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Isolation and Characterization of Acetylcholine Receptor Membrane-associated (Nonreceptor ν_2-Protein) and Soluble Electrocyte Creatine Kinases*

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Creatine kinase has been identified as a most prominent component of *Torpedo* electric organ and a minority constituent of the acetylcholine receptor (AChR) membranes obtained therefrom. Purification by low temperature ethanol extraction, precipitation of the Mg^{2+}-enzyme complex, and mercurial-agarose chromatography yield preparations of soluble kinase with specific activities >550 units/mg protein. Retention times in ion-exchange high performance liquid chromatography, electrophoretic behavior, immunochemical properties, tryptic mapping, and amino acid composition enable the comparison of creatine kinase isoenzymes. The denatured subunits of the predominant species have pI values of 6.3–6.8 and M_r = 40,000–42,000 characteristic of the so-called ν_2 proteins and show cross-reactivity with antibodies against the BB ("brain" type) creatine kinase. The MM ("muscle" type) antigens could be detected in the total electrocyte, but not in the AChR membranes; they have a slightly lower molecular weight and higher pI. The *in situ* membrane association of the BB isoenzyme is confirmed by immunocytochemistry.

The apparent K_m values for the substrate creatine phosphate are 2.2 mM for the AChR membrane-associated enzyme and 2.5 mM for the muscle form. The apparent K_m values for Mg^{2+}-ADP are 0.54 and 0.22 mM, respectively. Thus, a 2-fold higher affinity in the binding of ADP to the binary enzyme-creatine-P complex results from membrane association.

The electric tissue of fish is a mesodermal derivative. During course of development it passes through a period of differentiation in which many of the characteristics of muscle tissue are manifested in the electroblast (see Ref. 1 and references therein). Embryonic tissue explants from *Torpedo* recapitulate these properties; the electric tissue primordia possess spontaneously contracting myotubes, precursors of the mature electrocyte (2). The mature electrocyte (electroplax) appears to be a highly differentiated cell, almost monothematically specialized in the reception and processing of cholinergic stimuli. Optimization of this function within the physiologically relevant time scale of nerve transmission, and especially under conditions of repeated electrical discharge, would certainly be attained if a reliable mechanism for energy production existed. Furthermore, in view of the above mentioned embryological kindred between the electroplax and the skeletal muscle tissue, it is conceivable that evolution has conserved at least in part the molecular machinery which operates in muscle.

Creatine kinase (adenosine 5′-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2.) first described by Lohmann (3) is one of the phosphokinases that catalyze phosphoryl group transfer to a guanidine group. Specifically, it catalyzes the reaction:

$$MgADP^- + \text{phosphocreatine} + H^+ \longleftrightarrow MgATP^{2-} + \text{creatine} \quad (1)$$

Creatine kinase is of almost universal occurrence in skeletal and cardiac muscle, fish tissues being no exception. In trout muscle, a M_r = 42,000 has been reported for the subunits of this dimeric enzyme (4). In other species, the nonidentical nature of the two subunits has been documented, apparent M_r = 40,300 and 42,100 being found for each chain (5).

Several proteins in this molecular weight range, until recently named collectively the 43K protein (6, 7), the ν-peptide (8), the ν-doublet (9), or the 43,000-dalton proteins (10), together form a predominant component of the electrocyte and purified nicotinic AChR[1] membranes. It is becoming increasingly clear that these proteins probably bear no relation to one another aside from their very similar apparent mass (M_r = 40,000–43,000). However, since identification of all these proteins is neither totally unambiguous nor complete up to the present time, it is considered convenient to continue temporarily with the collective nomenclature and the ν_1, ν_2, ν_3 subdivision (10) in exclusive allusion to their respective *iso-*

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[1] The abbreviations used are: AChR, acetylcholine receptor; 2-EtSH, β-mercaptoethanol; CK, creatine kinase (adenosine 5′-triphosphate:creatine *N*-phosphotransferase, EC 2.7.3.2); Li-S, lithium di-iodosalicylate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; pCMB, p-hydroxymercuribenzoate; FITC, fluorescein isothiocyanate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 4-morpholinepropanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; G6P-DH, glucose-6-phosphate dehydrogenase.

electric point ranges. It is stressed that no structural or functional relationship between these proteins is implied.

