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Evidence for sequential expression of multiple AMP deaminase isoforms during skeletal muscle development

RAINER MARQUETANT*, NALINI M. DESAI*, RICHARD L. SABINA*†, AND EDWARD W. HOLMES*†

†Howard Hughes Medical Institute Laboratories and *Departments of Medicine and Biochemistry, Duke University, Durham, NC 27710

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ABSTRACT **AMP deaminase (myoadenylate deaminase; EC 3.5.4.6) is an integral part of the myofibril in skeletal muscle, and this enzyme plays an important role in energy metabolism in this tissue. We report here the identification of three AMP deaminase isoforms during skeletal muscle development in the rat. An embryonic isoform is expressed in the developing hindlimb of the rat between 7 and 14 days of gestation. This isoform is not unique to skeletal muscle or the embryo as it is also expressed in many nonmuscle tissues of the perinatal and adult rat. A perinatal isoform of AMP deaminase that is restricted to skeletal muscle is produced 4–6 days before birth and persists for 2–3 weeks of postnatal life. An adult, skeletal muscle-specific isoform of AMP deaminase appears at birth and reaches maximal levels after 3 weeks of postnatal development. We conclude from these studies there is a developmentally controlled program that leads to the sequential expression of AMP deaminase isoforms during the transition from embryonic to adult skeletal muscle.**

AMP deaminase (myoadenylate deaminase; EC 3.5.4.6) is the point of entry for AMP into the purine nucleotide cycle, a series of reactions that are important for energy production in skeletal muscle (1). Deficiency of this enzyme activity is associated with an exercise-related myopathy (2–4). Multiple isoforms of AMP deaminase have been identified in tissue surveys of many species (5–28) , and biochemical (5–9, 18, 19) and genetic (6, 18) data suggest there are muscle-specific isoforms of AMP deaminase. During differentiation of myoblasts into myotubes there is a 10- to 100-fold increase in AMP deaminase activity (ref. 29; unpublished data). These observations point to an important role for AMP deaminase in skeletal muscle function and suggest there may be tissue-specific expression of one or more AMP deaminase isozymes in skeletal muscle.

Studies in avian (5–10) and rodent (11–22) systems suggest there is more than one muscle-specific isozyme of AMP deaminase and there may be a switch in isozyme expression during muscle development *in vivo* (5, 6) and differentiation *in vitro* (6). There is precedent for isozyme switching in the expression of other proteins during muscle development—i.e., myosin heavy and light chains (30–32), actin (33–36), tropomyosin (55, 56), troponin (37–40), and creatine kinase (36, 41). The present study was undertaken to determine if there is more than one AMP deaminase polypeptide produced in skeletal muscle and whether there is differential expression of AMP deaminase polypeptides during muscle development. Rat was selected as a model system for these studies because of the information already available on isozyme expression during muscle development *in vivo* and myocyte differentiation *in vitro* in this species.

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MATERIALS

Male Sprague–Dawley LD strain rats were obtained from Charles River Breeding Laboratories. The protease inhibitors benzamidine, phenylmethylsulfonyl fluoride, leupeptin, soybean trypsin inhibitor, pepstatin, α_2-macroglobulin, chymostatin, aprotinin, and antipain were obtained from Sigma. *Staphylococcus aureus* strain V8 proteinase was obtained from Miles Laboratories. Electrophoresis reagents were supplied by Bio-Rad. Ampholytes were obtained from LKB. [^{14}C]AMP was purchased from New England Nuclear. Nitrocellulose sheets were purchased from Schleicher & Schuell. Goat serum and peroxidase-conjugated goat anti-rabbit IgG were obtained from Tago (Burlingame, CA). Pansorbin was purchased from Calbiochem. All other chemicals were obtained from Sigma.

