Purification of Homologous Protein Carboxyl Methyltransferase Isozymes from Human and Bovine Erythrocytes†

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Received December 17, 1987; Revised Manuscript Received March 2, 1988

ABSTRACT: We have purified the two major isozymes of the L-isoaspartyl/D-aspartyl protein methyltransferase from both human and bovine erythrocytes. These four enzymes all have polypeptide molecular weights of approximately 26 500 and appear to be monomers in solution. Each of these enzymes cross-reacts with antibodies directed against protein carboxyl methyltransferase I from bovine brain. Their structures also appear to be similar when analyzed by dodecyl sulfate gel electrophoresis for the large fragments produced by digestion with *Staphylococcus aureus* protease V8 or when analyzed by high-performance liquid chromatography (HPLC) for tryptic peptides. The structural relatedness of these enzymes was confirmed by sequence analysis of a total of 433 residues in 32 tryptic fragments of the human erythrocyte isozymes I and II and of the bovine erythrocyte isozyme II. We found sequence identity or probable identity in 111 out of 112 residues when we compared the human isozymes I and II and identities in 127 out of 134 residues when the human and bovine isozymes II were compared. These results suggest that the erythrocyte isozymes from both organisms may have nearly identical structures and confirm the similarities in the function of these methyltransferases that have been previously demonstrated.

Enzymes which catalyze *S*-adenosylmethionine-dependent protein carboxyl methylation reactions are widely distributed in nature (Clarke, 1985). In brain and erythrocytes, one class of these enzymes appears to recognize L-isoaspartyl and D-aspartyl residues but not normal L-aspartyl or L-glutamyl residues (McFadden & Clarke, 1982; Aswad, 1984; Murray & Clarke, 1984). Enzymes with similar properties have been described both in bacteria and in other eucaryotic cells and tissues (O'Connor & Clarke, 1985). These activities may function by initiating the repair or degradation of damaged proteins containing chemically modified aspartyl residues (Clarke, 1985).

When cellular extracts are fractionated by isoelectric focusing, multiple peaks of this type of protein methyltransferase activity have been found in several tissues. Aswad and Deight (1983) separated two of the major isozymes from bovine brain by DEAE-cellulose chromatography. The first isozyme (I) that eluted had an apparent isoelectric point of 6.5, while the second (II) was comprised of components with isoelectric points of 5.5 and 5.6. They found that the purified isozymes had very similar catalytic and structural properties. Two isozymes of p*I* = 6.1 and 6.4 were also found in the enzyme from *Torpedo* electric organ (Haklai & Kloog, 1987). On the other hand, the analogous protein carboxyl methyltransferase purified from human red cells (Kim et al., 1983) and from chicken red cells (Saido et al., 1987) has been reported as a single molecular species.

We have recently shown, however, that the L-aspartyl/D-isoaspartyl methyltransferase activity from human red cells can be resolved by DEAE-cellulose chromatography into two major isozymes with p*I* values of about 5.5 and 6.5 (Ota et al., 1988). In the present work, we have purified each of these isozymes and have performed sequence analysis on their tryptic peptides to understand the structural basis for the isozymes. We have also purified the two major isozymes from bovine erythrocytes and have performed sequence analysis on the more acidic isozyme II.

Materials and Methods

Purification of Protein Carboxyl Methyltransferase Isozymes I and II from Bovine and Human Erythrocytes. Human blood was obtained from healthy volunteers, and the red cell fraction was separated from leukocytes by Plasmagel (Laboratoire Roger Bellon, Neuilly Sur Seine, France). Bovine blood was obtained from the Acme Slaughterhouse (Vernon, CA). All steps were performed at 0–4 °C. Erythrocytes were pelleted by centrifugation and were washed 4 times in 5–10 volumes of phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.4). Care was taken to remove any buffy coat of white blood cells at the surface of the red cell pellet. Packed cells were lysed by being mixed with 5–6 volumes of buffer A [5 mM sodium phosphate, 5 mM disodium ethylenediaminetetraacetate (Na_2EDTA), 10% glycerol (w/v), 25 μM phenylmethanesulfonyl fluoride, and 15 mM 2-mercaptoethanol, pH 8.0 (the last two components were added immediately prior to use)] and frozen in a dry ice/2-propanol slurry. After thawing, membranes were removed by centrifugation at 27000*g* for 45 min at 4 °C. The cytosolic fraction was brought to 60% saturation with solid ammonium sulfate at 4 °C. The protein pellet, containing a considerable amount of hemoglobin, was collected by centrifugation at 27000*g* for 45 min and was resuspended in 55% ammonium sulfate made up in buffer B (see below). This procedure solubilizes much of the hemoglobin while leaving the bulk of

† This work was supported by Grant GM-26020 from the National Institutes of Health and by a Grant-in-Aid from the American Heart Association with funds contributed in part by the Greater Los Angeles Affiliate.