A series of spectroscopic studies revealed that extraction of the so-called ν-proteins gives rise to enhanced rotational motion of the AChR (11–13) and to alterations in the receptor packing habit (9). In the latter study, it was proposed that the extracted proteins played a role in processes like synapse formation during ontogenesis, receptor clustering, and stabilization of the adult synapse (9). This series of studies gave rise to the view that the so-called ν-proteins participate in the anchorage of the AChR in the membrane (see review in Ref. 14). Subsequently, a more dynamic role of these proteins was postulated, based on the observation that their presence or absence affected the stabilization of AChR dimers or higher oligomeric species, respectively, the thiol groups of the receptor being involved in this equilibrium (15, 16). A recent review deals with the structure and putative functions of the AChR membrane-associated ν-proteins (17).

Most recently, the function of one of the so-called ν-proteins was uncovered: using immunological and biochemical criteria, we identified creatine kinase activity with a ν-protein of intermediate pI value (6.5–6.8) (18, 19). Gysin *et al.* (20) have confirmed our results, basing their adscription on the enrichment of enzyme activity upon chromatofocusing of electric tissue cytosol extracts. We had also reported that only the BB type of antigen is associated with the AChR membranes (18), whereas both MM and BB isoenzymes are present in *Torpedo* skeletal muscle, brain, and adult and 70–99-mm embryonic electric tissue (19). In the present work, we show the different localization of the two isoenzymes in the intact *Torpedo* electrocyte, and the purification, enzyme kinetics, and some physicochemical properties of the creatine kinase from electric tissue, AChR membranes obtained therefrom, and muscle.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Localization of Creatine Kinase Isoenzymes in Situ by Immunofluorescence—Indirect immunofluorescence staining with anti-BB creatine kinase antibody of paraffin-embedded electrocytes from *Torpedo marmorata* leads to bright fluorescence of the electrocyte surface membranes, especially the ventral, innervated face of the electrocyte where the acetylcholine receptor (17) is located (Fig. 2*b*). Staining with anti-BB creatine kinase antibody at the noninnervated dorsal membranes was weaker and somewhat more diffusely distributed over the entire area of the dorsal face (Fig. 2*b*). Incubation with anti-MM creatine kinase antibodies, on the contrary, led to an overall staining of the whole electrocyte except the nuclei (Fig. 2*d*). Incubation with control antibody did not reveal unspecific fluorescence (Fig. 2*f*). Very similar results were obtained with *unfixed* (cryostat) sections (not shown).

Ethanol Extraction, Precipitation of the Mg^{2+}-Enzyme Complex, and Chromatographic Purification of Creatine Kinase from Torpedo Electric Organ and Dorsal Muscle—Attempts were made to apply the procedures normally used for the purification of creatine kinase from muscle tissues (see review in Ref. 38). One of the procedures adopted was based first on the ethanol extraction of the crude enzyme and low temperature precipitation of the Mg^{2+}-enzyme complex and second on the application of specific chromatographic procedures. In addition to Blue-Sepharose affinity chromatography (Fig. 3, see Miniprint Supplement), the ability of mercurial-agarose to retain creatine kinase has been reported (24). The method was first applied to partially purified ethanol extracts from *Torpedo* dorsal muscle (Fig. 4). Elution of the fraction having enriched creatine kinase activity was accomplished with 1 mM β-mercaptoethanol. Electrophoretically pure creatine kinase was also desorbed with higher β-mercaptoethanol concentrations, but at a much lower yield and with lower specific activities (Fig. 4). The SDS-PAGE pattern of the fractions is shown in Fig. 4, *inset*. The similarities between the polypeptide patterns of the starting material (homogenates) of *T. marmorata* dorsal muscle and of electric tissue are apparent, as is the purity of the fractions resulting from the pCMB-Sepharose chromatography in both cases (Fig. 4). The steps followed with *Torpedo* electric tissue and the quantitative

