in Table 1. Tadpoles were injected with [3H]Leucine at 11000 of the fifth day of treatment. After 6 hr labeling time, livers were homogenized and assayed for CPS-I activity and for leucine incorporation. 

* Means ± SD (five or six animals), as nmol/min per mg of protein.
† Mean values, as dpm/nmol of leucine. It was assumed that one-sixth of the radioactivity was lost in the ninhydrin reaction.
§ Calculated by using specific radioactivity of free leucine in liver after 6 hr of labeling.

Antipain action appears to be specific for T4-induced changes because antipain had no significant effect on the rate of total protein synthesis in tadpole liver or on the enzyme activities of control (T4-un-treated) animals. Among protease inhibitors other than antipain (inhibiting papain, trypsin, and cathepsins A and B), chymostatin (inhibiting chymotrypsins and cathepsin B) also strongly inhibited T4-stimulated enzyme synthesis. Leupeptin (inhibiting plasmin, trypsin, papain, and cathepsin B) was effective to a lesser extent, and pepstatin (inhibiting acid proteases, especially cathepsin D) was not effective. Therefore, it seems likely that a protease that is sensitive to antipain, chymostatin, and leupeptin (such as cathepsin B) is involved in T4-induced protein synthesis. If two proteases are involved, one is trypsin-like and sensitive to antipain and leupeptin and the other is chymotrypsin-like and sensitive to chymostatin.

Present knowledge does not permit identification of the cellular processes that involve proteolytic steps specifically related to thyroid hormone action, but the following possibilities are worthy of consideration: (i) intracellular transport of T4 and its binding to nuclear receptors (25, 26); and (ii) gene derepression. With respect to the latter, it is noteworthy that a chromatin- or nucleohistone-associated protease has been reported in many tissues and its possible role in gene derepression has been suggested (27–30).

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