Quantitation of Rat Liver Messenger Ribonucleic Acid for Malic Enzyme during Induction by Thyroid Hormone†

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ABSTRACT: The induction of cytosolic malic enzyme in rat liver by 3,5,3′-triiodo-L-thyronine (T_3) is due to an increase in the level of messenger ribonucleic acid (mRNA) coding for this protein. To investigate the mechanism of this hormone action, we measured the dose–response relationship between T_3 and malic enzyme mRNA and the kinetics of appearance and disappearance of malic enzyme mRNA following T_3 administration and withdrawal, respectively. Messenger RNA coding for malic enzyme was quantitated by using the mRNA-dependent rabbit reticulocyte lysate translational system. Identification of malic enzyme in the translational products was achieved by chromatography on N^6-(6-aminohexyl)adenosine 2′,5′-diphosphate–agarose, a biospecific affinity resin for NADP-dependent enzymes, followed by specific immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. With this procedure, levels of malic enzyme mRNA as low as 0.003% of the total translational products in normal, uninduced rat liver could be measured. Treatment of euthyroid rats with varying doses of T_3 led to proportional increases in both malic enzyme activity and specific mRNA coding for malic enzyme, indicating that hormonal action is due entirely to changes at a pretranslational level. Following acute administration of T_3 to normal rats, a lag time of approximately 2–3 h was found prior to the earliest effect on malic enzyme mRNA level. From the kinetics of mRNA appearance, a half-time of 12 h was estimated for malic enzyme mRNA in the presence of hormone. A similar half-time of 10 h was determined following withdrawal of animals from T_3 treatment. T_3 did not appear to act by stabilizing malic enzyme mRNA and, thus, must be acting at some step leading to increased production of mRNA. Based on the half-times of malic enzyme and its mRNA, a mathematical model of enzyme accumulation following T_3 induction was derived. This model demonstrates that no significant refractory period exists prior to the initiation of malic enzyme induction by T_3.

Rat liver malic enzyme [L-malate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40] responds markedly to the thyroidal status of the animal (Tepperman & Tepperman, 1964; Wise & Ball, 1964). Thus, the transition from the hypothyroid to the extremely hyperthyroid state results in a 20–30-fold increase in malic enzyme activity (Oppenheimer et al., 1977). The increase in enzyme activity has been shown to result from an increased rate of malic enzyme synthesis, leading to an induction in enzyme mass (Isohashi et al., 1971; Silpanata & Goodridge, 1971; Gibson et al., 1972; Murphy & Walker, 1974; Li et al., 1975). Recently, we have measured the level of hepatic messenger ribonucleic acid (mRNA)[1] coding for malic enzyme (Towle et al., 1980). Experimental hyperthyroidism induced by the injection of 15 μg of triiodothyronine (T_3)[1] (100 g of body weight)$^{-1}$ (day)$^{-1}$ for 7 days led to proportional increases in mRNA and enzyme activity for malic enzyme. Thus, the induction of malic enzyme by thyroid hormone was mediated by an increase in the cellular content of specific mRNA. To further elucidate the mechanism of induction of malic enzyme mRNA following T_3 administration, we have attempted to more closely define the response characteristics of this system. For this purpose, it was necessary to develop a more sensitive assay technique for detecting malic enzyme mRNA. This paper describes this technique, as well as the kinetics and dose–response relationship of the mRNA induction process. In addition, our studies allow us to prepare a mathematical model describing the relationship of malic enzyme to its mRNA at full receptor occupancy.

Experimental Procedures

Animals. Male Sprague-Dawley rats weighing 200–250 g were used in all experiments. Animals were fed standard chow diet (Ralston-Purina) ad libitum and maintained on a 12-h light–12-h dark cycle. T_3 (Sigma Chemical Co.) was dissolved in a minimum volume of 0.1 N NaOH, diluted to the appropriate concentration in standard saline, and injected intraperitoneally at the indicated doses. Animals were killed by exsanguination. Livers were rapidly removed, stripped of connective tissue, and rinsed in cold saline. A 2-g portion of liver was removed for determination of enzyme activity. The remainder was quickly frozen in liquid N_2 and stored at −80 °C until RNA extraction.

Malic Enzyme Assay. Freshly excised liver was homogenized in 9 volumes of 0.32 M sucrose, 3 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.6, and a sample was removed for DNA analysis (Giles & Myers, 1965). Homogenates were centrifuged at 130000g_{av} for 1 h at 4 °C. The supernatant fraction (cytosol) was assayed for malic enzyme by the method of Hsu & Lardy (1967). One unit of enzyme activity catalyzed the reduction of 1 nmol of $NADP^+$ in 1 min. Total protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Purification of malic enzyme and preparation of antibody to the purified enzyme were as previously described (Towle et al., 1980). The IgG fraction of serum was used in all

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[1] Abbreviations used: mRNA, messenger ribonucleic acid; DNA deoxyribonucleic acid; T_3, 3,5,3′-triiodo-L-thyronine; SaC, heat-killed, formaldehyde-fixed *Staphylococcus aureus* (Cowens I strain); NaDodSO_4, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; poly(A), poly(adenylic acid); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; TSH, thyroid-stimulating hormone.

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experiments. ^3H- or ^{14}C-labeled malic enzyme was prepared by the formaldehyde labeling procedure of Rice & Means (1971).

Isolation of Poly(A)-Containing RNA. Total cellular RNA was isolated from frozen liver samples by extraction with phenol–chloroform at pH 9 as described (Towle et al., 1980). Poly(A)-containing RNA was obtained from total cellular RNA by chromatography on oligo(dT)–cellulose (Collaborative Research).

