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AN ADENOSINE 3':5'-MONOPHOSPHATE-ADENOSINE BINDING PROTEIN FROM MOUSE LIVER: SOME PHYSICOCHEMICAL PROPERTIES

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Summary

A number of physiochemical properties of the cyclic AMP-adenosine binding protein of mouse liver (Ueland, P.M. and Døskeland, S.O. (1977) J. Biol. Chem. 252, 677—686) have been studied.

1. The specific extinction coefficient, $E^{1\%}_{280\,nm}$, was estimated to 13.0.

2. Amino acid and amide group analyses confirmed the acidic properties of the protein as determined by electrofocusing (pI = 5.7). Based on the estimated partial specific volume ($\bar{v}$ = 0.74 cm^3/g) the minimum molecular weight of the native, tetrameric protein was recalculated to be 185 000 ($s_{20,w}$ = 8.8 · 10^{-13} s and Stokes radius = 48 Å).

3. No NH_2-terminal amino acid was found by the dansyl method using [^{14}C]-dansyl chloride, indicating that the NH_2-terminal groups are blocked.

4. Amino acid analyses gave 6 half-cystine residues per subunit, and the same number of free sulfhydryl groups was found by titration of the denatured protein with 5,5'-dithiobis (2-nitrobenzoic acid).

5. The reactivity of the SH groups in the native protein with 5,5'-dithiobis (2-nitrobenzoic acid) revealed rapidly reacting (SH_I), sluggishly reacting (SH_{II}) and "masked" (SH_{III}) SH groups. ATP, adenosine, Mg^{2+} and KCl, factors known to affect the activation of cyclic AMP binding sites (Ueland, P.M. and Døskeland, S.O. (1978) Arch. Biochem. Biophys., in press) changed the reactivity of separate SH groups.

Abbreviations: DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

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Introduction

A protein which binds cyclic AMP and adenosine, has been isolated from mouse liver and purified to apparent homogeneity. The binding protein could be differentiated from both cyclic AMP dependent protein kinase and phosphofructokinase [1]. Experimental evidence has been presented indicating that cyclic AMP interacts with a site different from the adenosine binding site, and the binding capacity for cyclic AMP increases several-fold by incubating the protein in the presence of ATP [1,2]. This change in binding properties of the protein has tentatively been termed activation. The role of this class of protein in carrying out cyclic AMP effects has recently been discussed [3]. One of its functions may be to regulate the intracellular concentration of free adenine derivatives but the question still remains whether the protein also has an enzymic activity.

Previous studies [1] have revealed that the binding protein is a tetramer of molecular weight 180 000 calculated from sedimentation velocity and gel permeation chromatography, and a subunit molecular weight of approx. 45 000 (based on gel electrophoresis in the presence of dodecyl sulfate). In the present study further physicochemical studies on the binding protein are presented. Data is also given suggesting that the reactivity of the SH groups in the protein may be a useful probe to study its interaction with adenine derivatives under various experimental conditions in vitro.

Materials and Methods

Chemicals

Adenosine, ATP, 5,5′-dithiobis (2-nitrobenzoic acid) were obtained from the Sigma Chemical Co., St. Louis, U.S.A. Orcinol (monohydrate), periodic acid, potassium disulfite, and sodium diethyldithiocarbamate (trihydrate) were the products of Merck AG, Darmstadt. Basic fuchsin, dansyl amino acid reference collection, and polyamide sheets (manufactured by the Cheng Chin Trading Co.) were supplied by the British Drug Houses. Hydrochloric acid (ultrapure grade), dansyl chloride, and methanesulfonic acid (4 N, containing 0.2% 3-(2-aminoethyl) indole) were purchased from Pierce. *N*-Methyl[^{14}C]dansyl chloride (30.1 Ci/mol) was obtained from the Radiochemical Centre (Amersham).

Purification of cyclic AMP-adenosine binding protein

The binding protein was purified from mouse liver essentially as described [1]. When the protein was used for the determination of sulfhydryl groups it was purified in the absence of 2-mercaptoethanol.

Electrophoresis in the presence of dodecyl sulfate

Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate was performed as described previously [1], except that 2-mercaptoethanol was omitted in some experiments.

