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MUSCLE ALDOLASE: THE STRESS-DEPENDENT MODIFICATION OF CATALYTIC AND STRUCTURAL PROPERTIES BY RAT MUSCLE LYSOSOMAL CATHEPSIN B

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Stress-dependent variations in the properties of the rat muscle aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) have been linked to the corresponding changes in the levels of proteolytic activities in rat muscle. Whole-body X-irradiation of rat was shown to result in loss of muscle aldolase activity towards fructose 1,6-bisphosphate by 50% while fructose 1-phosphate activity remained unchanged (Pote, M.S. and Altekar, W. (1980) Ind. J. Biochem. Biophys. 17, 255–262). Incubation of muscle extract of irradiated rat with that from control rat or rabbit muscle aldolase caused similar changes in aldolase activity. The changes are attributed to the action of catheptic enzymes possessing latency characteristics and capable of using aldolase as a substrate; the time course of their increase after irradiation corresponds to that of loss in muscle aldolase activities. Exposure of rats to stress resulted in an increase in the 'free' proteolytic activity, and the concomitant loss of 'bound' activity in muscle lysosomes indicates labilization of lysosomal membrane. The observed degradation of aldolase in vivo by muscle lysosomes is shown to be due to the action of cathepsin B (EC 3.4.22.1) present in the proteolytic enzymes released into cytosol under stress. Inactivation of rabbit muscle aldolase and rat muscle aldolase by rat muscle cathepsin B is inhibited by leupeptin, antipain and iodoacetamide, but not by pepstatin. Inactivation is shown to be due to the release of C-terminal tyrosine if aldolase required for its catalytic activity. Cathepsin B acts as a rate-limiting enzyme in the degradation of aldolase. Such a proteolytic modification of aldolase in vivo could be relevant not only to the regulation of aldolase activity for glycolysis in muscle but also to the degradation of aldolase during stress conditions related to tissue damage and the maintenance of normal aldolase levels in the blood.

Introduction

The changes in the levels of tissue-specific aldolases that take place in rats subjected to whole-body X-irradiation were reported earlier [1,2]. Aldolase activity in skeletal muscle was found to be most susceptible to X-irradiation. There was a considerable loss in muscle activity, however, the loss was found to be towards FDP and the F1P activity remained unchanged. Increase in serum aldolase levels observed at the same time was possibly due to the leakage of muscle aldolase into serum. The observed effects appeared to have resulted from stress, rather than from radiation damage per se. Proteolysis by carboxypeptidase is known to modulate the activity of muscle aldolase into an enzyme with reduced FDP activity because of removal of its C-terminal tyrosine residues [3]. However, our results were suggestive of involvement of catheptic enzymes of lysosomal origin in the inactivation of rat muscle aldolase. Tissue proteases have been considered to play an important role in the degradation of intracellular proteins [4,5].

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Abbreviations: FDP, fructose 1,6-bisphosphate; F1P, fructose 1-phosphate; Bz-Arg-NH_2, *N*-benzoyl-L-arginine amide; Bz-Arg-NNap, α-*N*-benzoyl-DL-arginine 2-naphthylamide; Bz-Arg-Nan, α-*N*-benzoyl-DL-arginine-4-nitronilide.

The ability of cathepsin B to inactivate certain enzymes of glycolysis, including aldolase, was initially recognised by Otto and co-workers [6,7]. The inactivation of rabbit muscle aldolase by purified rabbit liver cathepsin B was reported to resemble the action of cathoxypeptidase on aldolase [8]. Recently, Bond and Barrett [9] in their detailed in vitro study have shown that human liver cathepsin B acts as a peptidyldipeptidase on rabbit muscle aldolase to release eight dipeptides from C-terminus and to reduce only FDP activity. Similar observations have not only strengthened the case for the involvement of lysosomal proteases in the degradation of intracellular proteins in general [10–12] but have prompted the suggestion of the possibility of regulation of aldolase activity in glycolysis through proteolytic degradation [8]. Although the studies with aldolase have helped in the elucidation of the mode of inactivation of aldolase by proteolysis, the manner in which the interaction could be achieved in vivo and be rate-limiting at the same time was not clear. This would be of primary importance if an endogenous protease is to be implicated as having a significant role in the control mechanism for aldolase through degradation. We have obtained evidence for an increased activity of lysosomal proteases in isotonic extracts prepared from rat muscle during stress conditions. In addition these endogenous, otherwise latent, proteases are shown to have the ability to modify the properties of muscle aldolase by converting it into a degraded enzyme with reduced enzyme activity.

Materials and Methods

Materials

Male Wistar rats, weighing 200–250 g and reared on laboratory stock diet were used.

Chemicals were purchased from the following sources: barium salts of FDP and F1P, NADH, dithiothreitol, Tris-HCl, Bz-Arg-NH_2, Bz-Arg-NNap, Bz-Arg-Nan from the Sigma Chem. Co., U.S.A.; hydrazine sulfate, 2,4-dinitrophenyl hydrazine, EDTA, β-mercaptoethanol, casein, Triton X-100, glycine-glycine and dimethylsulfoxide from British Drug Houses, U.K. A mixture of crystalline suspension of α-glycerophosphate dehydrogenase-triose phosphate isomerase from rabbit muscle was purchased from Sigma Chem. Co. A Worthington U.S.A. preparation of rabbit muscle aldolase of spec. act. 25 U/mg was used in experiments for the characterization of proteolytic degradation.