In Vitro Translational Assay. Translational assays were run by using the micrococcal nuclease treated rabbit reticulocyte lysate system (Pelham & Jackson, 1976). Treatment of lysate with 60 μg/mL nuclease (P-L Biochemicals) for 12 min at 20 °C led to a reduction in (−)RNA controls to 0.2–0.5% of untreated lysate. Addition of rat liver poly(A)-containing RNA led to stimulations of 15–20-fold over the (−)RNA backgrounds. Translational assays contained in 120–360 μL of volume 20 mM Hepes, pH 7.6, 80 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μg/mL creatine phosphokinase, 80 μM 19 unlabeled amino acids, and either 20 μCi of [^3H]leucine (120 Ci/mmol) or 67 μCi of [^{35}S]methionine (700 Ci/mmol) (New England Nuclear). Incubation was for 90 min at 23 °C. Following incubation, reactions were centrifuged at 130000g_{av} for 1 h at 2 °C, and the supernatant fraction was used for further analysis. In samples in which recovery was monitored, 10^4 cpm (~2 μg) of ^3H-labeled malic enzyme (for [^{35}S]methionine incorporation) or ^{14}C-labeled malic enzyme (for [^3H]leucine incorporation) was added to the reaction prior to centrifugation.

Quantitation of Malic Enzyme in Translational Products. To each sample was added 200 μL of a 25% (v/v) suspension of N^6-(6-aminohexyl)adenosine 2′,5′-diphosphate–agarose in 20 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, and 1 mM dithiothreitol (buffer A). After incubation for 60 min at 4 °C, the affinity resin was collected by centrifugation for 2 min in a Beckman microfuge. The supernatant was removed by aspiration and the resin was washed 4 times by resuspension in 500 μL of buffer A. After being washed, malic enzyme was eluted in 150 μL of 0.5 mM $NADP^+$ in buffer A (5 min at 4 °C). The eluted products were separated from the resin by centrifugation and then adjusted to 0.25% (v/v) NP-40. Samples were then further purified by immunoadsorption with specific antibody to malic enzyme and with heat-killed, formaldehyde-fixed *Staphylococcus aureus* cell wall preparation (SaC, Calbiochem), as previously described (Towle et al., 1980). Following $NaDodSO_4$–polyacrylamide gel electrophoresis on 7.5% gels, radioactivity comigrating with purified malic enzyme was quantitated, corrected for recovery, and expressed as a percentage of the total incorporation.

Results

Detection of Malic Enzyme mRNA Activity in Uninduced Animals. We have previously demonstrated that malic enzyme mRNA activity could be detected and quantitated in hepatic poly(A)-containing RNA isolated from hyperthyroid rats. For this purpose, RNA was translated in the nuclease-treated reticulocyte lysate system, and radiolabeled products were analyzed for malic enzyme by using specific antibody to the purified protein. Antigen–antibody complexes were collected by using SaC as an immunoadsorbent. With [^{35}S]methionine as the radiolabeled precursor, ~0.04% of the products translated from hepatic RNA were found as malic enzyme in these induced animals (Towle et al., 1980).

Efforts to quantitate malic enzyme mRNA from livers of normal, uninduced rats were largely unsuccessful. Since the RNA isolated from normal rats was not defective in its overall translational activity, we presumed that this difficulty reflected the extremely low levels of malic enzyme mRNA present in uninduced animals. Attempts to increase the synthesis of malic enzyme by scaling up the translational assay were of little value, since no enhancement of the signal to noise ratio was obtained. Therefore, a further purification step was added to the detection procedure. The biospecific affinity resin, N^6-(6-aminohexyl)adenosine 2′,5′-diphosphate–agarose, which had previously been used by Goodridge et al. (1979) for a similar purpose, was chosen. Only a limited number of $NADP^+$-dependent proteins, including malic enzyme, are capable of binding to this resin (Brodelius et al., 1974). Use of the affinity resin prior to immunoprecipitation greatly reduced (>10-fold) levels of nonspecific background with no substantial reduction in the level of specific malic enzyme (data not shown).

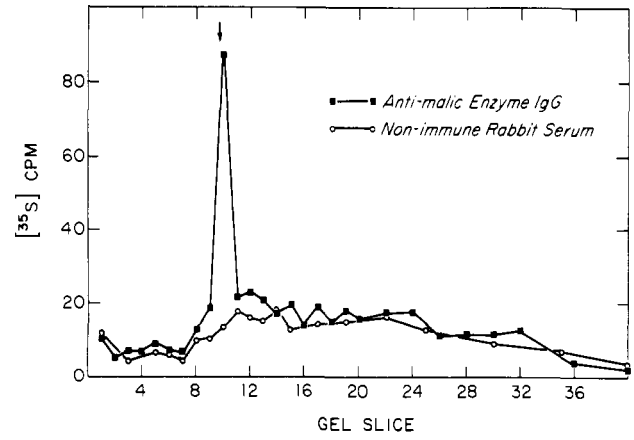

FIGURE 1: Translation of malic enzyme from euthyroid rat liver. Poly(A)-containing RNA was isolated from the liver of a euthyroid rat and translated in an assay of 360 μL by using [^{35}S]methionine. Products were analyzed by the combined affinity resin–immunoprecipitation procedure. Incorporation into newly synthesized polypeptides was 6×10^6 cpm. A control reaction was run in parallel in which nonimmune rabbit IgG replaced anti-malic enzyme IgG during the immunoadsorption (○).

Although the recovery of malic enzyme in each of the steps used for detection is quite high (>70%), potential variations could adversely affect attempts to quantitate malic enzyme mRNA activity. Therefore, the procedure has been adapted by addition of an internal standard for estimating recovery. When [^{35}S]methionine was used as precursor, ^3H-labeled malic enzyme was added to the translational products after incubation. $NaDodSO_4$–polyacrylamide gels were simultaneously counted for ^3H and ^{35}S. The recovery of ^3H-labeled malic enzyme was used to correct the amount of ^{35}S-labeled malic enzyme found in the translational products. The mean recovery of malic enzyme by the combined affinity resin–immunoadsorption procedure was 51.9% ($n = 12$) with a standard deviation of 9.6% (coefficient of variation = 18.5%). Intraassay variability was slightly less with a coefficient of variation of 9.4%.