METHODS

Antiserum Preparation. AMP deaminase was purified to apparent homogeneity, as judged by NaDodSO$_4$/PAGE, from adult rat skeletal muscle by the protocol of Smiley *et al.* (20). Two New Zealand White rabbits were initially immunized with 100 μg of AMP deaminase in complete Freund's adjuvant by intradermal injection. Booster injections of 100 μg in incomplete Freund's adjuvant were repeated at 2-week intervals by subcutaneous injections. Serum was collected 2 weeks after booster injections to test for antibody production by immunoprecipitation in immunodiffusion plates. Both rabbits produced antisera that demonstrated a line of identity between the purified enzyme and extract of adult skeletal muscle; only one precipitin line formed in the well with crude extract.

Tissue Preparation. Rats >1 week of age were sacrificed with a lethal dose of pentobarbital, and all available skeletal muscle was dissected from both hindlimbs and frozen immediately in liquid nitrogen. Newborn (<1 week of age) and fetal (up to 5 days before delivery) rats were sacrificed by decapitation, and hindlimbs as well as other organs were removed and frozen immediately in liquid nitrogen. In embryos of <15 days of gestation it was not feasible to harvest specific organs and the entire embryo was frozen in liquid nitrogen. Frozen tissues were homogenized under liquid nitrogen and extracted in 3 vol of the following buffer: 89 mM KP$_i$/180 mM KCl/1 mM 2-mercaptoethanol, pH 6.0, containing the following protease inhibitors: pepstatin, 5 μg/ml; leupeptin, 2 μg/ml; α_2-macroglobulin, 5 μg/ml; aprotinin, 2 μg/ml; antipain, 2 μg/ml; chymostatin 2 μg/ml; soybean trypsin inhibitor, 0.2 mg/ml; 1 mM benzamidine; 1 mM phenylmethylsulfonyl fluoride; and 1 mM EDTA. Homogenates were centrifuged at 16,000 × *g* at 4°C for 5 min. Aliquots of the supernatants were frozen immediately and used for PAGE as well as for isoelectrofocusing.

L-6 rat myoblasts, originally isolated by Yaffe (42), were obtained from Robert Smith (Harvard Medical School, Boston, MA) and maintained at subconfluent density in Eagle's

minimal essential medium with 10% horse serum. Monolayers of myoblasts were rinsed three times with 4°C phosphate-buffered saline and frozen *in situ* with liquid nitrogen. For immunoblots or peptide mapping studies the frozen monolayer was scraped with a rubber policeman and solubilized directly in $NaDodSO_4$ sample buffer. Protein determinations were conducted according to Bradford (43).

Gel Electrophoresis Procedures. Single-dimension $NaDodSO_4$ gels contained 8% acrylamide in the separating phase and were prepared according to the method of Laemmli (44). Protein was electrophoretically transferred from $NaDodSO_4$ gels to nitrocellulose sheets at 100 V for 1–2 hr using the method of Towbin *et al.* (45). Isoelectric focusing gels were prepared by the method of O'Farrell (46) using 3% acrylamide and ampholytes of pH 5–8 range. Protein was transferred from the isofocusing gel in the presence of urea at 30 V for 1–2 hr according to the method of Towbin *et al.* (45).

For two-dimensional gel analysis of protease or cyanogen bromide digests, extracts were first fractionated on Laemmli slab gels as described above, the region spanning M_r 60,000–85,000 was removed from each lane with a razor blade, and extracts were electrophoresed a second time using the method described by Cleveland *et al.* (47) and Bordier and Crettoli-Jaervinen (48) with the following modifications. For protease digestion, gel slices were incubated with 125 mM Tris·HCl (pH 6.8) containing 0.1% $NaDodSO_4$ for 40 min at room temperature and fixed in the second-dimension electrophoresis apparatus with 1% agarose in the same buffer. After solidification, the immobilized gel slices were overlayed with 50 mM Tris·HCl (pH 6.8) containing 2% $NaDodSO_4$, 10% glycerol, and the indicated amount of proteinase. Electrophoresis was carried out for a sufficient time for the tracking dye to reach the separating gel and the power was turned off for 15 min to permit proteolysis. Electrophoresis was then resumed and the resolved peptides were transferred to nitrocellulose sheets as described above. For cyanogen bromide cleavage, gel slices from the first dimension were soaked in 70% formic acid for 10 min and cyanogen bromide was added to a final concentration of 0.5%. The cyanogen bromide solution was gassed with nitrogen and incubated for 15 min in the dark at room temperature. The slices were then washed three times with 10% acetic acid, three times with 500 mM Tris·HCl (pH 6.8) containing 0.04% $NaDodSO_4$, and one time with 125 mM Tris·HCl (pH 6.8) with 0.1% $NaDodSO_4$ and 2% 2-mercaptoethanol. Gel slices were fixed in agarose for second-dimension electrophoresis as described above.