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the methyltransferase in the pellet (Kim et al., 1983). The pellet remaining after centrifugation of this material was resuspended as before in buffer B [20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 0.2 mM EDTA, and 10% glycerol (w/v), pH 8.0, containing 25 μM phenylmethanesulfonyl fluoride and 15 mM 2-mercaptoethanol as for buffer A). This material was dialyzed against buffer B containing 50 μM inositol hexaphosphate (Sigma) and chromatographed on a 4 cm diameter × 100 cm column of Sephadex G-75 in buffer B containing 50 μM inositol hexaphosphate. It was found that this latter compound improved the separation of methyltransferase activity from the residual hemoglobin, probably by shifting the dimer–tetramer equilibrium of hemoglobin to the tetramer (White, 1976). The active fractions eluting at an approximate M_r of 25 000 were then concentrated by ultrafiltration using an Amicon apparatus with a YM-10 filter.

DEAE-cellulose chromatography of this material in a column equilibrated in buffer B was performed as described by Ota et al. (1988). This procedures separates the methyltransferase isozyme I (pI = 6.5) from isozyme II (pI = 5.5). Each isozyme fraction was pooled and concentrated by Amicon ultrafiltration using a YM-10 filter.

These enzymes were judged to be approximately 30% pure by polyacrylamide gel electrophoresis in dodecyl sulfate (Laemmli, 1970). At this stage, homogeneous preparations of each isozyme could be obtained by affinity chromatography using a resin modified with *S*-adenosyl-L-homocysteine in a modification of the procedure described by Kim et al. (1978). We could elute the enzyme from the affinity resin by shifting the pH to more alkaline conditions, and thus, we replaced the elution with 10 μM *S*-adenosylmethionine to elution with 0.5 M ammonium bicarbonate, pH 9.00.

Alternatively, homogeneous material suitable for tryptic mapping and sequence analysis could be obtained directly by reverse-phase HPLC of the DEAE-cellulose fractions. Each isozyme was applied to a 4.6 mm i.d. × 250 mm Vydac C-4 column (300-Å pore, 5-μm spherical silica) equilibrated in 65% solvent B and eluted at a flow rate of 1.0 mL/min with a linear gradient of 65–75% solvent B over 45 min [solvent A is 0.1% (w/v) trifluoroacetic acid in water; solvent B is 0.1% (w/v) trifluoroacetic acid in 99% (v/v) methanol–0.9% (v/v) water]. Peak detection was accomplished by UV absorbance at 214 nm. The methyltransferase eluted at 24–28 min and was well separated from residual hemoglobin that eluted at approximately 48 min. Solvents were removed by vacuum centrifugation in a Savant Speed-Vac apparatus at room temperature. The purity of these fractions was confirmed by dodecyl sulfate–polyacrylamide gel electrophoresis. A single band of M_r 26 500 was observed with silver staining.

Assay Procedure for Protein Carboxyl Methyltransferase. Fractions were assayed for methyltransferase activity with *S*-adenosyl[*methyl*-^{3}H]-L-methionine as a methyl donor and ovalbumin as a methyl-accepting protein. Final concentrations in a 50-μL reaction mixture were 10 μM *S*-adenosylmethionine (Amersham, specific activity adjusted to about 50 cpm/pmol with the nonisotopically labeled HSO_4^- salt from Boehringer), 40 mg/mL ovalbumin (chicken, Sigma fraction V), and 0.1 M sodium citrate buffer at pH 6.0. After incubation at 37 °C for 10–20 min, the reaction was quenched by the addition of an equal volume (50 μL) of 0.2 N NaOH, 1.0% (w/v) sodium dodecyl sulfate. A total of 60 μL of this mixture was spotted on a 1 × 8.5 cm piece of thick filter paper (Bio-Rad 165-090) prefolded in an accordion pleat and placed in the neck of a 20-mL plastic scintillation vial containing 10 mL of ACS II counting fluor (Amersham) and capped. Ovalbumin methyl esters are hydrolyzed by the sodium hydroxide on the filter paper, and the product [^{3}H]methanol is transferred to the scintillation fluid via the vapor phase. After 2 h at room temperature, the transfer is complete (Murray & Clarke, 1986), and the filter paper was removed from the neck and the vial counted. A unit of activity is defined as 1 pmol of ovalbumin methyl ester formed per minute at 37 °C. Protein was determined in the initial hemoglobin-rich fractions (cytosol and 55% ammonium sulfate) under the assumption that 1 mg of hemoglobin/mL gives an absorbance of 2.4 at 280 nm. For other fractions, either a Coomassie dye binding assay was used (Bradford, 1976), or ultraviolet absorbance was determined at 280 nm, assuming that 1 mg of protein/mL gives an absorbance of 1.0.