Pepstatin, leupeptin and antipain were generous gifts from Dr. H. Umezawa, Microbial Chemistry Research Foundation, Tokyo, Japan.

Methods

Whole-body X-irradiation of rats. Irradiation was carried out at 600 R and skeletal muscle was obtained as described earlier [1]. Extraction of muscle by procedures described below was carried out at 0–4°C.

Preparation of muscle extract. Finely-minced muscle tissue (usually 5 g) was suspended in the ratio of 1 : 4 in 0.1 M EDTA, pH 6.0/1 mM β-mercaptoethanol. After stirring for 30 min and passing through muslin cloth the extract was centrifuged at $800 \times g$ 15 min and the supernatant was used. Muscle extract prepared by this method extracted aldolase and did not rupture lysosomes [1].

Disruptive treatments. Muscle suspension prepared as above was repetitively freeze-thawed in liquid nitrogen (four times) or ultrasonicated in an MSE model 60 W cell disintegrator at 15 Kc for 5 min [13]. The treated muscle extract was obtained after centrifugation as above.

Isolation of muscle lysosome-rich fraction. Lysosome-rich particulate fraction from rat muscle was isolated from rat muscle, homogenised under isotonic conditions according to the procedure of Bird [14]. Muscle extract was passed through muslin cloth and centrifuged at $800 \times g$ for 15 min and the post-nuclear supernatant was termed as the homogenate. The homogenate was centrifuged at $10\,000 \times g$ for 30 min to give 'supernatant' and a pellet containing lysosomes and mitochondria, which was suspended in a small volume of the extracting isotonic medium and termed as 'lysosomal particulate fraction'. No further purification of this fraction was undertaken.

Assay of aldolase. The colorimetric estimation of triose phosphates formed from FDP or F1P, adapted from Sibley and Lehninger [15] was described earlier [1]. Aldolase was also assayed spectrophotometrically in a coupled system [16].

Assay of lysosomal proteolytic enzymes in muscle extracts. 'Indirect' assay: The inactivation of aldolase (present in muscle extract or pure rabbit aldolase) was measured after incubation of appropriate reac-

tion mixtures containing either muscle extract prepared from irradiated rat or obtained by disruptive procedure as was described earlier [1]. The reduction in 0 min aldolase activity was taken as a measure of proteolytic activity.

'Direct' caseinolytic assay: The procedure of Kunitz [17] for caseinolytic assay, modified for chicken muscle lysosomal enzymes [13] was used. The liberated end products were estimated as equivalents of tyrosine [18].

Assay with synthetic substrates: Bz-Arg-NH_2, Bz-Arg-NNap and Bz-Arg-Nan were used as substrates which were incubated separately with solubilized lysosomal particulate fraction in phosphate buffer, pH 6.0, in the presence of 1 mM EDTA and dithiothreitol. The assays and analysis of products were an described previously [7,19].

Digestion of rabbit muscle aldolase by rat muscle lysosomal particulate fraction. Crystalline rabbit muscle aldolase was exhaustively dialysed in cold against 10^{-3} M EDTA at pH 7.6. The molar concentration of aldolase was determined from its absorbance ($A_{280}/0.91$ = mg aldolase/ml [20]) and its molecular weight of 160000 [21]. The assay of residual activity and determination of reaction products after digestion of aldolase by the lysosomal particulate fraction which was solubilized just before use are as described earlier.

Amino acid analysis. TLC on silica gel G plates was carried out employing *n*-butanol, acetic acid and water (80 : 20 : 30, v/v) as the developing system and ninhydrin for spray.

Analyses of reaction mixtures, before and after digestion with 6 N HCl, was also carried out with a Beckman/Spinco amino acid analyser [22]. These were mainly to detect the presence of small peptides and quantitation was not always considered necessary.

Total amino acids were determined as 'leucine equivalents' by the ninhydrin method [23]. Tyrosine determination employing Folin reagent [18] used in the caseinolytic assay was also used in 'aldolasolytic' assay.

The presence of tyrosine was also confirmed by ultraviolet fluorescence analysis in an Aminco-Bowman spectrofluorimeter as described by Altekar [24].

Protein was determined by the method of Lowry et al. [25].

Results

Inactivation of muscle aldolase by muscle extract of X-irradiated rat

Post-irradiation time-course of proteolytic activities was determined by subjecting a group of rats to whole-body X-irradiation at 600 R. Two animals were killed each at intervals of 0.5, 1, 2, 4, 6 and 24 h, respectively, and muscle extracts in EDTA-medium were prepared at each time of killing. The mixtures used for 'indirect' assays were (i) muscle extracts of irradiated and control rats, (ii) muscle extract from control rat and pure rabbit muscle aldolase, and (iii)

