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Arylamidase and Cathepsin-A Activity of Normal and Dystrophic Human Muscle[1] (39264)

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Previous studies have shown the presence of intracellular proteases in human skeletal muscle. In studying the role of these enzymes in muscle protein catabolism in human muscular dystrophies, earlier investigators have employed hemoglobin, myoglobin, or endogenous muscle proteins as substrate for these enzymes (1, 2). In particular correlative histological and biochemical studies using these substrates, we have been able to demonstrate that greatly increased activity of acidic protease and also of neutral and alkaline proteases are only present in severely degenerating dystrophic and other diseased muscles (2). It is evident, however, that precise knowledge about these proteases and other peptide hydrolases is necessary to gain further insight into their possible role, not only in enhanced protein catabolism in dystrophic muscle but also in normal muscle protein turnover. We have, therefore, initiated a series of studies on human muscle peptide hydrolases and proteases employing specific synthetic substrates in an effort to characterize these enzymes and to determine whether their activities are altered in human muscular dystrophies, especially during the early phases of disease. The present study reports on enzymes of normal and dystrophic human muscle that catalyze the hydrolysis of L-leucyl *p*-nitroanilide, a substrate for neutral arylamidase (3, 4) and *N*-carbobenzoxy-(CBZ)-glutamyl-L-tyrosine, a known cathepsin-A substrate (5, 6).

Materials and methods. Chemicals. L-Leucyl *p*-nitroanilide, CBZ-L-glutamyl-L-tyrosine, and cysteine hydrochloride were obtained from Sigma Chemical Co., St. Louis, Missouri. Dithioerythritol and *p*-chloromercuribenzoate were purchased from Cal Biochem, La Jolla, California. All other reagents were of analytical grade and obtained locally.

Muscle. Muscle specimens were biopsies of deltoid, gastrocnemius, or quadriceps obtained from 28 patients aged 5–68 yr, with various muscle and neuromuscular diseases, diagnosed on the basis of clinical, histological, and biochemical tests. Severely affected diseased muscles that were visibly abnormal were not employed in this study. Histologically normal samples of gastrocnemius and quadriceps muscle biopsies obtained from five patients aged 9–34 yr were used as controls. In all cases, biopsy samples were kept frozen at −18° for a period of 4 to 8 weeks. Storage for this length of time did not seem to significantly affect the activity of arylamidase and cathepsin A in several pilot studies.

The muscle was freed from any visible fat and connective tissue and was minced finely with scissors. The minced muscle was homogenized with 9 vol of ice-cold distilled and deionized water in an all-glass homogenizer for about 2 min with intermittent cooling in ice.

Enzyme assays. Arylamidase activity was assayed with L-leucyl *p*-nitroanilide as substrate. The standard reaction mixture contained 40 m*M* Tris–HCl buffer (pH 7.4), 0.8 m*M* substrate, and 0.05 ml of homogenate in a total volume of 1 ml. The mixture was incubated at 37° for 1 hr. The reaction was terminated with 0.1 ml of 1 *N* HCl and the mixture was centrifuged. An aliquot (0.5 ml) was diluted to 2.5 ml with distilled water, and the absorbance of the liberated *p*-nitroaniline was measured at 410 nm in a Beckman DU spectrophotometer. Controls, in which the enzyme was added after incubation, were included in each determination. The millimolar extinction coefficient of *p*-nitroaniline at 410 nm, according to Ellis and Perry (3), is 8.8, and this value is used in all calculations. The time-course of the reaction remained linear for at least 1 hr, and

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the observed activity was directly proportional to tissue weight (from 2.5 to 10 mg of muscle original wet wt/tube). Under the standard conditions about 17% of the substrate was hydrolyzed.

