TRANSFER RNA METHYLASE ACTIVITY IN NORMAL AND DYSTROPHIC CHICKEN MUSCLE

Bertrum Sheid and Lauri Pedrinan

Department of Pharmacology
State University of New York
Downstate Medical Center
450 Clarkson Avenue
Brooklyn, New York 11203

Received January 18,1977

SUMMARY: Adult-dystrophic chicken muscle had 30% higher tRNA methylase activity and 42% higher tRNA methylating capacity than normal-adult chicken muscle. Eighty percent of the tRNA methylase activity of the dystrophic muscle resulted in the synthesis of N^2-methylguanine, and 9% in the formation of N^2,N^2-dimethylguanine. From adult-normal muscle extracts, 33% of the tRNA methylase activity was due to the synthesis of N^2-methylguanine, and 45% to the formation of N^2,N^2-dimethylguanine. Eight other methylated bases accounted for 5-15% of the enzyme activity in both tissues. Dialyzed and nondialyzed adult-normal muscle extracts had equivalent tRNA methylase activity. However, the dialyzed extracts synthesized 22% more N^2-methylguanine and 18% less N^2,N^2-dimethylguanine than the nondialyzed extracts. Dialysis had no effect on the tRNA methylase activity or tRNA methylation pattern produced by adult-dystrophic muscle.

Considerable evidence has been accumulated to demonstrate that enzymatically induced modifications of tRNA after its initial synthesis play an obligatory role in the regulation of protein biosynthesis and, consequently, in the progression of normal growth and differentiation (1,2). Numerous experiments have shown that the enzymatic modifications of tRNA are necessary for the conformational and functional integrity of the individual tRNA isoaccepting tRNA species (1,3-10). As a consequence of these studies, it has been postulated that changes in a cell's constituent proteins, such as those which occur during differentiation and neoplasia, should be reflected by coinciding alterations in the modification of cellular tRNA. To support this hypothesis in relation to eukaryotic cells, investigators have demonstrated quantitative differences in the major and minor nucleotides of tRNA from the Morris hepatomas (11), and during erythroid differentiation of murine virus-induced leukemic cells (12). Other studies have demonstrated qualitative and quantitative changes in the tRNA modification enzymes from mitogen-stimulated lymphocytes (13), differentiating

ISSN 0006-291X

embryonic rabbit cells (14), and in various malignancies of chemical and viral etiology (1,5,15-17). From these data it has become apparent that if diversions from the normal pattern of modification of tRNA during normal growth and/or differentiation do occur, they may conceivably culminate in the synthesis of altered cellular constituents (16,17).

The most prominent modification of tRNA is the enzymatic synthesis of the methylated bases by a group of enzymes collectively known as the tRNA methylases (1,5). Although there have been numerous investigations concerned with the tRNA methylases in various mammalian tissues, none of these studies has involved differentiating muscle tissue. Since the most severe forms of human muscular dystrophy (the X-linked Duchenne type, and its variant, the slower growing Becker type) are believed to be due to aberrant maturation as a result of alterations in protein synthesis (18-20), the need to study the enzymatically induced modifications of tRNA from normal and diseased muscle tissue is apparent.

MATERIALS AND METHODS

New Hampshire Red normal and genetically determined dystrophic chickens (12-13 months old), which have been widely used as a model for human Duchenne muscular dystrophy (21), were kindly provided by Drs. A. Stracher and E.B. McGowan, of our institute. The diagnosis of muscular dystrophy was verified by the "flip test," whereby the chicken cannot right itself after being placed on its back (21). High-speed supernatant fractions (105,000 x g; 1 hr) of superior breast muscle from the test animals were prepared as previously described (17). A portion of each fraction was lyophilized, and stored until use. Other portions were either dialyzed against cold distilled water for 24 hr before lyophilization, or treated with $(NH_4)_2SO_4$ to 60% saturation before dialysis and lyophilization. Just prior to the enzyme assays, as many of the enzyme samples as needed were reconstituted with 1 ml of water per 0.5 g tissue. Initial experiments were performed to establish the fact that the enzyme preparations stored in this manner were 100% active for a period of at least one month. The standard reaction for tRNA methylase activity consisted of 10 µmoles Tris buffer, pH 8.9; 80 µmoles of ammonium acetate; 1 µmole of freshly prepared and neutralized reduced glutathione; 25 µg of *E. coli*$_B$ tRNA; 1 µCi of neutralized [H^3]S-adenosylmethionine; (11.6 Ci/mmole, New England Nuclear); and 50-2000 µg of protein from the 105,000 x g supernatant fraction in a total volume of 0.3 ml. After 45 min at 37°C, the reaction was terminated with 0.06 ml of 50% trichloroacetic acid and 0.02 ml of 6-N HCl. The precipitates were vigorously washed in the test tubes with 80% alcohol, 2 x with alcohol-ether (3:1) and ether, then dried *in vacuo*. They were then dissolved in alcoholic-hyamine at 70°C, neutralized, transferred to Bray's solution and counted. Enzyme blanks (one without tRNA and another without protein) were assayed concurrently with all of the samples. The tRNA methylation capacity was determined by the addition of increasing amounts of enzyme extract protein to the reaction mixture until no significant change in the total [^3H] incorporation per 25 µg tRNA could be detected. To determine the specific activity, the proportional range of enzyme activity was utilized. Utilizing this procedure, blanks

