Vol. 175, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1991 Pages 351-358

DISTINCT C-TERMINAL SEQUENCES OF ISOZYMES I AND II OF THE HUMAN ERYTHROCYTE L-ISOASPARTYL/D-ASPARTYL PROTEIN METHYLTRANSFERASE

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Received January 18, 1991

SUMMARY: We have purified the more acidic major isozyme (II) of the human erythrocyte L-isoaspartyl/D-aspartyl methyltransferase and compared its structure to that of the previously sequenced isozyme I. These isozymes are both monomers of 25,000 molecular weight polypeptides and have similar enzymatic properties, but have isoelectric points that differ by one pH unit. Analysis of 16 tryptic peptides of isozyme II accounting for 89% of the sequence of isozyme I revealed no differences between these enzyme forms. However, analysis of a *Staphylococcal* V8 protease C-terminal fragment revealed that the last two residues of these proteins differed. The -Trp-Lys-COOH terminus of isozyme I is replaced by a Asp-Asp-COOH terminus in isozyme II. Southern blot analysis of genomic DNA suggests that the human chromosome may contain only a single gene encoding the enzyme. We propose that the distinct C-termini of isozymes I and II can arise from the generation of multiple mRNA's by alternative splicing.

The type II protein carboxyl methyltransferases (E.C. 2.1.1.77) catalyze the methyl esterification of L-isoaspartyl and D-aspartyl residues in peptides and proteins that result from spontaneous decomposition of normal L-aspartyl and L-asparaginyl residues (1-4). These enzymes are present in procaryotic cells such as *Escherichia coli* and *Salmonella typhimurium* and in all eucaryotic cells and tissues examined so far (5,6). They have been postulated to recognize these altered residues in proteins in a reaction that can lead to either their degradation or repair (1,4, 7-10).

Fractionation of a variety of mammalian tissue extracts by ion-exchange chromatography or isoelectric focusing has revealed the presence of isozymes. For example, two major enzymes are separated by DEAE-cellulose chromatography in bovine brain that have isoelectric points of 5.6 and 6.5 (11). In rat testes the two major forms have isoelectric points of 6.1 and 7.35 (12), while at least four isozymes with isoelectric points of 5.4 to 6.5 have been described in rabbit brain, muscle, heart and

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Abbreviation used: HPLC, high performance liquid chromatography.

0006-291X/91 $1.50

liver (13). In human erythrocytes, two major enzymes of isoelectric points of 5.5 and 6.6 have been described (14). In all cases where the analysis was made, these isozymes have similar native and polypeptide molecular weights (about 25,000), substrate specificity, and kinetics (see Ref. 14 for a review).

Recently the amino acid sequence of the more basic major isozyme I of this enzyme has been determined from bovine brain (15) and from human erythrocytes (16). These studies showed that each enzyme contained 226 residues and were identical at all but 6 positions. The structure deduced from sequence analysis of a cDNA clone of a rat brain enzyme (17) was also very similar, with 10 amino acid changes from the human isozyme I and 9 amino acid changes from the bovine brain isozyme I, resulting in the net increase of two basic residues. The rat brain sequence would thus appear to be more similar to the human and bovine isozyme I rather than the more acidic isozyme II.

We have been interested in understanding the structural basis of the isozymes of these methyltransferases. Their polypeptide molecular weights determined by dodecyl sulfate gel electrophoresis were found to be identical (18). Proteolytic mapping of the human and bovine erythrocyte and the bovine brain enzymes with *Staphylococcus aureus* protease V8 showed identical patterns for isozyme I from bovine brain and erythrocytes and isozyme II from these two tissues, and only small differences between the two isozyme types (18). These results were supported by tryptic HPLC mapping and the sequence analysis of 12 corresponding tryptic peptides that revealed no amino acid differences between the human isozymes I and II at 112 compared sites (16,18). In this study, we now show that these human erythrocyte enzymes are identical at at least 202 of the 226 residues but do differ in their C-terminal two amino acid residues. Based on hybridization of cDNA probes to human genomic DNA, we suggest that alternative splicing of a single methyltransferase gene can create mRNA's that differ in the residues coded at the terminal two positions.

EXPERIMENTAL PROCEDURES

Purification of L-isoaspartyl/D-aspartyl methyltransferases from human erythrocytes. Isozymes I and II were purified as described by Gilbert et al. (18) with the modifications described in Ingrosso et al. (16).