Amino acid analysis

Lyophilized samples were hydrolyzed with 6 M HCl (1.0 ml) containing

0.05% (v/v) thioglycollic acid, under nitrogen and vacuum (Pierce Vacuum Reaction Tubes) for 24, 48 and 72 h at 105°C. The hydrolysates were analyzed in a Bio-Cal amino acid analyzer (type BC-200) according to the one column system of Dus et al. [4]. Total half-cystine was determined following performic acid oxidation [5]. Tryptophan and tyrosine were determined by the spectrophotometric method of Edelhoch [6].

NH_2-terminal amino acid determination

Samples of 0.3 mg of lyophilized protein were dansylated as described by Gros and Labouesse [7] using a reaction time of 120 min. Hydrolysis was carried out in 0.3 ml 6 M HCl for 16 h at 105°C or in 0.5 ml 4 N methanesulfonic acid for 4 h at 110°C. The dansyl amino acids were separated by two-dimensional thin-layer chromatography on 3.0 × 3.0 cm polyamide sheets developed in the solvent system of Gray [8]. Quantitation of the dansyl derivatives was performed using [^{14}C]dansyl chloride and 15 × 15 cm polyamide sheets as described previously [9].

Carbohydrate analysis

Analysis for carbohydrate was performed by an orcinol/H_2SO_4 method [10] (each required approx. 1 mg of protein) using glucose as a standard and by Periodic Acid Schiff staining of SDS polyacrylamide gels [11] (10—50 µg of protein per gel). Bovine gamma globulin was used as a positive control.

Sulfhydryl group determination

Sulfhydryl groups in the protein were estimated essentially as described by Habeeb [12] by measuring the absorbance change at 412 nm during the reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The protein (450 µg) was dissolved in 0.5 ml 80 mM sodium phosphate buffer, pH 8.0 which in some experiments also contained 300 mM KCl, 20 mM magnesium acetate, 6 mM ATP or 20 µM adenosine (for details, see Results). After preincubation for 30 min at 25°C, the same volume (0.5 ml) of 80 mM sodium phosphate buffer, pH 8.0 containing 0.65 mM DTNB, was added, and the absorbance at 412 nm was recorded by a Shimadzu multi-purpose spectrophotometer (Model MPS-50L) at 25°C.

The estimate of the total number of sulfhydryl groups in the protein was performed in the presence of 2% (w/v) of dodecyl sulfate [12].

Other analytical procedures

The amide content was determined in samples of about 0.2 mg of protein as described by Skotland and Ljones [13]. Protein was estimated by a modification of the biuret method as described by Klungsøyr [14] using bovine serum albumin as a standard.

Calculations

Based on the physiochemical parameters determined in previous [1] and present studies, the frictional ratio, axial ratio, partial specific volume and the minimum molecular weight were calculated. The frictional ratio was calculated according to the equation [15], $f/f_0 = a/(3\bar{v}M/4N)^{1/3}$ where a = Stokes radius,

N = Avogadro's number, M = molecular weight and $\bar{v}$ = partial specific volume. By using the model of a prolate ellipsoid, the axial ratio was determined [16]. The partial specific volume was calculated as described by Cohn and Edsall [17]. The minimum molecular weight of the monomer was calculated from the amino acid composition.

Results

Ultraviolet absorption and specific extinction coefficient

The ultraviolet absorption spectrum of the cyclic AMP-adenosine binding protein is characterized by an absorption maximum at 278 nm and the A_{280} : A_{260} ratio was found to be 1.38. This ratio did not change following incubation of the protein in the presence of charcoal (10 mg/ml) at 30°C for 30 min, suggesting that no adenine derivatives were bound to the isolated protein. The specific extinction coefficient, $E^{1\%}_{280\,nm}$, was found to be 13.0 based on protein determination by a modified biuret method [14] using bovine serum albumin as a standard. There was no indication of a chromophoric group absorbing in the visible region.

Carbohydrate content

No carbohydrate was detected by either of the two methods used (see Materials and Methods), and no amino sugar was detected by the amino acid analyzer.

Amino acid composition and amide content

The amino acid composition of the cyclic AMP-adenosine binding protein is summarized in Table I. All common amino acids are present, and the balance of charged residues (corrected for the amide content) indicates a rather acidic protein as expected from previous electrofocusing experiments (p*I* 5.7) [1]. The amino acid present in the lowest concentration was half-cystine (6 residues per monomer) followed by histidine and tryptophan. A partial specific volume of 0.74 cm^3/g was calculated from the amino acid composition. About 25 amide groups per monomer were found (Table I).