Table 1. Transfer RNA methylase activity in normal and dystrophic-chicken breast muscle.[a]

Tissue	Treatment	Methylation capacity (maximum amount of pmoles methyl-^{3}H incorporated/25 µg tRNA)[b]	Specific activity (pmoles methyl-^{3}H incorporated/200 µg protein/25 µg tRNA)[c]
Adult normal muscle	-	0.31	0.16
Adult normal muscle	Dialysis[d]	0.29	0.16
Adult dystrophic muscle	-	0.44	0.21
Adult dystrophic muscle	Dialysis	0.46	0.20
Embryonic normal muscle	-	1.64	0.54
Embryonic normal muscle	Dialysis	1.24	0.60

[a]These data are an average of 4 separate samples of each tissue assayed in duplicate. The individual results did not differ from each other by more than 6%.

[b]The amount of protein for maximal incorporation of (methyl-^{3}H) was between 1000-1250 µg for all of the samples.

[c]The proportional range for enzyme activity was 175-225 µg protein.

[d]Dialyzed and ammonium sulfate-treated muscle supernatant extracts had the same enzyme activity.

of 100-700 cpm for 50-2000 µg of muscle protein were obtained, while blanks of 1000-5000 cpm were found to be routine if the precipitates were first placed on filter paper discs before washing. Duplicate assays were reproducible within 5% of each other. The enzymatically synthesized methylated bases were identified and quantitated after two-dimensional thin-layer chromatography on microcrystalline cellulose (22). Protein was determined by the method of Lowry *et al.* (23). RNAase assays were performed by measuring the 260 nm adsorption of the acid-soluble oligonucleotides formed from tRNA after incubation with the muscle protein extracts (24).

RESULTS AND DISCUSSION

Table 1 demonstrates an approximate 30% increase in the tRNA methylase specific activity, and 42% increase in the tRNA methylation capacity in dystrophic-muscle extracts as compared to normal adult-muscle extracts. This increase in activity was not due to reduced RNAase activity in the dystrophic-muscle extracts, or to an increase in the metabolism of S-adenosyl-L-methionine by the normal-muscle extracts. Dialysis and/or ammonium sulfate treatment of the enzyme extracts from normal and dystrophic muscle had no apparent effect on their specific enzyme activity.

Table 2. Transfer RNA methylation patterns produced by enzyme extracts derived from normal (adult and embryonic) and dystrophic-chicken breast muscle.[a]

	Dystrophic adult muscle[b]		Normal nondialyzed adult muscle		Normal embryonic muscle[b]		Normal dialyzed adult muscle[c]	
	fmoles CH_3 incorp./ 100 µg tRNA	% total incorp.	fmoles CH_3 incorp./ 100 µg tRNA	% total incorp.	fmoles CH_3 incorp./ 100 µg tRNA	% total incorp.	fmoles CH_3 incorp./ 100 µg tRNA	% total incorp.
N^2-methylguanine	1810	79	392	33	1980	33	572	55
N^2N^2-dimethylguanine	205	9	532	45	2660	45	280	27
7-methylguanine	135	6	132	11	604	12	80	8
1-methylguanine	65	3	36	3	197	3	40	4
5-methyluracil	25	1	36	3	238	4	24	2
3 + 5-methylcytosine	25	1	44	4	184	2	36	3
1-methyladenine	tr[d]	tr	tr	tr	tr	tr	tr	tr
2-methyladenine	tr	tr	tr	tr	tr	tr	tr	tr
6-methyladenine	tr	tr	tr	tr	tr	tr	tr	tr
6-dimethyladenine	tr	tr	tr	tr	tr	tr	tr	tr

[a] Methylation patterns are an average of the results obtained for 3-5 specimens. 90-95% of all counts were recovered as methylated bases.

[b] Dialysis or $(NH_4)_2SO_4$ treatment had no effect on the methylation pattern produced by dystrophic muscle and normal embryonic tissue.

[c] The same tRNA methylation pattern was obtained whether the enzyme extracts from normal adult muscle was dialyzed or made 60% with respect to $(NH_4)_2SO_4$ before dialysis.

[d] tr equals trace (less than 1% of total).

Table 2 demonstrates that 80% of the tRNA methylase activity of dystrophic muscle resulted in the synthesis of N^2-methylguanine, while 9% resulted in the formation of N^2,N^2-dimethylguanine. In normal nondialyzed adult-chicken muscle, 33% of the enzyme activity resulted in the synthesis of N^2-methylguanine, while 45% resulted in the formation of N^2,N^2-dimethylguanine. The synthesis of eight other methylated bases accounted for the rest of the tRNA methylase activity in normal and dystrophic muscle.

