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PURIFICATION AND SOME MOLECULAR PROPERTIES OF PROTEIN METHYLASE II FROM EQUINE ERYTHROCYTES.

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Summary: Protein methylase II (S-adenosyl-L-methionine:protein carboxyl-O-methyltransferase; E.C. 2.1.1.24) has been purified 28 000 fold from equine erythrocytes. The purified enzyme is homogeneous on polyacrylamide gel electrophoresis performed either in presence or in absence of SDS, and on analytical ultracentrifugation. It appears constituted of a single polypeptidic chain of a molecular weight very close to 25 000 Daltons. Other enzymatic properties of the protein are quite similar to those previously reported for similar enzymes. The amino acid analysis of the enzyme is presented. The single cysteine residue, the enzyme contains, is essential for the enzymatic activity. Other amino acids apparentely involved in catalysis are tentatively identified.

Many proteins are subjected *in vivo* to various types of modification of their covalent structure. These modifications can be classified mainly in two types: a permanent one, which occurs generally shortly after protein biosynthesis, or a modification proceeding in a dynamic state, when the protein participes in a rapid adaptative function (1).

Methylation of proteins can be classified in either types. The methylation of lysines, arginines, and, perhaps, histidines, seems to be an irreversible modification devoid of any significant turnover (1, 2). On the other hand methylation of aspartic or, and, glutamic acid residues in proteins is a transitory modification leading to the formation of an unstable ester bond, which can be readily hydrolysed, even under mild conditions (3).

Protein carboxymethylation seems to be involved either in mammalian and in procaryotic cells in many important cellular events, such as the bacterial chemotaxis (4, 5) or the exocytotic secretion of exportable products (6). However, even if the biological importance of carboxymethylation is now well admitted, our knowledge of these reactions and of the enzymes catalysing them, remains still rudimentary.

In this paper we wish to report the purification of protein methylase II from equine erythrocytes to apparent homogeneity and some molecular and catalytic properties of this enzyme.

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MATERIALS AND METHODS

Equine blood was collected immediately after animal sacrifice. Coagulation was prevented by addition of dextrose citrate buffer.
All chemicals used were of the highest purity available. Most of the reagents used in the active site study were obtained from Sigma, who also supplied S-adenosyl-L-homocysteine. Carboxymethyl-cellulose (CM-22) was purshased from Whatman, Dextran gels and DEAE-Sephadex A-50 from Pharmacia and S-adenosyl-L-(methyl-^{14}C)-methionine from the Radiochemical Centre.
Enzymic activity was measured by the method described by Jamaluddin and al. (7) and by Axelrod (8), using either calf thymus protein mixture FP-100 (9) or bovine serum albumin. The specific activity of the enzyme is expressed as picomoles of methanol extracted per minute per milligram of protein under the experimental conditions used.
The detailed purification procedure, who mainly consists in an improvement and a prolongation of the method described by Kim (10), is illustrated in Table I. All buffers used contained 2 mM EDTA and 2 mM 2-mercaptoethanol.
The last step, an electrofocusing, was performed with a L.K.B. 110 ml electrofocusing column (pH 4-6) using synthetic ampholites (L.K.B.) and a density gradient of glycerol (0-50 % v/v). The column contained also 1 mM 2-mercaptoethanol, in order to stabilize protein methylase II.
Protein concentration was determined by the Lowry's method, using cristalline bovine serum albumin as a standard (11). The molar extinction coefficient was determined by measuring the absorbance of a solution of a known protein content.
Amino acid composition was obtained with an automatic amino acid analyser (Durrum) after acid hydrolysis of the protein, under vacuum, at 105°C for 24, 48, and 72 hours. In the calculations a molecular weight of 25 000 was used. Cysteine and tryptophane residues were respectively estimated by the procedures of Ellman (12) and of Pajot (13).
In the active site study, the enzyme was incubated with the appropriate reagent under the conditions described in Table III. Reactions were stopped by removing the excess of reagent by molecular sieving on a column (0.2 X 8 cm) of Sephadex G-25 or by annihilation of the reagent by its reaction with a specific molecule (i.e. histidine or imidazole when diethylpyrocarbonate was used). Blank controls, in which the enzyme is added after destruction of the reagent, were simultaneously run.
To ascertain a "true active site directed modification" the enzyme was also parallely incubated with the reagent and S-adenosyl-L-homocysteine, at variable concentrations, in order to detect a possible protection of the enzyme by this substrate analogue, against inactivation.
To prevent reaction of the unique cysteine residue during incubation with diethylpyrocarbonate, Koshland reagent II, tetranitromethane, acetylimidazole and Woodward's reagent K, this residue was protected by reaction with para-chloromercuribenzoic acid. This "protective group" can be removed from the enzyme by incubation in 10 mM 2-mercaptoethanol.