Cathepsin-A activity was determined according to the method of Iodice *et al.* (7). The reaction mixture contained 100 m*M* acetate buffer (pH 5), 5 m*M* CBZ-glutamyl-tyrosine, and 0.1 ml of homogenate in a total volume of 1 ml. The mixture was incubated at 37° for 2 hr. The reaction was terminated with 1 ml of 10% trichloroacetic acid, and the amount of tyrosine released was measured in the protein-free supernatant by a colorimetric ninhydrin procedure (2). Controls, in which the enzyme was added after incubation, were kept for each assay. The reaction remained linear for at least 2 hr, and the observed activity was proportional to about fourfold change in enzyme concentration. Under the standard conditions, about 1% of the substrate was hydrolyzed. The noncollagen protein was determined as described before (2).

Results. Variation of activity with pH. With homogenates of normal human quadriceps muscle, the activity against L-leucyl-*p*-nitroanilide over the pH range 3–9 using barbital-acetate buffer (50 m*M*) showed a pH optimum of 7.5, and the activity against CBZ-glutamyl-tyrosine in citrate buffer (50 m*M*) had a pH optimum of 5 (Fig. 1).

Effects of various added substances on muscle arylamidase activity. A number of substances are known to affect the activity of various mammalian arylamidases. Many of these were tested with the human muscle enzyme (Table I). The muscle enzyme was strongly inhibited by *p*-chloromercuribenzoate. Cysteine or dithioerythritol did not stimulate the activity. EDTA had slight inhibitory effect on the enzyme activity. The addition of 0.1 m*M* Co^{2+} resulted in slight stimulation (17%) of activity, whereas Mn^{2+}, Mg^{2+}, and Ca^{2+} at a concentration of 0.1 m*M* were slightly inhibitory. Hg^{2+} at 0.1 m*M* totally inhibited the enzyme activity.

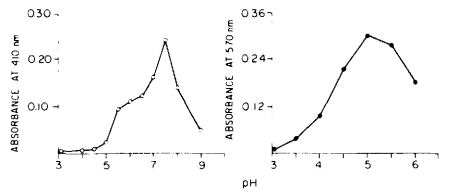

FIG. 1. Variation of activity with pH. Incubation systems were as described in the text except that 50 m*M* barbital-acetate buffer was used for arylamidase (○) and 50 m*M* citrate buffer for cathepsin A (●).

Arylamidase activity of human muscle. The data in Table II show that muscles from patients with Duchenne dystrophy and other major forms of muscular dystrophies had arylamidase levels comparable to those in controls. The arylamidase levels of muscles from patients with polymyositis and spinal muscular atrophies were also similar to those in controls.

Cathepsin-A activity of human muscle. The level of cathepsin A in muscle from control subjects and from patients with various diseases is shown in Table III. Among the muscular dystrophies that were exam-

TABLE I. SUBSTANCES AFFECTING THE ARYLAMIDASE ACTIVITY OF HUMAN MUSCLE.[a,b]

Substance	m*M*	control activity (%)
p-Chloromercuribenzoate	0.01	94
	0.1	0
Cysteine	0.1	92
	1.0	84
Dithioerythritol	1.0	86
EDTA	0.1	89
	1.0	86
Co^{2+}	0.1	117
Mn^{2+}	0.1	88
Mg^{2+}	0.1	87
Ca^{2+}	0.1	94
Hg^{2+}	0.1	0

[a] All metals were added in the form of Cl^- salts.
[b] Essentially similar results were obtained in two separate experiments.

TABLE II. ARYLAMIDASE ACTIVITY OF HUMAN MUSCLE HOMOGENATES.

Diagnosis	Arylamidase activity[a]
Control (5)[b]	135.6 ± 23.88[c]
Duchenne dystrophy (4)	180.6 ± 42.66
Limb girdle dystrophy (6)	176.4 ± 35.46
Facioscapulohumeral dystrophy (5)	186.0 ± 39.66
Myotonic dystrophy (4)	136.0 ± 6.00
Polymyositis (4)	157.8 ± 35.82
Spinal muscular atrophy (5)	166.8 ± 27.90

[a] Activity is expressed as nanomoles of *p*-nitroaniline released per milligram of noncollagen protein per hour at 37°.
[b] Number of cases studied.
[c] Mean ± standard error.