NH_2-terminal amino acid analysis

No fluorescent spots corresponding to NH_2-terminal amino acids were detected on the small polyamide sheets following hydrolysis of the dansylated protein with hydrochloric or methanesulfonic acid. Using [^{14}C]dansyl chloride and hydrolysis with hydrochloric acid, no radioactivity was found outside the spots corresponding to dansyl-ϵ-lysine, dansyl-*o*-tyrosine, dansyl-NH_2, and dansyl-OH. A maximum of 7650 cpm was recovered in the dansyl-ϵ-lysine spot (the background was 22 cpm). By assuming 28 residues of lysine per subunit (Table I) and a total recovery of the dansyl amino acid during hydrolysis, a free NH_2-terminal amino acid in the protein would have been expected to give a radioactive spot of about 270 cpm.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate revealed

TABLE I

AMINO ACID COMPOSITION OF THE CYCLIC AMP-ADENOSINE BINDING PROTEIN

Amino acid residues	μmol of amino acid residues			Average or extrapolated values	Amino acid residues per 46 500 mol. wt. of protein †	Nearest integral number of amino acid residues per 46 500 mol. wt. of protein
	24 h	48 h	72 h			
Aspartic acid	0.417	0.433	0.405	0.418	44.98	45
Threonine *	0.194	0.195	0.188	0.197	21.16	21
Serine *	0.126	0.120	0.109	0.135	14.47	15
Glutamic acid	0.359	0.371	0.360	0.364	39.08	39
Proline	0.173	0.157	0.170	0.166	17.89	18
Glycine	0.336	0.344	0.339	0.340	36.53	36
Alanine	0.344	0.357	0.353	0.351	37.76	38
Half-cystine **	0.055			0.055	5.94	6
Valine	0.260	0.287	0.294	0.280	30.15	30
Methionine	0.147	0.153	0.150	0.150	16.14	16
Isoleucine	0.253	0.275	0.281	0.270	28.99	29
Leucine	0.348	0.355	0.352	0.352	37.83	38
Tyrosine	0.126	0.111	0.124	0.120	12.92	13
Phenylalanine	0.113	0.097	0.117	0.109	11.69	12
Histidine	0.103	0.100	0.112	0.105	11.30	11
Lysine	0.288	0.246	0.241	0.258	27.75	28
Arginine	0.125	0.110	0.119	0.118	12.72	13
Tryptophan ***					10.77	11
Amide groups						25
Total residues						419

* Obtained by extrapolation to zero hydrolysis time by assuming first-order kinetics of destruction.

** Determined as cysteic acid following performic acid oxidation.

*** Determined spectrophotometrically.

† Figures are expressed as molar ratios using the average values for the following amino acids as integers: aspartic acid, glutamic acid, glycine, alanine, valine, phenylalanine, histidine, and lysine.

only a single band both in the absence (data not shown) and presence [1] of 2-mercaptoethanol; the reducing agent did not increase the mobility.

The reactivity of sulfhydryl groups

The time course of the reaction of the native protein with DTNB revealed a rapid initial phase (within the first few seconds) followed by an intermediate phase where the absorbancy increased more slowly. After 0.5—1 h a very slow phase was observed in which the absorbancy at 412 nm increased almost linearly as a function of time for at least 2 h. Upon the addition of SDS, which unfolds the protein and thus exposes masked SH groups, the absorbance increased abruptly reaching a steady-state level (Fig. 1A) which corresponds to 23.6 ± 0.7 (S.D., $n = 4$) sulfhydryl groups per tetramer using a molar extinction coefficient ϵ_{412} ($M^{-1} \cdot cm^{-1}$) = 13 600 for the nitromercaptobenzoate anion [12].