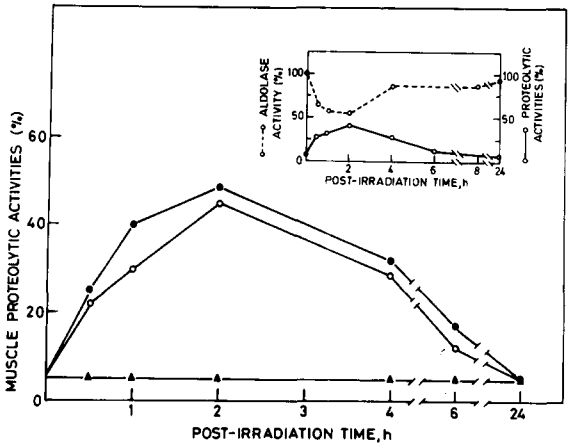

Fig. 1. Post-irradiation time course of muscle proteolytic activities and correlation to loss in muscle aldolase levels. Proteolytic activities were assayed indirectly with aldolase as the substrate. Preparation of muscle extracts at each time after irradiation and appropriate mixtures (in 0.1 M EDTA 1 mM β-mercaptoethanol, pH 6.0) is as described. Mixtures indicated below were prepared by mixing 0.25 ml each of two extracts; made to 1.0 ml with extracting medium; incubated for 30 min at 37°C and residual aldolase activity determined by colorimetric procedure [1]. Activity at 0 time incubation calculated as units/ml was considered 100 in the case of each mixture and the loss in aldolase after incubation was taken as proteolytic activity. The insert (o- - - - -o) shows activities determined directly on the muscle extract prepared at indicated post-irradiation times and calculated as units/g wet tissue and the value of 850 in muscle of unirradiated rat was considered as 100. One unit of aldolase cleaves 1 μmol FDP into 2 μmol trioses/10 min at 37°C. o———o, mixture of muscle extracts of control and irradiated rat, also reproduced in insert; •———•, mixture of muscle extract of irradiated rat and pure rabbit muscle aldolase; ▲———▲, mixture of muscle extract of control rat and pure rabbit muscle aldolase.

muscle extract from irradiated rat and pure rabbit muscle aldolase. The aldolase activities remaining after incubation of the mixtures are shown in Fig. 1. The capacity of muscle extract from irradiated rat to inactivate aldolase increased following irradiation. Activity losses of 40 and 32% were seen in mixtures of extract from irradiated rat with that from control rat and pure aldolase, respectively, at 1 h, and these values increased upto 2 h. After 2 h the proteolytic activities started decreasing slowly, the activities at 4 h being almost similar to those seen at 1 h and no appreciable loss in the activity of added aldolase, was detected at the end of 24 h. The aldolase activity towards F1P however, remained unaltered (data not shown). Pure aldolase was not inactivated to any appreciable extent in the presence of muscle extract from control rats. The insert shows good correlationship between the post-irradiation time-course of loss in rat muscle aldolase activity, demonstrated earlier [1], with the time-course in lysosomal proteolytic activities currently observed (Fig. 1).

Effect of disruptive treatments on inactivation of aldolase

The foregoing results were suggestive of changes in the latency characteristics of degradative enzymes induced by radiation as aldolase activity is easily extractable from muscle. Two procedures known to be disruptive to membranes [13] were employed for obtaining muscle extract from unirradiated rats. As a result of freeze-thawing treatment (Table I), less aldolase activity is present in the muscle extract. A further loss of 20% was seen in treated extract when incubated alone, whereas only a 3% loss was seen in control extract incubated alone. In the mixture of these two extracts 32% enzyme activity was lost. Similarly pure rabbit muscle aldolase also lost its activity (30%) in the presence of the treated extract as against the negligible loss in presence of control extract (5%).

Qualitatively similar results were obtained when freeze-thawed extract was replaced by sonicated extract in the above experiment. Liver extracts prepared by the two disruptive treatments were also found capable of inactivating added aldolase (unpublished data).

Thus, muscle extracts obtained under conditions deleterious for the integrity of lysosomal membranes were capable of inactivating rat and rabbit muscle aldolases. Moreover, the lower initial aldolase activity values in such extracts were indicative of in situ loss due to disruptive treatments. Similar effects of extraction procedures, especially on liver aldolase have been reported [26].

TABLE I

EFFECT OF DISRUPTIVE TREATMENT TO RAT MUSCLE ON ALDOLASE ACTIVITY

Freeze-thawing of minced muscle suspension and preparation of reaction mixtures were as in Materials and Methods. Mixtures of extracts (0.25 ml each) containing 1 mM EDTA/0.001 M β-mercaptoethanol (pH 6.0) were incubated in 1 ml vol. for 30 min at 37°C. Aldolase activity was determined by colorimetric procedure before and after incubation and expressed as units/ml incubation mixture.

Treatment	0 min	30 min	Percent of original activity
Control extract (No treatment)	27.8	27.0	97
Crystalline aldolase	33.7	33.0	–
Freeze-thawed extract	6.4	5.0	80
Control extract + freeze-thawed extract	29.3	19.9	68
Crystalline aldolase + control extract	37.8	36.4	95
Crystalline aldolase + freeze-thawed extract	35.2	25.0	70

Labilization of lysosomal proteases induced by whole-body X-irradiation of rat

The distribution of 'free', 'total' and 'bound' proteolytic activities in different muscle fractions obtained at different post-irradiation times was determined by the 'direct' method with casein which is a general substrate for proteases. The fractions were prepared and tested under isotonic conditions and the results are presented in Table II. It is evident that following irradiation the specific activities of 'free' proteases increased in the homogenate, supernatant and particulate fraction as compared to the corresponding fractions prepared from unirradiated rats. An increase of about 2-, 7- and 3-fold, respectively, was noticed at 2 h post-irradiation time (i.e., time of maximum rise). Concomitantly there was consider-

