Folate Status and Age Affect the Accumulation of L-Isoaspartyl Residues in Rat Liver Proteins[1,2]

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ABSTRACT Formation of atypical L-isoaspartyl residues in proteins and peptides is a common, spontaneous and nonenzymatic modification of aspartyl and asparaginyl sites. The enzyme protein-L-isoaspartyl methyltransferase (PIMT) catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) to these L-isoaspartyl sites, thereby allowing reisomerization and restoration of the original alpha peptide linkage. Because SAM is in part a product of folate metabolism, the present study was undertaken to determine the effects of folate deficiency on the presence of L-isoaspartyl residues in hepatic proteins. Young (weanling) and older (12 mo) Sprague-Dawley rats were fed a folate-sufficient (2 mg folate/kg diet) or folate-deficient (0 mg folate/kg diet) diet for 20 wk. Liver proteins were analyzed for L-isoaspartyl residues. This analysis was based on the PIMT-dependent incorporation of [^{3}H]-methyl groups from [^{3}H]-SAM and the subsequent (nonenzymatic) sublimation of these methyl groups into a nonaqueous scintillant. The amount of L-isoaspartyl residues in hepatic proteins was higher in younger folate-deficient than in folate-sufficient rats (deficient: 187 ± 71, sufficient: 64 ± 43 pmol/mg protein, $P < 0.025$). This difference, however, was not seen among the older groups of rats who instead exhibited a much larger accumulation of L-isoaspartyl residues in their hepatic proteins (deficient: 528 ± 151, sufficient: 470 ± 204 pmol/mg protein, $P = 0.568$). The importance of these observations is discussed. J. Nutr. 132: 1357–1360, 2002.

KEY WORDS: • folate • L-isoaspartyl • *S*-adenosyl-L-methionine • protein L-isoaspartyl methyltransferase • rats

Damaged and senescent proteins accumulate with aging and are associated with pathologic conditions such as Alzheimer's disease, diabetes and atherosclerosis (1–3). Damaged proteins can accumulate through spontaneous nonenzymatic modifications such as isomerization, deamidation and racemization. Such modifications have been observed to alter the molecular structure of the affected proteins and impair their biological function (4–7).

The enzyme L-isoaspartyl methyltransferase (PIMT,[4] EC 2.1.1.77) specifically recognizes L-isoaspartyl residues and catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) onto the α-carboxyl group of these residues (**Fig. 1**) (8,9). Methylation is followed by spontaneous demethylation, generating a cyclic imide. This imide is then hydrolyzed, resulting in a mixture of aspartyl and isoaspartyl peptides. The latter serve as a substrate for repeated rounds of methylation (10).

PIMT is a conserved and ubiquitous enzyme, which is involved in the repair of various proteins (11–16). *S*-Adenosyl-L-homocysteine (SAH), which accumulates intracellularly in the setting of folate deficiency, is a potent inhibitor of SAM-dependent methylation reactions, including those catalyzed by PIMT (17). Several cell culture and animal studies have confirmed that by pharmacologically inducing increases in SAH, there is an accumulation of L-isoaspartyl residues in proteins (11,18).

The present study was undertaken to assess the importance of folate status on the accumulation of L-isoaspartyl residues in proteins. Our premise was that folate deficiency would produce an accumulation of L-isoaspartyl residues either because of decreased availability of SAM, and/or a high level of SAH. Because aging is associated with the accumulation of these residues, we also examined how aging would affect this relationship.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University. Young (weanling) and 12-mo-old male Sprague-Dawley rats (Zivic-Miller, Zelienople, PA) were fed for 20 wk an amino acid defined diet (19,20) containing 2 mg folate/kg diet (folate-sufficient; 4 young and 8 old rats), or 0 mg folate/kg (folate-deficient; 4 young and 6 old rats) (Dyets, Bethlehem, PA). Rats were asphyxiated with carbon dioxide and livers were immediately removed and stored at −70°C until analysis.

Hepatic folate concentrations were determined by microbiological assay after conjugase treatment, using *Lactobacillus casei* as the test organism (21). Hepatic concentrations of SAM and SAH were determined by HPLC with UV detection (22).

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[4] Abbreviations used: DSIP, δ-sleep inducing peptide; PIMT, L-isoaspartyl methyltransferase; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine.