RESULTS AND DISCUSSION

Protein methylase II was purified from horse red blood cells by the procedure described in Table I. A preliminary purification of this enzyme from rat and human erythrocytes has already been reported by Kim (10). We have improved this method by introducing, after the ammonium sulfate precipitation, a batchwise treatement with carboxymethyl-cellulose CM-22, in 5 mM sodium phoshate buffer pH 6.3. This last step allows us to remove most of the still contaminant haemoglobin, which is absorbed on this chromatographical support, while

TABLE I : Purification scheme of protein methylase II from equine erythrocytes.

Preparation	Volume (ml)	Protein Total (mg)	Protein mg/ml	Specific activity (a)	Total activity	Purification	Yield %
Erythrocyte	5 000	490 000	98	0.022	IO 780	I	I00
Supernatant at I50 000 X g	4 700	440 000	94	0.02	9 592	0.99	89
58 % ($NH_4)_2SO_4$ precipitate	230	I2 000	52	0.54	6 46I	24.7	60
Chromatography on CM-22 (b)	900	I 650	I.83	2.8	4 6I0	I27	42.8
Sieving on Sephadex G-I00 (c)	350	I87	0.54	22.2	4 I55	I 0I0	38.5
Chromatography on DEAE-Seph. A-50 (d)	400	24	0.06	I26	3 024	5 730	28.I
Electrofocusing (e)	4.5	3.7	0.82	632	2 340	28 750	21.7

(a)-Specific activity is expressed as picomoles of methanol extracted per minute and per mg of protein under the experimental conditions used.

(b)-(4 X 30 cm) column, sodium phosphate buffer 5 mM pH 6.3.

(c)-(6 X I50 cm) column, sodium phosphate buffer 5 mM pH 6.3.

(d)-(2 X I0 cm) column, sodium borate buffer 5 mM pH 9.5.

(e)-Ampholites were removed by dialysis and finally by passage through a column (1 X 50 cm) of Sephadex G-50.

protein methylase II is not retained. Further purification is performed by gel filtration on a Sephadex G-100 column, a batchwise chromatography on DEAE-Sephadex A-50 and finally an electrofocusing.

An active enzyme, with a specific activity of 632 units, is obtained. It appears to be homogeneous by polyacrylamide gel electrophoresis performed at pH 4.3 and 6.5, by SDS polyacrylamide gel electrophoresis and by analytical ultracentrifugation (Figure 1).

The molecular weight of the enzyme is probably near of 25 000, since estimation by Sephadex G-75 molecular sieve gives a value of 26 000, SDS polyacrylamide gel electrophoresis 24 500, and finally, sedimentation and diffusion measurements ($s^{\circ}_{20,w}$ $2.8\ 10^{-13}$ sec^{-1} ; $D^{\circ}_{20,W}$ $9.8\ 10^{-7}$ cm^2 sec^{-1}) give an hydrodynamical weight of 25 400.

The enzyme seems to be composed of a single polypeptidic chain. Indeed, by SDS gel electrophoresis we have noticed a single proteinic band, corresponding to a molecular weight identical to that of the whole enzyme. Furthermore, no protein loss is evidenced after extensive dialysis of the enzyme in presence of 6 M urea and 20 mM 2-mercaptoethanol.