The 24 sulfhydryl groups in the native protein can be divided into 3 populations according to increasing reactivity towards DTNB, where $SH_I >> SH_{II} > SH_{III}$. About four of the groups may be classified as SH_I groups, about four as SH_{II} groups and the remaining 16 groups as SH_{III}. The possibility is considered that the reaction of DTNB with SH_{III}-groups is related to a slow denaturation

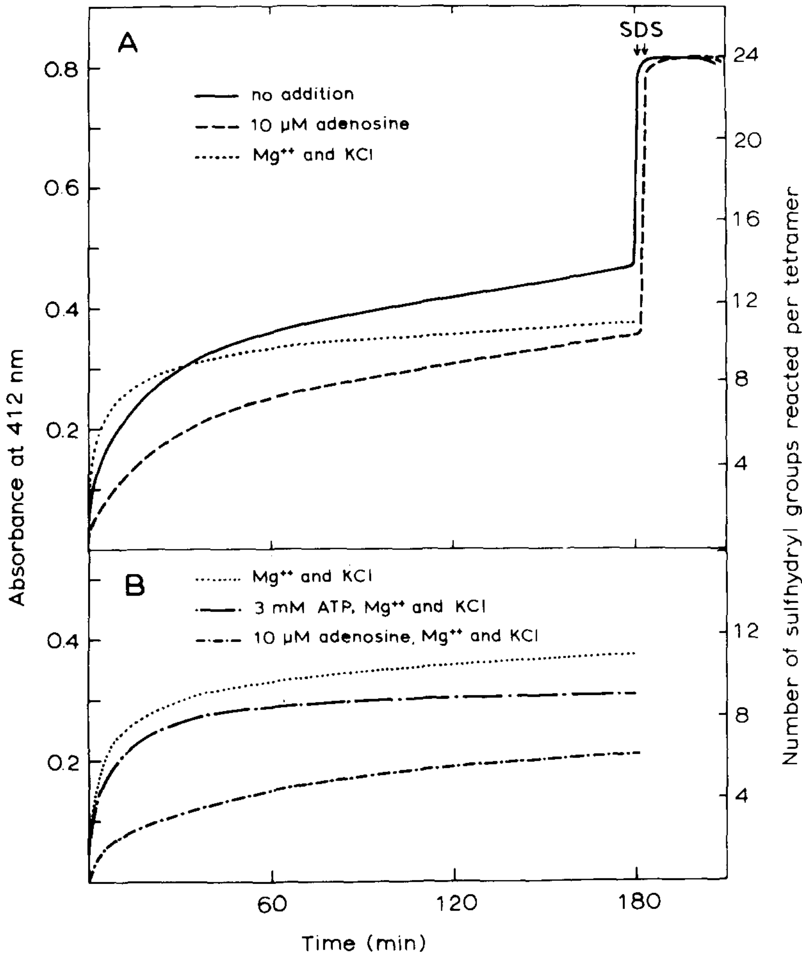

Fig. 1. The time course of the reaction of the cyclic AMP-adenosine binding protein with DTNB in the presence of various agents. The experiments were performed as described under Materials and Methods. The incubation mixture contained in a final volume of 1 ml: 450 µg of protein (concentration of protein = 2.5 µM), 10 µM adenosine, 3 mM ATP, 10 mM Mg^{2+}, 150 mM KCl and 0.2% (w/v) dodecyl sulfate.

of the binding protein. It should be noted, that the reaction with DTNB could not be followed more than 3 h due to precipitation of the protein in the medium used.

From Fig. 1A it is seen that adenosine suppressed the reaction of SH_I and SH_{II} groups with DTNB whereas the assessible SH_{III} groups were essentially unaffected. ATP alone was without effect (data not shown). Magnesium plus KCl increased the reactivity of SH_{II} groups and decreased that of assessible SH_{III} (Fig. 1A).

In the presence of KCl and Mg^{2+}, adenosine revealed principally the same effect as observed in the absence of these ions (Fig. 1B). ATP, on the other hand, lowered the reactivity of assessible SH_{III} groups and possibly of SH_{II} only in the presence of KCl and Mg^{2+} (Fig. 1B).

TABLE II

PHYSICOCHEMICAL PARAMETERS OF THE CYCLIC AMP-ADENOSINE BINDING PROTEIN

Parameter	Experimental basis of calculation	Estimated value
$E^{1\%}_{180nm}$	Spectrophotometry	13.0
$s_{20,w}$ (S)	Sedimentation velocity centrifugation	8.8
$s_{20,w}$ (S)	Density gradient centrifugation	9
Stokes radius (Å)	Gel filtration	48
$\bar{v}$ (cm^3/g)	Amino acid analysis	0.74
Mol. wt.	Sedimentation velocity centrifugation, gel permeation chromatography and amino acid analysis	185 000
Mol. wt. of subunit	SDS gel electrophoresis	45 000
Mol. wt. of subunit	Amino acid analysis	46 500
f/f_0	$s_{20,w}$, Stokes radius and $\bar{v}$	1.27
Axial ratio	$s_{20,w}$, Stokes radius and $\bar{v}$	5—6
pI	Isoelectric focusing	5.7
Hydrophobicity (cal/res)	Amino acid analysis	1 176
Number of sulfhydryl groups per tetramer	Reaction with DTNB	24
Number of half-cystine per subunit	Amino acid analysis	6
Disulfide bridges	Determination of half-cystine and sulfhydryl groups	0

