In vivo and in vitro Molecular Hybridization of Malate Dehydrogenase Isozymes

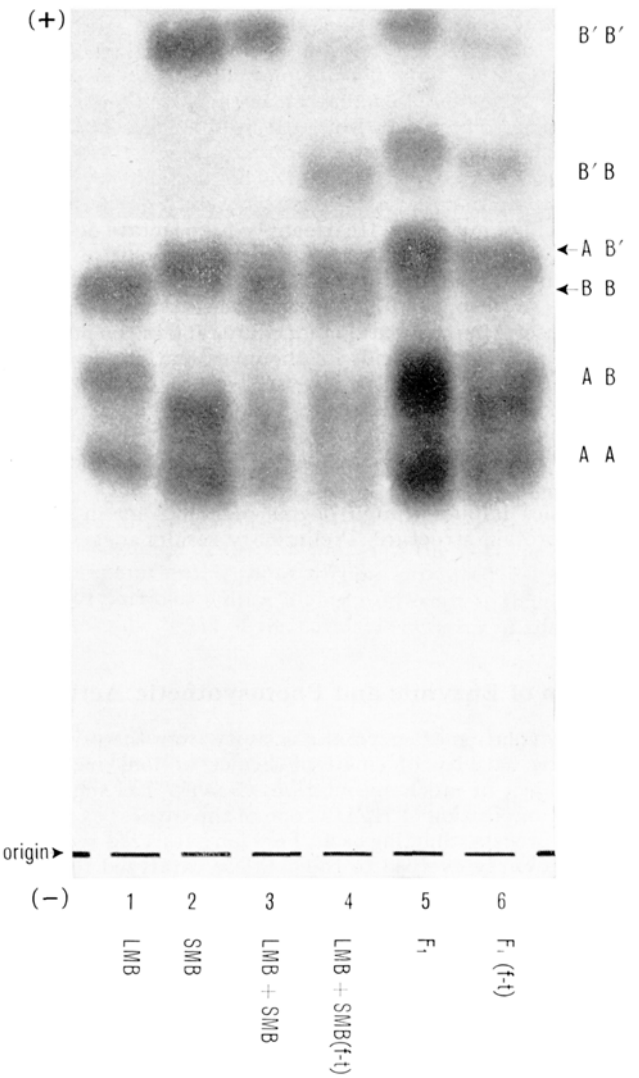
Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37) (MDH) commonly exists as a dimer with a molecular weight of the order of 60,000[1]. The MDH isozymes[2] in the mitochondrial and the supernatant fractions are under the control of separate genetic loci[3–8]. Additional isozymes may result from allelic variation[3–8] or from different conformational forms of the same enzyme[9,10]. The subunit composition of these isozymes may be studied by reversible denaturation, using guanidine-HCl, urea, or acid[6,9–12]. In the same manner, hybrid dimers have been prepared in vitro from subunits from different species as well as from subunits derived from mitochondrial and supernatant fractions of the same organism[13]. These molecular hybridization techniques have occasionally been useful in clarifying the results of genetic investigations[3–6,8], but the techniques are inconvenient, result in great loss of enzyme activity, and therefore have limited application. We have developed a simpler technique based upon the freeze-thaw procedure first employed for lactate dehydrogenase[14].

Our studies have been primarily concerned with MDH from the largemouth bass (*Micropterus salmoides*), the smallmouth bass (*M. dolomieui*), and the F_1 interspecific hybrid. Samples of skeletal muscle from each fish were homogenized in 2–3 volumes of 0.1 *M* sodium phosphate buffer, pH 7.5, and centrifuged twice at 48,000*g* for 30 min. The isozymes in the supernatant fraction were separated by vertical starch gel electrophoresis in a discontinuous *Tris*-citrate buffer system at pH 6.8[7]. Tetrazolium staining specific for MDH was used to visualize the enzyme[7].

The Figure shows the isozyme patterns of the 2 parental species and the hybrid. On the basis of genetic analyses to be reported elsewhere, we have demonstrated that the 3 bands of the largemouth and smallmouth bass result from the random assortment of the gene products of 2 distinct loci. In vitro molecular hybridization was used to investigate the subunit composition of the F_1 isozymes.

Equal volumes of crude enzyme extract from each parental species were mixed. The solution was brought to 0.5 *M* in NaCl by the addition of 0.14 ml of 4 *M* NaCl in 0.1 *M* sodium phosphate buffer, pH 7.5, per ml of combined extracts. Freezing in a dry ice-methanol bath was followed by storage at −70 °C for 2 h. After thawing slowly at room temperature, the mixture was centrifuged at 48,000*g* at 4 °C for 30 min, and the supernatant was subjected to electrophoresis as described above.