The increased tRNA methylase specific activity and tRNA methylating capacity of dystrophic muscle as compared to normal muscle may at first be considered a reflection of the increase in protein synthesis which is known to occur in adult dystrophic muscle (19,20). However, the fact that the two types of tissue, in the presence of an exogenous heterologous tRNA substrate, synthesize a different ratio of N^2-methylguanine:N^2,N^2-dimethylguanine may imply that, besides an increase in protein synthesis in dystrophic muscle, there may be alterations in specific proteins of the diseased tissue (16,17).

It is also demonstrated in Table 1 that dialyzed and nondialyzed normal-muscle extracts had the same tRNA methylase specific activity and tRNA methylating capacity. However, as seen in Table 2, although the different extracts synthesized approximately the same total amount of the two methylated guanine derivatives, the dialyzed extract resulted in the synthesis of 22% more monomethylguanine and 18% less dimethylguanine than the nondialyzed normal-muscle extract. Since dialysis and/or $(NH_4)_2SO_4$ treatment had no effect on the tRNA methylation pattern produced by dystrophic muscle as it did on normal muscle, it may be suggested that the qualitative differences in the synthesis of methylated bases between normal and dystrophic adult muscle are due to dialyzable, small molecular weight factor(s) present exclusively in the normal tissue.

It of course would be of interest to determine if the difference in the expression of the tRNA methylases between normal and dystrophic muscle is concomitant with an alteration in the base sequence of a specific isoaccepting tRNA in dustrophic muscle. Quantitation of the different methylated bases in nonfrac-

tionated tRNA may give misleading results. It is more reasonable to expect that if an altered tRNA is involved in muscular dystrophy, it will be limited to a specific isoaccepting tRNA, one which plays a pivotal role in muscle-cell maturation.

Table 1 also shows that the tRNA methylase activity in 15-day-old normal embryonic-chick muscle was 5 times higher than the enzyme activity in normal adult muscle. This is in agreement with previous data which described increased tRNA methylase activity in embryonic tissues (25). However, as seen in Table 2, the tRNA methylation patterns produced by normal-adult and normal-embryonic muscle were essentially equivalent to each other. These experiments imply that the different tRNA methylation pattern produced by adult dystrophic muscle as compared to adult normal muscle was not a reflection of muscle disuse as exemplified by embryonic muscle.

REFERENCES

(1) Borek, E. (1969). In: Exploitable Molecular Mechanisms and Neoplasia (M.D. Anderson Hospital and Tumor Institute Symposia), pp. 163-190. The Williams and Wilkins Co., Baltimore, Maryland.
(2) Zamecnik, P.C. (1971). Cancer Res. 31, 716-721.
(3) Soll, D. (1971). Science 173, 293-299.
(4) Goldberger, R.F. (1974). Science 183, 810-816.
(5) Borek, E. (1971). Cancer Res. 31, 596-597.
(6) Kearns, D.R., Wong, K.L. and Wong, Y.P. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 3843-3846.
(7) Marcu, K., Mignery, R., Raszelbach, R., Roe, B., Sirover, M. and Dudock, B.S. (1973). Biochem. Biophys. Res. Commun. 55, 477-483.
(8) Simsek, M. and RajBhandary, U.L. (1972). Biochem. Biophys. Res. Commun. 49, 508-515.
(9) Simsek, M., Siegenmeyer, J., Heckman, J. and RajBhandary, U.L. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 1041-1045.
(10) Petrissant, G. (1973). Proc. Natl. Acad. Sci. U.S.A. 70, 1046-1049.
(11) Randerath, E., Chia, L.L.S.Y., Morris, H.P. and Randerath, K. (1974). Cancer Res. 34, 643-653.
(12) Agris, P.F. (1975). Arch. Biochem. Biophys. 170, 114-123.
(13) Sharma, O.K. and Loeb, L.A. (1973). Biochem. Biophys. Res. Commun. 50, 172-178.
(14) Manes, C. and Sharma, O.K. (1972). Nature 244, 282-284.
(15) Nau, F. (1974). Biochem. 13, 1105-1109.
(16) Sheid, B., Wilson, S.M. and Morris, H.P. (1971). Cancer Res. 31, 774-777.
(17) Sheid, B., Lu, T. and Nelson, J.H., Jr. (1973). Cancer Res. 33, 2518-2523.
(19) Weinstock, I.M. and Murkiewicz, J. (1974). Biochim. Biophys. Acta 374, 197-206.
(20) Battelle, B.A. and Florini, J.R. (1973). Biochem. 12, 635-643.
(21) Asmundson, V.S. and Julian, L.M. (1956). J. Hered. 47, 248-252.
(22) Munns, T.W., Podratz, K.C. and Katzman, P.R. (1974). Biochem. 13, 4409-4416.
(23) Lowry, O.H., Rosebrought, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-275.
(24) Tuve, T.W. and Anfinsen, C.B. (1960). J. Biol. Chem. 235, 3437-3441.
(25) Hancock, R.L. (1967). Cancer Res. 27, 646-650.