Immunological Detection of Peptides on Nitrocellulose. Nitrocellulose sheets were incubated in a shaker in 2.5% bovine serum albumin and 10% fetal calf serum in saline (150 mM NaCl/10 mM Tris·HCl, pH 7.4) for at least 1 hr at room temperature. To this solution the AMP deaminase antiserum was added (1:500 dilution) and incubated for 1 hr at room temperature. The sheets were rinsed three times for 5 min with saline buffer and incubated for 15 min in 2.5% bovine albumin and 10% fetal calf serum in saline buffer. Peroxidase-conjugated goat anti-rabbit IgG was added at a dilution of 1:2000 and incubated for 30 min at room temperature. After washing three times for 10 min with saline buffer, the sheets were incubated with peroxidase reaction mixture [80 ml of saline buffer and 20 ml of 4-chloro-1-naphthol (3 mg/ml in methanol)]. The reaction was initiated by adding 33 μl of a 30% hydrogen peroxide solution and terminated by rinsing with water.

Densitometry. Densitometric analyses of immunoblots were performed using a soft laser scanning densitometer (Biomed Instruments, Fullerton, CA) to quantitate the amount of AMP deaminase detected at the different stages during development. Data presented are the average of three scans of each band and results are expressed in relative densities.

AMP Deaminase Assay. AMP deaminase activity was quantitated by determining the conversion of [^{14}C]AMP to [^{14}C]IMP. Routine assays were conducted at 37°C in 50 mM imidazole·HCl, 150 mM KCl, 1 mM 2-mercaptoethanol, and 10 mM [^{14}C]AMP (0.33 μCi/μmol; 1 Ci = 37 GBq); [^{14}C]AMP concentration was varied from 0.1 to 10 mM in experiments designed to determine the apparent K_m for AMP. Dilute solutions of AMP deaminase were stabilized by adding 2 mg of bovine serum albumin per ml to the reaction mixture. Five micromolar deoxycoformycin and 5 μM P^1,P^5-di(adenosine-5′)pentaphosphate were included in all assays to inhibit adenosine deaminase and myokinase activities, respectively. Tubes were frozen to terminate the assay. Twenty-microliter aliquots of the reaction mixture were spotted on PEI-cellulose TLC plates and the plates were developed in saturated $(NH_4)_2SO_4$/100 mM KP_i, pH 6.0/isopropanol, 75:18:2. IMP and AMP spots were localized with UV light, cut out, and assayed for radioactivity in toluene-based scintillation fluid. All assays were linear with respect to time and extract amount, and AMP disappearance equaled IMP formation.

RESULTS

Characterization of Antisera. This study utilized antisera raised in two rabbits immunized with AMP deaminase purified from adult rat skeletal muscle. Antisera from rabbit 1 only reacted with AMP deaminase obtained from adult skeletal muscle under all conditions we have tested, whereas antisera from rabbit 2 reacted with AMP deaminase obtained from adult and perinatal skeletal muscle as well as embryonic tissue and rat L-6 myoblasts grown in tissue culture (Fig. 1). No bands were detected on immunoblots of these tissues probed with control serum (Fig. 1). Both antisera detected a single protein band on immunoblots of these tissues (Fig. 1), and both antisera gave a strong signal on immunoblots of highly purified AMP deaminase from adult rat skeletal muscle (data not shown). These antisera, but not control sera, immunoprecipitated AMP deaminase activity from the above tissues (data not shown). Immunopurification of anti-AMP deaminase antibodies with an AMP deaminase affinity column yielded antisera that detected polypeptides of the same molecular weight as detected with crude antisera (data not shown). The antisera are capable of detecting AMP deaminase in muscle extracts from a number of species, including

