ISOLATION OF A DCCD-BINDING PROTEIN FROM BOVINE CHROMAFFIN-GRANULE MEMBRANES

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1. Introduction

Chromaffin granules, the secretory granules of the adrenal medulla, contain a membrane-bound, proton-translocating ATPase which, like the ATPase of mitochondria, chloroplasts and bacterial plasma-membranes, is inhibited by DCCD and by the alkyl tins [1,2]. The transmembrane proton-gradient and membrane potential established by this enzyme are utilized in the accumulation of catecholamines by the granules [3,4]. Treatment of purified bovine chromaffin-granule membranes with dichloromethane solubilizes an ATPase which is very similar, but not identical, to mitochondrial F_1-ATPase [5]. Since the solubilized chromaffin-granule enzyme is not inhibited by DCCD or alkyl tin, it is probably derived from a complex with a membrane-bound segment, containing a proton-conducting channel which is blocked by these inhibitors. This idea is supported by kinetic studies and by reconstitution experiments [2,6,7]; furthermore, treatment of chromaffin-granule membranes with [^{14}C]DCCD, followed by SDS electrophoretic separation of membrane components and autoradiography of the electrophoretograms, reveals the labelling of a low M_r polypeptide, with a greater electrophoretic mobility than the DCCD-binding protein of mitochondria [2]. We now report isolation of this protein, and determination of its amino acid content.

Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase (EC 3.6.1.3); DCCD, *N,N′*-dicyclohexyl carbodi-imide; Hepes, *N*-2-hydroxyethyl-*N′*-2-ethane sulphonic acid; SDS, sodium dodecyl sulphate; M_r, relative molecular mass

2. Materials and methods

Chromaffin granule membranes of high purity were prepared as in [5]. ATPase activity was measured at 30°C by following NADH oxidation in a coupled assay system containing 1.0 mM ATP, 10 mM $MgSO_4$, 0.5 mM phosphoenol pyruvate, 0.2 mM NADH, 5.5 units lactate dehydrogenase/ml and 4 units pyruvate kinase/ml, 50 mM KCl and 50 mM Hepes–KOH (pH 7.4). Since the NADH oxidase activity of the membranes was ~10 nmol . min^{-1} . mg $protein^{-1}$, no correction was necessary. Protein concentrations were estimated by the method in [8]. SDS–Polyacrylamide gel electrophoresis and autoradiography were performed as in [2].

Determination of N-terminal amino acids was attempted after treatment of the purified protein with dansyl chloride in SDS [9]. The dansylated protein was hydrolysed (6 M HCl, 20 h, 105°C), vacuum dried and redissolved in 50% aqueous pyridine. Dansyl amino acids were separated by chromatography on polyamide plates, the solvents being 1.5% formic acid in the first dimension, toluene–acetic acid (9:1) in the second dimension (run perpendicular to the first), and butyl acetate–methanol–acetic acid (30:20:1) for the third separation, run in the same direction as the second. Amino acid analysis was performed on ~10 nmol protein hydrolysed in 6 M HCl at 105°C for 40 h or 90 h. Amino acid separation was on a Locarte analyser, using a 3-buffer step elution system [10]. Cysteine was measured at cysteic acid after performic acid oxidation [11]. *N,N′*-Dicyclohexyl-[^{14}C]carbodi-imide (spec. act. 50 μCi/ml) was supplied by CEA (Gif-sur-Yvette).

3. Results and discussion

3.1. *Inhibition of chromaffin granule ATPase by DCCD*

Even high concentrations of DCCD (up to 150 nmol/mg protein) produce only partial inhibition of the ATPase [1,2], but it was noted that incorporation of label into a low M_r protein was greatly stimulated when ATP was included during the preincubations of the membranes with [^{14}C]DCCD. Fig.1 shows that ATP also increases the rate and extent of inactivation of ATPase by DCCD.

