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PROTEIN CARBOXYL METHYLTRANSFERASE FROM COW EYE LENS

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SUMMARY: Protein carboxyl methyltransferase activity (S-adenosyl-L-methionine: protein carboxyl-O-methyltransferase; E.C. 2.1.1.24) has been detected in crude soluble extracts of cow eye lens. The activity incorporates methyl groups from S-adenosyl-L-methionine into endogenous lens proteins in vitro, and several of these species co-migrate electrophoretically with lens crystallins. A 2600-fold purification of the enzyme free of endogenous substrates was achieved by gel filtration and affinity chromatography. The lens methyltransferase has a native molecular weight of approximately 27,000, and catalyzes the substoichiometric incorporation of highly alkali-labile methyl ester groups into a broad range of protein substrates. The lens enzyme appears to be similar to that found in human erythrocytes, which specifically recognizes and modifies D-aspartic acid residues in aged proteins in a postulated degradative or racemization-repair pathway (McFadden, P.N., and Clarke, S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 2460-2464).

Protein carboxyl methyltransferases from a wide variety of procaryotic and eucaryotic tissues and cells have been characterized with regard to their specificities toward protein substrates (1). From these studies it has become clear that two major classes of this activity exist. The first is found only in bacteria where membrane-associated chemoreceptors are specifically and stoichiometrically methylated in a process that serves to regulate bacterial chemotaxis (2). Glutamyl residues have been found to be sites of methyl esterification in these chemoreceptors (3). The second class of protein carboxyl methyltransferases has been found in the cytosol of all cells examined so far (1,4). The enzymes of this second class are peculiar in that each tested activity has been found to methylate a broad range of protein substrates including endogenous membrane proteins (5) as well as exogenous proteins such as ovalbumin and gelatin in reactions that are clearly non-physiological (1). Furthermore, the final level of methyl group incorporation into these substrate proteins is markedly substoichiometric and is typically on the order of one methyl group per 100 polypeptides or less (5,6).

In studying the nonspecific protein carboxyl methyltransferase in human erythrocytes, we have found that the enzyme catalyzes the formation of methyl

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esters of the β-carboxyl group of aspartyl residues (7). Surprisingly, these methylated aspartyl residues were found to exclusively have the uncommon D-configuration (8). This result provides an explanation for the non-specificity and the sub-stoichiometry of the enzyme reaction in that any protein that contains a small amount of D-residues may potentially be a substoichiometric substrate of the enzyme. Left unexplained however is the origin of the D-configuration itself since erythrocyte proteins are expected to be assembled from L-residues.

It has been established that the long-lived proteins of the mammalian eye lens slowly accumulate D-aspartyl residues as an apparent consequence of aging (9-11). The mechanism of formation of these D-residues in lens is presumed to to be through the spontaneous racemization of L-precursors. We have thus hypothesized that such intracellular "aging" reactions produce proteins that are enzymatically methylated as part of some metabolic process (8). We are now investigating whether protein carboxyl methylation plays a role in the metabolism of aging lens protein. In this report we establish the existence of a non-specific protein carboxyl methyltransferase in cow lens and we describe its isolation free of endogenous methyl acceptor proteins.

MATERIALS AND METHODS:

Domestic cattle lenses from 3 to 4 year old animals were excised from fresh eyes obtained at a local slaughterhouse. Other materials included S-adenosyl-L-[methyl-^{3}H]methionine (15 Ci/mmol; Amersham), S-adenosyl-L-methionine·HSO_4 (Boehringer Mannheim), S-adenosyl-L-homocysteine (Sigma), and chicken ovalbumin (Sigma; Grade V). S-Adenosyl-L-homocysteine was coupled to ω-aminohexyl Sepharose 4B (Sigma) as described (12).

Lens extracts -- Whole lenses were decapsulated and in most experiments the lens cortex (the outer 50% of the wet weight) was dissected away from the central nucleus with a razor and forceps. Lens tissue was homogenized with 10-15 strokes of a teflon mortar through a tight-fitting glass homogenization pestle in several volumes of 20 mM Tris·HCl/0.1 M NaCl/1 mM NaN_3 at pH 7.9. The crude suspensions were centrifuged (10,000 x g; 10 min) to remove insoluble material, and the supernatant (typically 40 mg/ml protein) was stored frozen at -20°C.