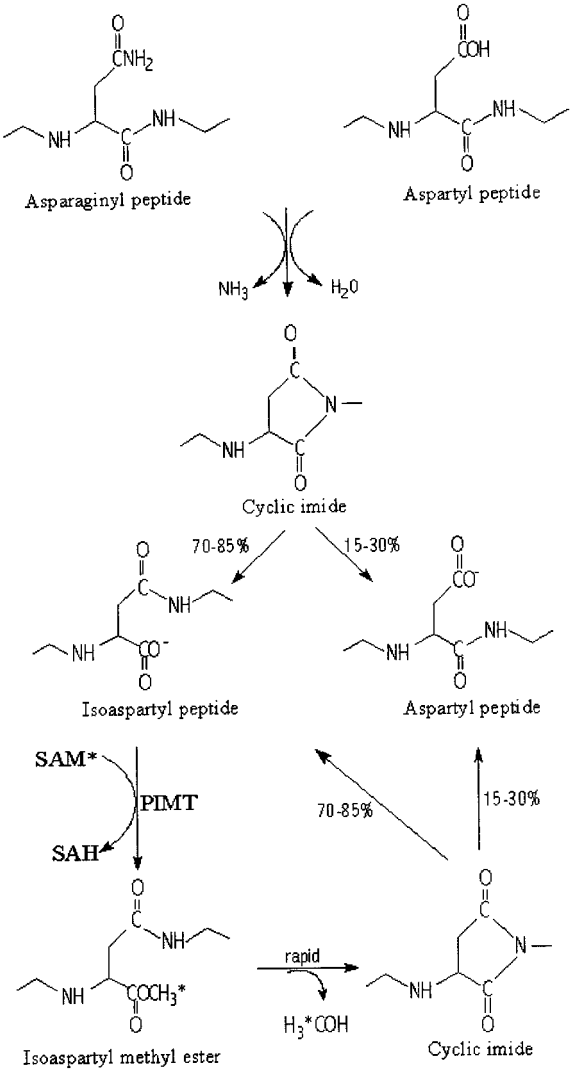

FIGURE 1 Formation of isoaspartyl residues in proteins, and PIMT-dependent conversion of isoaspartyl to aspartyl sites. SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; PIMT, Protein-L-isoaspartyl methyltransferase.

For the determination of L-isoaspartyl residues, liver samples (~15–30 mg) were sonicated for 30 s at 4°C in 1 mL of 5 mmol/L sodium piperazine-*N*,*N*′-bis-2-ethanosulphonic acid, pH 7, 2 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, 7 mmol/L 2-mercaptoethanol, 0.9 g/L leupeptin, and 10% (wt/wt) sucrose. The extract was then centrifuged at 20,000 × *g* for 60 min. The supernatant fraction was used to determine protein concentration (23). The supernatant was also used to assess L-isoaspartyl residues using the ISOQUANT protein deamidation detection kit (Promega, Madison, WI). In brief, samples (20–50 μg protein in 10 μL) were incubated for 30 min at 30°C in a reaction mixture containing PIMT (10 μL), 37 kBq [^{3}H]-SAM (37 kBq/nmol), and unlabeled SAM at a final concentration of 20 μmol/L. The reaction mixture was cooled in an ice bath and mixed with a 50 μL of stop solution (0.4 mol/L 3-[cyclohexamino]-1-propanesulfonic acid, pH 10, 5% SDS, 2.2% methanol, 0.1% *m*-cresol purple). A 50-μL aliquot of the mixture was adsorbed to a sponge attached to the cap of a scintillation vial. The vial was incubated at 40°C for 60 min to allow [^{3}H]-methanol diffusion into the scintillation mixture and the vial was subsequently counted. As a positive control, we used δ-sleep–inducing peptide (DSIP), which was provided in the ISOQUANT kit. This peptide contains one L-isoaspartyl residue per molecule. The counts generated per picomole of DSIP are equivalent to the counts per picomole of L-isoaspartyl residues. This enabled us to quantify the L-isoaspartyl residues in the liver samples.

The data are reported as means ± SD. Data were analyzed by two-way ANOVA with a post-hoc Bonferroni test. All statistical analyses were performed using SYSTAT 10 software (SPSS, Chicago, IL). Differences were considered to be significant if $P < 0.05$.

RESULTS

The dietary treatment used in this study is similar to that described earlier (20). The omission of sulfa drugs from the folate-deficient diet produced a moderate folate deficiency of insufficient magnitude to produce illness, anemia or weight loss. Liver folates were 75–90% lower in rats fed the folate-deficient diet than in those fed the folate-sufficient diet in both age groups ($P < 0.003$) (**Table 1**). Hepatic SAM concentrations in the younger rats fed the folate-deficient diet were half those of rats fed the folate-sufficient diet ($P < 0.003$). Overall, hepatic SAM levels were higher among the older rats ($P < 0.02$). However, as in the younger rats, the livers of the older rats who were fed the folate-deficient diet contained a much lower concentration of SAM than those fed the folate-sufficient diet ($P < 0.003$). The folate-deficient diet resulted in higher hepatic SAH levels than the folate-sufficient diet in both age groups ($P < 0.003$) (Table 1).

