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THE RAPID DISAPPEARANCE OF MUSCLE AMP AMINOHYDROLASE FROM BLOOD PLASMA OF NORMAL AND DYSTROPHIC CHICKENS

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SUMMARY: The levels of creatine kinase and pyruvate kinase are increased 22 and 9.3 fold respectively in the blood plasma of dystrophic chickens as compared to normal controls. AMP aminohydrolase levels are not increased despite their abundance in muscle tissue. When AMP aminohydrolase was injected into a blood vessel, its rate of disappearance from the plasma was rapid with 97% of the enzyme disappearing with a half-life of 3.3 minutes. In contrast, the rate of disappearance of pyruvate kinase from the blood plasma is relatively slow, following a biphasic exponential decay with half-lives of 113 min and 710 min. These data suggest that the rates of disappearance of enzymes from the blood plasma is an important factor in determining whether increased plasma levels of these enzymes are observed in muscular dystrophy.

Increased plasma levels of muscle associated enzymes are often used diagnostically in the identification of muscular diseases. In human Duchenne muscular dystrophy increased plasma levels of creatine kinase (1), pyruvate kinase (1,2), aldolase (3), and glutamic-oxalacetate transaminase (4) have been documented. On the other hand, the level of several other abundant muscle proteins including myoglobin (5), phosphofructokinase (5) and AMP aminohydrolase (6) are not increased in the plasma of dystrophic individuals. Several explanations for these observations have been suggested (7) including differential permeability of the sarcolemmal membrane to muscle proteins, association of some "soluble" proteins with intracellular structures, or differences in the rate of clearance of the enyzmes from the blood plasma.

In concert with the observations with plasma from human dystrophic patients, increased plasma levels of creatine kinase (8) and pyruvate kinase (9) have been noted in line 413 dystrophic chickens, when compared to normal chickens, line 412. In light of these results and as part of our plan to ascertain the origin of the reduced levels of pyruvate kinase and AMP

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aminohydrolase in dystrophic muscle (10), we have examined the rate of disappearance of these two enzymes from the plasma of normal and dystrophic chickens to determine the role of the plasma clearance rates of these enzymes on the regulation of their plasma levels.

MATERIALS AND METHODS

Materials: Fertile eggs from normal (line 412) and dystrophic (line 413) chickens were obtained from the Department of Avian Sciences at the University of California at Davis. Plasma enzyme levels and rates of disappearance of injected enzyme from plasma were determined with line 412 and 413 chicks at 4-6 weeks of age. Other materials were normal chicken breast muscle for enzyme preparation (Pel-Freez Biologicals); substrates and coupling enzymes for enzyme assays (Sigma Chemical Co.); Biogel A-1.5m (Bio Rad Laboratories); Aquacide I-A (Calbiochem-Behring Corp); 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril was a gift from Professor J.C. Speck; and carrier free $Na^{125}I$ in 0.1 N NaOH (ICN Chemical and Radioisotope Division). All other chemicals were reagent grade and all solutions were prepared in deionized distilled water.

Crude muscle extracts: Muscle crude extracts were prepared by homogenizing tissue in a Waring blender using 3.3 ml of 0.18 M KCl in 0.08 M potassium phosphate pH 6.5 per gm of tissue. The homogenate was centrifuged at 6000 X g for 20 minutes and the supernatant assayed for enzyme activity.

Collection of Blood Samples: Blood samples (0.5 ml) were collected from the subcutaneous vein of the wing elbow into glass heparinized tubes. The plasma was assayed for enzyme activity after removal of red blood cells by centrifugation.

Enzyme Assays: Phosphofructokinase was assayed as previously described (11); pyruvate kinase was assayed by coupling with lactate dehydrogenase (12); creatine kinase was assayed with the assay system supplied by Sigma Chemical Company (13). AMP aminohydrolase activity was determined by measuring changes in absorbance at 265 nm as AMP is deaminated (14) in an assay mixture containing 100 μM AMP in 0.15 M KCl, 50 mM Mes-Tris at pH=6.5. All assays were carried out at 30° C.