Staphylococcus Protease V8 Peptide Mapping. DEAE-cellulose-purified erythrocyte isozymes or purified brain isozymes [a gift of Dr. Dana W. Aswad, University of California, Irvine; cf. Aswad and Deight (1983)] were denatured in sodium dodecyl sulfate, and the M_r 26 500 polypeptide (about 2.4–4.2 μg of protein) was further purified by gel electrophoresis on a 1.5 mm thick slab gel containing 12.5% acrylamide and 0.43% methylenebis(acrylamide) in the buffer system described by Laemmli (1970). After being stained with Coomassie Blue, the protein band at M_r 26 500 was excised with a razor blade and subjected to proteolysis with *Staphylococcus aureus* protease V8 by the method of Cleveland et al. (1977) as modified by Josefsson and Randall (1983). Briefly, a separating gel containing 15% acrylamide and 0.52% methylenebis(acrylamide) was used with a 5-cm stacking gel; 0.35 μg of protease was added to each lane along with the excised band. Electrophoresis was performed until the dye band was 1 cm above the top of the resolving gel. At this point, the voltage was shut off for 30 min to allow proteolytic cleavage before the electrophoresis was restarted.

Trypsin Digestion and HPLC Peptide Mapping. Tryptic mapping was performed essentially as described by Ben-Avram et al. (1986). Briefly, HPLC-purified samples (1–2 nmol of polypeptide) were placed in 1.5-mL polyethylene centrifuge tubes, and the HPLC solvents were removed by vacuum centrifugation as described above. Freshly prepared solutions of 0.2 M ammonium bicarbonate (pH 8.0) and trypsin [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated from bovine pancreas (Sigma type XIII)] in the ammonium bicarbonate buffer were added to give a final volume of 200 μL and 1.5 μg of trypsin/2 nmol of polypeptide substrate. The samples were incubated with shaking for 18 h at room temperature and then were analyzed directly by HPLC on a 4.6 mm i.d. × 250 mm Vydac C-4 reversed-phase column 300-Å pore size, 5-μm spherical silica support) after the procedure of Ben-Avram et al. (1986). This column was equilibrated at room temperature in 0.1% (w/v) trifluoroacetic acid in water (solvent A) and was eluted at a flow rate of 1.0 mL/min with a linear gradient from 0 to 70% solvent B [0.1% (w/v) trifluoroacetic acid in 90% acetonitrile–9.9% water] over 90 min. Peptides were detected by their absorbance at 214 nm and were collected in 12 × 75 mm polypropylene tubes (Sarstedt).

Amino Acid Analysis. Samples were hydrolyzed in 6 × 50 mm glass tubes with 6 M HCl in the gas phase at 110 °C for 18 h in a Waters Pico-Tag work station. The tubes were cleaned prior to use by heating at 240 °C for 2 days. Analysis was done either by the phenylisothiocyanate method of Bidlingmeyer et al. (1984) or by the *o*-phthalaldehyde precolumn derivatization method. In the latter case, reagent solutions

Table I: Purification of Human Erythrocyte Protein Carboxyl Methyltransferase Isoenzymes

step[a]	vol (mL)	units/mL[b]	total units	yield (%)[c]	protein (mg/mL)	specific activity[d]	x-fold purifn
cytosol	660	170.5	112530	100	18.1	9.4	1.0
55% ammonium sulfate	14	1148	16060	14.3	5.6	204	21.7
Sephadex G-75	75	253	18980	16.9	0.431	587	62.5
DEAE isoenzyme I	65	88.9	5780	5.1	0.0045[e]	19930[e]	2120[e]
DEAE isoenzyme II	48	59.6	2860	2.5	0.0139	4270	454

[a] The polypeptide compositions of these fractions are shown in Figure 1. [b] Units are picomoles of methyl esters formed on ovalbumin per minute at 37 °C. [c] Not corrected for the small amount of sample reserved for assays and not applied at the next step. [d] Picomoles per minute per milligram of methyltransferase protein. [e] These values are only approximate because the protein concentration is near the lower limit of detection.

were prepared as described by Jones et al. (1981), and the buffer system of Pfeifer et al. (1983) was employed. Derivatized amino acids were separated on a Waters Resolve C-18 reverse-phase column (4.6 mm i.d. × 150 mm, 5-μm spherical silica) and quantitated on the basis of the fluorescence of 25-pmol standards of amino acids (Pierce Standard H).

Sequence Analysis. Sequence analysis was performed at the UCLA Protein Microsequencing Laboratory with an Applied Biosystems Model 470A gas-phase sequencer with on-line HPLC detection (120A PTH Analyzer).

Results and Discussion

Purification of Two Major Isozymes of Protein Carboxyl Methyltransferase from Human Erythrocytes. Previous work has demonstrated the purification of an apparent single species of protein carboxyl methyltransferase activity from human erythrocytes (Kim et al., 1983), while two isozymes were purified from bovine brain (Aswad & Deight, 1983). It now appears that erythrocytes also contain two isozymes that can be separated by isoelectric focusing or DEAE-cellulose chromatography (Ota et al., 1988). These isozymes are present in roughly equal amounts in human erythrocytes, while isozyme II is present at 2–4 times the level of isozyme I in bovine erythrocytes (data not shown). In the previous studies, no separations were performed on the basis of the charge of the protein, and this may explain why only a single species was found.

