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ARSENITE-INDUCED CHANGES IN METHYLATION OF THE 70,000 DALTON HEAT SHOCK PROTEINS IN CHICKEN EMBRYO FIBROBLASTS

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SUMMARY - Methylated lysyl and arginyl residues are present in the two major heat shock proteins, hsp70A and hsp70B, of chicken embryo fibroblasts. Here, we demonstrate that this methylation can be modulated by sodium arsenite, a chemical that increases the synthesis of hsp70. In particular, in hsp70A the amount of ε-N-trimethyl-lysine significantly decreases and the amount of ε-N-dimethyl-lysine and ε-N-monomethyl-lysine increases, while in hsp70B, the quantity of N^G-monomethyl-arginine is reduced fivefold after arsenite treatment. To determine the specificity of these changes in methylation the pool size of S-adenosyl-L-methionine (AdoMet) and the total cellular level of methylated protein was measured. After arsenite treatment, no significant change in AdoMet pool size and the level of protein methylation was observed with the exception of an apparent increase in N^G-monomethyl-arginine in total cellular protein. Thus, the arsenite-induced changes in methylation of hsp70 polypeptides are not a generalized phenomenon and may reflect a modulation in the structure or function of these two polypeptides after their induced synthesis by this chemical.

Exposure of Drosophila melanogaster, avian and mammalian tissue or cultured cells to temperatures higher than their normal growth temperature results in a profound change in protein synthesis. Under these conditions the translation of normal cellular proteins is suppressed, and the synthesis of "heat shock proteins" is enhanced (1-4). A similar set of polypeptides is induced upon exposure of cells to sodium arsenite and some other agents (3,4,5). Despite efforts for the analysis of the genes coding for these proteins, the function of heat shock proteins is not well understood.

Among all the heat shock proteins, a polypeptide(s) of an approximate molecular weight of 70,000 (hsp70) is the most commonly induced species. In chicken fibroblasts,

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the molecular weight of these polypeptides has been measured by this laboratory as 68,000 (4,6,7), while different molecular weights have also been reported for this protein by other laboratories (3,5). Hsp70 of chicken fibroblasts is composed of two major polypeptides with the same electrophoretic mobility but distinct isoelectric points (referred to as hsp70A and hsp70B), which are synthesized under normal growth conditions and whose rate of synthesis increases five to tenfold after arsenite treatment (4). We have previously demonstrated that both of these variants are methylated, presumably by S-adenosyl-L-methionine dependent methyl transferases (4). The methylated sites have been identified as lysyl and arginyl residues, and their methylation appears to be irreversible and coupled to the synthesis of these proteins (6). In this communication, we present evidence that the methylation of hsp70 is altered after sodium arsenite treatment. The intracellular S-adenosyl-L-methionine pool size and the total cellular protein methylation remain largely unchanged after arsenite treatment, suggesting that the changes in hsp70 methylation are specific for these two polypeptides.

MATERIALS AND METHODS

1. Analysis of Methylated Basic Amino Acids of hsp70. Chicken embryo fibroblasts were isolated from 10-12 day old chicken embryonic leg muscle. The cells were grown and maintained in Dulbecco's modified Eagle's medium (0.2 mM in L-methionine) supplemented with 10% calf serum, and subcultured at least three times before use to eliminate any contaminating muscle cells. Confluent 100 mm plates containing 10^7 cells each were incubated in methionine-free minimal essential medium with 0.63 μM [methyl-^{3}H]-L-methionine (New England Nuclear, specific activity: 80 Ci/mmole) and 2% calf serum for 6 to 12 hrs with or without 25 μM sodium arsenite. The hsp70 polypeptides were isolated by two-dimensional gel electrophoresis as previously described (4,6). The polypeptides were hydrolyzed with 6 N HCl at 108°C in an evacuated tube for 24 hrs. Then the HCl was removed by evaporation under reduced pressure, and the dried residue was resuspended in 0.2 N sodium citrate buffer, pH 2.0. It was subsequently applied to an amino acid analysis column (Beckman PA-35, 0.9 x 13 cm) equilibrated with sodium citrate buffer (0.35 N in Na^+, pH 5.28). The column was operated at 38°C at a flow rate of 67 ml/hr with the same buffer. Three ml fractions were collected, and [^{3}H]-radioactivity was determined by liquid scintillation counting.