Enzyme purifications: AMP aminohydrolase from frozen chicken breast muscle was purified as previously described (15). Samples were routinely concentrated to about 4 mg ml^{-1} using Aquacide I-A. Pyruvate kinase was purified from frozen chicken breast muscle (16) and stored at -20°C as a suspension in 50% saturated ammonium sulfate and 50% glycerol until use.

Radioiodination of Enzymes: Purified AMP aminohydrolase and pyruvate kinase were radioiodinated with $Na^{125}I$ using the iodinating agent 1,3,4, 6-tetrachloro-3a,6a-diphenylglycoluril by the method of Fraker and Speck (17). The specific radioactivity of radioiodinated AMP aminohydrolase was about 11 μCi/mg and pyruvate kinase was 51 μCi/mg. All radioactive samples were counted on a Beckman Biogamma Counter (Efficiency=65%). SDS polyacrylamide gel electrophoresis of the radioiodinated AMP aminohydrolase preparation revealed 67% of the bound iodine migrating with AMP aminohydrolase, with the remainder being incorporated into persistent trace contaminants in the AMP aminohydrolase preparation. Further purification of the enzyme before, or after, radioiodination on a 1.5 X 100 cm Biogel A-1.5m 200-400 mesh column eluted with 0.15 or .4

M KCl, 50 mM Mes-Tris pH=6.5, did not remove the contaminants. The radioactivity migrating with pyruvate kinase on SDS polyacrylamide gels was about 96% of the total incorporated label.

Measurement of the Rates of Disappearance of Enzymes from the Blood Plasma: Prior to injection, enzymes were dialyzed twice against 200 volumes of 0.9% NaCl, 10 mM sodium phosphate buffer pH=7.3. Insoluble material was removed by centrifugation and the supernatant used for injection. Injections were made in the subcutaneous vein of the wing elbow using 5 units of AMP aminohydrolase or 0.7 units of pyruvate kinase per gm of body weight, with or without radioactivity, in a total volume of 0.5 ml. Blood samples were collected from the subcutaneous vein of the wing elbow opposite to that injected at the time intervals given in the results. Following centrifugation of red blood cells, 50 µl of plasma was assayed for enzyme activity or ^{125}I.

The disappearance of enzyme activity and radioactivity of ^{125}I labelled enzymes followed a biphasic exponential decay and was fit to equation 1 with a non-linear curve fitting program (18).

$$A_t = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (1)$$

A_t is the total amplitude $(A_1 + A_2)$ of enzyme activity or radiolabel disappearing at rates, k_1 and k_2. The parameters were determined separately for each bird and averaged.

RESULTS AND DISCUSSION

The plasma levels of pyruvate kinase and creatine kinase given in Table 1 confirm results previously reported by others (8,9) namely dystrophic chickens have increased levels when compared to normals. The increased plasma level of

Table 1. Activities Of Several Enzymes In The Plasma And Breast Muscle Of Normal And Dystrophic Chickens. Experimental details are given in Materials and Methods.

Enzyme	Chicken Type[1]	Units/ml Plasma[2]	Units/g Breast Muscle	Percent of Total Units in Plasma[3]
Creatine kinase	N	.4 ± .2 (8)	93 ± 11 (4)	0.7 ± .5
	D	9.3 ± 4.6 (10)	76 ± 11 (4)	7.4 ± 3.8
Pyruvate Kinase	N	0.8 ± .2 (8)	106 ± 11 (4)	0.5 ± .1
	D	7.2 ± 2.6 (8)	43 ± 7 (4)	9.8 ± 4.0
AMP Aminohydrolase	N	.005 ± .002 (3)	36 ± 3 (4)	.009 ± .004
	D	.005 ± .002 (4)	13 ± 1 (4)	.025 ± .010
Phosphofructokinase	N	.07 ± .05 (4)	.95 ± .02 (4)	4.7 ± 3.1
	D	.05 ± .01 (4)	.53 ± .08 (4)	6.4 ± 1.4

[1] N designates normal line 412 and D, dystrophic line 413 chickens.

