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ACONITATE HYDRATASE ISOZYMES: SUBCELLULAR LOCATION, TISSUE DISTRIBUTION AND POSSIBLE SUBUNIT STRUCTURE

ANN L. KOEN AND MORRIS GOODMAN

Hawthorn-Plymouth Research Center, Northville, Michigan, and Department of Anatomy, Wayne State University, Detroit, Mich. (U.S.A.)

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SUMMARY

Aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) has been separated into two subcellular forms, cytoplasmic and mitochondrial. Tissue distribution of these two forms was investigated in mouse and Slow loris; spleen contains predominantly the cytoplasmic form, muscle the mitochondrial form, and other tissues varying proportions of each. Variation of the cytoplasmic isozyme was observed among Slow lorises; the patterns indicate that the subunit structure is either monomeric or polymeric without cross-association between the subunits.

INTRODUCTION

A growing number of enzymes have been shown to exist in multiple molecular forms (isozymes). For several enzymes this molecular heterogeneity is related to differences in subcellular location. For example, NAD^+-linked malate dehydrogenase (EC 1.1.1.37)[1,2], NADP-linked malate dehydrogenase (EC 1.1.1.40)[3], aspartate aminotransferase (EC 2.6.1.1)[4], creatine kinase (EC 2.7.3.2)[5] and isocitrate dehydrogenase (EC 1.1.1.42)[6] each exist in a mitochondrial and a cytoplasmic form.

Aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3) catalyses the interconversion of citrate, isocitrate and *cis*-aconitate in the tricarboxylic acid cycle. DICKMAN AND SPEYER[7] reported that most of the aconitate hyrdatase activity of rat liver was in the cytoplasmic fraction, and SHEPHERD AND KALNITSKY[8] found that in rabbit cerebral cortex most of the aconitate hydratase was bound to the mitochondria.

This report presents the electrophoretic characterization and tissue distribution of these two aconitate hydratase isozymes. Some tentative conclusions regarding subunit structure, based on isozyme variation, are also proposed.

MATERIALS AND METHODS

Animals used were ICR mice (*Mus musculus*) and Slow loris (*Nycticebus coucang*). The mice were killed by cervical dislocation and tissues were excised immediately. Mouse kidney was used for determination of subcellular distribution because pre-

liminary experiments had revealed that this tissue contained the two isozymes in approximately equal amounts. Cells were fractionated according to the method of HOGEBOOM[9] except that the microsomes were not separated from the cytoplasmic fraction. Precipitates were dissolved in 5.0 mM potassium phosphate (pH 7.0). The three fractions, nuclear, mitochondrial and cytoplasmic, were frozen, thawed and centrifuged until clear. The supernate was used as the sample.

Solutions were electrophoresed in starch gels made with 20.0 mM Tris–3.6 mM citric acid (pH 8.0), containing 50 g sucrose per l, to which 0.013 mg of $NADP^+$ were added per ml before degassing. After electrophoresis, gels were sliced horizontally and incubated in a substrate mixture containing potassium phosphate buffer (pH 8.0), *cis*-aconitic acid (sodium salt), $NADP^+$, commercially purchased isocitrate dehydrogenase, phenazine methosulfate, nitro blue tetrazolium and $MgCl_2$. Dark blue bands on the gel denoted the sites of aconitate hydratase activity.

Tissue distribution of aconitate hydratase isozymes was investigated in both mice and Slow lorises. Slow lorises were sacrificed by exsanguination. Tissues were removed, washed and frozen until use, which was usually within 7 days. Homogenates were prepared by grinding the tissues in 8.0 mM potassium citrate (pH 7.0) and freezing, thawing and centrifuging 3 times. The supernate was used as the sample. Electrophoresis and staining were carried out as described above. Tissues used were kidney, liver, heart, brain, muscle and spleen.

Extracts of kidneys from 24 Slow lorises were surveyed for electrophoretic variations. Some animals exhibited differences. Several tissues from these and from a few showing the usual pattern were also investigated, to confirm that the observed patterns were common to all tissues.

RESULTS AND DISCUSSION

When whole tissue extracts were subjected to electrophoresis in starch gels, two aconitate hydratase isozymes could be demonstrated. At pH 8.0, both migrated toward the anode; at pH 7.0, the more slowly-moving one migrated cathodally. Separation of ICR cells into component parts (Fig. 1) revealed that the more anodal form is cytoplasmic in origin, while the more cathodal form is mitochondrial. Although some of the cytoplasmic form remained with the mitochondria, this was attributed to incomplete separation.

It is interesting that in all the mammalian enzymes thus far shown to be separable into cytoplasmic and mitochondrial forms, the former migrates more anodally than the latter, indicating a more negative net charge. Although no significance has yet been attached to these comparative mobilities, it is possible that some physiological relationship exists.

Investigation of tissue distribution showed that in ICR tissues (Fig. 2), the spleen contains predominantly the cytoplasmic form of aconitate hydratase, kidney and liver about equal proportions of the mitochondrial and cytoplasmic forms, heart and brain predominantly the mitochondrial band, and muscle almost exclusively the mitochondrial band. Tissue distribution of the isozymes in Slow loris was essentially the same as in mouse.