2. Isolation of [^{35}S]-adenosyl-L-methionine. S-adenosyl-L-methionine (AdoMet) was isolated essentially by the method of Glazer and Peale (8). Confluent 60 mm plates of chicken fibroblasts (3.0 ± 0.5 x 10^6 cells) were incubated with [^{35}S]-L-methionine (New England Nuclear) to a final concentration of 20 μCi/ml in a total volume of 1.5 ml (specific activity: 100 Ci/mole). After incubating for 2 min to 5 hrs, the cells were rinsed three times with phosphate buffered saline and swollen in 1 ml of 10 mM HCl in the presence of 60 nmole of AdoMet (Sigma) added as a carrier. The cells were then scraped off the plates, an equal volume of cold 30% trichloroacetic acid (TCA) was added, and the suspension as mixed well. The supernatant was collected by centrifugation and the pellet was washed twice with 1 ml 5% TCA. All the supernatants were pooled together and TCA was subsequently extracted four times with an equal volume of ethyl ether. Thereafter, the sample was passed through a SP-Sephadex (C-25, Pharmacia) column (1 x 5 cm) equilibrated with 10 mM HCl. The column was washed extensively with 0.2 N HCl until both the OD_{256} and [^{35}S]-radioactivity of the effluent reached background levels. The AdoMet was eluted immediately after addition of 0.5 N HCl, and 3 ml fractions were collected. The [^{35}S]-radioactivity was determined by liquid scintillation counting with 10 volumes of Aquasol-2 (New England Nuclear).

In the experiments reported here, greater than 70% of the AdoMet standard, as assayed by its absorbance at 256 nm, was recovered from the column. The purity of [^{35}S]-AdoMet isolated by this method was further verified by thin layer chromatography, using two different solvent systems: n-butanol:acetic acid:H_2O (12:3:5) and ethanol:acetic acid:H_2O (65:1:34). We found that more than 85% of the [^{35}S]-radioactivity comigrated with the AdoMet standard.

3. Amino Acid Analysis of Cellular Protein After Labeling with [^{3}H]-L-methionine. Confluent chicken fibroblasts were incubated with 0.63 μM [methyl^{3}H]-L-methionine with or without 25 μM sodium arsenite for 1.5 to 24 hrs. After incubation, the cells were rinsed and harvested in phosphate buffered saline. Then the polypeptides were precipitated with 15% TCA. The pellet was collected by centrifugation, washed twice with 5% TCA followed by ethyl ether (twice). The final pellet was hydrolyzed with 6 N HCl and hydrolysates were analyzed by ion exchange chromatography (Beckman PA-35) as described in the previous paragraph.

RESULTS

1. Determination of [^{35}S]-AdoMet in Control and Arsenite-Treated Cultures. [^{35}S]-adenosyl-L-methionine (AdoMet) was isolated from acid extracts of [^{35}S]-L-methionine-labeled cells as described in Methods. The results in Figure 1 indicate that [^{35}S]-AdoMet is formed rapidly ($t_{1/2}$ = 0.4 h) and the radioactivity reaches a constant level, corresponding to a concentration of 1.5 x 10^{-5} M (see Figure Legend), within 1 hr after [^{35}S]-methionine is added extracellularly. In particular, arsenite has little effect on the intracellular level of AdoMet; hence, changes in methylation of hsp70 induced by arsenite (see below) cannot be attributed to changes in AdoMet pool size.

2. [^{3}H]-Radioactivity Incorporation into Total Cellular Protein. Chicken fibroblasts labeled metabolically with [methyl-^{3}H]-L-methionine incorporate [^{3}H]-radioactivity into polypeptides not only as methionine but also as methyl groups through [methyl-^{3}H]-S-adenosyl-L-methionine. The relative incorporation of methionine and methylated basic amino acid residues can be determined by amino acid analysis of labeled proteins (6). Table I shows the kinetics of incorporation of [^{3}H]-radioactivity into methyl histidinyl, methyl lysyl and methyl arginyl residues relative to the rate of incorporation into methionine. From the data, it appears that arsenite has little effect on basic amino acid methylation in general, since the frequency of methylated amino

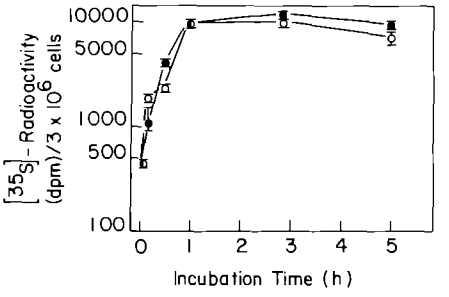

Fig. 1: Intracellular Levels of [^{35}S]-AdoMet. [^{35}S]-L-methionine was added extracellularly and, at a given time, [^{35}S]-AdoMet was isolated by the method described (8). Each point represents the average of duplicate samples. The dpm were determined by counting a set of standards. The intracellular pool size was estimated to be 45 pmole/3 x 10^{6} cells by assuming that the specific activity of intracellular [^{35}S]-AdoMet is identical to the [^{35}S]-L-methionine of the medium (100 Ci/mole) when steady state is reached. The concentration is calculated to be 1.5 x 10^{-5} M or 60 nmole/g protein by assuming that the cell volume is 1 pl (the cell volume of CHO-K1 was measured as 1.5 pl (25)), and the total cellular protein of fibroblasts is 250 pg/cell, as measured by the Lowry method. This value of concentration of AdoMet is similar to the AdoMet level obtained from tissue (26) and cultured cells (27).

