

## INTERACTION OF THYROXINE WITH LYSOZYME

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• *L-Thyroxine and hen's egg-white lysozyme form an insoluble complex at neutral pH. Previous reports have suggested that the enzyme and hormone also associate in solution. No evidence for a soluble interaction between thyroxine and lysozyme was obtained by using spectral, enzymatic, and immunological techniques.*

### INTRODUCTION

L-Thyroxine [3-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)alanine] interacts with a number of proteins in plasma such as  $\alpha$ -globulins and prealbumin.<sup>1</sup> It has also been reported to precipitate hen's egg white lysozyme from solution and to associate with the enzyme, perhaps at the active site.<sup>2-5</sup> We have examined the lysozyme-thyroxine interaction in more detail with the expectation of utilizing the hormone to probe the enzyme's active site and to develop a competitive protein-binding assay. Other small molecules have been used as probes, including Biebrich scarlet<sup>6</sup> and cobalt ion.<sup>7</sup> However, although thyroxine did precipitate lysozyme no evidence for an interaction in solution was found.

### MATERIALS AND METHODS

**Chemicals.** Twice-crystallized lysozyme (lot LYBAA) and dried *Micrococcus lysodeikticus* cells (lot ML0606-23) were obtained from Worthington Biochemical, L-thyroxine from Calbiochem, and DL-thyroxine from Mann Research Laboratories. The  $\beta(1\rightarrow4)$  linked trimer and hexamer of *N*-acetylglucosamine were prepared as described previously.<sup>8</sup> Other chemicals were of the highest purity commercially available.

**Concentration measurements.** Lysozyme concentration was determined spectrophotometrically<sup>9</sup> using  $\epsilon_{280} = 3.65 \times 10^4$ . Thyroxine concentration was measured in alkaline solution<sup>10</sup> using  $\epsilon_{326.5} = 6.45 \times 10^3$ .

**Enzyme assays.** Rates of *M. lysodeikticus* cell wall lysis were determined in 50 mM phosphate buffer, pH 7.1, using a Zeiss PMQII spectrophotometer equipped with a Varicord Photovolt model 43 recorder at 25°C. A stock suspension of cells was diluted into buffer with or without thyroxine to give a final volume of 3.1 ml and absorbance at 450 nm of 1.0. Enzyme (0.05 ml) was added to give a final concentration of  $10^{-6}$ - $10^{-7}$  M. Initial rates were determined from the absorbance trace of turbidity decrease over the first minute of lysis.

Hexa-*N*-acetylglucosamine hydrolysis was followed by using a Technicon automated system to measure the increase in ferri-ferrocyanide color at 410 nm associated with increase in reducing group concentration.<sup>11</sup> Hydrolysis was carried out with  $3.5 \times 10^{-7}$  M enzyme in 100 mM sodium phosphate, pH 7.1, at 25°C.

**Difference spectra.** These were measured with a Cary 15 spectrophotometer using 10- or 1-cm cylindrical quartz cells (Pyrocell) at 25°C in a series of experiments as follows: (I) 0.1 ionic strength universal buffer,<sup>12</sup> pH

7.1, 6.8  $\mu\text{M}$  L-thyroxine in 10-cm cell with buffer only in tandem 1-cm cell scanned 320-250 nm vs. same concentration of L-thyroxine in 10-cm cell and 68  $\mu\text{M}$  lysozyme in tandem 1-cm cell. (II) pH 11, 67  $\mu\text{M}$  enzyme and 80  $\mu\text{M}$  hormone in 1-cm cell with buffer in tandem 1-cm cell scanned vs. same concentration of enzyme and hormone in separate cells. (III) pH 11, 1-cm cells, 0.1 mM tri- $\beta$ -(1 $\rightarrow$ 4)-*N*-acetylglucosamine, 70  $\mu\text{M}$  lysozyme, 86  $\mu\text{M}$  L-thyroxine scanned vs. same concentration of enzyme and hormone in tandem separate cells. Saccharide binding was measured by the 293-289 nm peak-trough spectral perturbation.<sup>13</sup> (IV) Similar to III except L-thyroxine separate from enzyme and saccharide in the sample compartment also. (V) pH 11, 80  $\mu\text{M}$  L-thyroxine, 20% ethylene glycol in 0.1 ionic strength buffer in 1-cm cell scanned 400-300 nm vs. same concentration of hormone and diol in separate cells. (VI) Similar to II except samples scanned 400-300 nm.