A major problem faced in the purification of the red cell enzymes is the separation of the enzymatic activity from the very large amount of hemoglobin in these cells. To accomplish this, we utilized the ammonium sulfate backwash procedure suggested by Kim et al. (1983). Because contamination of the protein methyltransferases with dimeric hemoglobin was a problem in the gel filtration analysis, we performed the Sephadex G-75 chromatography in the presence of a low concentration of inositol hexaphosphate. This latter compound has been shown to stabilize the tetrameric form of hemoglobin, even in dilute protein solution (White, 1976), and its inclusion in the gel filtration buffer resulted in enhanced resolution of hemoglobin and methyltransferase activity.

A typical purification from human erythrocytes is shown in Table I, and the polypeptide composition of the partially purified and homogeneous isozymes is shown in Figure 1. The purification of the bovine erythrocyte enzyme gave similar results. The calculated polypeptide molecular weight of all four of these enzymes was 26500, and if we assume an average residue weight of 113, we would predict that each enzyme would contain about 234 residues. The amino acid composition of each of these isozymes was determined (Table II). In general, these analyses reveal that the compositions of the four isozymes are very similar to one another.

Analysis of the Similarities among the Enzymes by Immunological Cross-Reactivity and Staphylococcus aureus V8 Protease Mapping. Western blotting experiments (Burnette, 1981) demonstrated that antibody raised against the isozyme I of protein carboxyl methyltransferase from bovine brain (kindly provided by Dr. Dana Aswad, University of California, Irvine) cross-reacted with the M_r 26500 polypeptide of iso-

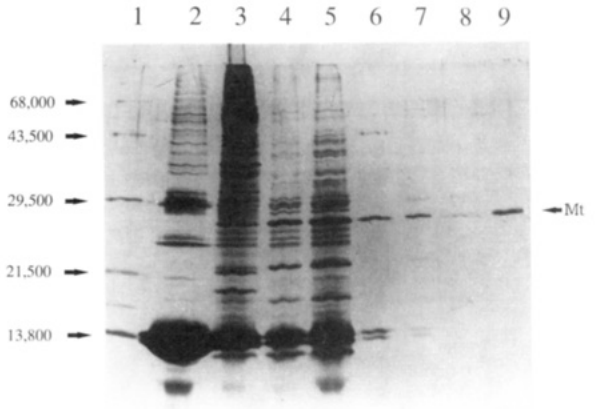

FIGURE 1: Purification of L-isoaspartyl/D-aspartyl protein methyltransferase isozymes I and II from human erythrocytes. Samples from a typical purification were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970) and the polypeptide bands visualized with silver stain. Lane 1 shows molecular weight markers including bovine serum albumin (68000), ovalbumin (43500), carbonic anhydrase (29500), soybean trypsin inhibitor (21500), and lysozyme (13800). Samples included crude cytosol (lane 2), 55% ammonium sulfate fraction (lane 3), Sephadex G-75 fraction (lane 4), concentrated Sephadex G-75 fraction (lane 5), isozyme I from DEAE-cellulose column (lane 6), isozyme II from DEAE-cellulose column (lane 7), affinity-purified isozyme I (lane 8), and affinity-purified isozyme II (lane 9). The position of the methyltransferase (Mt) band is indicated with an arrow at the right.

Table II: Amino Acid Composition of Protein Carboxyl Methyltransferase from Human and Bovine Erythrocytes[a]

	residues per enzyme[b]			
	human erythrocyte		bovine erythrocyte	
amino acid	I [3]	II [4]	I [1]	II [2]
Asx	24.8 (1.6)	24.0 (1.4)	21.0	22.1 (1.8)
Glx	20.0 (0.7)	20.5 (1.6)	19.0	20.2 (0.9)
Lys	16.7 (2.6)	17.0 (1.3)	20.0	17.9 (0.9)
Arg	7.2 (1.0)	8.4 (0.1)	9.2	8.3 (0.4)
His	8.5 (2.2)	6.7 (0.7)	7.9	6.7 (0.3)
Ser	22.6 (6.5)	14.9 (0.3)	15.8	14.5 (1.1)
Thr	11.2 (5.9)	5.9 (1.0)	7.1	5.9 (0.8)
Gly	16.6 (2.7)	21.0 (0.7)	16.9	19.9 (1.9)
Ala	19.2 (2.6)	21.7 (0.2)	21.3	21.7 (0.5)
Val	13.1 (0.8)	13.1 (1.1)	14.2	12.8 (0.8)
Ile	7.3 (1.7)	10.2 (0.9)	10.7	10.1 (0.6)
Leu	20.1 (2.8)	23.1 (1.0)	22.1	23.6 (0.9)
Tyr	7.2 (0.5)	7.3 (0.2)	7.4	7.5 (0.3)
Phe	8.2 (3.3)	4.4 (0.0)	4.5	4.5 (0.1)
Met	4.7 (8.0)	8.8 (3.4)	10.0	11.3 (2.3)

[a] Calculated under the assumption of 234 residues. The number of Pro, Cys, and Trp residues was assumed to be 27 on the basis of the proportional representation of codons for these amino acids. [b] Number in brackets indicates number of samples analyzed; standard deviations are given after each value (parentheses).