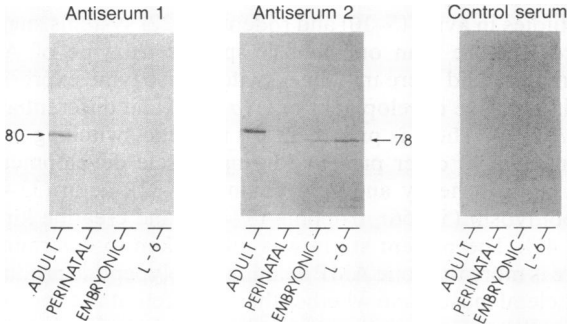

FIG. 1. Identification of AMP deaminase isoforms. Extracts from adult skeletal muscle, perinatal hindlimb, embryo, and L-6 myoblasts were prepared, and single-dimension $NaDodSO_4$ gel electrophoresis, transfer to nitrocellulose, and immunological detection of peptides were carried out. Two hundred micrograms of extract protein was loaded into each well; one filter was probed with antiserum from rabbit 1, one was probed with antiserum from rabbit 2, and the third was probed with preimmune serum. Molecular weights are indicated as $M_r \times 10^{-3}$.

human, and muscle extracts from patients with an inherited deficiency of AMP deaminase (2–4) have no signal on immunoblots screened with these antisera (unpublished observations). From these analyses we conclude that the antisera used under the conditions described in this study have adequate specificity for identifying AMP deaminase peptides in tissue extracts.

Identification of AMP Deaminase Isozymes. As seen in Fig. 1, extract from adult rat skeletal muscle contained a single AMP deaminase peptide with an apparent M_r of 80,000. In extract taken from perinatal hindlimb 2–3 days before birth, the AMP deaminase peptide had an apparent M_r of 77,500. Extract from embryos obtained 6–7 days before birth contained an AMP deaminase peptide of M_r 78,000. The difference in apparent molecular weight of AMP deaminase obtained from perinatal and embryonic tissues has been consistently observed in tissue samples from three sets of pregnant rats. Extracts from undifferentiated L-6 rat myoblasts contained an AMP deaminase peptide of the same size as that obtained from embryonic tissue (Fig. 1).

AMP deaminase isoforms in extracts from embryos, perinatal hindlimb, and adult skeletal muscle can also be distinguished on the basis of differential reactivity with various antisera. As shown in Fig. 1, antiserum 1 only reacted with the M_r 80,000 peptide found in adult skeletal muscle. Antiserum 2 reacted with peptides from tissues obtained at all stages of development. A third antibody raised against the AMP deaminase isoform in adult liver (18) quantitatively immunoprecipitated AMP deaminase activity in embryo extract, precipitated a fraction of AMP deaminase activity in perinatal hindlimb, but failed to precipitate any of the AMP deaminase activity in adult skeletal muscle extract (data not shown).

Peptide maps generated from limited proteolysis of AMP deaminase in extracts from the above tissues provide evidence suggesting a difference in primary structure among the embryonic, perinatal, and adult isoforms of AMP deaminase (Fig. 2). Based on preliminary experiments, tissue extracts were diluted to contain amounts of undigested AMP deaminase that yielded signals of approximately equivalent intensity on immunoblots. The first-dimension $NaDodSO_4$/PAGE was used to isolate AMP deaminase from the various tissues and this purified preparation was used in the second dimension for proteolytic digestion or cyanogen bromide cleavage (Fig. 2). The adult isoform yielded protease digestion and cyanogen bromide-cleavage patterns of immunoreactive peptides that were quite distinct from those of the other isoforms. The pattern of immunoreactive peptides was identical in embryonic and L-6 extracts with either protease digestion or cyanogen bromide cleavage. The perinatal isoform appears to be related to the embryonic and L-6 isoforms based on the presence of several immunoreactive peptides in perinatal extract that had mobilities in $NaDodSO_4$/PAGE identical to those of peptides in the embryonic and L-6 extracts. However, V-8 protease digestion and cyanogen bromide cleavage demonstrated multiple immunoreactive peptides in embryonic and L-6 extracts that were not identified in extract of perinatal muscle. Attempts to purify the embryonic, perinatal, and L-6 isoforms in sufficient quantities to generate peptide maps that can be analyzed independent of immunological methods have not been successful because of the limited amounts of embryonic and perinatal tissues available and because of the smaller amount of AMP deaminase in these tissues.