Using the improved detection procedure for malic enzyme, we attempted to translate hepatic poly(A)-containing RNA from the normal animal. The results are shown in Figure 1. A peak of radioactivity comigrating with malic enzyme was detected. No peak was observed when preimmune rabbit sera replaced anti-malic enzyme IgG in the immunoadsorption. In this assay, 6×10^6 cpm of [^{35}S]methionine was incorporated into total protein. Backgrounds were reduced to <20 cpm/gel slice and the level of malic enzyme mRNA activity, after correction for recovery, was 0.003% of the total.

Effect of Varying T_3 Doses on Levels of Malic Enzyme mRNA. One of the interesting features of malic enzyme

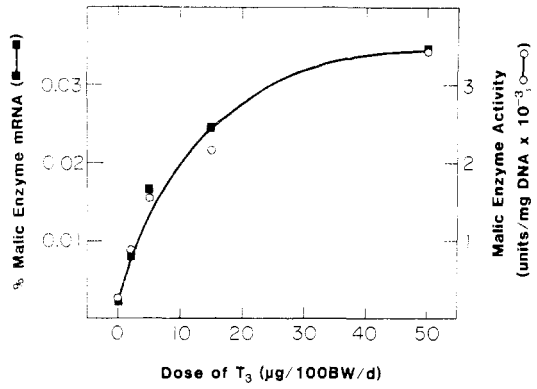

FIGURE 2: T_3 dose–response curve for malic enzyme activity and its mRNA. Normal rats were injected with the indicated doses of T_3 for a period of 7 days. Livers were then removed and assayed for malic enzyme activity (○) or mRNA (■). Translational assays contained 300 μL, and [^{35}S]methionine was used as the label. Malic enzyme was quantitated with the combined affinity resin–immunoprecipitation procedure.

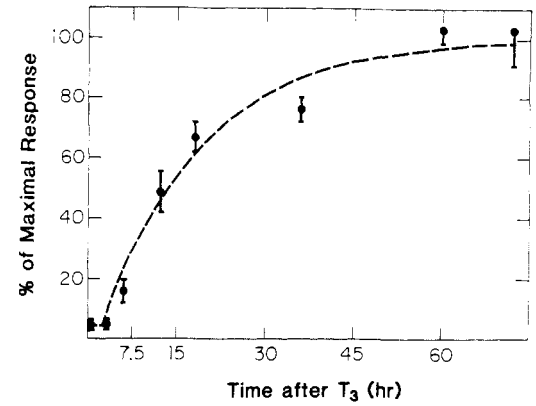

FIGURE 3: Time course of appearance of mRNA for malic enzyme following T_3 administration. Normal rats were injected with 200 μg of T_3 (100 g of body weight)$^{-1}$ at time zero, and then livers were removed at varying times. Poly(A)-containing RNA was extracted and analyzed for malic enzyme mRNA activity. Data points represent the normalized average of three separate experiments (mean ± standard error of the mean). The dashed line represents the theoretical accumulation curve for a product with a $t_{1/2}$ of 12 h and a lag time of 2 h.

induction by T_3 is the apparently nonlinear relationship to nuclear receptor occupancy (Oppenheimer et al., 1978). When T_3 sites are fully saturated, malic enzyme levels are 12–15-fold greater than in the euthyroid state, in which ~50% of nuclear sites are occupied. Many possibilities have been proposed to explain the amplified response of malic enzyme to T_3, including explanations involving T_3 effects on both mRNA production and translation. To better define the problem, we have attempted to determine whether the mRNA coding for malic enzyme also responds in an amplified fashion to T_3 receptor occupancy.

Euthyroid rats were injected with varying doses of T_3 [2–50 μg (100 g of body weight)$^{-1}$ (day)$^{-1}$] for a period of 7 days. At this time, livers were removed and assayed for both cytosolic malic enzyme activity and mRNA activity (Figure 2). As previously reported, malic enzyme activity underwent a dramatic increase over the euthyroid to hyperthyroid transition. At the highest dose of T_3, the level of enzyme activity (3480 units/mg of DNA) was 14.5-fold greater than that of euthyroid animals (350 units/mg of DNA). A similar induction was obtained if enzyme activity was expressed per milligram of cytosolic protein (210 units/mg of protein at 50 μg of T_3 dose; 12 units/mg of protein in euthyroid animal). Levels of malic enzyme mRNA increased proportionally with enzyme activity. Thus, the level of malic enzyme mRNA at the highest T_3 dose (0.035% of total products) was ~13-fold greater than the level in euthryoid animals (0.0027%). We have previously shown the hepatic poly(A)-containing RNA contents of hyperthyroid and euthyroid rat are essentially identical (Towle et al., 1980). Consequently, the percentage of malic enzyme activity is proportional to the level of specific mRNA in these states. The parallel response of both malic enzyme activity and its specific mRNA to T_3 indicates that enzyme induction is due exclusively to hormonal action on mRNA levels. Both responses appear to be amplified with respect to receptor occupancy.

