A cis-active regulatory gene in the mouse: Direct demonstration of cis-active control of the rate of enzyme subunit synthesis

(mitochondrial malic enzyme/brain/isoelectric focusing/antibody)

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ABSTRACT Mouse mitochondrial malic enzyme [l-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] is a tetrameric protein. Two alleles of the structural gene (Mod-2) are known which code for electrophoretically distinct enzyme subunits: Mod-2a and Mod-2b. A regulatory gene (Mdr-1), closely linked to Mod-2 on chromosome 7, determines the rate of mitochondrial malic enzyme synthesis in brain. Two alleles of Mdr-1 are known: Mdr-1a (high activity) and Mdr-1b (low activity). By pulse-labeling with [35S]methionine, immune precipitation, and isoelectric focusing under dissociating conditions, we have measured the relative rates of synthesis of the two types of enzyme subunit in animals of genotypes Mdr-1a Mod-2a/Mdr-1a Mod-2b and Mdr-1b Mod-2a/Mdr-1b Mod-2b. The results show that in the former animals both types of subunit are made at an identical rate, whereas in the latter animals the Mod-2b gene product is synthesized at a rate 2.5 times that of the Mod-2b-coded subunit. Thus we have unambiguously demonstrated that Mdr-1 is cis-active in its control of the expression of the Mod-2 structural gene.

Mitochondrial malic enzyme [l-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40] in mouse brain has been shown to be regulated by a gene (Mdr-1) that is closely linked to the gene (Mod-2) that codes for the enzyme (1, 2). Inbred strains of genotype Mdr-1a/Mdr-1a have a 2- to 3-fold higher specific enzyme activity than do Mdr-1b/Mdr-1b animals. The difference in enzyme activity is quantitatively accounted for by the rates of enzyme synthesis in brain (2). It is of special interest that Mdr-1 has no effect on the expression of Mod-2 in heart (1).

The clear establishment of regulatory activity in cis or trans configurations is a central point in discerning the mechanisms by which enzyme synthesis is genetically regulated in mammals (3–5). Since the regulation of Mod-2 expression in brain by Mdr-1 (1, 2) has been shown to occur by an effect on the rate of enzyme synthesis, a direct demonstration of cis or trans action of Mdr-1 consists in measuring the relative rates of synthesis of enzyme subunits coded for by Mod-2a and Mod-2b genes in heterozygotes that differ in their genetic relationship to the Mdr-1 alleles. The results of such experiments are reported here. The conclusions reached do not depend on assumptions about the stability and catalytic activity of the isozenzymic forms of a multimeric enzyme that are synthesized in animals heterozygous for subunit structural genes.

MATERIALS AND METHODS

Mice. The Mod-2a allele was from strain SM/JR1, which is Mdr-1a. Mod-2b was from either stock 4 animals (Mdr-1) or strain FS/E1R1 (Mdr-1b). The appropriate crosses gave F1 animals of the required genotypes.

Determination of pI of Mitochondrial Malic Enzyme. Mitochondrial pellets were disrupted as described (6), centrifuged at 17,600 X g for 10 min, and heated at 50°C for 5 min. After centrifugation for 10 min at 12,000 rpm, the supernatant was applied to a column of ADP-Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl, pH 7.5/25 mM KCl/1% Triton X-100. After the column was washed with equilibration buffer, the enzyme was eluted with NADP+ at 500 μg/ml, dialyzed, and applied to a 110-ml isoelectric focusing column containing a 5–50% sucrose gradient and 1.1% Ampholines. Focusing was carried out for 48 hr at an initial setting of 500 V and 4°C. Determinations of pH and enzyme activity were made on each fraction.

Enzyme Assay. Enzyme activity was determined by following spectrophotometrically the reduction of NADP+ in a reaction mixture containing 40 mM triethanolamine-HCl (pH 7.5), 4 mM MnCl2, 0.34 mM NADP+, and 5 mM potassium malate (pH 7.6). One enzyme unit catalyzes the reduction of 1 μmol of NADP+ per min. Specific activity is presented as units/mg of protein; protein determinations were by a modification of the method of Lowry et al. (7).

Immune Precipitation of Enzyme. For immune titrations of enzyme from animals carrying different alleles at Mod-2, centrifuged mitochondrial extracts were incubated with varying amounts of antibody (1) for 60 min at 57°C and then on ice for 4 hr. Samples (200 μl) were layered over a 30% (wt/vol) sucrose cushion (2) and centrifuged for 5 min in an Eppendorf tabletop centrifuge. Enzyme remaining in the supernatant was determined by enzyme assay.