TABLE II

CHANGES IN ACTIVITIES AND DISTRIBUTION OF LYSOSOMAL PROTEASES BETWEEN SOLUBLE AND PARTICULATE FRACTIONS OF MUSCLE FROM IRRADIATED RATS

Homogenate, supernatant and lysosomal particulate fractions from muscle were prepared fresh in isotonic sucrose solution from rats killed at indicated post-irradiation times as described in Materials and Methods. Triton X-100 (0.2%, v/v) was added for lysosomal disruption in homogenate and particulate fraction before assay, for determination of 'total' activity. Protease activity was assayed with casein as the substrate. Reaction mixture (1.0 ml) contained 0.5 ml of 1.5% casein dissolved in 0.1 M EDTA, pH 6.0 and muscle fraction in sucrose (final pH 6.0 and 0.25 M sucrose). Incubated for 30 min at 37°C. After precipitation with 2 ml 10% TCA, supernatant was tested for products of proteolysis as tyrosine equivalents. 'Free' and 'total' activities in the absence and presence of Triton X-100 are expressed as nmol tyrosine liberated/min per mg protein. The difference between 'total' and 'free' was considered as 'bound' or 'latent' activity shown in the parentheses and was obtained as ((Total-Free)/Total) × 100. The values represent the average obtained from four fractionation experiments.

Caseinolytic activities in fraction	Post-irradiation time (h)					
	0	1	2	4	6	24
I. Homogenate						
'Free'	4.1 ± 1.03	10.3 ± 0.39	10.5 ± 0.16	7.0 ± 0.48	6.0 ± 0.30	5.4 ± 0.82
	(78)	(70)	(69)	(75)	(72)	(73)
'Total'	19.4 ± 2.1	34.0 ± 0.53	33.7 ± 1.15	28.3 ± 1.1	21.8 ± 0.90	19.0 ± 0.65
II. Supernatant						
'Free'	3.5 ± 0.65	13.2 ± 1.40	22.3 ± 0.80	7.7 ± 0.73	6.7 ± 0.52	3.7 ± 0.50
III. Particulate fraction						
'Free'	11.8 ± 1.9	32.6 ± 1.0	34.1 ± 2.0	18.8 ± 1.2	18.3 ± 1.2	13.8 ± 2.0
	(54)	(25)	(30)	(31)	(33)	(41)
'Total'	25.8 ± 1.9	43.3 ± 1.25	49.0 ± 0.3	27.3 ± 0.95	27.3 ± 1.21	23.6 ± 2.0

able loss in 'bound' activity in the particulate fraction. The exposure of rats to X-irradiation thus resulted in increase in the specific activities of 'free' lysosomal proteolytic enzymes in the supernatant which could be attributed to the labilization of lysosomes. It appears that 'total' activity in the particulate fraction and homogenate also increased significantly. Normal levels were almost regained after 24 h. The decrease in the percent of 'bound' activity in the particulate fraction, inspite of its increase in the 'free' and 'total' activity, suggested that the lysosomes after irradiation may be less stable or more permeable and released their proteolytic enzymes even when prepared in isotonic solution.

Effect of X-irradiation on the K_m and V values for aldolase

The values of K_m and V for muscle aldolase following irradiation are shown in Table III. Muscle was removed for extraction at 0, 2 and 48 h after irradiation of rats. K_m values for FDP showed changes from a value of $1.1 \cdot 10^{-5}$ M to $3.5 \cdot 10^{-5}$ M at 2 h and returned to $1.2 \cdot 10^{-5}$ M at 48 h. However, the V value for FDP was lowered by 50% at 2 h after irradiation and near normal value was observed after 48 h. The K_m and V for F1P remained practically the same. A slight alteration in K_m for FDP and a drastic change in V for FDP without any change in F1P values can be considered as indicative of proteolytic

TABLE III

KINETIC PROPERTIES OF MUSCLE ALDOLASE FOLLOWING WHOLE-BODY IRRADIATION OF RAT

Muscle aldolase activities were determined in extracts prepared at indicated times by the colorimetric procedure.

	Control	Irradiated	
		2 h post-irradiation	48 h post-irradiation
K_m FDP	$1.1 \cdot 10^{-5}$ M	$3.5 \cdot 10^{-5}$ M	$1.2 \cdot 10^{-5}$ M
K_m F1P	$3.57 \cdot 10^{-4}$ M	$3.12 \cdot 10^{-4}$ M	$3.8 \cdot 10^{-4}$ M
V FDP	12.6	6.0	11.4
V F1P	0.63	0.63	0.60

degradation of aldolase. These results from in vivo studies are in agreement with earlier reports on the kinetic behaviour of pure rabbit muscle aldolase treated with proteinases such as carboxypeptidase, subtilisin and chymotrypsin [27].

Proteolytic activity with synthetic substrates for cathepsin

Rat muscle fractions were tested for hydrolysing activity towards synthetic substrates as shown in Table IV. Enzymes capable of hydrolysing Bz-Arg-NH_2, Bz-Arg-NNap and Bz-Arg-Nan which are usually employed as substrates for the detection and assay of cathepsin B activity were found to be present in all fractions except supernatant from control rats.