Kinetics of Malic Enzyme mRNA Appearance during T_3 Induction. The time course of accumulation of malic enzyme mRNA activity following T_3 administration is demonstrated in Figure 3. In this experiment, rats were injected with a high dose of T_3 [200 μg of T_3 (100 g of body weight)$^{-1}$] to ensure a maximal rate of mRNA production. The data represent the normalized results of three separate experiments, two using [^{35}S]methionine as the labeled precursor, in which the maximum level of malic enzyme mRNA activity was ~0.04% of the total incorporation, and one using [^{3}H]leucine, in which a final value of 0.10% was reached. (The reason for this difference presumably reflects the relative ratio of [^{35}S]-methionine to [^{3}H]leucine in malic enzyme vs. all other rat liver proteins being synthesized.) Little change can be seen for the first few hours, but by 6 h a 4-fold increase in specific mRNA activity is evident. The half-maximal response is obtained after 14 h, and by 60 h the level of malic enzyme mRNA activity appears to reach a plateau. Extrapolation of the accumulation curve back to the normal euthyroid level of mRNA leads to an estimate of a 2–3-h lag time prior to the earliest T_3 effect. We have previously estimated a similar lag time of 2 h from data on the in vivo relative rate of malic enzyme synthesis (Mariash et al., 1980).

Based on the generally accepted model for the kinetics of induction processes (Segal & Kim, 1963; Berlin & Schimke, 1965; Kafatos, 1972), an estimate of the $t_{1/2}$ of malic enzyme mRNA can be obtained from the accumulation curve. This model assumes that the amount of functional mRNA is determined by a zero-order rate of synthesis and a first-order rate of degradation and that the rate of change of mRNA production from the normal to the T_3-induced state is rapid, relative to the overall time course. In addition, it is assumed that the kinetics of mRNA appearance are dictated by the $t_{1/2}$ of the mRNA and not an unknown intermediate acting in the pathway of T_3 induction of malic enzyme mRNA. The dashed curve in Figure 3 represents the theoretical accumulation of malic enzyme mRNA, assuming a $t_{1/2}$ of 12 h and a lag time of 2 h before T_3 effects are observed. This theoretical accumulation curve provides a reasonable fit to the experimental data within the limits of the assay sensitivity.

Kinetics of Disappearance of Malic Enzyme mRNA following T_3 Withdrawal. The loss of malic enzyme mRNA activity in total cellular poly(A)-containing RNA following withdrawal from T_3 is shown in Figure 4. In this experiment, animals were stimulated by injection with 8 μg of T_3 (100 g of body weight)$^{-1}$ every 12 h for 3 days. This regimen was chosen to produce a near maximally stimulating T_3 dose that would be rapidly cleared from the animal. T_3 plasma levels, measured by radioimmunoassay, had fallen to normal levels by 20 h after the last T_3 injection. Somewhat surprising was the observation that plasma T_3 continued to fall well below euthyroid values and remained low through an additional 40 h. Apparently, the capability of the normal animal to respond

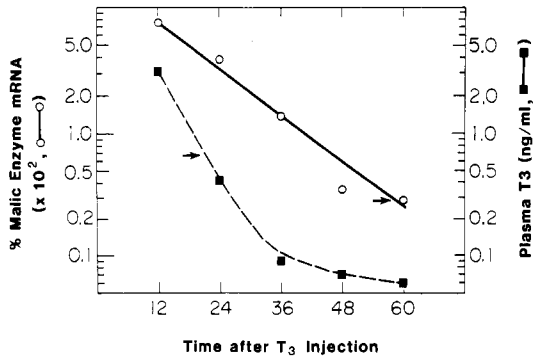

FIGURE 4: Time course of disappearance of malic enzyme mRNA following T_3 withdrawal. Normal rats were injected with 8 μg of T_3 (100 g of body weight)$^{-1}$ every 12 h for 3 days and then withdrawn from hormone treatment for varying periods of time. Plasmas were drawn for measurement of T_3 levels by radioimmunoassay (■), and livers were removed for analysis of malic enzyme mRNA activity (○). Arrows indicate normal levels found in euthyroid animals.

to low plasma T_3 levels with TSH release is transiently blocked following a period of extreme hyperthyroidism (Oppenheimer et al., 1977). The mechanism of this suppression is unknown.

Considering the highly amplified response of the malic enzyme mRNA to T_3, levels of mRNA synthesis should be extremely low in the latter stages of T_3 withdrawal. It is, therefore, possible to obtain an estimate of the $t_{1/2}$ of malic enzyme mRNA from the disappearance curve. The same assumptions discussed for determining the $t_{1/2}$ from the kinetics of appearance apply to this estimate. From the data in Figure 4, we estimate a $t_{1/2}$ of 10 h. This value is in fairly good agreement with that obtained previously from the accumulation curve. Thus, T_3 does not appear to cause a major change in the stability of malic enzyme mRNA.

Model for Enzyme Induction. From the $t_{1/2}$ of malic enzyme mRNA observed in these studies and the previously determined $t_{1/2}$ for malic enzyme itself, it is possible to derive a model for enzyme accumulation. The time course of specific mRNA accumulation is described by

$$\frac{dM_t}{dt} = \alpha q - \lambda_1 M_t \tag{1}$$

where α is the zero-order rate of mRNA production, q is the fractional occupancy of T_3 receptor, λ_1 is the first-order rate of mRNA degradation, and M_t is the induced level of malic enzyme mRNA at any time t. For the purposes of this derivation, we will assume that q is 1 throughout (i.e., all sites remain saturated with T_3), and thus α is a constant. Integration of this equation, with the condition that at $t = 0$, $M_t = 0$, leads to

$$M_t = \frac{\alpha}{\lambda_1}(1 - e^{-\lambda_1 t}) \tag{2}$$

It is likewise possible to express the change of malic enzyme with time by a similar formulation

$$\frac{dE_t}{dt} = \beta M_t - \lambda_2 E_t \tag{3}$$

where β is a proportionality constant between the level of specific mRNA (M_t) and the zero-order rate constant of enzyme production, λ_2 is the first-order rate of enzyme degradation, and E_t is the induced level of malic enzyme at any time t. This formulation assumes that the rate of enzyme synthesis is directly proportional to the level of its mRNA, a reasonable, but untested, assumption. In addition, λ_1 and λ_2 are assumed