Proteolytic digestion and isolation of peptide fragments. Isozyme II was reduced and carboxymethylated with iodo[2-^{14}C]acetic acid as described by Ingrosso et al. (16) and digested with trypsin and fractionated on reverse phase HPLC as described by Gilbert et al. (18). Staphylococcus aureus V8 protease digestion of underivatized isozymes I and II and HPLC fractionation was performed as described previously (16).

N-terminal sequencing. Sequence analysis was performed at the UCLA Protein Microsequencing Facility by Dr. Audree Fowler. Tryptic fragments were selected for sequencing based on their amino acid compositions and analyzed with an Applied Biosystems Model 470A gas-phase instrument with on-line HPLC detection (120A PTH Analyzer). V8 peptides were analyzed with a Porton Model 2090E gas phase sequencer.

Southern blot analysis of human genomic DNA. Human placental genomic DNA (Clontech Laboratories) was digested with restriction endonucleases and the fragments were separated by electrophoresis through gels containing 0.5 to 0.8% agarose (19). The DNA was transferred onto Millipore Immobilon-N PVDF membranes, fixed, hybridized, and washed according to the manufacturer. The probes utilized were derived from restriction enzyme digests of a pGEM7ZF(+) plasmid kindly provided by Dr. Clare O'Connor (Worcester Foundation for Experimental Biology, Shrewsbury, MA) that contains a 1.6 kb cDNA insert of the mouse testes L-isoaspartyl/D-aspartyl methyltransferase (20). Probe I was a 800 bp *Hae*III fragment encompassing the coding sequence of the enzyme. Probe II was a 700 bp *Hae*III fragment encompassing the 3' noncoding region of the methyltransferase cDNA. Probes were labelled to a specific activity of > 1 X 10^9 cpm/μg with the PRIME-IT random priming kit (Stratagene), using 100 ng of DNA and 200 μCi of [α-^{32}P]-dCTP (3,000 Ci/mmol; ICN). Bands were visualized by autoradiography.

RESULTS AND DISCUSSION

Comparison of tryptic peptides of isozymes I and II of the human erythrocyte L-isoaspartyl/D-aspartyl methyltransferase. In Fig. 1 we present the sequences of the