Inhibition of cathepsin B mediated aldolase inactivation by protease-inhibitors

Effect of leupeptin, pepstatin, antipain and iodoacetamide on the inactivation of aldolase was tested. Inhibitor was omitted from the control digestion mixture. Residual aldolase activity was determined after 30 min of mixing with the treated muscle extract and the results are given in Table V. Leupeptin and antipain, known inhibitors of cathepsin B, [28–30] inhibited the degradation of aldolase. The control without any inhibitor showed 50% loss in aldolase activity while 90% activity was retained in the presence of leupeptin and antipain. Pepstatin was without any effect. Iodoacetamide was inhibitory for the proteolytic action.

The foregoing results indicated that among the proteolytic enzymes released, the activity of rat muscle lysosomal cathepsin B capable of using rat muscle aldolase as well as rabbit muscle aldolase as substrates for degradation increased under the stress. At this stage we could not obtain a homogeneous preparation of aldolase from rat muscle in a reasonable quantity. Therefore, rabbit muscle aldolase, whose structure resembles that of rat muscle aldolase [31] was used for characterization of the proteolytic action of rat muscle cathepsin B.

Loss of aldolase activity in presence of lysosomal cathepsin B

When a solution of aldolase was incubated with the lysosomal preparation at pH 6.0, an incomplete and time-dependent loss of aldolase activity towards FDP was seen. About 50% loss of original activity was seen within 30 min as shown in Fig. 2. The rate but not the extent of activity loss was changed by variation of aldolase: proteases ratio was extended incubation of aldolase: proteases ratio and extended incubaother hand F1P activity was not altered during the incubation (data not shown).

TABLE IV

CATHEPTIC ACTIVITY WITH SYNTHETIC SUBSTRATES IN RAT MUSCLE FRACTIONS BEFORE AND AFTER IRRADIATION

Rat muscle fractions were prepared before and after irradiation (600 R, 2 h) as described in Table II. Supernatant-(1.5 mg protein) or Triton X-100-treated lysosomal fraction (9.0 mg protein) was incubated separately with indicated quantities of the three substrates for 30 min at 37°C in pH 6.0, 0.2 M phosphate buffer containing 1 mM each of EDTA and dithiothreitol (vol. 1.0 ml). Assays of products are as given and the values given are corrected for controls.

Substrate and final concentration	Determination of reaction product	Activity, nmol product/30 min			
		Control		Irradiated	
		Supernatant	Lysosomal fraction	Supernatant	Lysosomal fraction
Bz-Arg-NH_2 20 mM	Ninhydrin reaction E 570 nm	14.3	106	91	201
Bz-Arg-Nan 0.6 mM	Absorbance E 410 nm	traces	130	112	246
Bz-Arg-NNap 0.4 mM	Fluorescence E_m 403 nm (E_x 339 nm)	traces	102	92	193

TABLE V

EFFECT OF SPECIFIC INHIBITORS ON RAT MUSCLE CATHEPSIN MEDIATED ALDOLASE INACTIVATION

Dialysed rabbit muscle aldolase (70 units) was digested in separate reaction mixtures (1 ml) containing rat muscle lysosomal particulate fraction (2.7 mg) which was solubilized and preincubated with indicated quantities of inhibitors for 15 min in buffer, except for pepstatin. Digestion was carried out under conditions as in Fig. 2 and aliquots were assayed for aldolase activity spectrophotometrically at 0 and 30 min. Aldolase in rat muscle extract (200 units) prepared in 0.1 M EDTA, pH 6.0, was digested in a similar experiment conducted separately.

Inhibitor and concentration	Rabbit muscle aldolase activity		Rat muscle extract aldolase activity	
	At 30 min digestion	% Inactivation [a]	At 30 min digestion	% Inactivation [b]
Nil	45	36	94	53
Leupeptin $3.3 \cdot 10^{-5}$ M	70	nil	193	nil
Antipain $2.2 \cdot 10^{-5}$ M	70	nil	200	nil
Pepstatin $4.8 \cdot 10^{-6}$ M	45	36	94	53
Iodoacetamide 10^{-3} M	70	nil	193	nil

[a] With respect to 70 units at 0 min.
[b] With respect to 200 units at 0 min.

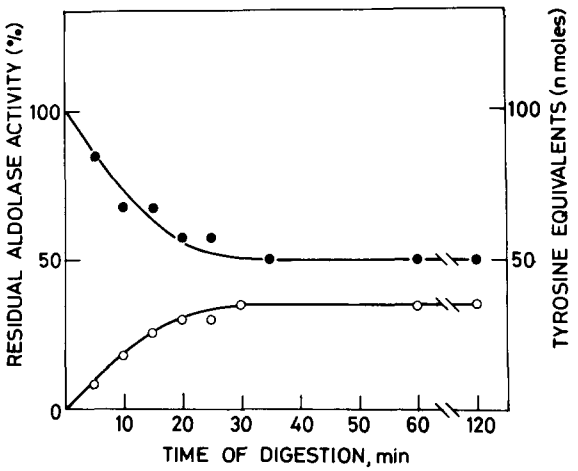

Fig. 2. Correlation of catalytic activity loss and tyrosine release by cathepsin action on aldolase. 16 mg dialysed crystalline rabbit muscle aldolase was incubated with Triton X-100-treated rat muscle particulate fraction (10 mg protein) in a volume of 4.5 ml containing 0.1 M EDTA/0.001 M β-mercaptoethanol in 0.2 M acetate buffer, pH 6.0 at 37°C. At the indicated times aliquots were removed for immediate spectrophotometric assay of catalytic activity. Tyrosine released was estimated in aliquots removed at same time after removal of protein by TCA. ●———●, residual aldolase activity; ○———○, tyrosine equivalents.