[2] The number of chickens tested is in parentheses.

[3] These values were calculated assuming the plasma volume to be 6.0% of the total body weight (20), using measured values of body weight and breast muscle weight.

pyruvate kinase is attributed to loss from muscle tissue since the muscle isozyme predominates in the plasma as determined by kinetic methods (data not shown)and isoelectric focusing (19). On the other hand as is the case with human dystrophic patients (5,6) no significant differences in the plasma levels of phosphofructokinase and AMP aminohydrolase are observed. The results in Table 1 also show that the percent of the total enzyme activity found in the blood plasma when compared to the total activity in the breast muscle and the blood plasma combined is much lower for AMP aminohydrolase than for the other enzymes examined. These data suggest that either AMP aminohydrolase is not released from dystrophic muscle tissue as rapidly as the other enzymes examined, or that it is more rapidly inactivated or removed from the blood plasma.

Following injection of native or radioiodinated AMP aminohydrolase as described in Materials and Methods, both enzyme activity and radioactivity were

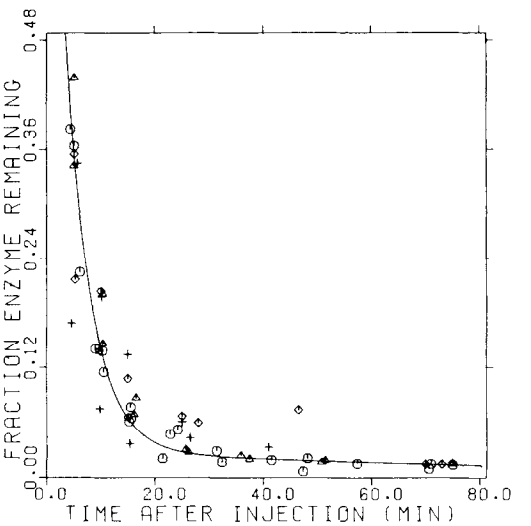

Figure 1. Disappearance of AMP aminohydrolase from the blood plasma of normal and dystrophic chickens. Experimental details are in Materials and Methods. The contribution by contaminants in the [^{125}I]-AMP aminohydrolase preparation has been subtracted to give the values shown. The theoretical curve was calculated with equation 1 using the average amplitude and rate constants given in Table 2.

○ -Disappearance of AMP aminohydrolase in normal chickens.
△ -Disappearance of [^{125}I]-AMP aminohydrolase in normal chickens.
+ -Disappearance of AMP aminohydrolase in dystrophic chickens.
◇ -Disappearance of [^{125}I]-AMP aminohydrolase in dystrophic chickens.

Table 2. Rates of Disappearance of AMP Aminohydrolase and Pyruvate Kinase From Plasma of Normal and Dystrophic Chickens. Experimental details are given in Materials and Methods.

Enzyme	Chicken Type	k_1, min^{-1} ($t_{\frac{1}{2}}$, min)	k_2, min^{-1} ($t_{\frac{1}{2}}$, min)	Percent lost in Rapid Phase[4]
AMP Aminohydrolase	N(3)	.24 ±.05 (2.9)		96
[^{125}I]-AMP Aminohydrolase	N(3)	.23 ±.13 (3.0)		—
AMP Aminohydrolase	D(3)	.17 ±.01 (4.1)	See Footnote[2]	97
[^{125}I]-AMP Aminohydrolase	D(3)	.20 ±.061 (3.5)		—
Average Rates of Disappearance of AMP Aminohydrolase	—	.21 ±.03 (3.3)		97
Pyruvate Kinase	N(3)	.0051 ± .0024 (138)	.00085 ± .00008 (820)	39
[^{125}I]-Pyruvate Kinase	N(4)	.0059 ± .0023 (120)	.00097 ± .00022 (720)	66
Pyruvate Kinase[3]	D(5)	———	———	—
[^{125}I]-Pyruvate Kinase	D(2)	.0074 ± .0034 (96)	.0011 ± .0002 (610)	71
Average Rates of Disappearance of Pyruvate Kinase	—	.0061 ± .0012 (113)	.00099 ± .00014 (710)	