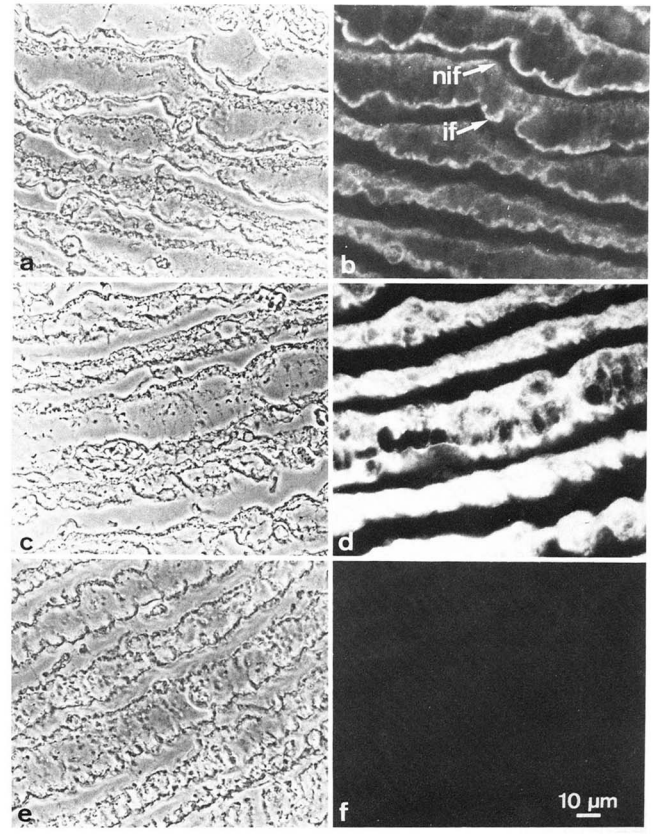

FIG. 2. **Immunofluorescence localization of creatine kinase isoenzymes in *in situ*.** Sections of paraffin-embedded electric organ from *T. marmorata* stained by indirect immunofluorescence for BB creatine kinase (*a* and *b*) and for MM creatine kinase antibodies (*c* and *d*), respectively. Controls incubated with the same concentration of preimmune IgG (*e* and *f*) followed by 5-isothiocyanatofluorescein-conjugated goat anti-rabbit IgG as second antibody. Phase contrast (*a, c,* and *e*) and immunofluorescence (*b, d,* and *f*) pictures. Note the bright fluorescence after staining for BB creatine kinase at the membranes, especially the ventral innervated face of the electrocyte stacks (*b*) and the overall staining after incubation with anti-MM creatine kinase antibody sparing the nuclei (*d*); *if*, innervated face; *nif*, noninnervated face. Given the lack of information on the homology between *T.* and chicken creatine kinase, and their corresponding cross-reactivities with rabbit anti-creatine kinase antibodies, one cannot infer the amount of each isoenzyme present from the relative intensity of the immunofluorescence staining.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1, 3, 5, and 12, and Equations 2–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-0306, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

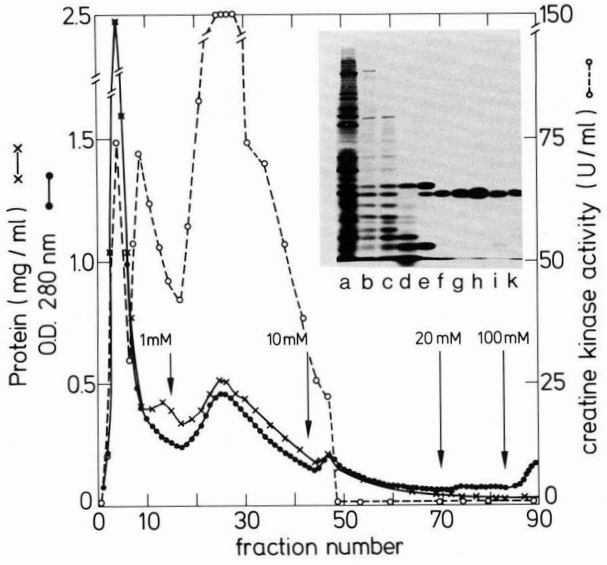

FIG. 4. **Purification of creatine kinase from dorsal muscle and electric tissue of *T. marmorata*.** Samples containing about 20 mg of protein were loaded onto the pCMB-Sepharose column (1.8 ml) equilibrated with 50 mM Tris-HCl buffer, pH 7.4. After elution of nonadsorbed protein, pulses of increasing β-mercaptoethanol concentration were applied (*arrows*). Highest specific activity (fraction 26) eluted with 1 mM β-mercaptoethanol. *Inset,* the corresponding polypeptide patterns in SDS-PAGE: the starting material, the total homogenates of electric tissue, and dorsal muscle of *T. marmorata* are shown in *a* and *b*, respectively; *c*, supernatant of the first low-speed centrifugation of the muscle homogenate *b*. *d*, product of the successive ethanol extractions from this homogenate. *Tracks e–h* correspond to fractions 4, 13, 25, and 47 of the pCMB column. *Track k* shows the corresponding highest specific activity fraction from electric tissue, eluting with 100 mM β-mercaptoethanol (see Table I). Rabbit muscle creatine kinase is shown in *track i* for comparison. Between 5 and 15 μg of protein were loaded in each lane. The two-dimensional SDS-PAGE pattern of the purified enzymes is shown in Fig. 8.

aspects of the purification procedure are given in detail in Table I.