Qualitative identification of amino acids released

The amino acids released during the digestion of aldolase were identified in aliquots removed at different time intervals (Table VI).

(i) TLC showed simultaneous appearance of two amino acids in the early stages of digestion. They were tyrosine and alanine. As the digestion proceeded further, two new amino acids at a time, appeared almost simultaneously. Firstly, aspargine and histidine, followed by isoleucine, serine and phenylalanine and leucine.

(ii) Half of the deproteinised aliquot was subjected to automatic amino acid analysis without any prior treatment. The other half of the aliquot was first hydrolysed with HCl and then analysed for amino acids. No differences were noticed at any time between the unhydrolysed and hydrolysed samples which was considered an indication of the absence of peptides in the reaction mixture. Tyrosine and alanine were released first, followed initially by aspargine and histidine and later by isoleucine, serine, leucine, phenylalanine and glutamic acid. The quantities of tyrosine and alanine showed equal increase with time and the same was true for aspargine and histidine, serine and isoleucine; and phenylalanine and leucine (unpublished data).

(iii) As an independent check, the presence of

TABLE VI

QUALITATIVE DETECTION OF AMINO ACIDS RELEASED BY CATHEPSIN ACTION OF ALDOLASE

Dialysed rabbit muscle aldolase (64 mg) was digested by rat muscle lysosomal fraction (45 mg) in a volume of 11.0 ml as described in Fig. 2. Suitable aliquots were removed at indicated times for chromatographic analysis by TLC and automatic amino acid analysis (same quantities analysed before and after 6 N HCl hydrolysis) as described.

Time of digestion (min)	Thin layer chromatography	Analysis by column chromatography	
		Before acid hydrolysis	After acid hydrolysis
5	Tyr, Ala	–	–
10,30	Tyr, Ala	Tyr, Ala	Tyr, Ala
60	Tyr, Ala His, Asn	Tyr, Ala His, Asn	Tyr, Ala His, Asn
90	Tyr, Ala His, Asn Ser, Ile	Tyr, Ala His, Asn Ser, Ile	Tyr, Ala His, Asn Ser, Ile
120	Tyr, Ala His, Asn Ser, Ile Phe, Leu	Tyr, Ala His, Asn Ser, Ile Phe, Leu	Tyr, Ala His, Asn Ser, Ile, Phe, Leu

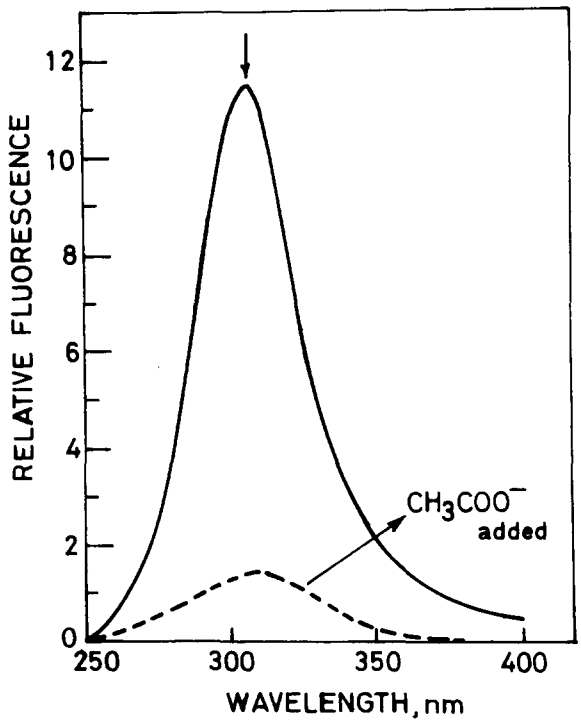

Fig. 3. Fluorescence characteristics of amino acid released from aldolase. Digestion of dialysed rabbit muscle aldolase (16 mg) by rat muscle lysosomal fraction was carried out as in Fig. 2, and the reaction was stopped with 50% perchloric acid at the end of 5 min. Deproteinised supernatant was used for fluorescence spectra by exciting at 275 nm. Arrow on the solid curve indicates the tyrosine fluorescence peak (303 nm) and the broken line shows the quenching of the same in the presence of 2 M acetate.

tyrosine as one of the first products of degradation was confirmed by fluorescence analysis of digestion mixture obtained after 5 min. The maximum of the single peak in the ultraviolet fluorescence spectrum (Fig. 3) at 303 nm upon excitation at 275 nm indicated it to be due to tyrosine. Tryptophan was not present. This was confirmed by quenching of the fluorescence peak by acetate, which was earlier shown to be a specific quencher of tyrosine [24].

Correlation of amino acid release with activity loss

Quantitative determinations of tyrosine released during digestion were carried out using Folin reagent [18]. The results incorporated into Fig. 2 for comparison show that a nearly linear relationship exists between loss of aldolase activity and release of tyrosine. The maximum loss of 50% is reached when tyrosine release is also at the maximum and this value does not change upon further incubation upto 2 h.