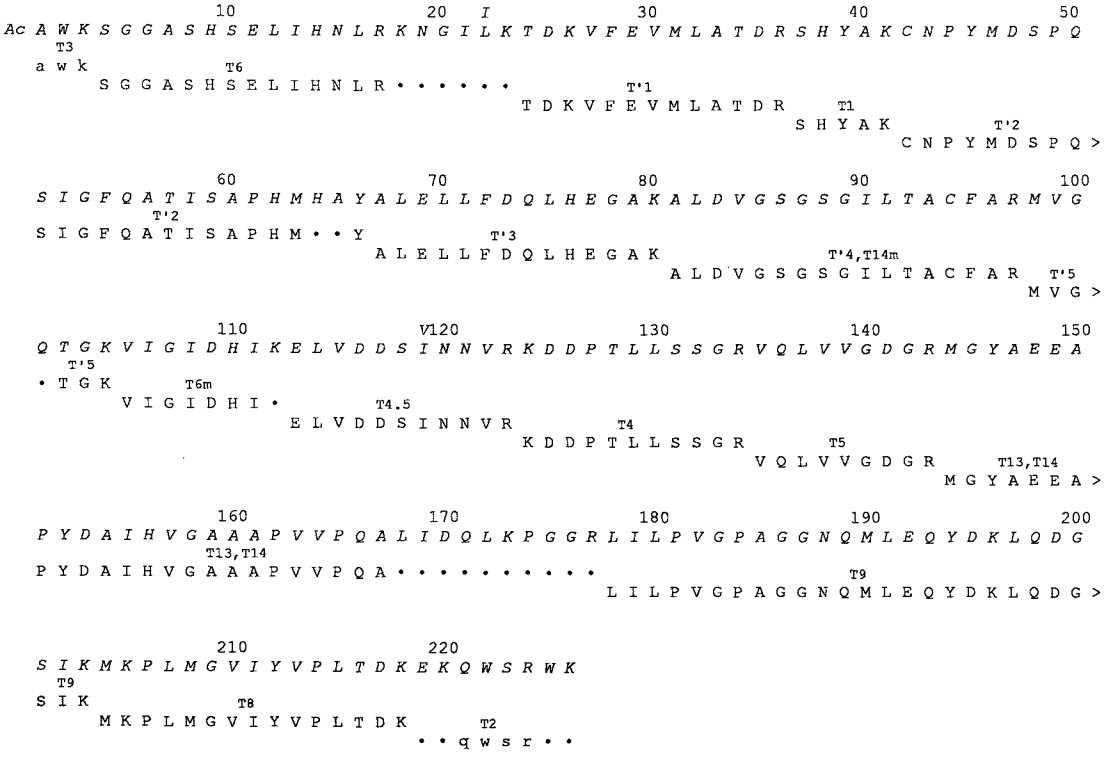

Fig. 1. Partial sequence of human erythrocyte L-isoaspartyl/D-aspartyl methyltransferase isozyme II. The sequences of tryptic peptides determined for isozyme II are compared with the complete sequence of isozyme I (*italics*, Ref. 16). Peptides T1-T14 are taken from the work of Gilbert et al. (18). The sequences of isozyme II peptides T2 and T3 are shown in lower case letters because their structure was inferred from their comigration on reverse phase HPLC with the corresponding peptides from isozyme I and their amino acid composition (18). Peptides T'1 to T'5 were isolated and sequenced as described in "Experimental Procedures" (Table I).

TABLE I

Sequences of tryptic peptides of isozyme II of the human erythrocyte L-isoaspartyl/D-aspartyl protein methyltransferase[a]

Cycle Number	T'1[b] (pmol)	T'2 (pmol)	T'3 (pmol)	T'4 (pmol)	T'5 (pmol)
1	Thr (78)	CMC[c]	Ala (390)	Ala (335)	Met (91)
2	Asp (70)	Asn (12.1)	Leu (302)	Leu (265)	Val (64)
3	Lys (58)	Pro (9.0)	Glu (239)	Asp (203)	Gly (89)
4	Val (34)	Tyr (6.6)	Leu (170)	Val (176)	?[d]
5	Phe (32)	Met (4.0)	Leu (243)	Gly (191)	Thr (83)
6	Glu (23)	Asp (8.7)	Phe (154)	Ser (107)	Gly (33)
7	Val (17)	Ser (10.9)	Asp[c]	Gly (165)	Lys (18)
8	Met (15)	Pro (5.7)	Gln (83)	Ser (95)	
9	Leu (30)	Gln (6.1)	Leu (88)	Gly (122)	
10	Ala (20)	Ser (8.2)	His (49)	Ile (90)	
11	Thr (16)	Ile (4.0)	Glu (65)	Leu (90)	
12	Asp (25)	Gly (19.6)	Gly (56)	Thr (75)	
13	Arg (6.4)	Phe (3.8)	Ala (42)	Ala (92)	
14		Gln (4.3)	Lys (5)	CMC (51)	
15		Ala (5.5)		Phe (58)	
16		Thr (4.2)		Ala (68)	
17		Ile (3.0)		Arg (13)	
18		Ser (6.4)			
19		Ala (4.8)			
20		Pro (2.1)			
21		His (0.6)			
22		Met (0.8)			
23		?[d]			
24		?[d]			
25		Tyr (1.8)			

[a]Analysis was performed by Dr. Audree Fowler at the UCLA Protein Microsequencing Laboratory. CMC is carboxymethylcysteine.
[b]An additional peptide, consisting of residues 4-13, was also found.
[c]Quantitation not available.
[d]It was not possible to unambiguously assign a residue at this step.

tryptic fragments of isozyme II that have been determined by Gilbert et al. (18) and in this study (Table I). The N-terminal peptide for isozyme II (T3) has the identical HPLC elution position and amino acid composition of the N-Ac-Ala-Trp-Lys peptide of isozyme I (16,18) suggesting that the amino terminus of these isozymes are identical. Although we have not found the small tryptic peptides corresponding to residues 18-23 and 219-220 of isozyme II and were not able to obtain complete sequences for four other peptides (T2', T'5, T6m, and T13/T14), there appear to be no differences between the isozymes at any of the 202 compared positions within the polypeptide chain.