1. N designates normal line 412 and D dystrophic line 413. In parentheses is the number of chickens for which measurements were made.

2. The rate constant for the second phase of AMP aminohydrolase disappearance is small with a half-life greater than 1 hour.

3. Satisfactory convergence could not be attained for data from single birds, or combined data due to fluctuations in background pyruvate kinase activity.

4. These values were calculated by extrapolation of the data collected to time zero (time of injection). The values for [^{125}I] AMP aminohydrolase could not be determined due to contaminants in the preparation which were slowly removed from the plasma.

lost from the plasma at about the same rate in normal or dystrophic chickens (Figure 1 and Table 2). Not apparent in Figure 1 is the fact that 95% of the injected AMP aminohydrolase was lost from the blood plasma prior to the collection of the first blood sample 4 to 5 minutes after injection. This is estimated assuming plasma volume of 6.0% of the body weight (20). In fact, extrapolation of the data shown in Figure 1 to time zero (time of injection)

indicates less than 10% of the enzyme was lost at the measured rate. Thus the disappearance is at least triphasic with an abrupt phase and two slower phases with half-lives of 3.3 min and over one hour. About 97% of the AMP aminohydrolase activity loss that was observed after the beginning of data collection occurred in the rapid phase with a half-life of 3.3 minutes.

The ^{125}I labeled contaminant of the AMP aminohydrolase preparation as noted in Materials and Methods disappeared very slowly from the plasma. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of blood plasma samples taken 25 minutes after injection of radioiodinated AMP aminohydrolase preparations showed 83% of the remaining activity migrating on gels to the same positions as the impurities in the radioiodinated AMP aminohydrolase preparation.

No loss of enzyme activity was observed when AMP aminohydrolase was incubated in plasma or whole blood at 37° for 2 hrs at concentrations similar to those used in the in vivo experiments. Therefore, the loss of enzyme activity from the blood plasma is not due to inactivation of the enzyme by some plasma component.

When pyruvate kinase was injected, its kinetics of disappearance also followed a biphasic exponential decay (decay curve not shown). Furthermore, extrapolation of the data to zero time resulted in enzyme levels expected based on the amount injected and a plasma volume equivalent to 6% of the total body weight (20). In contrast to the results obtained with AMP aminohydrolase, pyruvate kinase disappeared very slowly from the plasma with half- lives of 113 and 710 min for the 2 phases (Table 2). The rate of loss of pyruvate kinase enzyme activity from the plasma of dystrophic chickens following injection could not be fit by the non-linear curve fitting program because of large fluctuations in the high background pyruvate kinase activity in these birds. Since the rates of disappearance of pyruvate kinase from normal and dystrophic birds are similar, the data indicate that the increased pyruvate kinase activity in the blood plasma of dystrophic chickens is due to increased loss

from muscle tissue, and not due to an inability of the dystrophic chicken to clear the enzyme from the blood plasma.

The mechanism for the rapid removal of AMP aminohydrolase from the circulation is not known. Rates of disappearance of proteins from chicken plasma have not been measured. However, asialoglycoproteins are removed from mammalian plasma with half-lives of a few minutes while sialoglycoproteins are removed very slowly from plasma with half-lives of several hours (21). Since AMP aminohydrolase is a glycoprotein containing glucose, N-acetylneuraminic acid and N-acetylglucosamine (22), it may be removed from the circulation by an interaction with hepatic receptors (23). In fact, the highest concentration of [^{125}I]-AMP aminohydrolase was recovered in the liver and spleen (24).