Physiochemical parameters of the cyclic AMP-adenosine binding protein

The physiochemical properties so far determined for the binding protein are summarized in Table II.

Discussion

Based on previous and present physiochemical studies on the cyclic AMP-adenosine binding protein from mouse liver, a rather detailed picture of its molecular properties has emerged. The protein is a globular (f/f_0 = 1.27), acidic (pH = 5.7) oligomer consisting of four subunits of equal size [1]. By assuming a partial specific volume of 0.73 cm^3/g, the molecular weight was previously calculated to be 180 000 [1]. However, using the partial specific volume (0.74 cm^3/g) calculated from the amino acid composition, a slightly higher molecular weight is obtained, i.e. 185 000. A subunit molecular weight of 45 000 as determined by SDS polyacrylamide gel electrophoresis [1], is in good agreement with the minimum molecular weight of 46 500 calculated from the amino acid composition (Table II). The identity of the subunits is so far only supported by the presence of a single band on SDS gel electrophoresis [1] as well as the observation that no free NH_2-terminal amino acid was detected with the dansyl method although the hydrolysis was carried out in methanesulfonic acid in order not to destroy NH_2-terminal tryptophan [18]. From the high recovery of dansyl-ϵ-lysine one would expect that a free NH_2-terminal should have been easily detected using [^{14}C]dansyl chloride. Further studies are, however, required to establish the tentative conclusions that the subunits are identical.

When the amino acid composition of 207 known proteins [19] was compared with that of the cyclic AMP-adenosine binding protein, the most charac-

teristic feature of the protein is a rather high content of methionine, tryptophan, and isoleucine. Furthermore an average hydrophobicity of 1176 cal/residue was calculated [20] which is in the upper range reported for water soluble proteins.

The finding that the native protein dissociates completely into subunits in the presence of dodecyl sulfate only, is in accordance with the finding of no disulfide bridges in the native protein. This conclusion is based on the finding that the total number of free SH groups, i.e. 24 per 185 000 daltons as measured by the reaction of the denatured protein with DTNB, equals the number of half-cystine residues, i.e. 6 per 46 500 daltons. If the subunits are identical, this result would mean that each subunit contains 6 free SH groups.

The reactivity of the SH groups in the native protein with DTNB varies over a wide range, and they can be divided into rapidly reacting (SH_I), sluggishly reacting (SH_{II}), and "masked" or "buried" (SH_{III}) residues according to Barron [21].

The reactivity was also measured in the presence of Mg^{2+}, KCl, ATP and adenosine since these factors affect the activation of the cyclic AMP binding sites [2]. ATP had no effect on the reactivity of any of the SH groups in the absence of Mg^{2+} and KCl which correlates well with the finding that ATP in the absence of these ions induces no activation of cyclic AMP binding sites [2]. Mg^{2+} and KCl alone increased the reactivity of SH_I and SH_{II} and decreased the reactivity of assessable SH_{III} groups. The effect introduced by these ions is probably a prerequisite for the activation by ATP [2]. In the presence of Mg^{2+} and KCl, ATP further decreased the reactivity of SH_{III} groups (and possibly of SH_{II}), but had no effect on SH_I. Under these conditions adenosine displayed principally the same effect as in the absence of the ions, i.e. decreased the reactivity of SH_I and SH_{II} whereas SH_{III} was unaffected. These results suggest that the activation process is related to an increased reactivity of SH_{II} groups whereas inhibition of activation is correlated to a decreased reactivity of SH_I and SH_{II}. Whether the altered reactivity of the SH groups are essential for the activation process or binding, or whether they are secondary to conformational changes of the protein during these processes, remain to be established. Further studies on the sulfhydryl groups should await a more detailed knowledge on the interaction between this protein and adenine derivatives as well as the functional role of this protein.

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