TABLE I

[^{3}H]-Methyl Groups Incorporated into Basic Amino Acids of Chicken Embryonic Fibroblast Total Cellular Protein[a]

Incubation time	methyl-lysines[b]		3-N-methyl-histidine[b]		dimethyl-arginine[b]		N^G-monomethyl-arginine[b]	
	Control	Arsenite	Control	Arsenite	Control	Arsenite	Control	Arsenite
1.5 hr	1.5%	1.4%	0.37%	0.35%	2.3%	2.7%	0.33%	0.44%
3 hr	1.8%	1.8%	0.38%	0.38%	3.2%	3.6%	0.42%	0.68%
6 hr	2.2%	2.3%	0.45%	0.44%	3.8%	4.3%	0.31%	0.80%
12 hr	2.2%	2.2%	0.39%	0.46%	3.4%	3.2%	0.24%	0.53%
24 hr	2.3%	-[c]	0.46%	-[c]	3.2%	-[c]	0.20%	-[c]

[a][^{3}H]-methionine incorporated into polypeptide backbone is arbitrarily assigned as 100%. The numbers given here are the averages of two determinations.
[b]The maximum range of the measurements for methyl-lysines, 3-N-methyl-histidine, dimethyl-arginine and N^G-monomethyl-arginine is 0.2%, 0.09%, 0.2%, 0.12%, respectively.
[c]Not determined.

acids in total cellular protein is not altered appreciably after arsenite treatment. A notable exception is the [^{3}H]-radioactivity incorporated into N^G-monomethyl-arginine which increases after arsenite treatment.

From the results presented above, we can conclude that the intracellular pool size of AdoMet and the total amount of protein methylation remain largely unchanged in the presence of sodium arsenite. Therefore, we proceeded to study whether arsenite has any effect on the methylation of the hsp70 polypeptides in chicken fibroblasts.

3. Methylation of hsp70A and hsp70B. Figure 2 shows the elution profile from an amino acid analysis column of the hydrolysate of hsp70A from a sodium arsenite treated culture. Under these conditions, the methyl lysines and N^G-monomethyl-arginine are separated from the other methylated amino acids, but the three different methyl lysine species are not resolved from each other. Three different peaks of [^{3}H]-radioactivity were found, corresponding to methionine, methyl lysine and N^G-monomethyl arginine. Virtually identical results were obtained with hsp70A isolated from control cells. The profile obtained from hsp70B is similar, except that the level of N^G-monomethyl-arginine content of hsp70B drops sharply after sodium arsenite treatment. Quantitative analysis of these column profiles is shown in Table II.

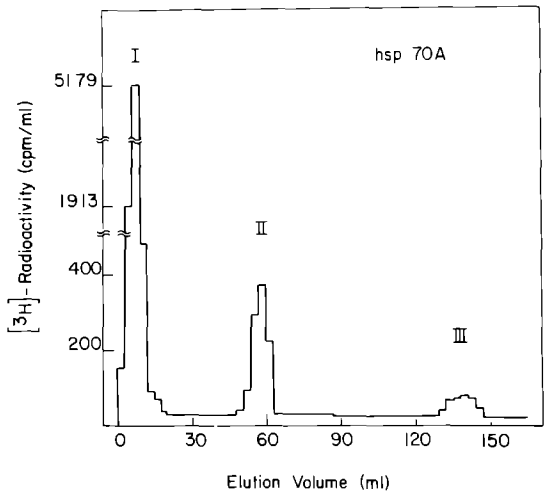

Fig. 2: Ion Exchange Chromatography of hsp70A hydrolysate. Chicken embryo fibroblast hsp70A was isolated and chromatographed as described in Materials and Methods. Three ml fractions were collected and 1 ml was analyzed for radioactivity. Peaks I, II, and III coeluted with the amino acid standards of L-methionine, methyl-lysines and N^G-monomethyl-arginine, respectively.

To further examine any changes in the distribution of three methylated lysine species after arsenite treatment, the methyl lysines were desalted and further resolved by thin layer chromatography (6). No significant differences were found in the ratio of methyl-lysines in hsp70B from arsenite and control treated cultures (data not shown). However, the percentage of ε-N-monomethyl-lysine, dimethyl-lysine and trimethyl-lysine of hsp70A changes from 15%, 6% and 79% to 26%, 5% and 49% respectively after arsenite treatment. In other words, in hsp70A the relative amount of ε-N-trimethyl-lysine decreases, while ε-N-monomethyl-lysine and ε-N-dimethyl-lysine increase after sodium arsenite treatment.