*Radioimmunoassay.* This was performed

with the standard kit from ICN Medical Laboratories used for quantitative determination of human thyroxine in serum at pH 8.6.<sup>14</sup> Lysozyme and thyroxine concentrations ranged from  $10^{-7}$  to  $10^{-4}$  M and  $10^{-8}$  to  $10^{-7}$  M respectively.

## RESULTS

*Effect of thyroxine on lysozyme solubility and activity.* When micromolar thyroxine solutions were mixed with micromolar lysozyme solutions at neutral pH and room temperature, immediate precipitation occurred with approximately 40-60% of the total protein precipitating. This phenomenon was previously observed by Litwack and Sears.<sup>3</sup> At slightly lower lysozyme concentrations that did not lead to precipitation, no inhibition of lysozyme catalysis by thyroxine was detected by bacterial cell wall lysis assay (Table I). Litwack<sup>2</sup> reported that the hormone inhibited lysozyme by about 70% under similar conditions. Because rates of solubilization of

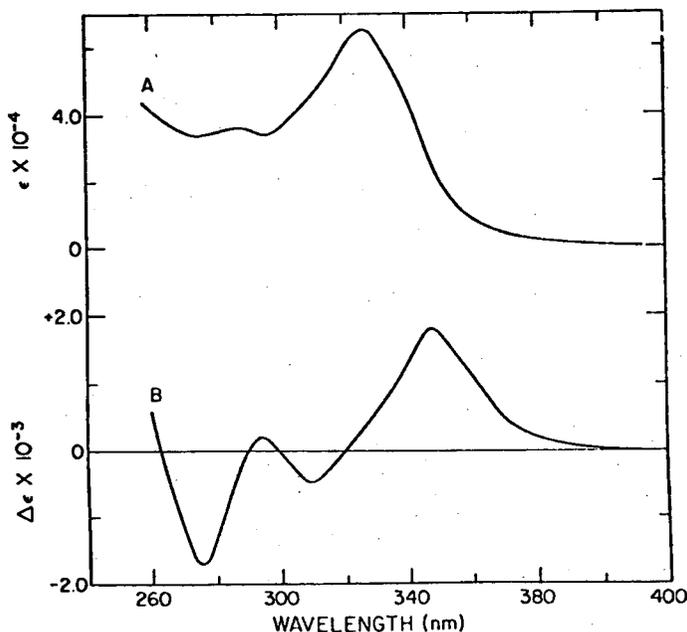


FIGURE 1. Lysozyme-catalyzed hydrolysis of the  $\beta$ (1 $\rightarrow$ 4) linked hexamer of *N*-acetylglucosamine in the presence (○) and absence (●) of  $8.6 \times 10^{-6}$  M L-thyroxine. See MATERIALS AND METHODS for experimental detail.