As seen in the Figure, the simple addition of enzyme extracts, without freezing, from smallmouth and largemouth bass does not produce the second most anodal isozyme (BB′) of the hybrid. However, when this same mixture is subjected to a freeze-thaw cycle, an isozyme band is generated which is identical in electrophoretic mobility to the characteristic band (BB′) of the hybrid. This isozyme is absent in samples of parental extracts frozen separately, and the freeze-thaw cycle apparently does not significantly alter the pattern of the F_1 hybrid. These results strongly suggest that the second most



Malate dehydrogenase. Freeze-Thaw hybridization. Starch gel electrophoresis of crude muscle homogenates followed by specific tetrazolium staining for MDH. 1. Largemouth bass. 2. Smallmouth bass (the isozyme band anodal to the AA homopolymer in this species appears to be the result of a conformational variant of this isozyme). 3. Simple mixture of parental extracts without freezing. 4. Mixture of parental extracts and salt subjected to a freeze-thaw cycle. 5. F_1 hybrid. 6. F_1 hybrid extract mixed with salt and subjected to a freeze-thaw cycle. Note the generation of the BB′ hybrid dimer in the freeze-thaw mixtures and in the F_1 extract, but not in the simple mixture of parental extracts.

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anodal band of the F_1 hybrid and of the freeze-thaw mixture of parental extracts are identical and that this band represents a hybrid dimer (BB') composed of 1 subunit from each of the most anodal parent dimers. Thus, this in vitro technique apparently produces a molecular hybrid identical to that synthesized in the cells of the interspecific hybrid organism.

Preliminary investigations of our experimental conditions have revealed some similarity between the hybridization of malate dehydrogenase and of lactate dehydrogenase. Hybridization does not occur when salt is omitted from the mixture, when the tissue is homogenized in distilled water, or when 0.1 *M* *Tris*-HCl, pH 8.0, is substituted for the phosphate buffer. There is no apparent difference between quick freezing the mixture and slow freezing by storage at −20 °C for 4 h. This behavior is analogous to that reported for lactate dehydrogenase[15], suggesting a similarity in the mechanism of subunit assembly in these dehydrogenases.

The freezing and thawing process does not greatly reduce the amount of enzyme activity detected by staining. It is possible that an even better recovery of enzyme activity will be obtained by the use of coenzyme as a stabilizing agent as in the freeze-thaw hybridization of lactate dehydrogenase[15]. NADH has markedly enhanced the recovery of malate dehydrogenase during reactivation after acid or guanidine treatment[13]. Like lactate dehydrogenase, variation in the concentration or nature of the hybridization promoting ions may also increase the yield[15]. With optimal conditions, this technique should prove useful in purification procedures and in the preparation of molecular hybrids for the investigation of subunit interactions[16].

Molecular hybridization can be useful in studying protein evolution by examining the affinity of monomeric polypeptides from widely divergent organisms in forming multimeric enzymes[12]. The formation of such hybrid molecules implies a high degree of homology in higher order protein structure. Preliminary results suggest that evolutionary analyses of MDH polypeptides can be performed by the freeze-thaw technique. We have also obtained evidence that the supernatant malate dehydrogenase subunits from the sheep and the largemouth bass assemble into the same dimer during freeze-thaw hybridization.

The present investigation is, to our knowledge, the first demonstration that the freeze-thaw technique produces molecular hybrids of malate dehydrogenase isozymes. This convenient and economical procedure should prove useful in the study of genetics, enzyme structure, and molecular evolution[17].

Résumé. Les hybrides moléculaires des isozymes de la déshydrogénase de malate de différentes espèces de poissons peuvent être engendrés in vitro en gelant puis dégelant les isozymes en présence du sel. L'isozyme hybride est identique à celle observée in vivo dans l'hybride interspécifique F_1.

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Origin of Enzymic and Photosynthetic Activity in a Prebiotic System

The evolution of enzymatic activity from the primitive catalytic activity of small molecules or ions has been the subject of much speculation. CALVIN[1] has suggested the decomposition of H_2O_2 as one of the earliest examples of this process. Starting as an Fe^{+3} ion catalyzed reaction, it presumably evolved to the catalase catalyzed reaction by successive addition of increasingly complex organic ligands to the iron.

We report that UV-irradiation of a solution of NH_4SCN, glycine, and several salts produces insoluble microspheres having peroxidase activity. Glycine has been produced in many prebiotic experiments[1,2], and may be presumed to have been present in a primitive Earth environment. NH_4SCN is a product of juvenile volcanic gases[3]. We use artificial sea water[4] as a plausible ionic medium and ferrous ammonium sulfate a convenient source of iron.

In a typical experiment, 40 ml of sea water containing 0.04 moles each of glycine and NH_4SCN and 0.001 moles of $Fe(NH_4)_2(SO_4)_2$ was subjected to UV-irradiation at 254 nm for 3 h from a submerged pen lamp[5]. Particles appeared close to the surface after only 2 or 3 min of irradiation. The reaction was left standing for 14 h; during this time the particle color changed from beige to grey, suggesting that dark reactions occurred. The particles were separated from the solution on a 0.22 μm filter and were washed repeatedly with distilled water.

Microscopic examination of the particles revealed that they were spherical, 0.2–1.0 μm in diameter, and that the average diameter increased with irradiation time. Morphological integrity remained after heating in boiling water and freezing. Thus stability under geological conditions seems plausible. A scanning electron micrograph is shown in Figure 1.

Because the microspheres were insoluble in common solvents, we used an aqueous suspension in the peroxidase

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