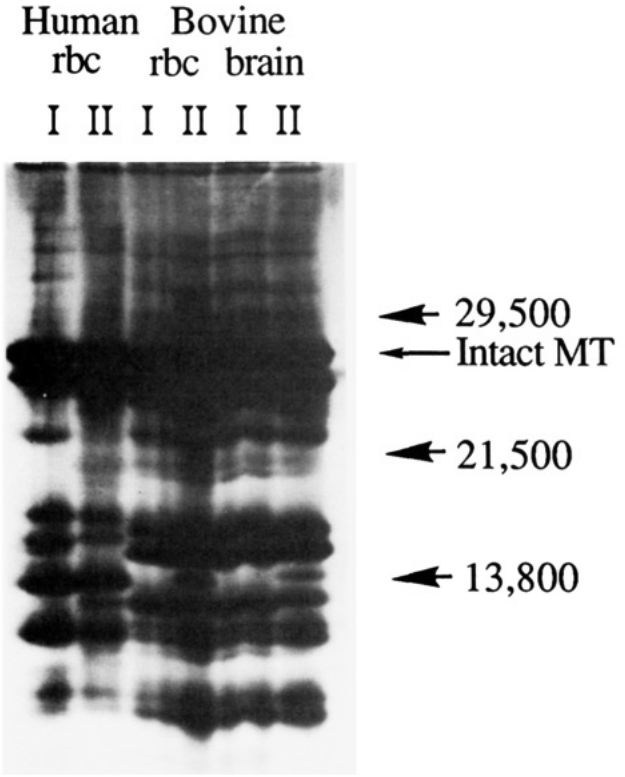

FIGURE 2: Comparison of bovine and human erythrocyte protein carboxyl methyltransferase by proteolytic mapping with *S. aureus* V8 protease as described under Materials and Methods. Samples of isozyme I and isozyme II were prepared as described from human and bovine erythrocytes, and samples from brain were a kind gift of Dr. Dana Aswad (University of California, Irvine). The positions of molecular weight standards are indicated by arrows at the right (see Figure 1) as well as the position of the undigested methyltransferase (Intact MT). Control experiments showed that none of these polypeptides were derived from the V8 protease itself.

zymes I and II of the methyltransferase from both human and bovine erythrocytes (data not shown). Furthermore, when each isozyme was digested with the V8 protease from *S. aureus* by the method of Cleveland et al. (1977) as modified by Josefsson and Randall (1983), the pattern of fragmentation was also similar (Figure 2). The distribution of cleaved polypeptides was very similar when isozyme I was compared to isozyme II from each tissue, while essentially identical patterns were found when isozyme I from bovine brain was compared to isozyme I from bovine erythrocytes and when isozyme II from bovine brain was compared to isozyme II from bovine erythrocytes. These latter results suggest that there may be little or no tissue differences in the bovine isozymes.

Tryptic Mapping of Erythrocyte Methyltransferase Isozymes: Comparison of Sequences of Tryptic Peptides. Figure 3 shows the HPLC profiles of the tryptic digests of the methyltransferase isozymes I and II from human and bovine erythrocytes. Although each pattern is unique, many of the fragments eluted at similar positions, suggesting that similar sequences are present. We performed automated Edman sequencing on each of the numbered peptides shown in Figure 3 for the human isozymes I and II and for the bovine isozyme II as described under Materials and Methods. We were able to obtain sequence information on 133 residues of the human isozyme I, 135 residues of the human isozyme II, and 165 residues of the bovine isozyme II (we did not have sufficient material to analyze the peptides of the bovine isozyme I) (Table III). In all cases, these assignments were found to be consistent with the amino acid compositions of the peptides

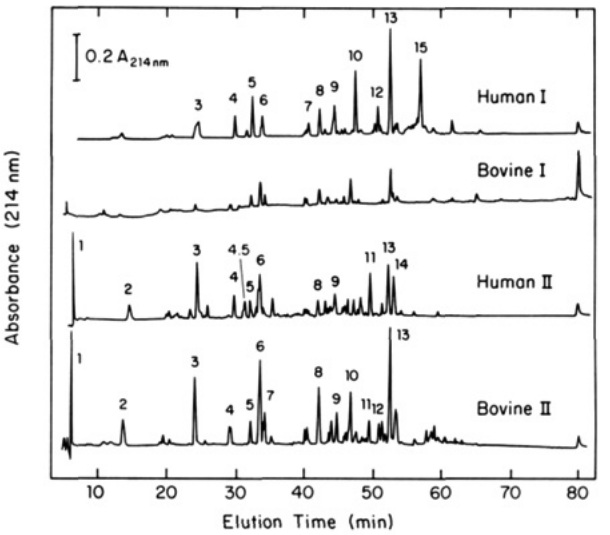

FIGURE 3: Tryptic peptide mapping of human and bovine erythrocyte protein carboxyl methyltransferase isozymes. Purified proteins were digested with trypsin and the fragments separated by reverse-phase HPLC as described under Materials and Methods. The scale of absorbance is indicated by the bar in the upper left for all of the chromatographs except for the isozyme I from bovine erythrocytes. For this case, a smaller amount of material was loaded, and the bar represents 0.05 absorbance unit. Peaks are numbered in order of their elution. In control experiments, we found no detectable peaks from the autodigestion of trypsin; we also found that undigested enzyme eluted from 62 to 64 min under these conditions.

that we determined in separate experiments (data not shown). Thus, these sequences represent a large fraction of these enzymes, estimated to contain a total of about 234 residues from the polypeptide molecular weight.