Subsequent purification of creatine kinase from the electric tissue of *Discopyge tschudii* (Table II) circumvented the $(NH_4)_2SO_4$ precipitation initially used with *T. marmorata* tissue (Table I), and the yield increased. Recovery from dialysis was also improved by including 50% glycerol in the medium. A 10-fold higher yield was also accomplished in the chromatographic step by overnight, batchwise elution of creatine kinase. Specific activities in the order of 400–500 units/mg (pooled fractions) were obtained from the South American *Torpedinidae* (Table II). Highest specific activity fractions had 550–600 units/mg protein.

Attempts to further purify the resulting material by a subsequent chromatography in thiol-agarose with 2,2′-dithiobispyridyl or dithiothreitol elution were not successful (Table II). Gel filtration through Sephadex G-150 in the presence of 5 mM β-mercaptoethanol yielded an apparent M_r ~ 80,000 for the purified soluble creatine kinase. The purity of the enzyme preparations was judged to be more than 95% by densitometric scanning and computer analysis (see Ref. 18) of overloaded SDS gels under nonreducing and reducing conditions (Figs. 4 and 5). In the latter case, a single band in the M_r = 40,000–42,000 region was observed. Two-dimensional SDS-PAGE resolved this band into its subunit components (see below).

Creatine Kinase Activity in AChR Membranes and Nonreceptor Polypeptide Extracts—AChR-rich membrane fragments, prepared under conditions not necessarily optimal for the preservation of creatine kinase activity, nevertheless display low but measurable enzyme levels. Control experiments were conducted to ensure that the NADPH production in the coupled enzyme assay (see "Methods") was not due to glutathione reductase (EC 1.6.4.2) or myokinase (EC 2.7.4.3) activities, which are present in the total electric organ but not in the AChR membranes. The membrane-bound creatine kinase activity diminished upon submitting the AChR membranes to the alkaline stripping procedure (7) or the lithium diiodosalicylate extraction (21). Concomitantly, increased creatine kinase activity was found in the resulting soluble extracts, in parallel with their relative enrichment in proteins of M_r = 40,000–43,000 (Table IV).

The mode of association of creatine kinase with the AChR membranes was also studied by following the enzymatic activity associated with and released from the membranes by high performance liquid chromatography. The system employed an anion-exchange resin (Fig. 1) to which the more basic (MM) isoenzyme was not adsorbed and which retained the more acidic BB isoenzyme (see Fig. 5). Fluorescence detection of NADPH revealed two main peaks of creatine kinase activity associated wtih *native* AChR membrane extracts, eluting at 6.7–8.0 min (~2%) and between 18 and 21 min (~98%) (Fig. 6). A third minor peak (<1% area) eluted at about 10 min. Chromatography of skeletal muscle and brain extracts and creatine kinase purified from these sources was used to identify the creatine kinase isoenzyme activity corresponding to each peak (not shown). The fast eluting component corresponded to the MM isoenzyme; the BB

TABLE I

Purification of creatine kinase from T. marmorata electric organ by sequential elution from pCMB-agarose

Step	Volume	Activity	Total activity	Protein concentration	Specific activity	Recovery from previous step
	ml	*units/ml*	*units*	*mg/ml*	*units/mg*	%
1. Extraction[a]	310	58	17,980	1.0	58	100
2. 50% EtOH supernatant	560	27	15,120	0.15	180	84
3. 70% EtOH extraction, pellet	110	155	17,050	0.5	310	100
4. 70% $SO_4(NH_4)_2$ precipitation	7.5	432	3,240	1.4	309	19
5. Dialysate	7.5	178	1,335	1.2	148	41
6. pCMB-agarose						
I. Elution, 100 mM β-mercaptoethanol	0.8	58	46	0.42	138	3.5
II. Elution, 100 mM β-mercaptoethanol (overnight)	2	16	32	0.053	302	2.4
III. Elution, 2 mM pCMB (dialysate, Tris/glycerol)	2	0.6	1.2	0.01	60	0.1

[a] Starting material was 5 g of wet tissue, specific activity 600 units/g.

TABLE II

Comparison of pCMB-agarose and thiol-agarose chromatographic procedures for the purification of creatine kinase from total electric tissue of D. tschudii (Torpedinidae)

Step	Volume	Activity	Total activity	Protein	Specific activity	Recovery from previous step
	ml	*units/ml*	*units*	*mg/ml*	*units/mg*	%
1. Extraction[a]	22	102	2,244	1.1	93	100
2. 50% EtOH supernatant	45	45	2,025	0.42	107	90
3. 70% EtOH extraction, pellet	2.3	637	1,466	4.9	130	72
4. Dialysate (β-mercaptoethanol)	2.9	490	1,421	4.1	120	97
5a. pCMB-agarose[b]						
I. 2 mM pCMB elution	5.5	8.8	48.4	0.052	169	6.8
II. 2 mM pCMB elution	7	1.8	12.6	0.022	82	1.8
III. 20 mM dithiothreitol elution	1.6	180	288	0.44	409	41
5b. Thiol-agarose[b] (2,2'-dithiobis pyridyl) unbound	2.6	201	522	1.4	144	73.5
20 mM dithiothreitol eluate	3.6	8	29	0.158	51	4.1

[a] The starting material was 5.2 g of wet tissue, with a specific activity of 435 units/g.
[b] The column was loaded with 1.45 ml (710 units) of the material resulting from step 4 in each case.