Distinct amino acid sequence at the C-terminus of isozymes I and II. To ask whether the structure of the carboxyl terminus was similar in each isozyme, we digested purified preparations of isozyme I and II with protease V8 from *Staphylococcus aureus*. and separated the peptides by HPLC with UV detection at

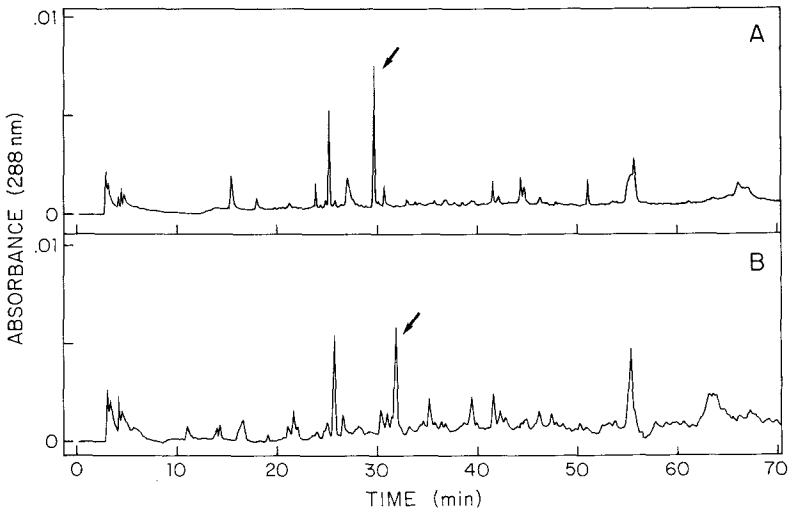

Fig. 2. Isolation of the C-terminal fragments of isozymes I and II from human erythrocytes. Purified methyltransferase isozymes were digested by *Staphylococcus aureus* V8 protease and fractionated by reverse phase HPLC. The upper panel (A) shows the result with isozyme I; the lower panel (B) for isozyme II. The HPLC gradient was 0-70% solvent B over 90 min (cf. Ref. 16). The *arrow* marks the position of the distinct C-terminal peptide.

288 nm to selectively monitor Trp-containing peptides (Fig. 2). The N-terminal undecapeptide fragment containing Trp-2 eluties at about 25 min and is present in digests of each isozyme. On the other hand, there is no fragment eluting at about 30 min in the isozyme II digest at the position of the C-terminal heptapeptide fragment of isozyme I that contains Trp-222 and Trp-225. A new UV-absorbing peak is present, however, at 31.7 min in the digest of isozyme II suggesting that the C-termini of these isozymes differ structurally. This hypothesis was confirmed by direct sequence analysis of the 31.7 min peptide of isozyme II (Table II). These results show a sequence (Lys-Gln-Trp-Ser-Arg-Asp-Asp) that has the same amino-terminal five residues as the isozyme I C-terminal heptapeptide but with the terminal Trp-Lys replaced by two Asp residues. The absence of a protease V8-sensitive glutamate residue in this peptide indicates that it represents the C-terminus of isozyme II. The loss of a positive charge of the C-terminal Lys in isozyme I and the gain of two negatively charged Asp residues in isozyme II would result in a difference of three net charges between these species and is consistent with the lower isoelectric point of isozyme II.

Number of human genes for L-isoaspartyl/D-aspartyl methyltransferase isozymes. The apparent absence of amino acid changes except at the C-terminus of the protein suggests that one gene may direct the synthesis of both isozymes via alternative splicing of mRNA transcripts (21). It is already clear that multiple mRNA species for this methyltransferase are produced in rat brain (17) and in various mouse tissues (20). Selection of alternative 3'-terminal exons encoding the last amino acid residues, the stop codon, and the 3' untranslated region would result in the synthesis of isozymes identical except at the C-terminus.

TABLE II

Sequence of the Staphylococcus aureus C-terminal fragment of isozyme II of the human erythrocyte L-isoaspartyl/D-aspartyl protein methyltransferase[a]

Cycle Number	Experiment I[b] (pmol)	Experiment II (pmol)
1	Lys (47.2)	Lys (47.5)
2	Gln (37.7)	Gln (82.1)
3	Trp[c]	Trp (27.9)
4	Ser (14.4)	Ser (18.6)
5	Arg (24.1)	Arg (8.1)
6	Asp (17.8)	Asp (37.3)
7	Asp (13.7)	Asp (30.0)

[a]Analysis was performed by Dr. Audree Fowler at the UCLA Protein Microsequencing Laboratory. Sequences were determined on two different preparations of this peptide.

[b]A minor sequence was also present that reflects a contaminating peptide eluting just prior to the major A_{288nm} peak at 31.8 min (see Fig. 2).

[c]Quantitation not available.