The total amino acids released were assayed at the same time intervals as above and the relationship between total amino acids released to tyrosine released is shown in Fig. 4. Tyrosine does not appear to be the only amino acid released in the early stages of digestion, since more leucine equivalents than can be accounted for by tyrosine alone were present. This could be attributed to the presence of alanine which was shown to be present in the early stages of incubation by TLC and amino acid analyser. At the time of 50% inactivation of aldolase, about 3.8 equivalents of leucine were released of which tyrosine would account for 2 equivalents [3]. The values for tyrosine show that while the release of tyrosine was limited at 2 equivalents, other amino acids were still being released upto 2 h. No further loss in aldolase activity, however, took place though a release of 7.6 leucine

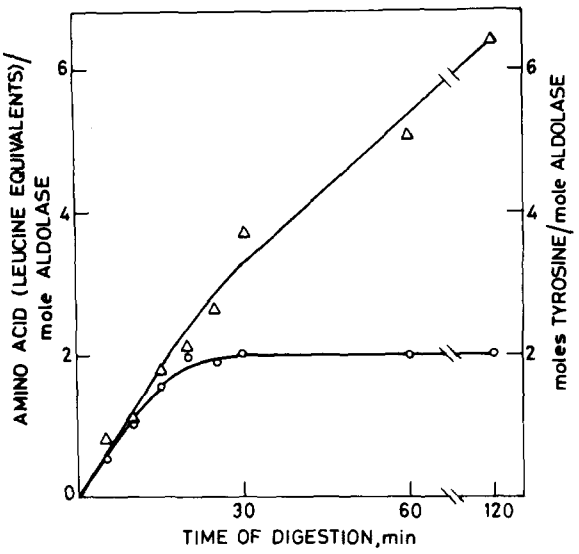

Fig. 4. Correlation of total amino acid and tyrosine release by cathepsin action on aldolase. Digestion of dialysed rabbit muscle aldolase (64 mg) by rat muscle lysosomal fraction (40 mg) was carried out in a volume of 11.0 ml as in Fig. 2. Suitable aliquots were removed at indicated times into 10% TCA and deproteinised supernatants were used for determinations of tyrosine [18] and total amino acids as leucine equivalents [23]. △———△, amino acid (leucine equivalents)/mol aldolase; ○———○, mol tyrosine/mol aldolase.

equivalents in 120 min was observed. These amino acids could be accounted for as the presence of histidine, aspargine, serine, isoleucine, leucine and phenylalanine as per the qualitative analysis (Table VI). Their sequence in the C-terminus of aldolase is established as Leu-Phe-Ile-Ser-Asn-His-Ala-Tyr [32]. They do not appear to be necessary for aldolase activity. activity.

Discussion

The data presented in this paper elucidate the role of lysosomal proteases in the stress-dependent modification of catalytic and structural properties of aldolase in rat muscle. The levels of protease capable of inactivating aldolase increased following irradiation and were found to commensurate with decreases in the aldolase levels. The recovery of muscle aldolase activity started at the time when protease levels started to decline.

The release of proteolytic enzymes into the cytosol fraction of muscle extract of irradiated animals was confirmed by caseinolytic assay performed under isotonic conditions. Increased activity of the proteolytic enzymes implicated here following treatment with Triton X-100 points to their structure-linked latency and lysosomal localization. The maximum increase in 'free' proteolytic activity was seen at the time of maximum loss of aldolase activity following irradiation and was most pronounced in the soluble fraction. At the same time considerable changes in the distribution of activities were seen in the lysosomal particulate fraction; these were a loss in percent of 'bound' activity and an increase in 'total' activity. The loss could be attributed to increased fragility of lysosomes, induced by radiation, leading to membrane permeability resulting in an increase of 'free' proteolytic activity in the soluble fraction. Changes in distribution of different lysosomal enzymes in different tissues following irradiation are known [33,34] and have been attributed to the damage of membrane due to production of free radicals and lipid peroxides [35–37]. The possibility of increased synthesis as an indirect effect of liberation of hormones inducing protein synthesis cannot be ruled out [38]. In muscle extracts prepared in EDTA, the medium used for the extraction of aldolase, 'free' proteolytic activity was negligible. Nonetheless, 'free' activity appeared in the EDTA extract also after irradiation indicating release of proteolytic enzymes. The decrease in muscle aldolase levels following irradiation is, therefore, attributed to the concomitant increase in the 'free' proteolytic activity in the same tissue. Rabbit muscle aldolase was also inactivated. The changes observed in the kinetic parameters of rat aldolase also supported the involvement of proteolytic enzymes in the inactivation. Degradative enzymes are released from lysosomes during pathological conditions and degrade other proteins present in the same tissue [39]. Increases in both 'free' and 'total' activities of lysosomal enzymes in chicken and rabbit skeletal muscles during muscular dystrophy were reported [40,41]. Pontremoli et al. [42–44] showed that under the stress of exposure to cold and starvation of rabbit, the increased lysosomal proteases were capable of altering properties of liver fructose bisphosphatase and modulation of gluconeogenesis through such changes was suggested.