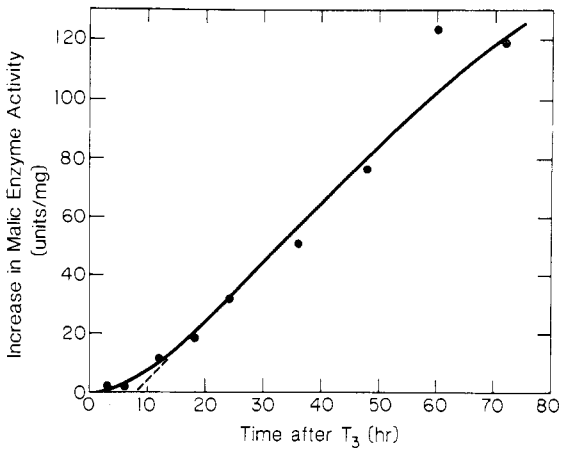

FIGURE 5: Predicted time course for induction of malic enzyme. Based on the known $t_{1/2}$ of malic enzyme (2.8 days) and the $t_{1/2}$ determined for its mRNA (10 h), a theoretical accumulation curve for malic enzyme induction by T_3 was determined. Data points are experimentally determined values of malic enzyme activity obtained from the experiment described in Figure 3. The dotted line represents an extrapolation of the "linear" portion of the curve previously used to estimate a refractory period of 8 h for T_3 action on malic enzyme (Oppenheimer et al., 1977).

to be independent of T_3, as indicated by this study for mRNA and previous work for the enzyme (Oppenheimer et al., 1977). Substituting eq 2 into 3 and integrating with the same limiting condition yields

$$E_t = \frac{\alpha\beta}{\lambda_1\lambda_2} - \left[\frac{\alpha\beta}{\lambda_1(\lambda_2 - \lambda_1)}\right]e^{-\lambda_1 t} + \left[\frac{\alpha\beta}{\lambda_1(\lambda_2 - \lambda_1)} - \frac{\alpha\beta}{\lambda_1\lambda_2}\right]e^{-\lambda_2 t} \tag{4}$$

This equation can clearly be solved if we determine the constants α and β. To estimate these values, we can use the steady-state level of E as $t \to \infty$ in the presence of T_3 (E_{max}). Under steady-state conditions (from eq 1 and 3)

$$E_{max} = \frac{\alpha\beta}{\lambda_1\lambda_2} \tag{5}$$

Solving for $\alpha\beta$ and substituting back into eq 4 yields the final expression:

$$E_t = E_{max} - \left(\frac{\lambda_2 E_{max}}{\lambda_2 - \lambda_1}\right)e^{-\lambda_1 t} + \left(\frac{\lambda_2}{\lambda_2 - \lambda_1} - 1\right)E_{max}e^{-\lambda_2 t} \tag{6}$$

Substituting in the experimentally determined values of λ_1 ($0.0693/h$), λ_2 ($0.0103/h$), and E_{max} (275 units/mg of protein), the accumulation curve shown in Figure 5 was calculated. The experimental points were obtained in the T_3 induction experiment shown in Figure 3.

Although it is undoubtably somewhat simplified, the purpose of this exercise was to examine the initial time course of enzyme induction. Previous studies suggested a refractory period of 8 h prior to enzyme accumulation (Oppenheimer et al., 1977). This value was estimated by extrapolating the "linear" portion of the enzyme accumulation curve in the presence of T_3. Likewise, extrapolation of the theoretical accumulation curve in Figure 5 to the abscissa yields a similar value (dotted line). Nevertheless, evaluation of the accumulation curve reveals that the apparent refractory period is due to the prior requisite of mRNA accumulation; the curve actually has an immediate inflection point. It is not necessary, therefore, to

postulate any discrete refractory period for the action of T_3 on malic enzyme.

Discussion

The intracellular mechanism of action of thyroid hormone remains largely unknown. Currently, many of the actions of thyroid hormone are thought to be initiated through interaction of hormone with a specific receptor protein located in the nucleus [for review, see Samuels (1978) and Oppenheimer (1979)]. Subsequent to hormone binding, the chain of events leading to altered physiological action are unclear. Tata and co-workers suggested in the early 1960s that nuclear RNA synthesis was altered as a primary response to thyroid hormone (Tata & Widnell, 1966). This suggestion was based on studies measuring whole nuclear RNA polymerase activities and rates of incorporation of precursor into RNA in rat liver following administration of T_3 to thyroidectomized animals. Further development, however, was hampered by the lack of specific models analogous to the induction of ovalbumin in chick oviduct (O'Malley & Means, 1974; Palmiter, 1975; Hynes et al., 1977) or vitellogenin in roosters (Tata, 1976; Burns et al., 1978; Jost et al., 1978) by estrogen. Recently, three such potential models have been developed for studying thyroid hormone action. In rat pituitary or cell lines derived from rat pituitary tumors, the production of growth hormone and its mRNA has been shown to be dependent on the levels of thyroid hormone (Seo et al., 1977, 1979; Martial et al., 1977a). Glucocorticoids appear to act synergistically to influence growth hormone production (Martial et al., 1977b; Shapiro et al., 1978; Samuels et al., 1979). In rat liver, the production of the protein α_{2u}-globulin requires the presence of at least four hormones: testosterone, glucocorticoid, growth hormone, and thyroid hormone (Kurtz & Feigelson, 1978). The presence of thyroid hormone has been shown to be necessary, but not sufficient, for maintaining mRNA levels for this protein (Kurtz et al., 1976; Roy et al., 1976). Finally, as demonstrated by Goodridge and co-workers with avian hepatocytes in culture (Silpanata & Goodridge, 1971; Goodridge & Adelman, 1976; Goodridge, 1978; Goodridge et al., 1979) and by us in rat liver (Towle et al., 1980), malic enzyme production provides another potential model system for the study of thyroid hormone action.