To test this hypothesis, we probed restriction fragments of human genomic DNA with mouse L-isoaspartyl/D-aspartyl cDNA probes. The mouse 3'-non-coding probe was found to hybridize to a single *Bam*H1, *Pst*I, *Kpn*I, *Bst*XI, *Bgl*I, and *Hae*III fragment, while the 5'-coding probe hybridized to a single *BamH*1 and *Bst*XI fragment (Table III). However, three *Pst*I and *Kpn*I fragments and two *Sac*I, *Hind*III, *Bgl*I, and *Hae*III fragments hybridized with the coding probe (Table III). Although it is possible that these multiple sites may represent hybridization of the probe to more than one gene, the presence of restriction sites within exons or introns of a single gene is also consistent with the data. Our recent isolation of a human cDNA clone that contains *Hae*III, *Hind*III, and *Pst*I restriction sites supports the latter interpretation.

Mechanism of isozyme formation for the L-isoaspartyl/D-aspartyl methyltransferase The simplest interpretation of the data shown in this paper is that a single human gene encodes the L-isoaspartyl/D-aspartyl protein methyltransferase and that the two major isozymes result from a distinct pair of C-terminal residues encoded by separate exons. Two similar cases of isozyme generation have been described. In *Drosophila*, one gene encodes the glycerol-3-phosphate dehydrogenase isozymes GPDH-1 and GPDH-2 . These proteins have identical amino acid sequences except for the C-terminal 3 and 10 amino acid residues, respectively (22). Isozyme GPDH-1 arises when exon 6 is spliced to exon 8 whereas GPDH-2 arises when exon 6 is spliced to exon 7. In the rat α-tropomyosin gene, similar alternative 3'-terminal exon use results in smooth muscle and striated muscle isozymes that contain 26 distinct C-terminal amino acid residues but are otherwise identical (23). Our results, however, do not completely exclude the possibility that the isozymes are products of distinct genes nor the possibilities that isozyme I and II may

TABLE III

Southern hybridization analysis of human genomic DNA fragments with probes of the mouse testes L-isoaspartyl/D-aspartyl protein methyltransferase cDNA[a]

Probe	Size of hybridizing fragment(s) (kb)[b]							
	*Bam*HI	*Sac*I	*Hind*III	*Pst*I	*Kpn*I	*Bst*XI	*Bgl*I	*Hae*III
Full-length	20+	16	19	11	20-		17.8	3.0
		15	16	3.3	6.9		11.9	1.5
		8.7	11	2.4	4.9		1.4	1.0
		1.7	8.7	0.7	3.3			
5'-coding region	16	13	20+	3.3	20-	1.3	17	1.5
		1.6	20-	2.3	7.2		1.5	1.0
				0.7	4.9			
3'-noncoding region	28		9.2	11	3.3	3.8	17	3.4
			6.5					

[a]Human placental DNA was cleaved with the indicated restriction endonuclease and the fragments separated on gels containing from 0.5% to 0.8% agarose.

[b]Sizes were determined in comparison to markers of *Bst*EII digests of lambda DNA electrophoresed in parallel lanes. The "20+" designation is used for fragments near the top of the gel that are larger than the resolving limits of the gel electrophoresis. The "20-" designation is used for fragments that migrated slightly ahead of the "20+" fragments.

also vary by additional alternative splicing reactions in the interior of the coding sequence (21) or by differential posttranslational modification reactions (phosphorylation, etc.). Our inability to detect the amino acid residue corresponding to Gln-150 in peptide T'5 (Table II) suggests that this may be an additional site of difference between the isozymes.

The physiological rationale for having both isozymes produced in erythrocytes is not clear. For the human red cell enzymes, no differences in their kinetic properties or *in vitro* substrate specificity have been found so far (14), although the situation in intact cells has not been investigated. It is possible that distinct 3'-noncoding mRNA sequences may allow differential translational efficiency in response to cellular conditions. On the other hand, distinct C-terminal amino acid sequences may result in differences in enzyme stability during cellular aging or when intracellular proteases are activated.

ACKNOWLEDGMENTS

This work was supported by Grant GM-26020 from the National Institutes of Health. R.M.K. was supported in part by United States Public Health Service Training Grant GM 07185. Special thanks are due to Dr. Clare O'Connor (Worcester Foundation for Experimental Biology, Shrewsbury, MA) for her generous gift of a full length cDNA clone of the mouse testes L-isoaspartyl/D-aspartyl methyltransferase and for sharing unpublished results. We are also grateful to Dr. Audree Fowler for expert

sequence analysis at the UCLA Protein Microsequencing Facility, supported in part by a BRS Shared Instrumentation Grant RR05554 from the National Institutes of Health.

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