Assay for protein carboxyl methyltransferase -- The standard assay mixture for measuring reaction velocities contained S-adenosyl-L-[methyl-^{3}H]methionine (10 μM; 400-1000 dpm/pmol), sodium citrate buffer (0.14 M Na^+, pH 6.0), ovalbumin (0-40 mg/ml), and 30 μl of enzyme solution in a final volume of 100 μl. Incubations were performed at 37°C and were quenched within the linear portion of the initial velocity curve (10-30 min) by withdrawing an aliquot (80 μl) from the reaction vessel, spotting it onto individually marked pieces of absorbent filter paper (0.5 x 1.0 cm; BioRad Cat. No. 165-0921), and then dropping the paper into a container of 10% trichloroacetic acid (100 ml). The filter papers were washed three times (30 min each) with fresh changes of 10% trichloroacetic acid, soaked overnight in a fourth change, rinsed with methanol (to remove trichloroacetic acid), and transferred to scintillation vials containing 0.1 M NaOH (1 ml). The vial was briefly vortexed, and after standing for 10 min, 10 ml of counting fluor (ACS II; Amersham) was added. Liquid scintillation measurements were corrected for a measured counting efficiency (25-27%) that was unaffected by the presence of the filter paper laying flat against the bottom of the vial.

RESULTS:

Detection of protein carboxyl methyltransferase in lens -- Methyl groups from S-adenosyl-L-[methyl-^{3}H]methionine are incorporated into both endogenous lens protein and exogenously added ovalbumin by an activity in cow lens extracts (Fig. 1A). We have used several criteria to identify this activity as a protein carboxyl methyltransferase. First, the activity can be saturated by ovalbumin, a known substrate of mammalian protein carboxyl methyltransferase. Secondly, the reaction is inhibited by 98% in the presence of 1 mM S-adenosyl-L-homocysteine, an end product of the reaction. Finally, over 95% of the incorporated radioactivity is recovered as methanol when the protein is treated with base. This identification was made on the basis of the co-distillation of radioactivity with authentic methanol from fractionally distilled methanol-

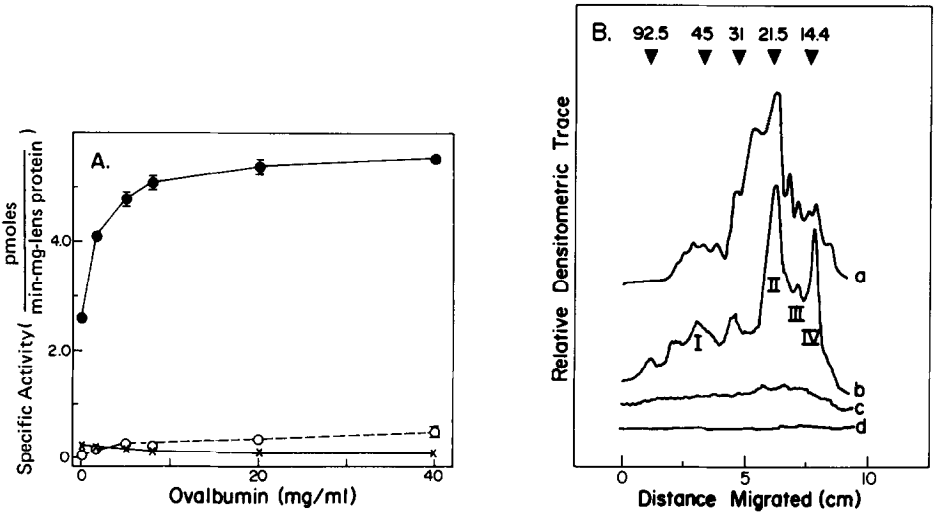

Fig. 1. Protein Carboxyl Methylation in Crude Extracts of Bovine Eye Lens. Top, the velocity of protein methylation in lens cortical extract (1.3 mg protein/ml) was determined in the presence of increasing concentrations of ovalbumin (•) (see Materials and Methods for assay procedure). Controls were performed where 1 mM S-adenosyl-L-homocysteine was present (x), and where the acid precipitated, methylated protein was base treated (0.1 M Na borate, pH 10; 30 min; 25°C) and lyophilized (o). All assays were performed in duplicate, and the average values ± range are shown. Bottom, extracts of a decapsulated lens (10 mg protein/ml) were incubated at pH 6 in the presence of S-adenosyl-L-[methyl-^{3}H]-methionine (3.2 μM; 15 Ci/mmol) for 2 hr at 37°C. Samples (200 μg protein) were then denatured with dodecyl sulfate sample buffer (pH 2.4) and electrophoresed in a 10% polyacrylamide slab gel as described (5). The gel was fixed and stained with Coomassie Blue, and radioactive species were visualized by autofluorography as described (5). Densitometric traces are shown of the Coomassie-staining pattern, a, and the fluorogram, b, of the same gel lane; the fluorogram of a lane where the original sample incubation contained 1 mM S-adenosyl-L-homocysteine, c; and the fluorogram obtained when a stained gel lane was treated in base (0.1 M NaOH; 30 min; 25°C), and then re-acidified before autofluorography, d. Each densitometric trace was recorded by a Quickscan R&D (Helena), and the autofluorogram traces (b-d) were recorded at identical instrument settings. Migration distances of protein standards (BioRad) are indicated along with their polypeptide molecular weights (x 10^{-3}).