Rates of Subunit Synthesis. Brains of anesthetized animals were injected with [35S]methionine (Amersham) as described (2); 3 hr later the mice were killed and their brains were homogenized. Mitochondrial extracts were prepared, heated 8 min at 50°C, and centrifuged. The supernatant was applied to a column of NADP+-agarose (P-L Biochemicals) equilibrated in 50 mM Tris-HCl, pH 7.5/25 mM KCl/5 mM MgCl2/1% Triton X-100. The enzyme was eluted with NADP+ at 500 μg/ml. Deoxycholate was added to 1%, followed by an amount of antibody sufficient to precipitate more than 90% of the enzyme present (1). After incubation for 60 min at 37°C and overnight on ice, the precipitate was collected by centrifugation and washed three times. It was then dissolved in 9.5 M urea/3% (wt/vol) Nonidet P-40/2% (wt/vol) 2-mercaptoethanol/0.05% sodium dodecyl sulfate, in which it was frozen and thawed two or three times. The sample was applied to an isoelectric focusing gel containing 9.0 M urea, 4.5% (wt/vol) acrylamide, 2% Nonidet P-40, and 2% Ampholines (LKB; 4 vol at pH 6–8; 1 vol at pH 3–10). Focusing was carried out overnight at 400 V and then for 1 hr at 500 V. Gels were removed from their tubes, frozen, sliced, oxidized overnight in 30% H2O2 at 70°C, and assayed in Aquasol (New England Nuclear) in a liquid scintillation spectrometer.

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After focusing, enzyme activity was assayed for enzyme activity (O) and pH (●).

RESULTS

An electrophoretic variant of Mod-2 (Mod-2a) carried by strain SM/J (8) was compared with the more common Mod-2b gene product by preparative isoelectric focusing of active (tetrameric) mitochondrial malic enzyme. The difference in isoelectric point between the two forms was only about 0.3 pH unit (Fig. 1). This value is consistent with a single charge difference between them. The results essentially rule out the possibility of resolving the five species of tetramer expected in a heterozygous animal (Mod-2a/Mod-2b). Nevertheless, it seemed possible that, if the enzyme could be completely dissociated into subunits, one could resolve the Mod-2a polypeptide product from its Mod-2b counterpart by isoelectric focusing in a narrow pH gradient in polyacrylamide gels.

The specific activities of malic enzyme in brain mitochondria from animals of different genotypes are given in Table 1. The question to be answered is whether the intermediate specific activity (0.029 unit/mg of protein) of the doubly heterozygous class (Mdr-1a Mod-2a/Mdr-1b Mod-2b) reflects an equal but intermediate rate of synthesis of both types of subunit, implying some trans action, or rather represents a Mod-2a gene that produces more gene product than does the Mod-2b due to its linkage to Mdr-1a (cis activity of Mdr-1).

To resolve this question we pulse-labeled brains of animals of different genotypes with [15S]methionine, partially purified the enzyme, and immunoprecipitated it. When the washed precipitate was dissolved under strongly dissociating conditions and focused in polyacrylamide gels, only one peak of radioactivity was found in precipitates from Mod-2a/Mod-2a animals and one of different mobility was found in precipitates from Mod-2a/Mod-2b animals (Fig. 2 A and B). Our previous work (2) has shown that the relative rate of enzyme synthesis is lower in Mdr-1a Mod-2b/Mdr-1a Mod-2b animals than in Mdr-1a Mod-2b/Mdr-1b Mod-2b strains and that this difference accounts totally for the difference in enzyme activity levels. It is possible to measure the relative rates of synthesis of the two types of subunit produced in Mod-2 heterozygotes. The heterozygotes in Fig. 2 C were Mdr-1a Mod-2a/Mdr-1a Mod-2b; the ratio of their rates of synthesis is 0.98. In contrast to this result, the heterozygotes in Fig. 2D, which were Mdr-1a Mod-2a/Mdr-1b Mod-2b, showed a 2.2-fold excess of synthesis of the subunits coded by Mod-2a relative to those coded by Mod-2b. Each of these results was consistently obtained in repeated experiments.