There were more L-isoaspartyl residues in liver proteins of younger rats fed the folate-deficient diet than in those fed the folate-sufficient diet (**Figure 2**; $P < 0.025$). The levels of hepatic L-isoaspartyl residues in the older rats differed from those of the younger rats in two respects. There were no differences between the folate-deficient and folate-sufficient groups and, irrespective of diet, L-isoaspartyl residues in the livers of the older rats were several-fold higher than in the livers of the younger rats.

DISCUSSION

Formation of L-isoaspartyl residues is one of several mechanisms that contribute to the senescence of proteins with age

TABLE 1

Levels of folate, S-adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) in livers of weanling young and old (12 mo) rats fed a folate-deficient (0 mg folate/kg diet) or -sufficient (2 mg folate/kg diet) diet for 20 wk[1]

	Younger rats		Older rats	
	Deficient	Sufficient	Deficient	Sufficient
n	4	4	6	8
	nmol/g			
Folate	2.7 ± 1.3	17.0 ± 2.4	1.8 ± 0.6	17.0 ± 5.2[a]
SAM	5.1 ± 2.0	11.6 ± 1.4[a]	7.9 ± 3.4[c]	32.9 ± 6.0[ab]
SAH	10.9 ± 0.3	7.7 ± 1.1[a]	15.0 ± 7.1	6.2 ± 2.0[a]

[1] Values are means ± SD; [a] $P < 0.003$, different from folate deficient rats within each age group; [b] $P < 0.002$, [c] $P < 0.02$, different from younger rats within each diet group.

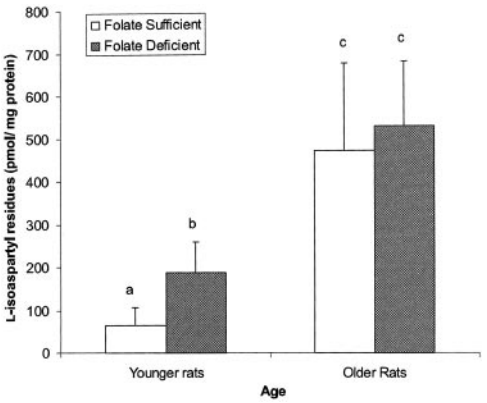

FIGURE 2 Hepatic protein L-isoaspartyl residues in weanling young and old (12 mo) rats fed folate-sufficient or -deficient diets for 20 wk. Values are means ± SD; (*n*, younger rats: 4 and 4; older rats: 8 and 6, for folate sufficient or deficient diet, respectively). Means without a common letter differ; a vs. b, $P < 0.025$; a vs. c, $P < 0.0001$; b vs. c, $P < 0.0001$).

(8,9). The repair of these age-damaged proteins by PIMT is vital, as recently demonstrated with knockout mice that lack this enzyme (24–26). These mice accumulate abnormal L-isoaspartyl residues in brain proteins and die at an early age of seizures. Perturbation of PIMT-dependent activity has also been achieved through changes in the intracellular concentrations of SAH, either as a result of the administration of adenosine diacetaldehyde (17) or as a result of kidney disease (27), and in both instances, there is an accumulation of damaged proteins.

The present study was undertaken to determine whether folate deficiency would affect PIMT-dependent protein methylation, a proxy measure of the accumulation of L-isoaspartyl residues. L-Isoaspartyl accumulation in hepatic proteins was higher in the folate-deficient than in the folate-sufficient young rats. Because L-isoaspartyl residues in proteins were determined as a function of PIMT-dependent methyl accepting capacity, these data are consistent with the possibility that folate deficiency produces an in vivo impairment of this methylation.

The older rats differed both quantitatively and qualitatively. First, the levels of L-isoaspartyl residues per unit of protein were several-fold higher in the older rats, regardless of folate status. Second, in the older rats, folate deficiency produced no further increase in the levels of L-isoaspartyl residues. These differences between the younger and older rats cannot be attributed to excessive SAH or lower SAM concentrations. In fact, the SAM concentration in the older rats fed the normal diet was threefold the value in the younger rats.

One plausible interpretation of the high level of L-isoaspartyl residues in the older rats is based on two prior observations: *1*) the isomerization of aspartyl and asparginyl into isoaspartyl (and D-aspartate) residues is limited to those sites that are linked to serine or glycine and are located in flexible and unstable areas of the protein (9); 2) the spontaneous isomerization at different protein sites differ and can range from a few hours to a number of days (28). Thus, in older rats, L-isoaspartyl accumulation in hepatic proteins may be at maximum levels and therefore cannot be further enhanced by folate deficiency. Accordingly, we speculate that aging has greater effects on the accumulation of L-isoaspartly residues than folate deficiency, at least under the conditions of this experiment. Whether the apparent maximal accumulation of residues with age is due to slower protein turnover, which allows for greater opportunity for this isomerization to take place, or to ineffective PIMT-dependent methylation, is not known, nor do we know how folate deficiency and other perturbations of one-carbon metabolism affect this methylation in other tissues.

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