water mixtures, the co-azeotropic distillation (bp 53°) from methanol-chloroform mixtures, and by the formation of radioactive crystals (mp 108°) of 3,5-dinitrobenzoic acid methyl ester from 3,5-dinitrobenzoyl chloride. Under the assay conditions described here, little or no activity from other types of macromolecular methyltransferases is detected.

Endogenously methylated proteins from a cow lens extract were separated by sodium dodecyl sulfate gel electrophoresis under acidic conditions in order to preserve base-labile ^{3}H-methyl groups (Fig. 1B). Several methylated species (regions I-IV) comigrated with Coomassie-stained polypeptides, the latter corresponding to subunits of lens crystallins based on their abundance in the extract and their relative migration distances in the gel. The incorporated methyl groups were demonstrated to be in base-labile linkages with the proteins (trace d), and the methylation of all species is inhibited by S-adenosyl-L-homocysteine (trace c).

Isolation of cow lens protein carboxyl methyltransferase -- The activity can be isolated free of endogenous substrate protein by a two-step purification scheme (Fig. 2, Table I). Activity applied to a gel filtration column (Fig. 2A) in the form of a crude lens extract elutes as a symmetrical peak between protein peaks corresponding to the β_L-crystallin (42-62K) and the γ-crystallin (16K)(13). Assuming a spherical enzyme molecule, this elution behaviour is consistent with a native molecular weight for the enzyme of 27,000 $\pm$ 3,000. The enzyme from the gel filtration step (in pH 7.9 buffer) does not bind to the affinity-gel-beads used to purify the enzyme from sources other than lens (12). However, lowering the buffer pH to 6.6 results in the selective binding of the lens enzyme to the beads, and after washing away unbound protein, the activity can be eluted as a sharp peak by raising the buffer pH (Fig. 2B).

The affinity-purified enzyme is not detectably contaminated by methyl acceptor protein (Fig. 2C), and it is saturable by ovalbumin (Km = 1.6 mg/ml). In addition to ovalbumin the isolated enzyme substoichiometrically methylates ribonuclease, glucagon, casein, adrenocorticotropic hormone, gelatin, and purified lens crystallins (data not shown). Methyl groups incorporated into ovalbumin by the isolated methyltransferase show similar base lability as those incorporated by crude enzyme preparations. The rapid hydrolysis half-times at 37°C for these groups (30 min at pH 8; less than 5 min at pH 10) suggests that they are β-methyl esters of aspartyl residues rather than the relatively base stable methyl esters of glutamyl residues (14). Finally, the affinity-purified enzyme eluted from a calibrated TSK-3000 gel permeation column in a manner that confirmed the native molecular weight value cited above.

We have also purified and characterized a protein carboxyl methyltransferase from human lens tissue by these techniques. The properties of the human enzyme appear to be identical to those of the bovine enzyme.