TABLE I. Rates of Cell Wall Lysis in the Presence of Thyroxine.<sup>a</sup>

Lysozyme × 10 <sup>7</sup> (M)	Thyroxine × 10 <sup>6</sup> (M)	Relative lytic activity (%)
5	DL, 5	97±3
5	DL, 5	96±4 <sup>b</sup>
6	DL, 3.4	100±4
6	DL, 3.4	100±5 <sup>b</sup>
3	L, 8	96±2
3	L, 8	95±2 <sup>b</sup>

<sup>a</sup>0.05 M phosphate buffer, pH 7.1, 25 or 30°C. The optical density of the cell wall suspension was 1.0 at 450 nm. The lytic activity is given relative to controls that contained no thyroxine. <sup>b</sup>Thyroxine was preincubated with lysozyme for 30–60 min at the temperature of the assay.

bacterial cells are rather difficult to interpret mechanistically due to the complex chemical nature of the cell wall,<sup>15</sup> a smaller and less complex substrate was utilized and rates of hydrolysis of hexameric  $\beta(1\rightarrow4)$  *N*-acetylglucosamine catalyzed by thyroxine-incubat-

ed lysozyme were measured (Fig. 1). This substrate filled the entire active site and was cleaved at only one bond under the experimental conditions. No inhibition of saccharide hydrolysis was observed even at higher hormone concentrations than were used in the cell wall lysis experiments. The kinetic data support the hypothesis that thyroxine cannot bind to lysozyme in such a way as to interfere with catalytic activity.

*Effect of ligand binding on lysozyme and thyroxine spectra.* It was thought possible that thyroxine might bind to lysozyme at a region apart from the active site. Crystallographic analysis has shown that lysozyme has six tryptophan and three tyrosine residues distributed throughout the three-dimensional structure. Binding of any ligand would be expected to perturb the ultraviolet spectrum, particularly if the association was in the active site where three tryptophan residues are present.<sup>18</sup> Using difference spectroscopy, no adsorbance change was detected upon addition of thyroxine to lysozyme at either neutral or alkaline pH. The putative

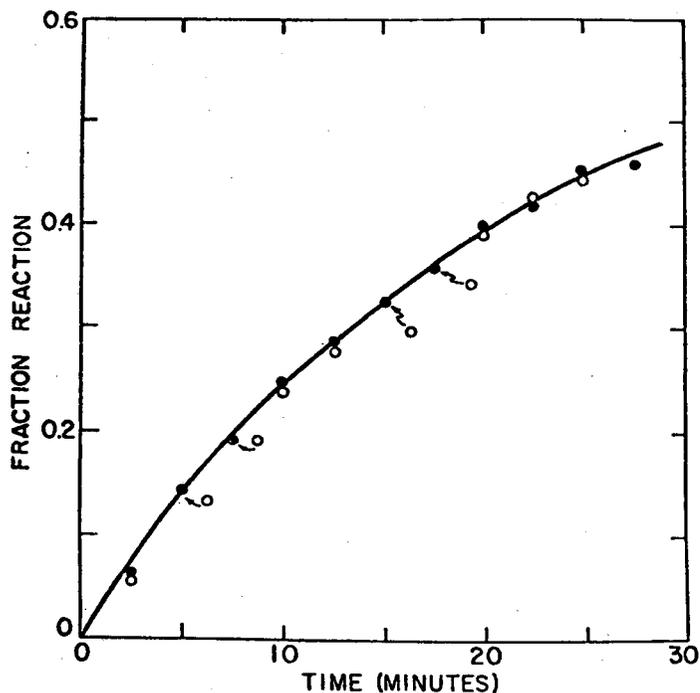


FIGURE 2. L-Thyroxine ultraviolet spectrum at pH 11 (A) and difference spectrum produced by 20% ethylene glycol (B). See MATERIALS AND METHODS for experimental detail.

$\Delta A_{293-289}$  peak-trough difference was less than  $2 \times 10^{-3}$  adsorbance units when essentially equimolar concentrations of enzyme and hormone were mixed together (experiments I and II in MATERIALS AND METHODS). Thus thyroxine did not strongly interact with any aromatic residue in lysozyme, even those outside the active site.