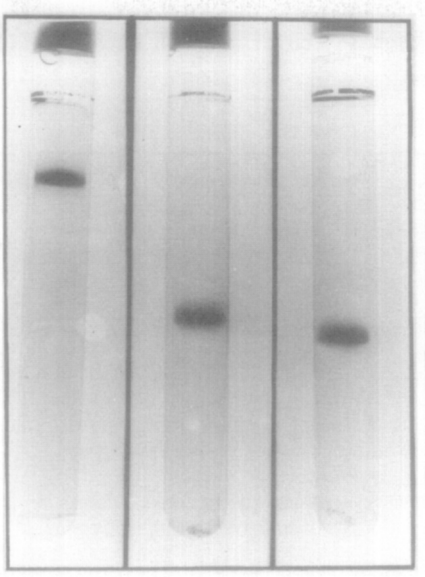

FIGURE 1 : Disc gel electrophoretic patterns of purified protein methylase II. From left to right, gels without SDS at pH 4.3, 6.5 and, in presence of SDS, at pH 7.2.

The characteristics of protein methylase II from equine erythrocytes we have obtained are in good agreement with those reported by Kim for a partially purified preparation of rat and human blood enzymes (10). Indeed the molecular weight estimated by Kim is near of 26 000. Furthermore, catalytic properties, as the Km for S-adenosyl-L-methionine (Km 1.1 μ M), the Ki for S-adenosyl-L-homocysteine (Ki 0.7 μ M) and the optimal activity pH (6.5, using many proteins as substrates) we found, are very similar to the values reported by Kim (10), and by Paik and Kim (14) for other protein methylases II. However, at the opposite of Kim, who found many active fractions during electrofocusing of the partially purified rat blood enzyme (10), we could evidence a single activity peak, of an isoelectric point of 5.6.

Also, it is interesting to note that Paik (14) has reported that calf thymus protein methylase II exists in various molecular forms with subunits, of a postulated molecular weight of 8 000. Erythrocyte protein methylase II would be, so, structurally very different from the calf thymus enzyme.

Table II lists the amino acid composition of equine erythrocytes protein methylase II. The enzyme has no disulfide bridges, and a single cysteine residue has been evidenced. The aromatic amino acid content is fully compatible with the molar extinction coefficient at 280 nm of 26 000 $\pm$ 1 500 l $mole^{-1}$ cm^{-1}

TABLE II : Amino acid composition of protein methylase II from equine erythrocytes.

Amino acid	Residues found (a)	Estimated
Lysine	I3.9	I4
Histidine	8.2	8
Arginine	7.95	8
Aspartic acid	22.93	23
Threonine	II.6	I2
Serine	24.97	25
Glutamic acid	27.3	27
Proline	I4.76	I5
Glycine	25.26	25
Alanine	I8.I	I8
Cysteine	0.94 (b)	I
Valine	I5	I5
Methionine	TRACES	-
Isoleucine	7.74	8
Leucine	I8.7I	I9
Tyrosine	4.7	5
Phenylalanine	7.59	3
Tryptophane	3.87 (c)	4
Molecular Weight :		25 200

(a)-Values corrected for hydrolytic losses,and average of six determinations over two distinct preparations.
(b)-By the Ellman's method (12).
(c)-By the Pajot's method (13).

we obtained. The four tryptophane residues seem to be quite buried in an apolar environment, since fluorescence measurements revealed an emission wavelenght maximum of 334 nm, an exitation wavelenght optimum at 290 nm almost independent from the emission wavelenght, and a quantum yield of 0.1. These figures are quite typical of tryptophane residues in an apolar environment (15). By the method of Scott (16), the protein was found to be a glycoprotein containing 2 % of neutral hexoses, using galactose and fucose as standards.

As very few, or none, of the catalytic properties of protein methylases, in

TABLE III : Inhibition study of protein methylase II from equine erythrocytes by group specific reagents.