3.2. *Extraction of the DCCD-binding protein*

The DCCD-binding proteins from many sources have been purified after extraction into chloroform–methanol mixtures [12] or into butanol [13]. The protein from chromaffin-granules is extracted by chloroform–methanol (2:1) (though not butanol) but the high lipid content of the chromaffin-granule membrane hinders subsequent isolation of the protein from the chloroform–methanol extract. This problem was overcome by first extracting lipids from the membrane by treatment with acetone–ethanol (1:1, v/v) then extracting the DCCD-binding protein from

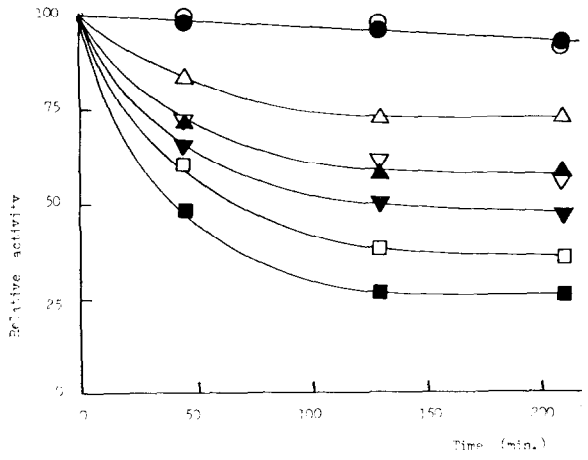

Fig.1. Effect of ATP on inhibition of chromaffin-granule membrane ATPase activity by DCCD. Chromaffin-granule membranes (1.0 mg protein/ml, in 0.1 M Hepes–NaOH (pH 7.0)) were incubated at 25°C with various concentrations of DCCD, and samples removed at intervals for assay. The initial ATPase activity was 420 nmol . min^{-1} . mg protein^{-1} at 30°C. Solid symbols, 10 mM ATP present during incubation; open symbols, no ATP. DCCD concentrations were: 0 (○,●), 10 (△,▲), 25 (▽,▼) and 100 (□,■) nmol/mg protein.

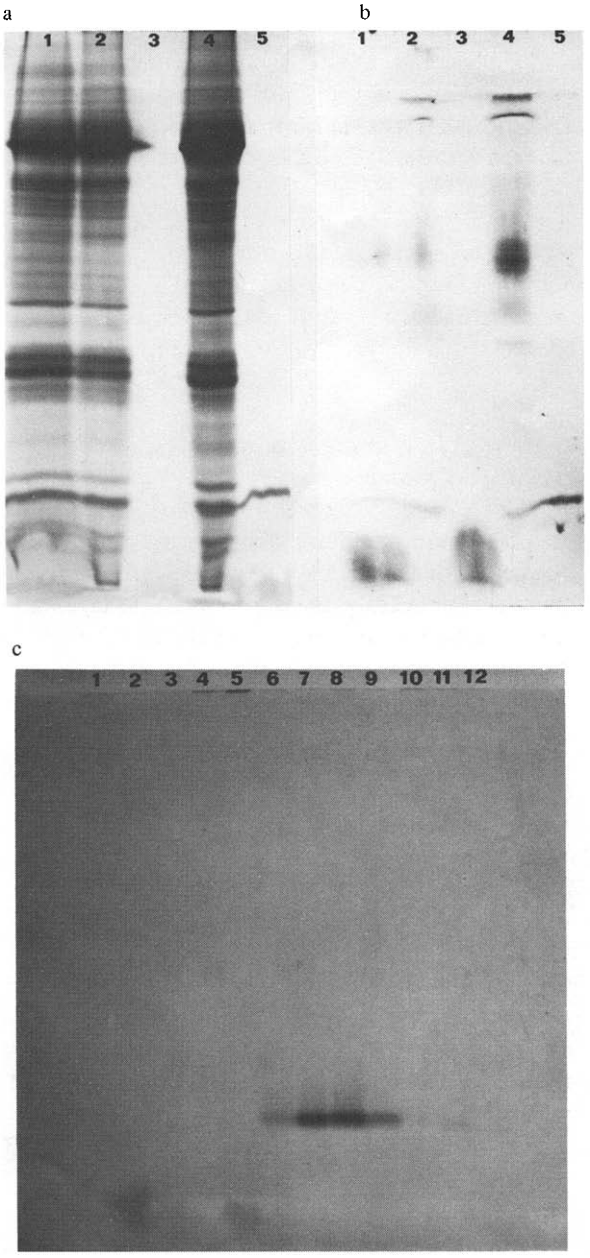

Fig.2. SDS–polyacrylamide gel electrophoresis. (a) Extracts of chromaffin-granule membranes which had been treated with [^{14}C]DCCD; gel stained for protein with Kenacid blue: (1) labelled membranes; (2) residue from acetone–ethanol extraction; (3) supernatant from acetone–ethanol extraction; (4) residue from chloroform–methanol extraction; (5) supernatant from chloroform–methanol extraction. (b) Autoradiograph of a gel similar to that in (a). (c) Successive fractions of elute from the Sephadex LH-20 column used to purify the DCCD-binding protein. Gel stained with Kenacid blue.