An interaction of lysozyme with thyroxine would be expected to perturb the adsorption spectrum of thyroxine, perhaps in a manner similar to the perturbation produced by addition of organic solvent. Figure 2 shows the thyroxine absorption spectrum in aqueous solution and the difference spectrum generated in 20% ethylene glycol. A red shift was produced by the change in solvent polarizability with  $\Delta A_{350}/\Delta A_{326} = 2.9 \times 10^{-2}$  adsorbance units (experiment V). No comparable effects were observed upon addition of lysozyme to L-thyroxine; i.e.,  $\Delta A_{350}/\Delta A_{326} < 1 \times 10^{-3}$  (experiment VI). There was no detectable interaction in solution between lysozyme and the aromatic ring system of the thyroxine molecule.

The hypothesis that thyroxine does not bind to the active site of lysozyme was supported further by an experiment on substrate binding. The binding of trimeric  $\beta(1 \rightarrow 4)$  linked *N*-acetylglucosamine to lysozyme should be interfered with by any ligand interacting in the upper half of the active site.<sup>15</sup> L-Thyroxine did not displace trimer present at a concentration that gave about 75% of the enzyme in the complex. The  $\Delta A_{293-289}$  peak-trough difference generated by trimer association was  $6.5 \pm 0.2 \times 10^{-2}$  absorbance units in the absence and in the presence of hormone (experiments III and IV).

Thus the trisaccharide binding contacts occurring in the upper half of the active site were not altered significantly by thyroxine.

*Effect of lysozyme on thyroxine radioimmunoassay.* Any association of lysozyme with thyroxine should be detected by the

highly sensitive technique of thyroxine radioimmunoassay.<sup>14</sup> At  $10^{-7}$ - $10^{-4}$  M enzyme, lysozyme did not compete with rabbit anti-thyroxine serum for radiolabeled or unlabeled hormone at  $10^{-8}$ - $10^{-7}$  M. Some precipitation of the enzyme did occur in experiments where the protein concentration was greater than  $10^{-5}$  M. This was due in part to the pH of the incubation mixture being near the isoelectric point of lysozyme.

## DISCUSSION

The spectral, catalytic, and immunological data reported here support the hypothesis that there is no interaction in solution between thyroxine and lysozyme. The rate studies with hexamer and bacterial cells relate to the entire active site, the measurements of trisaccharide binding to the upper half of the site, and the measurements of thyroxine spectrum and immunoglobulin binding to the hormone molecule itself. Binding of hormone to enzyme had been suggested on the basis of results from precipitation experiments,<sup>3</sup> inhibition of lytic activity,<sup>2</sup> and direct binding and fluorescence measurements.<sup>4</sup> At pH 10, the fluorescence quenching experiment yielded an association constant of ca.  $10^4$  M<sup>-1</sup>. However, it had been pointed out that iodotyrosines are highly absorbant at the wavelengths used in the fluorescence study. This necessitated a correction being made in the experiment for an inner filter effect and there was little evidence of any kind of association when appropriate corrections are made for chromophore absorption.<sup>16</sup> These results suggest that if there is a soluble interaction it must be a weak one and, depending on conditions, it either dissociates rapidly or denatures to an insoluble complex.

Conclusions based solely on inhibition of lytic activity or on precipitation effects should be interpreted cautiously. For exam-

ple, changes in lytic activity may be caused by a variety of effects that need not include association reactions of the enzyme in solution.<sup>15</sup> Likewise, precipitation of a protein upon addition of a ligand may indicate that a highly aggregated, insoluble form of the protein binds the ligand. Thyroxine at low ionic strength has been shown to precipitate lysozyme at neutral pH, which suggests a strong interaction with aggregated

enzyme (see ref. 3; also, note present study). Fluorescence polarization experiments with dansylated lysozyme have indicated that thyroxine promotes self-aggregation of the enzyme.<sup>17</sup> Thyroglobulin, a glycoprotein-thyroxine complex, has been found to interact with lysozyme in solution.<sup>18</sup> However, this behavior probably reflects binding of the protein to elements of the globulin such as *N*-acetylamino sugar moieties. □

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