Reagent	Conditions	Molar ratio reagent/enzyme	Notes	Probable site of action	Ref.
Complete inhibition :					
Parachloromercuribenzoate (pCMB)	Tris-HCl 10 mM; pH 7 37°C; 20 min.	20 to 50	full reversibility[a] by 2-mercaptoethanol	Cys,(His)	(20)
Mercuric chloride	idem	10 to 20	idem	Cys,(His)	
Diethylpyrocarbonate	Phosphate buffer 10 mM pH 6; 20°C; 10 min.	10 to 100	reversible by NH_2OH 0,1M [a] [b]	His,(Cys)	(21)
Diazotetrazole	Carbonate buffer 0,1 M pH 8,8; 20°C; 30 min.	100	reversibility not tested [a] [b]	His,Tyr, Cys	(22)
Tetranitromethane	Phosphate buffer 10 mM pH 8; 20°C; 20 min.	100 to 300		Tyr,(Cys)	(23)
Woodward's reagent K	Phosphate buffer 10 mM pH 6,4; 20°C; 10 min.	100	not reversible by NH_2OH 0,1 M [a] [b]	Glu,Asp, (Cys,Tyr)	(24)
Acetylimidazole	Phosphate buffer 10 mM pH 7,5; 20°C; 30 min.	1 000	only 40 % inhibition [a] [b]	Tyr,Cys	(25)
No inhibition :					
Phenylmethane sulfonyl fluoride	Phosphate buffer 0,1 M pH 7; 37°C; 60 min.	1 000		Ser	(26)
2-4 Pentanedione	Phosphate buffer 0,1 M pH 7; 30°C; 240 min.	10 000		Lys,(Arg)	
1-2 Cyclohexanedione	Borate buffer 0,1 M pH 9; 30°C; 240 min.	1 000 to 40 000		Arg	(27)
N-Bromosuccinimide and Koshland's reagent II	Acetate buffer 0,1 M pH 5,5; 20°C; 60 min.	1 000		Try,(Tyr, Cys,Met...)	(28) (29)
Iodoacetamide Bromoacetic acid Aziridin	Tris-HCl 0,1 M; pH 8 20°C; 60 min.	1 000	no inhibition without previous denaturation of the enzyme	Cys,(His, ...)	(30)
N-Ethylmaleimide	Tris-HCl 0,1 M; pH 7 20°C; 120 min.	1 000	idem	Cys	(20)

[a]-Protection of the enzyme against this reagent is provided by S-adenosyl-L-homocysteine

[b]-Inhibition by this reagent is noticed even when cysteine is protected during reaction by pCMB. pCMB is removed by incubation in 10 mM 2-mercaptoethanol.

general, and of protein methylase II, in particular, are known, we found useful to study the active site of this enzyme using specific reagents. The results of this study are presented in Table III. We have checked that incubation of the enzyme with S-adenosyl-L-homocysteine prevents inactivation by most of the reagents tested. This protection is dependent on the concentration of this substrate analogue and became effective ($\pm$ 50 % protection) for con-

centrations close to that of the Ki value (0.7 μ M).
This argument strongly suggests that para-chloromercuribenzoate, diethylpyrocarbonate, tetranitromethane, and Woodward's reagent K (N-ethyl-5-phenylisoxazolium-3'sulfonate) inactivate the enzyme by a modification of the active site and probably not by a gross conformational change.
However, since only minute quantities of the enzyme were available, it is still difficult to assess what kind of residues are modified by these reagents. Since incubation with hydroxylamine restores the enzymic activity after reaction with diethylpyrocarbonate, and that this reagent inactivates the enzyme even when its cysteine is reversibly protected with para-chloromercuribenzoate, we can surmise that one or more histidines are located in the active site (21). By similar arguments it can be argued that one or more tyrosines and aspartic or glutamic acids participate to catalysis.
We can also suggest that the single cysteine residue is necessary for the enzymatic activity of protein methylase II. The lack of reactivity of this residue towards iodoacetamide, bromoacetic acid, N-ethylmaleimide and aziridine, failing in inhibiting the enzyme without its previous denaturation, can be tentatively explained by a particular buried and apolar microenvironment.
It is interesting to note that Borchardt (17) has pointed out that an SH group is also involved in the catalytic activity of a Catechol-O-MethylTransferase purified from rat liver. Furthermore, some indirect evidences can suggest the participation of such a functional group in many other methyltransferases (10, 18, 19).

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