the insoluble residue into chloroform–methanol. A polyacrylamide gel of fractions from each stage of this double solvent extraction (fig.2a) and the autoradiograph of a similar gel (fig.2b) show that the DCCD-binding protein is completely insoluble in acetone–ethanol, and is extracted into chloroform–methanol. The protein so extracted appears to be almost pure by the criterion of polyacrylamide gel electrophoresis (fig.2a (5)), although it contains some low M_r impurities which are probably lipids. Further purification was achieved by chromatography on Sephadex LH-20, yielding the pure material shown in fig.2c.

3.3. *Purification of the chromaffin-granule DCCD-binding protein*

The following procedure was adopted for purification of the protein. When performed on membranes preincubated with [^{14}C]DCCD, it yielded labelled protein; alternatively, the protein could be labelled after isolation, by incubation with [^{14}C]DCCD in chloroform–methanol solution.

Pure chromaffin-granule membranes (200 mg protein, suspended in 24 ml 10 mM Hepes–NaOH (pH 7.0)) were treated with 600 ml acetone–ethanol (1:1). After 5 min at 25°C the mixture was centrifuged (10 min, 15 000 rev./min, 4°C, in a Beckman JA20 rotor, g_{av} = 18 000), the supernatant discarded, and the pellet resuspended in 24 ml water. Chloroform–methanol (2:1) 600 ml, was then added, and the mixture stirred slowly for 5 h at 4°C, then centrifuged as before. The pellet was discarded, and the supernatant rotary-evaporated almost to dryness. The extracted material was redissolved in 10 ml chloroform–methanol (2:1 containing 4% (v/v) water) and applied to the top of a 30 cm × 5.1 cm^2 column of Sephadex LH-20 pre-equilibrated with chloroform. The protein was eluted at room temperature with a 500 ml linear gradient of chloroform to chloroform–methanol (1:1), and 35 ml fractions collected. Individual fractions were concentrated by rotary evaporation and the protein contents examined by SDS–polyacrylamide gel electrophoresis. Those fractions containing DCCD-binding protein of the highest purity were pooled, rotary-evaporated to dryness, redissolved in 2 ml chloroform–methanol (2:1) and washed 3 times with 0.5 ml water. The purified protein was readily soluble in chloroform–methanol (2:1, containing 4% water), and was stored as a solution at 4°C. The entire procedure yielded 30–50 nmol pure protein.

3.4. *N-Terminal amino acid determination*

Attempts to determine the identity of the N-terminus of the protein have been unsuccessful, the only dansyl amino acids in hydrolysates of the dansyl chloride-treated protein being N^6-dansyl lysine and *O*-dansyl tyrosine. This suggests that the amino-terminus is blocked, a result confirmed by the failure of the Edman degradation procedure to yield phenylthiohydantoin derivatives of amino acids (W. Sebald, personal communication). The amino-termini of DCCD-binding proteins from *Saccharomyces cerevisiae* mitochondria and spinach chloroplasts are also blocked; in each case the N-terminus is formylmethionine, a result of the intra-organellar synthesis of the protein [14]. However, this reason would not apply to the protein from chromaffin-granules, and it is noteworthy that the DCCD-binding proteins of beef heart, mouse liver and *Neurospora crassa* mitochondria, all of which are probably cytoplasmically synthesized, have unblocked N-termini [14,15].

Table 1
Amino acid content of DCCD-binding protein from chromaffin-granule membranes

Amino acid	Analysis 1	Analysis 2	Integral figure
Asp	3.25	2.99	3
Thr	2.22	2.20	2
Ser	9.69	9.56	10
Glu	4.44	3.85	4
Pro	3.32	2.98	3
Gly	7.85	8.32	8
Ala	9.24	9.14	9
Cys	0.11	–	0
Val	4.29	6.20	6
Met	3.17	2.19	3
Ile	3.45	5.03	5
Leu	5.81	7.17	7
Tyr	1.16	1.07	1
Phe	2.81	3.15	3
His	0.09	0.06	0
Lys	2.00	2.00	2
Arg	2.13	2.17	2
Try	–	–	–