The experiments shown in Fig. 3 demonstrate that the antibody precipitates enzyme from both types of Mod-2 heterozygote identically. Thus, no selection for tetramers containing more or less of one type of subunit occurs. It should also be noted that the results in Fig. 2C, clearly demonstrating equal incorporation into both types of subunit when both Mod-2 genes are associated with Mdr-1, rule out the possibility of the data in Fig. 2D being accounted for by an unequal number of methionine residues in the two subunits. We conclude that Mdr-1

Table 1. Specific activity of mitochondrial malic enzyme in mice carrying different combinations of alleles at Mdr-1 and Mod-2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Specific activity, units/mg protein</th>
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<tbody>
<tr>
<td>Mdr-1a Mod-2a/Mdr-1a Mod-2a</td>
<td>0.050 ± 0.005 (3)</td>
</tr>
<tr>
<td>Mdr-1a Mod-2a/Mdr-1b Mod-2b</td>
<td>0.051 ± 0.004 (3)</td>
</tr>
<tr>
<td>Mdr-1a Mod-2b/Mdr-1b Mod-2b</td>
<td>0.029 ± 0.003 (3)</td>
</tr>
<tr>
<td>Mdr-1b Mod-2b/Mdr-1b Mod-2b</td>
<td>0.016 ± 0.001 (2)</td>
</tr>
</tbody>
</table>

* Each value represents the mean (±SEM) of the number of determinations given in parentheses.
regulates the rate of synthesis of the Mod-2 gene product by action in the cis configuration.

**DISCUSSION**

The tissue-specific action of Mdr-1 (1) in controlling the expression of Mod-2 is a key feature of this locus. Previous characterization of the enzyme from heart and brain (1), the demonstration that c-locus deletions including Mod-2 give an identical gene dosage effect on the enzyme in heart and brain (2), and the results given in this report showing that an electrophoretic variant of the enzyme known to be expressed in heart is the form expressed in brain provide proof that both heart and brain express the same structural gene. Furthermore, Mod-2b-coded subunits may be associated with either Mdr-1a or Mdr-1b. It is thus unlikely that any coding sequences of Mod-2 are involved in the regulation of subunit synthesis.

Previous experiments designed to detect cis or trans activity of regulatory loci in maize (9), Drosophila (10), and the mouse (11, 12) were based on histochemical staining of gels. In each case the criterion for regulation in cis was the occurrence of a shift in the isozyme pattern which indicated an excess of one type of enzyme subunit over the other. Such experiments are inherently qualitative, particularly when total resolution of the isozyme bands cannot be achieved. Furthermore, interpretation of the results requires the assumptions that enzyme subunit associations proceed randomly and that all of the isozyme forms visualized on the gel have identical catalytic properties (only enzyme activity is noted). There is no reason to believe that either of these assumptions should always hold. For example, the $a_2 \beta^+ \beta^d$ molecule is not present in the blood of animals heterozygous at Hbb (Hbb/Hbb). The catalytic properties of the various tetramers studied in the mouse (11, 12) have not been documented. Because our experiments do not draw conclusions from the frequency of the various tetrameric forms of the enzyme in Mod-2a/Mod-2b animals, but rather measure directly the relative rates of synthesis of the two polypeptide chains, the interpretation of the results is not dependent upon undetermined factors concerning the assembly of the tetrameric enzyme.

The key factor to be considered in the present work is whether the two peaks observed are in fact the malic enzyme subunits, because catalytic activity is not demonstrable under the focusing conditions used. Both the magnitude and direction of the difference in $pI$ between the peaks are totally consistent with the $pI$ values determined for the active enzyme (Fig. 1). Sodium dodecyl sulfate gel electrophoresis of the immune precipitates shows only one peak of radioactivity, as was described for Mod-2b/Mod-2b (2) whose mobility corresponds exactly to that of the subunit from the pure enzyme (1, 2). Two peaks are seen only in Mod-2a/Mod-2b heterozygotes (Fig. 2). Taken together, these observations establish that the two peaks seen in heterozygotes are enzyme subunits. In addition, they provide support for the idea that the enzyme in Mod-2 homozgyotes consists of four identical polypeptide chains, because both types of homozygote show only one band on gels that separate on the basis of either charge or molecular weight.

Although our results have conclusively demonstrated cis-active regulation of the rate of enzyme subunit synthesis, the mechanism by which Mdr-1 functions is not understood at present. One can imagine several possible means by which it exerts its effect on Mod-2, including controlling the rate of Mod-2 transcription, determining the rate of Mod-2 mRNA processing, and affecting the stability of Mod-2 mRNA or the rate at which it is translated. The evidence supports the idea that Mdr-1 lies outside the coding sequences of Mod-2, but we have not yet determined the positions of these two sites relative to one another. This is a question of some importance with respect to the mode of action of Mdr-1. For example, it is even possible that Mdr-1 lies within Mod-2 in a noncoding region of the gene. The fact that Mdr-1 does not exert its effect in heart (1) makes it more likely that it is the quantity of Mod-2 mRNA that is influenced by Mod-1 than that the quality of the message differs in Mod-1a/Mod-1a and Mod-1b/Mod-1b animals. Further experiments are required to determine the precise means by which Mdr-1 controls the level of Mod-2 expression in brain.

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References: