Note

Free and Bound-form N^{ϵ} -Methylhistidines in Acid-soluble Fraction of Rat Muscle: The Effect of Starvation, Protein Deprivation or Hydrocortisone-treatment

Naoyuki NISHIZAWA, Tadashi NOGUCHI,
Shin-ichi HAREYAMA and
Ryuhei FUNABIKI*

Department of Agricultural Chemistry, Iwate
University, Morioka, Iwate, Japan

*Department of Agricultural Chemistry,
Tokyo Noko University,
Fuchu-shi, Tokyo, Japan

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Recently, the rates of catabolism of myofibrillar proteins have been estimated by many workers from the urinary excretion of N^{ϵ} -methylhistidine (3-methylhistidine; Me-His).¹⁻⁶⁾ We also applied this method to animals under various nutritional conditions. The results clearly showed that the rates of synthesis and catabolism are regulated in different way under the physiological situations studied in our laboratories.⁷⁻⁹⁾ However, metabolic fates of Me-His in the myofibrillar proteins are not fully elucidated. Since peptide-form Me-His is not excreted into urine,¹⁰⁾ the peptide containing Me-His is supposed to be hydrolyzed to amino acids at some place in the body, presumably within muscle cells. If the peptide containing Me-His is hydrolyzed within muscle cells, small peptides containing Me-His and free Me-His (f-Me-His) will be found in muscle. Furthermore, by estimating these Me-Hises, the mechanism of degradation of myofibrillar proteins will be elucidated more extensively. Therefore, in the present investigation, free and total Me-Hises in the acid-soluble fraction of rat muscle were estimated.

Donryu male rats weighing 200~250 g were used for all experiments. They were caged individually and nourished with a 20% casein diet as described previously¹¹⁾ with the exception of vitamin and salt mixtures. Vitamin and salt mixtures were prepared according to National Academy of Sciences.¹²⁾ This diet will be referred to as casein diet. Animals were divided into four experimental groups of each five rats as follows: (I) casein diet group, given casein diet for 5 days; (II) protein-free diet group, given a diet without protein for 5 days; (III) hydrocortisone-treated group, injected subcutaneously 3 mg hydrocortisone (Wako Pure Chemical Industries Co., Ltd., Osaka, Japan)/100 g

body weight and thereafter received casein diet for 3 days; (IV) starvation group, deprived food for 5 days. Food and/or water were available at any time. Numbers of days from dietary or hormonal treatments to sacrifice of animals were selected from our experiments with the exception of casein diet and protein-free diet groups, since urinary Me-His excretion was maximum on the 5th day after deprivation of food or on the 3rd day after administration of hydrocortisone.¹³⁾ After these experimental periods, animals were anesthetized with diethylether and killed by exsanguination. The hind limbs were removed and immediately frozen in liquid nitrogen. It took 1 min to exsanguinate and excise hind limbs. It is very important to excise and freeze the hind limbs as rapidly as possible in order to measure Me-His amount in intact muscle cells. Frozen limbs were stored at -20°C until they were used for analysis. Frozen muscle of 6~11 g was cut into small pieces using scissors and homogenized in ice-cold 1% picric acid using a Polytron homogenizer (Kinematika Co., Ltd., Switzerland). These procedures were carried out in a cold room (4°C). The homogenates were left to stand at 4°C overnight and centrifuged for 10 min at $400\times g$. The supernatants obtained thus were passed through a column of Dowex 1X8 of 200~400 mesh in order to remove picric acid. The filtrates were reduced to dryness using a rotary evaporator and dissolved in 4.2 ml of water. One-half of the samples was used to isolate f-Me-His in muscle according to the method of Nishizawa *et al.*¹⁴⁾ The other part was hydrolyzed in 6 M HCl for 24 hr at 110°C and used to isolate total Me-His including bound-form Me-His (b-Me-His). Me-His obtained thus was measured by an amino acid analyzer (JEOL-5AH; Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan) operated at high sensitivity. In order to estimate the amount of purified Me-His as quickly as possible, amino acid analyzer was operated as follows. Using a column (8×120 mm) of JEOL LC-R-2 resin, chromatography was started at 30°C with 0.38 M sodium citrate buffer (pH 4.21). After 75 min, the column temperature was raised to 57°C . By this method, histidine and Me-His were separated completely. The former appears after 107 min and the latter 117 min. Although Me-His fraction of pyridine column chromatography¹⁴⁾ may not be perfectly free from carnosine and/or anserine, subsequent application of the present method excluded contamination of these peptides. Duplicate determinations showed the recovery of Me-His to be $98\pm 1\%$ (mean $\pm$ SE, n 8), when $0.75\sim 3.00\text{ }\mu\text{g}$ of Me-His was estimated. The results were evaluated statistically by Student's t test.¹⁵⁾

Starving rats contained the most f-Me-His in their muscle among the groups of rats (Table I). The f-Me-His content in muscle of this group of rats was significantly higher than that of rats fed casein diet. Although the rats deprived protein from their diet showed higher

TABLE I. THE AMOUNT OF FREE AND BOUND-FORM *N*^ε-METHYLHISTIDINES (f-Me-His, b-Me-His) IN ACID-SOLUBLE FRACTION OF RAT MUSCLE UNDER SOME NUTRITIONAL OR HORMONAL CONDITIONS^a

Feeding regimen		f-Me-His	Total Me-His	b-Me-His ^b
Casein	(5)	0.546±0.050	0.601±0.044	0.055±0.016
Starvation	(5)	0.785±0.070 ^{1c}	1.04±0.06 ²	0.261±0.019 ³
Hydrocortisone-treatment	(4)	0.435±0.047	0.546±0.046	0.111±0.019
Protein deprivation	(5)	0.677±0.051	0.848±0.045 ¹	0.170±0.040 ¹

^a μg/g muscle: Mean±SE; No. of rats in parentheses.

^b b-Me-His=total Me-His-f-Me-His.

^c Significant difference from casein diet: ¹*p*<0.05; ²*p*<0.01; ³*p*<0.001.

f-Me-His content in their muscle than the rats given casein diet, the difference was not statistically significant. Injection of hydrocortisone did not affect both f-Me-His and b-Me-His contents in muscle (Table I), although this corticosteroid is known to enhance the catabolism of muscle proteins. Starving rats contained the highest amount of b-Me-His in their muscle. Rats fed protein-free diet also showed high b-Me-His content. The content of b-Me-His in these two groups of rats was significantly higher than that in groups of rats given casein diet (Table I). If we suppose that b-Me-His is the peptide-form Me-His, it is concluded that starving rats or protein deprived rats contained higher amount of the peptide-form Me-His than normal rats fed enough amount of food and protein. However, the mechanism of increase of b-Me-His under these two nutritional conditions might be different, since catabolism of myofibrillar proteins increases under starvation and decreases under protein deficiency.^{8,13} Although an explanation of physiological meaning of these results is difficult at present, our recent results suggest that total amount of peptides (excluding carnosine, anserine and glutathione) in skeletal muscle also increases under the nutritional conditions bringing about loss of muscle mass (Noguchi *et al.* unpublished results).

Since recent studies show that Me-His is localised at the inner part of peptide chain of myosin and actin,^{16,17} the acid-soluble peptides containing Me-His might probably be the products of catabolism of myofibrillar proteins. However, we have to take into account the possibility that b-Me-His is derived from other derivatives of Me-His: *e.g.* *N*-acetyl-Me-His. Although it is known that a part of Me-His in the urine is *N*-acetyl Me-His,¹⁰ the organ or tissue which acetylates Me-His is not established yet.

The previous study¹¹ showed that, taking into account the contribution of skin and gastrointestinal to daily urinary output of Me-His,¹¹ fractional flux rate of Me-His in skeletal muscle protein is 2.29%/day (half-life of 30 days) (Fig. 1). From these results on the rate of muscle protein catabolism and the present investigation, the turnover time of f-Me-His and b-Me-

His was calculated to be 6 hr (fractional flux rate of 17%/hr) and 0.6 hr (fractional flux rate of 167%/hr) from the following equation:

turnover time (hr)

$$= 24 \left(\frac{\text{amount of f- or b-Me-His in muscle}}{\text{daily amount of Me-His released from muscle}} \right)$$

FIG. 1. Summary of the Pathway of *N*^ε-Methylhistidine (3-Methylhistidine; Me-His) of Tissues into Urine in Rats Given a 20% Casein Diet.

Figures are presented as mg/g fresh tissue per 100 g body weight. Me-His in skin and gastrointestinal is total amount in their muscle.¹¹ Pool-size of Me-His in skeletal muscle protein was calculated on the basis that 90% of total amount (4.65 mg/100 g body weight) in the body is originated from muscle.⁹ Pool-size of free and peptide-form Me-Hises in skeletal muscle was calculated from Table I and on the basis that skeletal muscle mass is 45% of body weight.¹⁸ Flux rate of Me-His from skeletal muscle (0.096 mg/day) was calculated from urinary flux rate¹¹ and that of skin and gastrointestinal.¹¹

Figure 1 summarizes the results of the present work with our previous results,^{9,11} showing the flux rates of Me-His in the body. Further, studies on the chemical properties of peptides containing Me-His and direct measurement of turnover of f-Me-His using radioisotope are now progress in our laboratories.

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