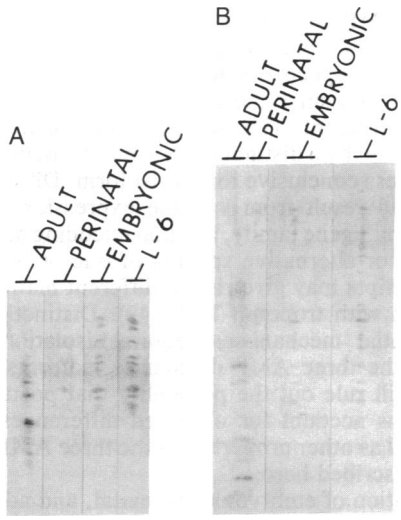

FIG. 2. Peptide maps of AMP deaminase isoforms. Extracts of embryonic, L-6, perinatal, and adult skeletal muscle were screened for immunoreactive AMP deaminase peptides as illustrated in Fig. 1. An amount of extract that produced a signal of approximately equivalent intensity was applied to each well in the first-dimension $NaDodSO_4$/PAGE—1.0 mg of protein for embryonic tissue and L-6 extract, 1.5 mg of perinatal hindlimb extract, and 0.15 mg of adult skeletal muscle extract. The M_r 60,000–85,000 region of each lane from the first-dimension gel was isolated and peptides were generated by V8 protease digestion (*A*) or cyanogen bromide cleavage (*B*). Transfer to nitrocellulose and immunoblotting were then carried out. Antiserum 2 was used for these studies.

Isoelectrofocusing gels revealed a single AMP deaminase peptide in extracts from embryos, perinatal hindlimb, adult skeletal muscle, and L-6 myoblasts. AMP deaminase in adult rat muscle has an isoelectric point of 5.6 (mean of two determinations), similar to that reported for AMP deaminase purified from adult rabbit skeletal muscle (49). In perinatal hindlimb, embryonic tissue, and L-6 myoblasts, AMP deaminase has an isoelectric point of 6.2 (mean of three determinations for each tissue).

The apparent K_m for AMP was determined on two occasions in dialyzed extracts of adult skeletal muscle (0.9–1.0 mM), perinatal hindlimb (2.4–2.7 mM), embryo (3.7–3.9 mM), and L-6 myoblasts (3.5–4.0 mM).

Changes in AMP Deaminase Isoforms During Development. Fig. 3 illustrates changes in expression of AMP deaminase isoforms during intrauterine and postnatal development. Embryos of <15 days' gestation were too small to isolate individual organs, and consequently the entire embryo was extracted. Only the M_r 78,000 isoform was identified in embryo extracts of 5–15 days' gestation (16 to 7 days prior to birth) (Fig. 3*A*). The M_r 77,500 isoform was first detected in hindlimb between 15 and 17 days of gestation, reached its maximum 2–3 days before birth, and disappeared by 2–3 weeks of postnatal life (Fig. 3). The increase and decrease in the perinatal isoform (Fig. 3 *A* and *B*) coincided with a rise and fall in AMP deaminase activity in the hindlimb (Fig. 3*C*).

Between 15 and 20 days of gestation, the fetus is of sufficient size to isolate individual organs, permitting an analysis of AMP deaminase isoforms in specific tissues. A M_r 78,000 isoform was produced in heart, liver, and lung of the 15- and 20-day-old fetus (data not shown). The perinatal isoform (M_r 77,500) was not seen in any of these tissues from the 20-day-old fetus, yet it was present in maximal concentrations in hindlimb at this time (Fig. 3). Several tissues of the adult rat, including heart, liver, lung, spleen and thymus produced an AMP deaminase isoform with a mobility on $NaDodSO_4$/PAGE identical to that of the M_r 78,000 isoform (data not presented).