From the results of the Edman degradation analyses, there appears to be a very large degree of sequence identity between the isozymes of the human erythrocyte enzymes and between both human isozymes and bovine isozyme II (Table IV). From the data shown in Table III, amino acid residues were identical or probably identical at 111 of the 112 residues compared between the human isozymes I and II. The only potential difference that we found was indicated by our detection of both proline and valine at position 24 of peptide T13 for isozyme I while an alanine residue was present in isozyme II. A similar comparison of 134 corresponding residues in human and bovine isozymes II revealed that all but five residues were identical or probably identical. These differences include the substitution of a methionine residue for a threonine residue in peptide T4, compensating substitutions of isoleucine and valine residues in peptides T10 and T12, and the replacement of a glutamic acid residue by an alanine residue in peptide T13. Each of these differences can be accounted for by a single base change on the DNA level.

It remains to be established whether the very high degree of sequence identity established for these tryptic fragments will also be found in the remaining unsequenced regions of these three methyltransferase species. By focusing our analysis on the tryptic peptides with the highest UV absorbances, we have probably only obtained sequence information from the fragments that are either large or contain aromatic amino acids. Nevertheless, we were able to find corresponding sequences for the three isozymes in the majority of the cases. Coupled with the previously determined functional and structural similarities of the erythrocyte (O'Connor et al., 1984; Ota et al., 1988) and the bovine brain enzymes (Aswad & Deight, 1983; O'Connor et al., 1984), the present data suggest that these enzymes are in fact very similar.

Table III: Amino Acid Sequences of Tryptic Peptides from Erythrocyte Protein Carboxyl Methyltransferases[a]

Peptide[b]	Protein	Sequence
T1	Human II	S H Y A K
T1	Bovine II	S H Y A K
T4	Human I	K D D P T L L S(S)G(R)
T4	Human II	K D D P T L L S S G R
T6(minor)	Bovine II	K D D P M L L(W)- G
T5	Human I	V Q L V V G D G R
T5	Human II	V Q L V V G D G R
T5	Bovine II	V Q L V V G D G R
T6	Human I	(S)G G A(S)H S E L I H N L R
T6	Human II	S G G A S H S E L I H N L R
T6	Bovine II	(S)G G A(S)H S E L I H N L R
T8,T9	Human I	M K P L M G V I Y V P L T D K E
T8	Human II	M(K)P L M G V I Y V P L T D K E
T8	Bovine II	M K P L M G V I Y V P L T D K E K
T10	Human I	L I L P V G P A G G N Q M L E Q Y D K L
T9	Human II	L I L P V G P A G G N Q M L E Q Y D K L Q D G S I(K)
T10	Bovine II	L I L P V G P A G G N Q M L E Q Y D K L Q D G(S)V
T13	Human I	M G Y A E E A P Y D A I H(V)G(A)A A P V V(P)Q([c])L I
T13,T14	Human II	M G Y A E E A P Y D A I H V G A A A P V V P Q A
T13	Bovine II	M G Y A A E A P Y D A I H V G A A A P V V P Q A L I D Q L W P G G R
T12	Human I	V I G I D H I([d])E L V D D S V N N V R
T6(minor)	Human II	V I G I D(H)I
T4.5	Human II	E L V D D S V N N V R
T6(minor)	Bovine II	V I G I(D)H I([d])
T7	Bovine II	E L V D D S I N N V R
T14 (minor)	Human II	A L D V G S G S(G)I L T
T12	Bovine II	A L D V G S G(S G)I L T A
T15	Human I	G N P Y M D S P Q S I G F Q A T I - A
T11	Bovine II	(A)N P Y M D(S)P Q(S I)G F Q A T I S A P

[a] The one-letter code is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. Residues in parentheses are tentative assignments; a dash indicates that no clear identification of a residue could be made in this cycle of the Edman degradation. Differences between the isoenzymes or between the human and bovine forms are indicated by underlining. [b] Peptide T2 from human and bovine isoenzymes II and peptide T3 from both human isoenzymes and from bovine isoenzyme II were found to be blocked on the amino terminus, and no Edman sequence information was obtained. The amino acid composition of T2 includes stoichiometric amounts of glutamate, serine, and arginine. Tryptophan may also be present and account for the large relative peak of absorbance at 214 nm—this amino acid would be destroyed by the acid hydrolysis procedure. The amino acid composition of T3 from each of the three methyltransferases indicated stoichiometric amounts of alanine and lysine; tryptophan may also be present in this peptide to account for its ultraviolet absorbance. The blocking group may be an acetylated N-terminal residue common to eucaryotic cytosolic proteins (Tsunasawa et al., 1985) or, for peptide T2, may represent a cyclized glutamate or glutamine residue. [c] Both proline and valine were detected at this cycle. [d] Both lysine and tryptophan were detected at this cycle.