TABLE II

$[^3H]$-Methyl Groups Incorporated into Lysyl and Arginyl Residues of hsp70 of Chicken Embryo Fibroblasts[a]

	hsp70A		hsp70B	
	(-)[b]	(+)[b]	(-)[b]	(+)[b]
Methyl-lysines	10 ± 1%[c]	11 ± 1 %	9 ± 1%	9 ± 1%
N^G-monomethyl-arginine	3.4 ± 0.1%	2.9 ± 0.2%	2.3 ± 0.2%	0.45 ± 0.15%

[a]The cells were labeled for longer than 6 hrs to minimize any differences caused by asynchrony in the kinetics of $[^3H]$-Met-$tRNA^{Met}$ and $[^3H]$-AdoMet labeling as shown in Table I.
[b](-): control cells; (+): arsenite-treated cells.
[c]$[^3H]$-radioactivity incorporated into methionine is arbitrarily assigned as 100%.

DISCUSSION

Methylation of basic amino acids appears to occur as a posttranslational event. It has been reported that the methylation of cultured chicken muscle cell proteins is coupled to their synthesis (9). In the present work, experiments were performed to determine the relationship between protein synthesis and basic amino acid methylation in chicken fibroblasts. The results (Table I) are consistent with the interpretation that the majority of these methylation events are coupled to protein synthesis. We calculate that approximately six methyl groups are incorporated into polypeptide backbones; this is compatible with the results obtained by amino acid analysis of mammalian and chicken tissues (10). In addition, arsenite treatment does not change the amount of the methylated lysine species in total cellular protein, although the synthesis of the methylated lysine-containing hsp70 polypeptide inreases five to tenfold. Therefore, we conclude that the methylation of basic amino acids should be common to many proteins.

It is not clear why the level of N^G-monomethyl-arginine in total cellular protein increases after arsenite treatment (Table I). This increase may be due to the increased synthesis of hsp70. It is possible that the decrease in the level of N^G-monomethyl-arginine observed in hsp70B after arsenite treatment may be compensated by the increased number of polypeptide chains synthesized. Alternatively, the increase may result from an increase in methylation of N^G-monomethyl-arginine on some as yet unidentified polypeptide(s) after arsenite treatment.

Hsp70A and hsp70B are two distinct polypeptides, though some homology among them may exist (4,5). We reported previously that both polypeptides are methylated at lysyl and arginyl residues and that their methylation is coupled to their synthesis (6). Here, we have demonstrated that the amount of N^G-monomethyl-arginine in hsp70B is reduced fivefold after arsenite treatment, while in hsp70A, the level of arginyl methylation remains unchanged. Furthermore, the change in the distribution among three different methyl lysines of hsp70A by sodium arsenite is also evident. Since the functional role of hsp70 in fibroblasts is unknown, the significance of these changes in methylation cannot be easily verified. In addition to inducing the synthesis of heat shock proteins, arsenite may alter certain enzymatic functions (11,12). It remains to be determined whether or not the changes in methylation after arsenite treatment is a

general phenomenon for all agents which can induce the 70,000 dalton heat shock proteins.

The use of larger amounts of material and slightly different experimental protocols reported here have allowed us to obtain a better quantitative estimate than before of the degree of protein methylation (6). Assuming that the amino acid composition of hsp70 of chicken fibroblasts is the same as that of HeLa cells (13), we calculate that there are, on the average, 0.2 methylated lysines and 0.1 methylated arginine per hsp70 polypeptides, i.e., the methylation may be substoichiometric.

Methylation of several different basic amino acid residues in proteins has been documented; on most occasions, the methylation is irreversible and its functional significance is unknown. For example, the level of methylation of fungal elongation factor εF-1α changes during morphogenesis, but the enzymatic activity does not parallel these changes (14). Similarly, histone methylation occurs mainly near the end of the DNA replication phase of the cell cycle (15), but both methylated and unmethylated histon H4 can assemble equally well into nucleosomes (16). Perhaps, cytochrome c is the only case where a functional role has been assigned to its lysyl methylation. It has been suggested that methylation may facilitate the binding of cytochrome c to mitochondria (17,18) without affecting electron transport (18).

The localization of heat shock proteins has been intensively studied. In Drosophila, hsp70 is located in nuclei shortly after heat treatment (19,20,21); however, a fraction of it may also be cytosolic (20,21). By immunofluorescence, hsp70 is a cytoplasmic protein in chicken fibroblasts (22); a portion of it may be associated with the cytoskeleton or nuclei, since it is insoluble in Triton-KCl (7). A fraction of it may be associated also with a membrane protein (23) and microtubules (7,24) in cultured mammalian cells. As we have shown in this study, hsp70 can exist in several different mthylated forms; each form may possibly have a preferential intracellular localization and the changes in their methylation observed after arsenite treatment may reflect a redistribution in the cytoplasmic localization of these proteins.

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