The adult isoform was first detected 1 day prior to birth and this AMP deaminase isoform increased in amount until about 3 weeks of age, when adult levels are reached (Fig. 3). There is a positive correlation between the increase in the amount of the adult isoform of AMP deaminase at various stages of postnatal development, as quantitated by densitometric anal-

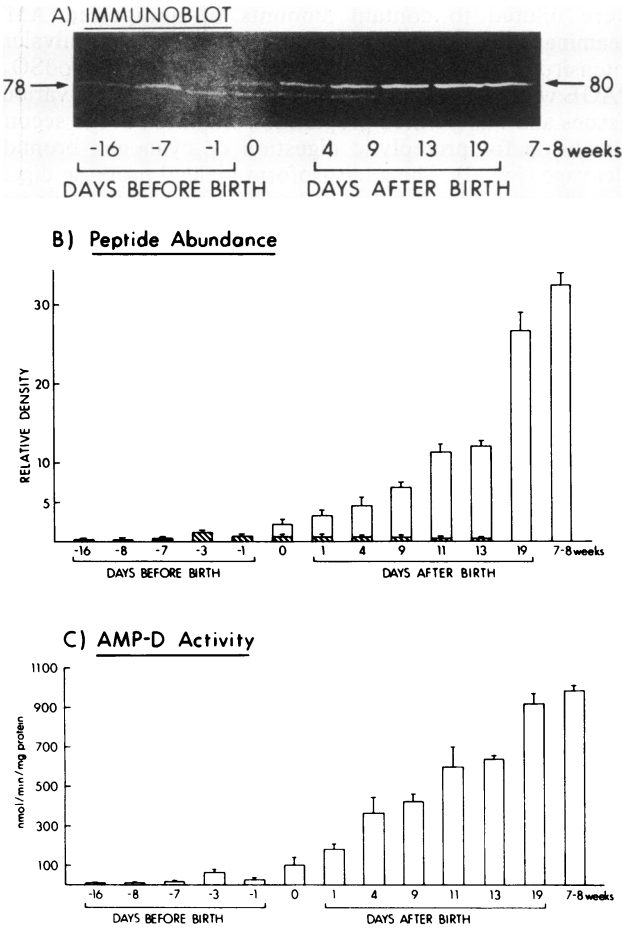

FIG. 3. Expression of AMP deaminase isoforms during development. Extracts were prepared from embryos 16, 8, and 7 days prior to delivery (5, 13, and 14 days of gestation, respectively); perinatal hindlimb, 3 and 1 day prior to delivery as well as on the day of delivery; and skeletal muscle, 4, 9, 13, and 19 days of postnatal development. Skeletal muscle was also obtained from 7- to 8-week-old adults. (*A* and *B*) Two hundred micrograms of extract protein was loaded in each well and displayed by single-dimension $NaDodSO_4$ gel electrophoresis; transfer to nitrocellulose and immunoblots were then performed. Antiserum 2 was used for these studies. (*A*) Immunoblot of the extracts obtained at the indicated time points. Molecular weights are indicated as (*B*) $M_r \times 10^{-3}$. Relative density (mean of three determinations) of the AMP deaminase peptide bands shown at the indicated times. Solid bars, embryonic isoform; hatched bars, perinatal isoform; open bars, adult isoform. (*C*) AMP deaminase (AMP-D) activity (mean of three determinations) measured in each of the extracts used in *A* and *B*.

ysis of immunoblots (Fig. 3*B*), and the increase in AMP deaminase activity determined in these extracts (Fig. 3*C*) ($r = 0.95$). Immunoblots of skeletal muscle, brain, liver, and heart extracts probed with antisera specific for the adult isoform (Fig. 1) demonstrated this isoform was only expressed in skeletal muscle of the adult rat (data not shown).