The fact that malic enzyme is influenced by a direct action of T_3 on the liver, rather than through a secondary action such as the stimulation of pituitary growth hormone production, is supported by several lines of investigation. First, Goodridge has shown in hepatocytes derived from chick embryos that the addition of thyroid hormone alone to the culture media can increase the synthesis of malic enzyme up to 100-fold over base line levels (Goodridge & Adelman, 1976). Second, treatment of hypophysectomized rats with thyroid hormone leads to a similar increase in hepatic malic enzyme activity as seen upon treatment of thyroidectomized rats; growth hormone treatment did not have any effect on malic enzyme activity (Ruegamer et al., 1965; Simat et al., 1980). Finally, the lag time of approximately 2–3 h between hormone administration and changes in malic enzyme mRNA levels resembles the lag time seen for many other hormonally responsive systems (Palmiter et al., 1976).

Certain reservations must be made when using a cell-free translational assay to quantitatively measure specific mRNA levels. One limitation concerns the possibility of varying translational efficiencies among individual mRNA species in the reticulocyte system. If mRNA coding for malic enzyme were translated either more or less efficiently than the average liver mRNA molecule, the percentage of malic enzyme mRNA could be overestimated or underestimated, respectively. However, it would seem reasonable to assume that malic enzyme mRNA, isolated free of cellular proteins from varying thyroidal states of the rat, would not vary in its inherent translational efficiency and, thus, the relative changes in mRNA levels would be valid. A second consideration in the quantitation of malic enzyme mRNA is whether the purified, native, tetrameric malic enzyme used as a standard to quantitate recovery is recognized to the same extent as the translated malic enzyme polypeptide by affinity resin and antibody to purified malic enzyme. Again, if a difference did exist, the absolute quantity of malic enzyme mRNA could be over- or underestimated to some degree. Nevertheless, relative levels of specific mRNA compared between different animals should be quantitatively appropriate.

Keeping these reservations in mind, we have attempted to follow the response of malic enzyme mRNA levels to thyroid hormone administration. Studies of the relationship between receptor occupancy and response are useful in attempting to understand hormone action. For malic enzyme, a highly amplified relationship has been described (Oppenheimer et al., 1978). Most of the response to thyroid hormone occurs as receptor occupancy increases above 50%. The parallel nature of the dose–response curves for malic enzyme activity and its mRNA indicates that the signal for amplification is occurring at a pretranslational level. This finding effectively eliminates several postulated explanations for amplification that we had previously suggested (Oppenheimer et al., 1978). For instance, the possibility that thyroid hormone may stimulate both malic enzyme mRNA and elements of the translational machinery leading to a multiplicative effect can be ruled out. In such a case the relative increase in malic enzyme activity would have to be greater than that for the mRNA activity. Also, postulates involving concentration effects on the assembly of the subunits of this tetrameric protein can be argued against on similar grounds. The explanation for the amplified response of malic enzyme mRNA to T_3, however, remains unknown. The influence of postreceptor modulation of the malic enzyme response must be considered as a possibility. Malic enzyme in rat liver is a lipogenic enzyme which can be induced by high-carbohydrate, fat-free diets (Fitch & Chaikoff, 1960; Pande et al., 1964; Tepperman & Tepperman, 1964; Wise & Ball, 1964). When animals are fed such diets, the dose of T_3 required to achieve 50% of the maximal response is reduced ~5-fold compared to animals maintained on standard laboratory chow (Mariash et al., 1980). We have previously shown that the induction of malic enzyme by the high-carbohydrate, fat-free diet is accompanied by a proportional increase in mRNA for malic enzyme (Towle et al., 1980). Thus, the regulation of malic enzyme mRNA involves not only hormonal but also dietary factors. We have previously suggested that the regulatory pathway of T_3 for malic enzyme interacts with an unknown signal generated in response to the high-carbohydrate, fat-free diet (Mariash et al., 1980). If T_3 were to also influence independently the formation of the diet-induced signal, the interaction of the two stimuli would result in an amplified response.

The induction of malic enzyme mRNA by thyroid hormone could result from either an increased rate of formation of specific mRNA or a decreased rate of mRNA degradation. Several examples of hormonal influences on specific mRNA stability have been reported. The induction of casein mRNA by prolactin was found to be due to a 20-fold increase in the half-life of specific mRNA in the presence of hormone, coupled with a 2–4-fold increase in the rate of mRNA formation (Guyette et al., 1979). Likewise, ovalbumin mRNA in chick

oviduct had a half-life of 24–36 h in the presence of estrogen, but in the absence of hormone it is degraded completely within 4 h (Palmiter & Carey, 1974; Cox, 1977). Both of these proteins represent differentiation-specific products made in great abundance in the hormone target tissue. In such cases, the maximal production of the product from a limited set of gene copies may have required control at many levels.

To explore the possibility that T_3 might influence the degradation of malic enzyme mRNA, we studied the kinetics of mRNA appearance following hormone administration and disappearance following hormone withdrawal. In the appearance study, T_3 was present at high levels throughout the time course, whereas in the disappearance study, T_3 was rapidly reduced to below normal levels. In either case, the $t_{1/2}$ of malic enzyme mRNA was estimated at between 10 and 12 h. The $t_{1/2}$ would have to change by over 10-fold to account for the accumulation of mRNA based solely on a T_3-induced change in mRNA stability. It is important to recognize that the estimate of $t_{1/2}$ from the kinetics of appearance and disappearance is an indirect measurement. This method assumes that the kinetics are being determined by the $t_{1/2}$ of malic enzyme mRNA. It is conceivable that these kinetics are being dictated by an unknown intermediate in the pathway between the T_3–receptor interaction and malic enzyme mRNA production with a $t_{1/2}$ of 10–12 h. In such a case, malic enzyme mRNA would have a shorter $t_{1/2}$. Since we know of no direct evidence for such an intermediate, we tentatively conclude that the $t_{1/2}$ of malic enzyme mRNA is being measured and is not affected by T_3 levels of the animal. Final proof of this hypothesis awaits direct measurement of the mRNA for malic enzyme by hybridization techniques.