The 24 Slow lorises which were surveyed for electrophoretic variation were captured in Thailand, transported alive and sacrificed shortly before use. There were

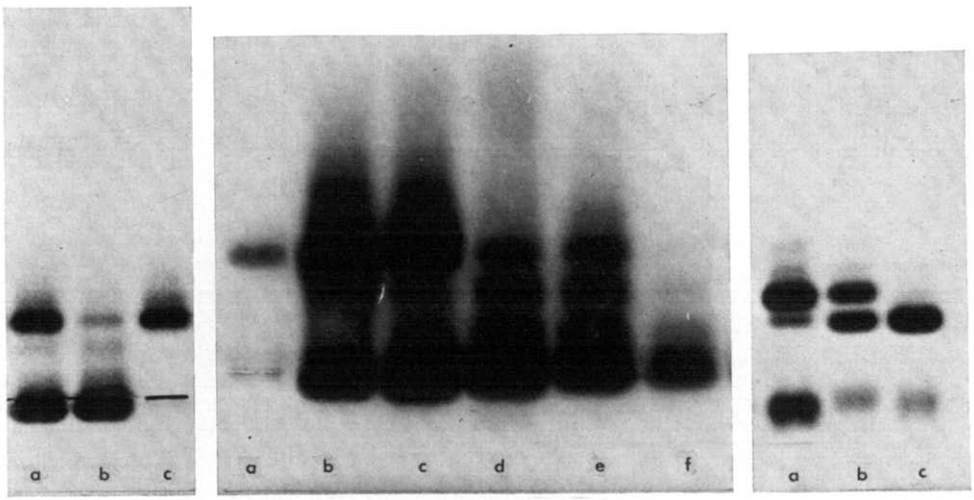

Fig. 1. Intracellular distribution of aconitate hydratase isozymes. a. Whole kidney extract. b. Mitochondria. c. Cytoplasm.

Fig. 2. Tissue distribution of aconitate hydratase isozymes in ICR mice. a. Spleen. b. Liver. c. Kidney. d. Heart. e. Brain. f. Muscle.

Fig. 3. Cytoplasmic isozyme variation in Slow loris. a. Male (No. 6.) b. Male (No. 3). c. Female (No. 2). Tissue is kidney.

15 males and 9 females. Three different electrophoretic patterns were observed among the 24 animals (Fig. 3). 17 animals, both males and females, exhibited a slow (S) band; one (No. 6), a male, possessed a lighter band at the S site and a faster migrating, heavier staining band (F). In 6 animals, 4 males and 2 females, the F and S bands were present at approximately equal intensity. These respective patterns were also present in other tissues of the same animals, indicating that these were not kidney-specific variations.

The 17 Slow lorises in which only the S band was present were presumably homozygotic at that genetic locus. The 6 in which the F and S band were of equal intensity were presumably heterozygotes, since this pattern is consistent with the heterozygotic condition. The single male (No. 6) which exhibited a heavy F band and a lighter S band is not so easily classifiable. A true homozygote for the F band should exhibit no band at the S site; conversely, if he were a true heterozygote, there is no known reason why his pattern should not be the same as that of the 6 unequivocal heterozygotes. One explanation may be that the fainter band at the S site is not an S isozyme, but a conformational isomer of the F band which, by chance, has the same electrophoretic mobility as the slower isozyme. This is the generally accepted explanation for the faint, more anodal bands (*e.g.*, in all three patterns in Fig. 3) which are commonly seen with many enzymes.

Another possibility is that the faint band at the S site in No. 6 represents a small dosage of this isozyme. We can postulate that there are actually four structural alleles at two loci coding for the cytoplasmic isozyme. Although in the S homozygote the products of these genes are electrophoretically indistinguishable, we designate

them as SS and ss. We assume that the SS alleles produce a greater amount of the enzyme than the ss alleles, and that mutation of an S allele to an F allele has occurred, leaving the ss alleles unaffected. The genotypes which give rise to the respective patterns are then: SS + ss; SF + ss and FF + ss. Thus, only in the FF homozygote would the product of the ss alleles be discernable.

SHAW[10] has reviewed the use of electrophoretic variation of enzymes in analysis of subunit structure. The presence of "hybrid" bands in heterozygous animals has been taken to indicate polymeric structure; the extra bands are formed by random association of the parent molecules. Thus, the lack of hybrid bands between the F and S aconitate hydratase bands of the heterozygous Slow lorises may indicate either that this is a monomeric enzyme or a polymeric one in which random association of the subunits does not occur.

According to present knowledge of subunit structure, the appearance of intermediate bands between the cytoplasmic and mitochondrial aconitate hydratase isozymes in the mouse (Fig. 2) indicates that random association has occurred between these molecules. This is presumed to have taken place during the tissue preparation procedures (freezing, thawing, *etc.*). Since these two isozymes are capable of forming hybrid bands, it seems that at least one of them must be polymeric in nature.

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