Table IV: Differences in Bovine and Human Methyltransferase Amino Acid Sequences

	residues					
comparison	identical	probably identical[a]	unknown[b]	probably different[a]	different	identity[c]
human I vs human II	102	9	0	1	0	99.1
human II vs bovine II	119	8	2	1	4	94.8
human I vs bovine II	115	9	3	3	3	93.2

[a] This category includes cases where one or both of the compared residues represent tentative assignments (residues in parentheses in Table III). [b] This category includes cases where no comparison was possible due to the absence of an identifiable residue (dashed residues in Table III). [c] Calculated with the fraction of all residues compared that were either identical or probably identical.

Table V shows the divergence of amino acid sequences in a selection of corresponding bovine and human proteins. The 4% divergence in the tryptic peptides (Table IV) of the bovine and human methyltransferase isozyme II represents a very low value and suggests that this sequence has been more conserved than that of proteins such as growth hormone or serum albumin.

The sequence results obtained here also allow us to speculate on the origin of the isozymes. Our finding of only one potential amino acid sequence difference between human erythrocyte

Table V: Differences in Bovine and Human Amino Acid Sequences[a]

protein	% difference in amino acid residues
protein carboxyl methyltransferase II (partial tryptic fragments)[b]	4
ubiquitin	0
histone III	0
calmodulin	0
adenylate kinase	4
cytochrome *c*	10
hemoglobin (α chain)	12
dihydrofolate reductase	13
cytochrome oxidase subunit III	13
proenkephalin precursor	15
hemoglobin (β chain)	16
myoglobin	19
cytochrome b_5	20
superoxide dismutase	20
lactalbumin	23
carbonic anhydrase II	24
serum albumin	25
factor X (Stuart factor)	32
growth hormone	39

[a] Data were taken from Dickerson and Geis (1983), Dayhoff (1972), and Version 7.0 of the NBRF/PIR protein sequence data base (National Biomedical Research Foundation, Washington, DC). [b] Data from the tryptic fragment comparisons in Table IV, using the fraction of the total residues that were classified as either different or probably different.

isozymes I and II indicates that posttranslational modifications may account for the presence of the two isozymes if this difference is not confirmed by further analysis. It is also possible that the differences in the isozymes result from the presence of two closely related (duplicated?) genes for the methyltransferase or from the differential splicing of the RNA transcript of a single gene. Finally, we should note that the one potential amino acid sequence change observed between the fragments of the human erythrocyte isozymes I and II studied here does not involve a charged residue and cannot explain the difference of about 1 pH unit in the isoelectric point of these species. The changes in the structure responsible for the relative acidity of isozyme II either are located in the portions of the molecule that we have not yet sequenced or are due to posttranslational modifications.

Sequence Homologies with Other Proteins. We have compared the sequences of the tryptic peptides in Table III to the sequences of the 4750 proteins in Release 12.0 (March 1987) of the National Biomedical Research Foundation/Protein Identification Resource (NBRF/PIR, Washington, DC) using the FASTP program of Lipman and Pearson (1985). Although we found sequences in some of these proteins that showed marginal homology with individual tryptic peptides, we did not find any protein sequences with shared homologies to multiple tryptic fragments from our samples. To focus more closely on whether there may be general homologies in the subclass of *S*-adenosylmethionine-dependent methyltransferases, we compared the sequences of the tryptic fragments with those of a group of 30 methyltransferase sequences, many of which were not yet represented in this protein bank. This group included three enzymes that methylate small molecules, two related enzymes that methylate L-glutamyl residues, a set of nine related and one unrelated enzymes active on 23S ribosomal RNA, methyltransferases for a 16S ribosomal and a transfer RNA, a group of six related DNA cytosine methyltransferases, and seven partially related DNA adenine methyltransferases. Because each of these enzymes utilizes a common substrate, *S*-adenosylmethionine, and because many share structural features as well, they may contain common sequences that bind this cofactor. However, we did not detect any significant homologies between these groups using the FASTP algorithm. In particular, we could detect no distinctive homology with the protein carboxyl methyltransferases from *Escherichia coli* (Mutoh & Simon, 1986) or *Salmonella typhimurium* (Simms et al., 1987). These latter enzymes methylate L-glutamyl residues in a group of membrane chemoreceptor proteins and might be expected to be the enzymes most closely related to the L-isoaspartyl/D-aspartyl methyltransferases we have studied here.