The determination of a $t_{1/2}$ of 10 h for malic enzyme mRNA disappearance following T_3 withdrawal is inconsistent with the previous suggestion of a "long-lived" mediator of thyroid hormone action. The long-lived intermediate was inferred from published data which indicated that the hepatic effects of thyroid hormone decay with a common $t_{1/2}$ varying between 3 and 6 days (Oppenheimer et al., 1972). Clearly the $t_{1/2}$ of malic enzyme mRNA disappearance is much shorter. The similarity previously observed in $t_{1/2}$'s of T_3 responses may simply reflect the similarity in degradation constants of many enzymes in rat liver. The participation of a long-lived mediator was also suggested by experiments with α-amanitin, an inhibitor of RNA polymerase II (Dillmann et al., 1977). Administration of α-amanitin to hypothyroid or euthyroid rats together with T_3 was found to inhibit the normal induction of malic enzyme for the first 24 h. Following this inhibition, however, an unexpected and striking rise in the rate of enzyme appearance occurred. Residual T_3 from the initial injection of the animal was not sufficient to account for the delayed enzyme appearance, and, thus, the results were interpreted as being consistent with the long-lived mediator of thyroid action. Since the $t_{1/2}$ of malic enzyme mRNA disappearance is inconsistent with the long-lived intermediate, an alternate explanation of the observations with α-amanitin must be evoked. At the present time, this explanation is unknown.

The assignment of a $t_{1/2}$ of 10–12 h has allowed us to test the validity of a formal model defining the relationship of malic enzyme to its specific RNA under conditions of full nuclear receptor occupancy. This model, implicit in eq 1 and 3, holds that the fractional rate of accumulation of mRNA at any given level of nuclear occupancy will depend on the fractional removal rate of the mRNA and that the rate of formation of malic enzyme is dependent on both the rate of mRNA accumulation and the fractional rate of removal of malic enzyme. The constancy of fractional removal rates of mRNA and malic enzyme during the course of the experiment has been shown. The model assumes that the rate of malic enzyme synthesis is directly proportional to the concentration of its mRNA. The excellent correspondence between the theoretical projection and the experimentally determined points illustrated in Figure 5 strongly supports the underlying assumptions made.

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References

Berlin, C. M., & Schimke, R. T. (1965) *Mol. Pharmacol. 1*, 149–156.

Brodelius, P., Larsson, P. O., & Mosbach, K. (1974) *Eur. J. Biochem. 47*, 81–89.

Burns, A. T. H., Deeley, R. G., Gordon, J. I., Udell, D. S., Mullinex, K. P., & Goldberger, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1815–1819.

Cox, R. F. (1977) *Biochemistry 16*, 3433–3443.

Dillmann, W. H., Schwartz, H. L., Silva, E., Surks, M. I., & Oppenheimer, J. H. (1977) *Endocrinology (Philadelphia, PA) 100*, 1621–1627.

Fitch, W. M., & Chaikoff, I. L. (1960) *J. Biol. Chem. 235*, 554–557.

Gibson, D. M., Lyons, R. T., Scott, D. F., & Muto, Y. (1972) *Adv. Enzyme Regul. 10*, 187–204.

Giles, K. W., & Myers, A. (1965) *Nature (London) 206*, 93.

Goodridge, A. G. (1978) *Mol. Cell. Endocrinol. 11*, 19–29.

Goodridge, A. G., & Adelman, T. G. (1976) *J. Biol. Chem. 251*, 3027–3032.

Goodridge, A. G., Civelli, O., Yip, C., & Scherrer, K. (1979) *Eur. J. Biochem. 96*, 1–8.

Guyette, W. A., Matusik, R. J., & Rosen, J. M. (1979) *Cell (Cambridge, Mass.) 17*, 1013–1023.

Hsu, R. Y., & Lardy, H. A. (1967) *J. Biol. Chem. 242*, 520–526.

Hynes, N. E., Groner, B., Sippel, A. E., Nguyen-Huu, M. C., & Schutz, G. (1977) *Cell (Cambridge, Mass.) 11*, 923–932.

Isohashi, F., Shibayama, K., Maruyama, E., Aoki, Y., & Wada, F. (1971) *Biochim. Biophys. Acta 250*, 14–24.

Jost, J. P., Ohno, T., Panyim, S., & Schuerch, A. R. (1978) *Eur. J. Biochem. 84*, 355–361.

Kafatos, F. C. (1972) *Curr. Top. Dev. Biol. 7*, 125–191.

Kurtz, D. T., & Feigelson, P. (1978) *Biochem. Actions Horm. 5*, 433–455.

Kurtz, D. T., Sippel, A. E., Ansah-Yiadom, R., & Feigelson, P. (1976) *J. Biol. Chem. 251*, 3594–3598.

Li, J. J., Ross, C. R., Tepperman, H. M., & Tepperman, J. (1975) *J. Biol. Chem. 250*, 141–148.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mariash, C. N., Kaiser, F. E., Schwartz, H. L., Towle, H. C., & Oppenheimer, J. H. (1980) *J. Clin. Invest. 65*, 1126–1134.

Martial, J. A., Baxter, J. D., Goodman, H. M., & Seeburg, P. H. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1816–1820.

Martial, J. A., Seeburg, P. H., Guenzi, D., Goodman, H. M., & Baxter, J. D. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4293–4295.

Murphy, G., & Walker, D. G. (1974) *Biochem. J. 144*, 149–160.

O'Malley, B. W., & Means, A. R. (1974) *Science (Washington, D.C.) 183*, 610–620.