Cathepsin B is known to be present in rat skeletal

muscle lysosomes [6,19]. We have confirmed the presence of cathepsin B in rat muscle lysosomal fraction as well as in soluble fraction after irradiation by their ability to hydrolyse synthetic substrates normally employed for the assay of cathepsin B activity. The dependence on free SH groups and EDTA in degradation (a requirement of cathepsin B) was demonstrated earlier [1]. Lysosomal carboxypeptidase (cathepsin B_2) hydrolyses only the amide and does not inactivate aldolase (Ref. 19 and Ninjoor, V., personal communication). Participation of cathepsin L which shares this activity is ruled out here since it reportedly lacks activity against synthetic substrates of cathepsin B apart from the general substrate Bz-Arg-NH_2 [12]. Moreover, inactivation of aldolase is employed as a routine test for the detection and assay of cathepsin B in addition to hydrolysis of Bz-Arg-NNap and Bz-Arg-Nan [6,7,12,19]. We have further shown that the inactivation of aldolase was inhibited in the muscle fraction treated with leupeptin, antipain and iodoacetamide which are known inhibitors of cathepsin B [28–30]. Therefore, the inactivation of aldolase due to the protease found in the soluble part of muscle extract from irradiated rat can be ascribed to the action of cathepsin B present.

For the elucidation of the changes caused by rat muscle cathepsin B, at molecular level rabbit muscle aldolase provided an excellent model system because of its known primary structure of subunits. Besides, muscle aldolases from rabbit and rat have been shown to be structurally quite similar [31]. Rat muscle aldolase was also shown to have C-terminal tyrosine residues which are released during inactivation by carboxypeptidase. The time-dependent changes in the catalytic properties of rabbit aldolase by cathepsin B were similar to those observed earlier for rat muscle aldolase activity following irradiation, in that, only the FDP activity was reduced without any change in F1P activity. Tyrosine was one of the first products of degradation along with alanine. The inactivation of aldolase is attributed to the hydrolysis of its tyrosine residues. That the C-terminal tyrosine residues of aldolase are involved in its catalytic function and their removal leads to loss in FDP activity was first demonstrated by carboxypeptidase treatment of aldolase [3]. The neighbouring amino acids are reportedly not important in catalytic function. Tyrosine release is limited to 3 mol/mol aldolase when nearly 95% activity is lost and this is followed by the sequential release of other amino acids. In our experiments, neither did the value of 2 mol tyrosine/mol aldolase increase nor did the activity loss go beyond 50%, in spite of additional release of 5–6 amino acids. The incomplete loss may be attributed to the presence of cathepsin B inhibitors known to be present in muscle [12,19]. That the loss does not reach completion means that a good amount of aldolase structure including some C-terminal portion, still remains. Only a single dipeptide Ala-Tyr was reported to be released during proteolysis of rabbit muscle aldolase by purified cathepsin B from rabbit liver [8]. During the course of this work Bond and Barrett [9] published the details of cathepsin B degradation of aldolase. Eight dipeptides including Ala-Tyr were shown to be sequentially released from the C-terminal of aldolase by the action of human liver cathepsin B; leupeptin and iodoacetamide inhibited the proteolytic action which otherwise resulted in FDP activity loss in aldolase. The data presented in this paper which were obtained with rat muscle cathepsin B are thus in general agreement with these results [9] obtained by the action of pure liver cathepsin B on rabbit muscle aldolase. Though no dipeptides were detected in our reactions, some amino acids were detected and they were apparently released after tyrosine and alanine. The quantities of released amino acids (unpublished data) were suggestive of initial cleavage at the known dibasic sequence from C-terminal of aldolase [32] and similar to that already reported [9]. The absence of dipeptides in our experiments can be attributed to further degradation by the other proteases which are present in the lysosomal fraction used. At the same time it is surprising that no further degradation of the already degraded aldolase was observed in 2 h.

Most studies on the degradation of aldolase by cathepsin B were carried out with enzymes derived from different tissues [6,8,9]. Though limited proteolysis was suggested [8] as a means of regulation of aldolase activity, how this interaction could be achieved in vivo was not made clear. One of the ways by which regulation of one protein by another in the same tissue can be achieved is due to the latency characteristics of one of them. This would mean that either the increase in the activity of the degrading enzyme was likely to be detrimental to the cells or that there is an altered sub-cellular localization or

lysosomal membrane function associated with these changes, and which in turn could be linked to the changes in the levels of other enzymes in the same tissue. Lysosomal processes have been demonstrated to be quantitatively important in the overall degradation of endogenous proteins in many tissues [10,11], but they fail to indicate whether the degradative actions are normally involved in a rate-limiting or regulatory step. A great variety of cellular substrate proteins other than enzymes are available for degradation e.g., collagen, glucagon and contractile proteins, for cathepsin B action [45,46]. Therefore, any regulation of activity of the substrate protein may depend on the differential involvement of the amino acid residues which are removed, in the catalytic function of the substrate enzymes. In other words if the amino acids removed by hydrolysis are not required for the function of the enzyme which acts as a substrate, the protease need not have any role in its regulation. This point is relevant because from the earlier work on the glycolytic enzymes as substrates [6] it is not clear, whether in the case of enzymes shown as not inactivated by cathepsin B, the activity was retained in spite of any degradation of the substrate enzyme since similar interactions as described here are possible. The data presented in the foregoing have contributed evidence which focuses on the above aspects. Aldolase and cathepsin B, are both muscle myofibrillar proteins [47,48]. Cathepsin B fulfils the requirements of a rate-limiting enzyme in the degradation of aldolase. The stress-dependent proteolytic degradation of aldolase in vivo strongly supports the idea that the activity of aldolase can be enzymatically regulated by this process. Such lysosomal proteolytic action could be relevant not only to the regulation of aldolase activity for glycolysis in muscle but also to the degradation of aldolase during stress conditions related to tissue damage and the maintenance of normal aldolase levels in the blood [2].

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