ACKNOWLEDGMENTS

We are grateful to Chad Miller and Dr. Jack Shively (Division of Immunology, Beckman Research Institute of the City of Hope Medical Center, Duarte, CA) for performing preliminary sequence analysis. We thank Dr. Dana Aswad (University of California, Irvine) for many helpful discussions and for providing us with purified brain protein carboxyl methyltransferase isozymes and antibodies against isozyme I. We also thank Dr. Diego Ingrosso for his contributions to this work. A portion of this study was performed while S.C. was visiting Dr. Jeffry Stock's laboratory at Princeton University, and his support and hospitality are well appreciated.

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(Iodoacetamido)fluorescein Labels a Pair of Proximal Cysteines on the Ca^{2+}-ATPase of Sarcoplasmic Reticulum†

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Received November 3, 1987; Revised Manuscript Received February 1, 1988

ABSTRACT: Previous energy transfer studies [Squier, T. C., Bigelow, D. J., de Ancos, J. G., & Inesi, G. (1987) *J. Biol. Chem. 262*, 4748–4754] have utilized fluorescent iodoacetamide derivatives covalently bound to the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR), using labeling conditions that completely modify the most reactive of the protein's surface sulfhydryls to a final level of 9 nmol/mg of SR protein. Unambiguous interpretation of these results requires localization of these labeling sites with respect to the primary structure of the Ca^{2+}-ATPase. In the present study, we have used the probe 6-(iodoacetamido)fluorescein (IAF) as a marker for these sites. The IAF-labeled Ca^{2+}-ATPase was completely proteolyzed with trypsin, followed by centrifugation to remove (unlabeled) membrane-associated portions. The soluble IAF-labeled tryptic peptides were purified by size-exclusion and reverse-phase high-performance liquid chromatography. Two IAF-peptides resulted. The major (4.1 nmol of IAF/mg of starting protein) and minor (1.9 nmol/mg) IAF-peptides were sequenced and were identified, respectively, as Ala_{673}-IAF-Cys_{674}-Cys_{675}-Phe_{676}-Ala_{677}-Arg_{678} and as Glu_{668}-Ala_{669}-IAF-Cys_{670}-Arg_{671}. A model is proposed to explain the selectivity of IAF for Cys_{670} and Cys_{674} of the ~14 surface sulfhydryls of the Ca^{2+}-ATPase. The labeling region, Arg_{667} through Arg_{678}, has been predicted to be α-helical; Cys_{670} and Cys_{674} would be adjacent in the helix and imbedded in an Arg cluster. The Arg residues would both attract the anionic IAF and enhance sulfhydryl reactivities by lowering their pK values.

The Ca^{2+}-ATPase of sarcoplasmic reticulum (SR)[1] has been extensively studied to determine the kinetic mechanism of ion transport and, more recently, the causative structural features thereof [see Inesi (1985) for a recent review]. One widely used avenue has been through the use of sulfhydryl-directed spin-label and fluorescence reagents, primarily iodoacetate and maleimide derivatives, which modify a limited number of the surface cysteinyl residues of the protein under certain conditions [see Ikemoto (1982) for a review].

Of the 24 cysteinyl residues of the Ca^{2+}-ATPase (Brandl et al., 1986), roughly 14 are on the surface of the protein and accessible to modification reagents (Murphy, 1976, 1978; Thorley-Lawson & Green, 1977; Ikemoto et al., 1978; Reithmeier & MacLennan, 1981). These authors have found that the 14 cysteines can be grouped into about 3 reactivity classes spanning a range of 1–2 orders of magnitude in their chemical reactivities to different sulfhydryl-directed reagents. Microenvironmental influences presumably account for the differing reactivities. Selective modification of only one to three cysteinyl residues per Ca^{2+}-ATPase has been achieved, in all cases, by using a lower pH (6.0–7.0), where the difference between reactivity classes is more marked and, in some cases, by using limiting amounts of modifying reagent. Some sulfhydryl-directed probes used to study the Ca^{2+}-ATPase have included the following: conformationally sensitive iodoacetamide and maleimide spin-labeles (Landgraf & Inesi, 1969; Coan & Inesi, 1977; Coan et al., 1979; Coan & Keating, 1982; Yasuoka-Yabe et al., 1983); conformationally sensitive fluorescent probes, such as (anilinonaphthyl)maleimide, [(benzimidazolyl)phenyl]maleimide, [[(dimethylamino)-methyl]coumarinyl]maleimide, and pyrenylmaleimide (Miki et al., 1981; Yasuoka-Yabe & Kawakita, 1983; Yasuoka-Yabe et al., 1983; Kurtenbach & Verjovski-Almeida, 1985); and fluorescent probes used for distance measurement, such as

† This work was supported by grants from the National Institutes of Health (HL 27867) and the Muscular Dystrophy Association of America and by a postdoctoral fellowship to T.C.S. from the American Heart Association.

[1] Abbreviations: SR, sarcoplasmic reticulum; IAF, 6-(iodoacetamido)fluorescein; IAF–SR, covalent adduct from reaction of IAF with SR; IAEDANS, 5-[[2-(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; AUFS, absorbance units full scale; FITC, fluorescein isothiocyanate.

0006-2960/88/0427-5233$01.50/0 © 1988 American Chemical Society