The rapid rate of disappearance of AMP aminohydrolase from the blood plasma in addition to accounting for low plasma levels of the enzyme in dystrophic as well as normal chickens, may contribute to reduced levels of this enzyme in the muscle tissue as well. If there is an increased permeability of the sarcolemmal membrane in dystrophic animals the loss from muscle to plasma may result in a significant loss from muscle tissue.

These experiments suggest that all soluble muscle proteins may be released at increased rates from dystrophic muscle tissue, but that the rate at which these proteins are removed from the plasma may be the limiting factor which determines whether these proteins will be present in increased levels in the blood plasma of dystrophic animals.

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REFERENCES

1. Okinaka, S., Kumagai, H., and Fbashi, S. (1961) Arch. Neurol. 4, 64-69.
2. Harano, Y., Adair, R., and Vignos, P.J., Jr., Miller, M. and Kowal, J. (1973) Metabolism 22, 493-501.
3. Schapira, G., Joly, M., and Schapira, F. (1953) Sem. Hop. Paris 29, 1917-1920.

4. Dreyfus, J.C., and Schapira, G. (1955) C. Rend. Soc. Biol. 149, 1934-1935.
5. Rowland, L.P., Layzer, R.B., Kagan, L.J. (1968) Arch. Neurol. 18, 272-276.
6. Pennington, R.J. (1969) Disorders of Voluntary Muscle, pp. 385-410, J. and A. Churchill Ltd., London.
7. Pennington, R.J.T. (1977) Pathogenesis of Human Muscular Dystrophies, pp. 341-349, Excerpta-Medica, Amsterdam-Oxford.
8. Wilson, B.W., Randall, W.R., Patterson, G.T., Enriken, R.D (1979) Ann. N.Y. Acad. Sci. 317, 224-246.
9. Barnard, E.A., and Barnard, P.J. (1979) Ann. N.Y. Acad. Sci. 317, 374-399.
10. Husic, H.D., Young, R., Suelter, C.H., and McConnell, D.G. (1979) Fed. Proc. 38, 667.
11. Bucher, T., and Pfleiderer, G., (1955) Methods in Enzymology 1, pp. 435-440.
12. Emerk, K., and Freiden, C. (1975) Arch. Biochem. Biophy 168, 210-218.
13. Rosalki, S.B. (1967) J. Lab. Clin. Med. 69, 696-700.
14. Smiley, K.L., Berry, A.J., and Suelter, C.H. (1967) J. Biol. Chem. 247, 2502-2506.
15. Suelter, C.H., Thompson, D., Oakley, G., Pearce, M., Husic, H.D., and Brody, M.S. (1979) Biochem. Med. 21, 352-365.
16. Cardenas, J.M., Blachly, E.G., Ceccotti, P.L., and Dyson, R.D. (1975) Biochem. 14, 2247-2252.
17. Fraker, P.J., and Speck, J.C., Jr. (1978) Biochem. Biophys. Res. Comm. 80, 849-857.
18. Dye, J.L., and Nicely, V.A. (1971) J. Chem. Ed. 48, 443-448.
19. Liu, P.K.S., Barnard, E.A., Barnard, P.J. (1980) Exp. Neurol. 67, 581-600.
20. Sturkie, P. (1965) Avian Physiology, pp. 109, Cornell University Press.
21. Ashwell, G. and Morrell, A.G. (1974) Adv. Enzymol. Relat. Areas Mol. Biol. 41, 99-128.
22. Oakley, G., Lamport, D.T.A., Macher, B., and Suelter, C.H. (1979) Fed. Proc. 38, 337.
23. Lunney, J., and Ashwell, G. (1976) Proc. Nat. Acad. Sci. USA 73, 341-343.
24. Husic, H.D., and Suelter, C.H., unpublished observations.