TABLE III. CATHEPSIN-A ACTIVITY OF HUMAN MUSCLE HOMOGENATES.

Diagnosis	Number of cases	Cathepsin-A activity[a]
Control	5	28.7 ± 3.83[b]
Duchenne dystrophy	4	49.4 ± 3.49 (72)[c] $P < 0.001$
Limb girdle dystrophy	4	43.8 ± 3.24 (53) $P < 0.01$
Facioscapulohumeral dystrophy	4	32.1 ± 3.77 (12) NS
Polymyositis	4	48.3 ± 5.83 (68) $P < 0.02$
Spinal muscular atrophy	5	44.4 ± 6.24 (55) $P < 0.05$

[a] Activity is expressed as nanomoles of tyrosine released per milligram of noncollagen protein per hour at 37°.
[b] Mean ± standard error.
[c] Denotes percentage increase above normal.

ined, Duchenne and limb girdle types of dystrophy had significantly increased muscle cathepsin-A activity. Muscles from patients with polymyositis and spinal muscular atrophies also had elevated levels of this enzyme.

Discussion. The results of the present study demonstrate the presence in human muscle of an active arylamidase that catalyzes the hydrolysis of L-leucyl-*p*-nitroanilide. The level of this enzyme in normal human muscle is about 3.7-fold higher than that of cathepsin D (2). Arylamidases, hydrolysing amino-acid-substituted β-naphthylamides and *p*-nitroanilides are usually referred to as N, B, or A according to whether the substrate possesses a neutral, basic, or acidic amino acid. Arylamidase *N* hydrolysing leucyl β-naphthylamide or leucyl *p*-nitroanilide has been extensively studied in many tissues (8–11), while arylamidase A (hydrolysing glutamyl or aspartyl-β-naphthylamide) have been studied particularly in nervous tissues (11–13). The human muscle arylamidase in the present studies is similar in some respects to the rat brain enzyme described by Marks *et al.* (11). Both enzymes require sulfhydryl groups for activity as indicated from the inhibition observed with *p*-chloromercuribenzoate (0.1 m*M*). Unlike the brain enzyme, however, the muscle arylamidase is neither stimulated by thiol compounds nor inhibited by EDTA. It is only slightly stimulated by 0.1 m*M* Co^{2+}. Hg^{2+} is found to strongly inhibit the enzyme activity at 0.1 m*M*.

The physiological substrates for tissue arylamidases are unknown, and no functional role for these enzymes has yet been proposed. Along with the previous evidence that skeletal muscle contains a variety of active peptide hydrolases capable of hydrolysing various dipeptides, tripeptides, and polypeptides (14), it seems likely that these enzymes and arylamidase(s) act sequentially in the degradation of polypeptides in this tissue. The present study shows that muscle neutral arylamidase activity is not significantly altered in human dystrophies and also in certain denervating diseases (Table II). Further studies are needed to ascertain whether the basic or the acidic arylamidases are altered in human dystrophic muscle.

The present study further demonstrates the presence in human muscle of cathepsin A that catalyzes the release of tyrosine from CBZ-glutamyl-tyrosine at acid pH. Cathepsin A activity of normal human muscle is almost similar to that of cathepsin D (2). Its level has been found to be significantly elevated in muscles from patients with various muscular dystrophies and also with certain denervating diseases (Table III). A significantly higher level of this enzyme has also been found in the dystrophic muscle of chicken at the earliest stage of the disease (7). Cathepsin A was one of the first components of the lysosomal cathepsin complex of bovine spleen to be described (15, 16). It has been shown that cathepsin A acts mainly as an exopeptidase resembling carboxypeptidase in its specificity (17, 18) and displays only limited endopeptidase activity (19). Cathepsin A has been reported to partially degrade glucagon and other polypeptides (7). It seems likely therefore that cathepsin A may modify or inactivate a small but functionally important protein and thereby initiate the sequence of events leading to muscle wasting. It may be mentioned that myofibrillar proteins are shown to be not attacked by lysosomal catheptic enzymes (20). It is interesting to note that based on studies with a variety of lysosomal marker enzymes

(21–23) including cathepsin D (2), we have proposed that lysosomal enzymes are most likely involved in protein degradation only during gross cellular autophagy. The higher level of cathepsin A as observed in this study may indicate that an activation of this enzyme occurs rather early in muscle diseases characterized by wasting and atrophy.