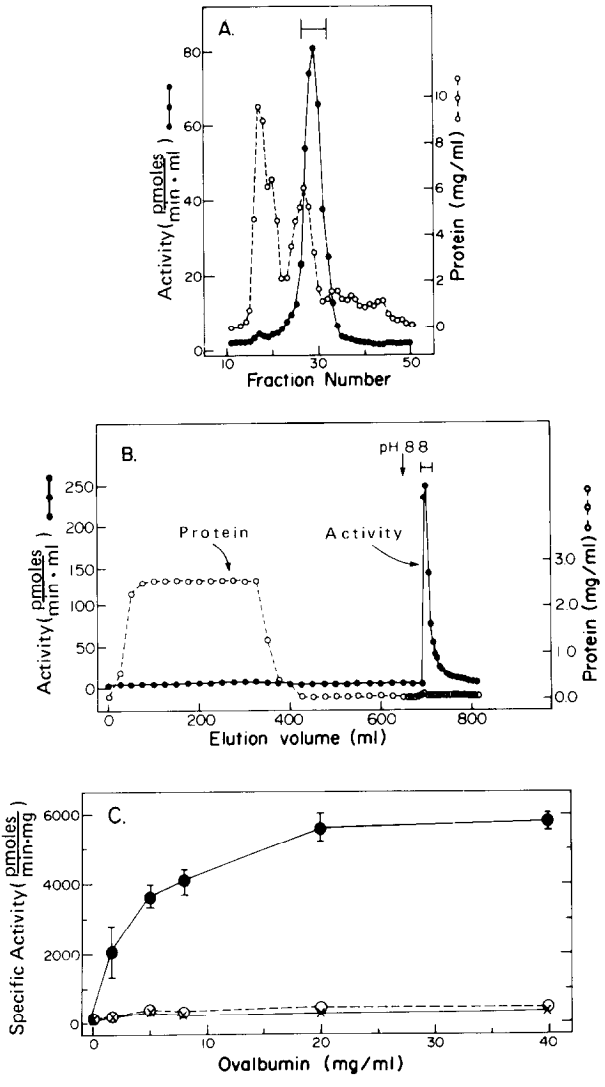

Fig. 2. Isolation of Bovine Lens Protein Carboxyl Methyltransferase. All steps were performed on ice or in a 4°C cold room. Protein concentrations were estimated by assuming an extinction coefficient for a 1 mg/ml solution at 280 nm of 1.0. Top, cortical extract containing 1.0 g of soluble protein was applied to a column (2.5 x 100 cm) of Sephacryl S-200 equilibrated in 20 mM Tris·HCl, 0.1 M NaCl, pH 7.9. Elution (0.9 ml/min) was with the same buffer, and fractions (10 min) were collected and assayed for protein (o) and enzyme activity(•) in the presence of 40 mg/ml ovalbumin. Fractions pooled for the affinity chromatography step are indicated by the bar. Middle, enzyme-containing fractions from four gel filtration steps were combined (180 ml), and the solution pH was lowered to 6.6 by slow addition of 1 M phosphoric acid before applying it at 1 ml/min to a column (1.5 x 15 cm) of S-adenosyl-L-homocysteine-Sepharose 4B resin (12) which had been equilibrated in 10 mM sodium phosphate, 100 mM NaCl, pH 6.6. The column was washed (1 ml/min) with this same buffer, and bound enzyme was then eluted (0.5 ml/min) with 50 mM Tris·HCl, pH 8.8. Fractions were collected and assayed for protein (o) and enzyme activity (•) as above. The indicated fractions (bar) were pooled and stored frozen at -20°C. Bottom, affinity-purified enzyme was assayed in the presence of increasing concentrations of ovalbumin (•). Results of control assays are shown where 1 mM S-adenosyl-L-homocysteine was added to the assay mixture (x), and where the methylated ovalbumin was treated at pH 10 and lyophilized prior to counting (o), as described in Fig. 1. All assays were performed in duplicate, and average values ± range are shown.

TABLE I

PURIFICATION OF PROTEIN CARBOXYL METHYLTRANFERASE FROM BOVINE EYE LENS CORTEX

STEP	VOLUME ml	PROTEIN mg	SPECIFIC ACTIVITY[a] pmol/min/mg	YIELD %	FOLD PURIFICATION
Crude Extract	40	4000	4.4	100	1
Sephacryl S-200[b]	180	556	35	110	8
Affinity purified	35	0.36	11,400	23	2600

[a]Measured with 40 mg/ml ovalbumin.

[b]Material combined from four gel filtration runs, each of which processed 10 ml of crude extract

DISCUSSION:

The protein carboxyl methyltransferase from cow lens described here resembles the corresponding enzyme from other mammalian sources in several respects. The apparent native molecular weight of the cow lens enzyme is close to that determined for the enzyme from calf brain (15), and human and rat erythrocytes (16). The cow lens enzyme also demonstrates similar purification properties to these other enzymes (12,15) in that it specifically binds to an affinity column derived from the end-product of the methylation reaction, S-adenosyl-L-homocysteine. The results described here also indicate that the cow lens enzyme, as is typical for this class of enzyme, methylates a broad range of proteins (1). We have determined that the lens enzyme methylates these substrates to substoichiometric levels even after extended reaction periods in the presence of S-adenosyl-L-methionine (data not shown).

Another important similarity between the lens enzyme and the methyltransferases from other mammalian tissues is the extreme base lability of the methyl ester linkages formed by the enzymes. This susceptibility to hydrolysis, even at neutral pH, has been shown to be characteristic of β-methyl esters of protein-bound aspartyl residues (14); thus it is likely that the lens enzyme recognizes and methylates aspartyl residues. In the case of methylated aspartyl residues formed by the human erythrocyte protein carboxyl methyltransferase there is clear evidence that the methylated residues have the uncommon D-configuration (8,17). We are currently investigating the optical configuration of the residues methylated by the lens methyltransferase to determine whether this enzyme may play a role in the metabolism of age-racemized lens proteins in a similar fashion as that described for erythrocytes (8,17,18).

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