DISCUSSION

Several lines of evidence suggest distinct AMP deaminase isozymes are produced sequentially during development in the rat. In the embryo of <15 days of gestation, the only form of AMP deaminase identified has an apparent subunit M_r of 78,000, an isoelectric point of 6.2, and apparent K_m for AMP of 3.8 mM. Skeletal muscle obtained from the fetus 4–6 days before birth contains another AMP deaminase isoform with apparent subunit M_r of 77,500, isoelectric point of 6.2, and apparent K_m for AMP of 2.5 mM. Peptide maps of the perinatal isoform demonstrate several peptides with mobilities in $NaDodSO_4$/PAGE identical to those of peptides in the embryonic isoform, but there are multiple peptides not shared by these two isoforms. At or just prior to birth a third isoform of AMP deaminase appears in skeletal muscle. This isoform has an apparent M_r of 80,000, an isoelectric point of 5.6, selective reactivity with rabbit antiserum 1, apparent K_m for AMP of 1.0 mM, and a pattern of peptides distinct from that of either the embryonic or perinatal forms of AMP deaminase.

The embryonic isoform, so called because it is first detected in the embryo, is not unique to skeletal muscle or embryonic tissues. A peptide of similar molecular weight is also expressed in several organs of the perinatal and adult animal—i.e., spleen, lung, liver, thymus, and blood. This isoform is expressed in tissue destined to become skeletal muscle, as evidenced by its presence in fetal hindlimb and L-6 myoblasts.

The perinatal and adult isoforms of AMP deaminase appear to be unique to skeletal muscle since they are not observed in other perinatal or adult tissues. Embryonic and perinatal isoforms are present at the same time in the hindlimb of the fetus; perinatal and adult isoforms are present contemporaneously in skeletal muscle of newborn animals. Better antibody reagents are needed to determine through histochemical studies whether a single cell contains more than one isoform of AMP deaminase as opposed to the simultaneous presence of cells at different stages of development that express only one isoform of AMP deaminase.

Three isoforms of myosin heavy chain are expressed in rat skeletal muscle with time courses similar to those for AMP deaminase (30), suggesting the production of AMP deaminase and myosin heavy chain isoforms may be regulated by common developmental signals. Coordinate expression of these two proteins during myocyte development may be important physiologically since AMP deaminase binds tightly and specifically to myosin heavy chains (50–52) and is localized to the end of the A band in skeletal muscle (51, 52).

A number of mechanisms may account for the appearance of multiple AMP deaminase isoforms during development. The adult, skeletal muscle-specific isoform and the embryonic, nonmuscle-specific isoform are likely to be encoded by different mRNAs since the peptide maps of these isoforms are entirely different. The isoform in perinatal muscle may also be encoded by a distinct mRNA but the peptide mapping studies are less conclusive for this isoform. Different mRNA species could result from variable expression of an AMP deaminase multigene family, like that encoding myosin heavy chains (53), or alternative splicing of one or more primary RNA transcripts may give rise to different mature mRNAs, as observed with troponin T (37–40). Distinction between these potential mechanisms requires isolation of cDNA clones for the three AMP deaminase isoforms. The latter approach will rule out the possibility that posttranslational modifications account for observed differences in peptide maps as well as other properties of the three AMP deaminase isoforms described here.

Identification of embryonic, perinatal, and adult isoforms of AMP deaminase and developmental control of the expression of these isozymes may be relevant to understanding the molecular bases of inherited and secondary deficiencies of AMP deaminase (3, 4). Patients with inherited deficiency of AMP deaminase have no detectable adult isozyme in their skeletal muscle (unpublished data), yet primary myoblast cultures from these patients have normal AMP deaminase activity, presumably reflecting the expression of an embryonic isoform of the enzyme (54). In light of the results of the present study, we postulate that the loss of the adult isoform of AMP deaminase with retention of normal capability for expression of the embryonic and/or perinatal isoforms re-

flects either a structural defect in the gene or an exon unique to the adult isoform or a defect in the developmental program that controls the sequential expression of the various isoforms of this enzyme. Further evaluation of these patients may help to clarify the mechanisms responsible for switching of AMP deaminase isoforms during normal muscle development.

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