Each analysis is the average of results derived from 40 h and 90 h hydrolyses, with the following exceptions: threonine and serine were extrapolated to zero hydrolysis time, 90 h hydrolysis values were used for leucine, isoleucine and valine, and the 40 h hydrolysis value for methionine. The methionine value is in agreement with that for methionine sulphone in the 40 h hydrolysate of performic acid-oxidized protein; this analysis was also used for cysteine

3.5. *Amino acid analysis*

The amino acid content of the protein, determined from analysis of total acid hydrolysates and expressed in mol/2 mol lysine, is shown in table 1. The integral value of 2 for lysine was chosen as this gave a M_r-value in agreement with that suggested by the mobility of the protein on SDS–polyacrylamide gels; it also results in approximately integral values for the other amino acids (table 1). The total number of amino acids is 68, compared to 75 in the beef mitochondrial protein [14]: this may account for the higher electrophoretic mobility of the protein from chromaffin granules [2]. The proportion of hydrophilic amino acids is somewhat higher than that found in other DCCD-binding proteins [14], although the content of asparagine and glutamine is unknown, and the 10 serine residues make a major contribution. This apparently high content of serine could derive from contamination of the protein with phosphatidyl serine, although no dansyl serine was found in hydrolysates of dansyl chloride-treated protein.

4. Conclusion

The protein purified from chromaffin granules is very similar in its properties to the low M_r, DCCD-binding subunits of proton-translocating ATPase from the membranes of mitochondria, chloroplasts and bacteria. Nonetheless, the differences in amino acid content and electrophoretic mobility between the DCCD-binding proteins of chromaffin granules and mitochondria are great enough to exclude the possibility that the former is of mitochondrial origin. A number of lines of evidence suggest that it is a subunit of the proton-translocating ATPase complex of chromaffin granule membranes:

(i) Increasing inhibition of ATPase activity by DCCD correlates with increasing incorporation of label from [^{14}C]DCCD into the protein; both inhibition and labelling are potentiated by ATP;

(ii) Treatment of resealed granule 'ghosts' with low concentrations of DCCD appears to decrease the proton-permeability of the membrane, suggesting that the inhibitor blocks a proton channel [2];

(iii) The DCCD-binding protein co-elutes with ATPase activity, when chromaffin granule membrane components are solubilized with a non-denaturing detergent, and separated by exclusion chromatography (unpublished).

It therefore appears that the structural similarity found for the F_1 portions of chromaffin-granule and mitochondrial ATPases [5] may extend to the membrane segments of these enzymes.

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References

[1] Bashford, C. L., Casey, R. P., Radda, G. K. and Ritchie, G. A. (1976) Neuroscience 1, 399–412.
[2] Apps, D. K., Pryde, J. G., Sutton, R. and Phillips, J. H. (1980) Biochem. J. 190, 273–282.
[3] Knoth, J., Handloser, K. and Njus, D. (1980) Biochemistry 19, 2938–2942.
[4] Phillips, J. H. and Apps, D. K. (1980) Biochem. J. 192, 273–278.
[5] Apps, D. K. and Schatz, G. (1979) Eur. J. Biochem. 100, 411–419.
[6] Buckland, R. M., Radda, G. K. and Wakefield, L. M. (1979) FEBS Lett. 103, 323–327.
[7] Giraudat, J., Roisin, M.-P. and Henry, J.-P. (1980) Biochemistry 19, 4499–4505.
[8] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[9] Gray, W. R. (1972) Methods Enzymol. 25, 121–138.
[10] Spackman, D. H. (1963) Fed. Proc. Am. Soc. Exp. Biol. 22, 244.
[11] Hirs, C. H. W. (1967) Methods Enzymol. 11, 197–199.
[12] Fillingame, R. H. (1976) J. Biol. Chem. 251, 6630–6637.
[13] Sigrist-Nelson, K. and Azzi, A. (1979) Biochem. J. 177, 687–692.
[14] Sebald, W., Hoppe, J. and Wachter, E. (1979) in: Functional and Molecular Aspects of Biomembrane Transport (Klingenberg, E. M. et al. eds) pp. 63–74, Elsevier/North-Holland, Amsterdam, New York.
[15] Aroskar, V. A. and Avadhani, N. G. (1979) Biochem. Biophys. Res. Commun. 91, 17–23.