Oppenheimer, J. H. (1979) *Science (Washington, D.C.) 203*, 971-979.
Oppenheimer, J. H., Surks, M. I., & Schwartz, H. L. (1972) *J. Clin. Invest. 51*, 2796-2807.
Oppenheimer, J. H., Silva, E., Schwartz, H. L., & Surks, M. I. (1977) *J. Clin. Invest. 59*, 517-527.
Oppenheimer, J. H., Coulombe, P., Gutfeld, N. W., & Schwartz, H. L. (1978) *J. Clin. Invest. 61*, 987-997.
Palmiter, R. D. (1975) *Cell (Cambridge, Mass.) 4*, 189-197.
Palmiter, R. D., & Carey, N. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2357-2361.
Palmiter, R. D., Moore, P. B., Mulvihill, E. R., & Emtage, S. (1976) *Cell (Cambridge, Mass.) 8*, 557-572.
Pande, S. V., Khan, R. P., & Venkitasubramanian, T. A. (1964) *Biochim. Biophys. Acta 84*, 239-250.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247-256.
Rice, R. H., & Means, G. E. (1971) *J. Biol. Chem. 246*, 831-832.
Roy, A. K., Schiop, M. J., & Dowbenko, D. J. (1976) *FEBS Lett. 64*, 396-399.
Ruegamer, W. R., Newman, G. H., Richert, D. A., & Westerfeld, W. W. (1965) *Endocrinology (Philadelphia, PA) 77*, 707-715.
Samuels, H. H. (1978) *Recept. Horm. Action 3*, 35-74.
Samuels, H. H., Stanley, F., & Shapiro, L. E. (1979) *Biochemistry 18*, 715-721.
Segal, H. L., & Kim, Y. S. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 912-918.
Seo, H., Vassart, G., Brocas, H., & Refetoff, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2054-2058.
Seo, H., Refetoff, S., Martino, E., Vassart, G., & Brocas, H. (1979) *Endocrinology (Baltimore) 104*, 1083-1090.
Shapiro, L. E., Samuels, H. H., & Yaffe, B. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 45-49.
Silpanata, P., & Goodridge, A. G. (1971) *J. Biol. Chem. 246*, 5754-5761.
Simat, B. M., Towle, H. C., Schwartz, H. L., & Oppenheimer, J. H. (1980) *Endocrinology (Baltimore) 107*, 1338-1344.
Tata, J. R. (1976) *Cell (Cambridge, Mass.) 9*, 1-14.
Tata, J. R., & Widnell, C. C. (1966) *Biochem. J. 98*, 604-620.
Tepperman, H. M., & Tepperman, J. (1964) *Am. J. Physiol. 206*, 357-361.
Towle, H. C., Mariash, C. N., & Oppenheimer, J. H. (1980) *Biochemistry 19*, 579-585.
Wise, E. M., & Ball, E. G. (1964) *Proc. Natl. Acad. Sci. U.S.A. 52*, 1255-1263.

Kinetic and Equilibrium Studies of Concanavalin A Activation by Calcium Ions†

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ABSTRACT: The kinetics of conversion of concanavalin A (Con A) to the saccharide-binding conformation by Ca^{2+} alone were studied as a function of pH by monitoring the fluorescence quenching of 4-methylumbelliferyl α-D-mannopyranoside. In the pH range 6.0-7.2, the protein conformational change associated with metal ion binding occurs upon binding of only one Ca^{2+} per P monomer. When sufficient Ca^{2+} is present to saturate the single Ca^{2+} site on P, the rate constant for the locking process, k_2, is independent of pH between 6.4 and 7.2 and furthermore is identical within experimental error with k_2 for Ca^{2+} in the presence of Mn^{2+}. Equilibrium dialysis studies demonstrate that only one Ca^{2+} is bound in the final CPL conformation with a $K_D = 23 \pm 5$ μM at 25 °C. Ca^{2+} has been completely removed from CPL by gel filtration techniques, and the resulting metal-free PL structure has no measurable affinity for saccharides. The near-UV circular dichroism spectrum of CPL is identical with that of native Con A (CMPL) while the spectrum of PL is different from those of both P and CPL. The activation energy for the Ca^{2+}-induced locking process is 14 kcal/mol at both pH 6.4 and pH 7.4 and is completely unaffected by the presence of Mn^{2+} at the higher pH. These data are consistent with a recent crystallographic report [Shoham, M., Yonath, A., Sussman, J. L., Moult, J., Traub, W., & Kalb, A. J. (1979) *J. Mol. Biol. 131*, 137-155] of minor structural differences between native and demetalized Con A. We propose that Ca^{2+} binding at S2 produces a general ordering of the ligands at this site which in turn orders those side-chain residues involved in saccharide recognition.

Concanavalin A (Con A)[1] is a metalloprotein isolated from the jack bean which has been used extensively in the study of cell surfaces. Con A is composed of identical subunits and undergoes a pH-dependent association from a primarily dimeric form below pH 6 to a tetrameric form above pH 7. The metal ions Mn^{2+} and Ca^{2+} are known to play a fundamental role in maintaining the conformational form of Con A which possesses a specific saccharide-binding site. Many of the early metal binding studies were carried out below pH 6 to avoid possible complications arising from the dimer ⇄ tetramer equilibrium (Kalb & Levitzki, 1968; Shoham et al., 1973; Sherry & Cottam, 1973). The results of this early work led

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[1] Abbreviations used: Con A, concanavalin A; EDTA, ethylenediaminetetraacetate; MUM, 4-methylumbelliferyl α-D-mannopyranoside; CD, circular dichroism; C, divalent calcium; M, divalent manganese or any other divalent first-row transition-metal ion where noted; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)-ethanesulfonic acid; αMeMann, methyl α-D-mannoside.

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