Summary. Human skeletal muscle homogenate has been shown to contain enzymes that catalyze the hydrolysis of L-leucyl *p*-nitroanilide and carbobenzoxyglutamyl-L-tyrosine, known substrates, respectively, for arylamidase and cathepsin A. The muscle arylamidase was found to be inhibited by *p*-chloromercuribenzoate. Addition of Co^{2+} resulted in slight stimulation of its activity. Neither ethylenediamine tetraacetate nor thiol compounds had any appreciable effect on the enzyme. When compared to controls, no significant differences in muscle arylamidase levels were observed in patients with muscular dystrophies and certain selected neuromuscular diseases. Cathepsin A was, however, increased in muscles moderately affected by muscular dystrophy and denervating diseases.

1. Pennington, R. J. and Robinson, J. E., Enzymol. Biol. Clin. **9,** 175 (1968).
2. Kar, N. C., and Pearson, C. M., Enzyme **13,** 188 (1972).
3. Ellis, S., and Perry, M., J. Biol. Chem. **241,** 3679 (1966).
4. Park, D. C., Parsons, M. E., and Pennington, R. J., Biochem. Soc. Transc. **1,** 730 (1973).
5. Lichenstein, N., and Fruton, J. S., Proc. Nat. Acad. Sci. USA **46,** 787 (1960).
6. Iodice, A. A., Leong, V., and Weinstock, I. M., Arch. Biochem. Biophys. **117,** 477 (1966).
7. Iodice, A. A., Chin, J., Perker, S., and Weinstock, I. M., Arch. Biochem. Biophys. **152,** 166 (1972).
8. Hopsu, V. K., Makinnen, K. K., and Glenner, G. G., Arch. Biochem. Biophys. **114,** 557 (1966).
9. Smith, E. E., and Rutenburg, A. M., Science **152,** 3726 (1966).
10. Nachlas, M. M., Goldstein, T. P., and Seligman, A. M., Arch. Biochem. Biophys. **97,** 223 (1964).
11. Marks, N., Datta, R. K., and Lajtha, A., J. Biol. Chem. **243,** 2882 (1968).
12. Marks, N., Int. Rev. Neurobiol. **11,** 57 (1968).
13. Mezei, C., and Palmer, F. B. St. C., J. Neurochem. **23,** 1087 (1974).
14. Smith, E. L., J. Biol. Chem. **173,** 553 (1948).
15. Shibko, S., and Tappel, A. L., Biochem. J. **95,** 731 (1965).
16. Coffey, J. W., and deDuve, C., J. Biol. Chem. **243,** 3255 (1968).
17. Iodice, A. A., Arch. Biochem. Biophys. **121,** 241 (1967).
18. Barrett, A. J., and Dingle, J. T., *in* "Tissue Proteinases" (A. J. Barrett, and J. T. Dingle, eds.), p. ix. North Holland, Amsterdam (1971).
19. Logunov, A. I., and Orekhovich, N. O., Biochem. Biophys. Res. Commun. **46,** 1161 (1972).
20. Martins, C. B., and Whitaker, J. R., J. Food Sci. **33,** 59 (1968).
21. Pearson, C. M., and Kar, N. C., *in* "Clinical Studies in Myology" (B. A., Kakulas, ed.), p. 89. Excerpta Medica, Amsterdam (1973).
22. Kar, N. C., and Pearson, C. M., Clin. Chim. Acta **40,** 341 (1972).
23. Kar, N. C., and Pearson, C. M., Clin. Chim. Acta **45,** 269 (1973).

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