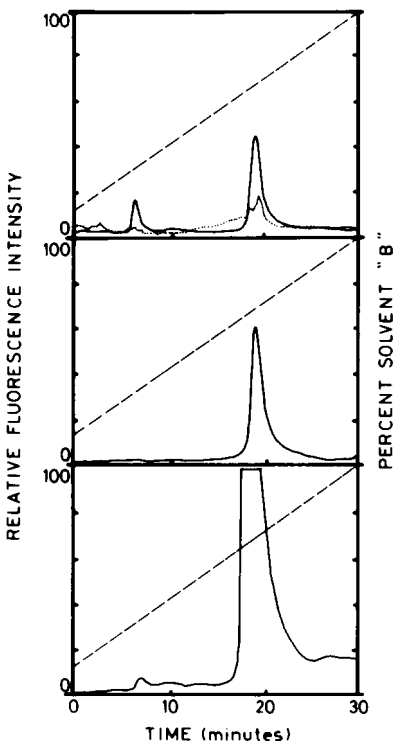

FIG. 6. **Identification of creatine kinase activity associated with AChR membranes by HPLC on Synchropak AX-300.** The simultaneous detection of protein profile and enzyme activity through fluorogenic-coupled enzyme reaction is given in Fig. 1 and Equations 2–4 in the text. *Top*, native AChR-rich membrane extracts. *Dotted line*, absorbance at 280 nm; *full line*, NADPH fluorescence (enzyme activity); *dashed line*, per cent solvent B. *Middle*, the same, after treating with low ionic strength buffer (20 mM Tris-HCl, pH 7.4) for 30 min. *Bottom*, supernatant of 1% Triton X-100 extract of AChR membranes. The actual fluorescence patterns of the creatine kinase activity eluted 3.48 min later (*i.e.* the delay involved in passing through the enzyme reactor). Buffer A, 20 mM Tris-HCl, pH 7.4; buffer B, 0.5 M NaCl in 0.02 M sodium phosphate buffer, pH 7.8. Membranes were solubilized in 0.5% Triton X-100 in buffer A.

needed concentrations of NaCl above 200 mM to be released from the HPLC column. The third minor component might correspond to the hybrid (MB) isoenzyme. SDS-PAGE experiments (see Fig. 7) and amino acid analyses (Table III) of the electric tissue creatine kinase, having the same R_F value as the membrane-associated creatine kinase, provided a reasonable explanation for the ability of the more basic MM type to pass unretained and the more acidic BB type to be retained by the anion-exchange resin.

Native AChR membranes of high specific activity in terms of receptor sites (3.5–4.5 nmol of [^{3}H]α-toxin/mg of protein) displayed, therefore, at least two isoenzyme activities. However, such membranes are purified in a relatively high ionic strength buffer (see "Methods"), and at least part of the creatine kinase activity might occur in the membranes merely as a contaminant. Indeed, a single washing of the membranes with low ionic strength buffer (20 mM Tris-HCl) for 30 min sufficed to release all the MM creatine kinase activity from the membranes; within the limits of detection, only BB type of activity associated with the membranes (Fig. 6, *middle*) remained. MM creatine kinase activity was recovered in the low-salt wash. Finally, when this treatment failed to release any further activity, extraction with 1% Triton X-100 was performed on the washed AChR membranes. The activity of the detergent-soluble extract of a low-salt washed membranes consisted of more than 99% BB isoenzyme (Fig. 6, *bottom*). No significant differences were observed in the total or specific activity of the enzyme associated with the AChR membranes upon addition of 1–2% sodium cholate. It was determined that this and other detergents (CHAPS, sodium salts of cholate and deoxycholate) solubilized more than 80% of the particulate creatine kinase activity. All these experiments reinforce the view the BB creatine kinase does indeed represent the membrane-associated form of the enzyme, in agreement with its immunofluorescence localization (Fig. 2).

Purification of the Membrane-associated Creatine Kinase—The pCMB-agarose column chromatography procedure previously applied to the AChR membranes (18) was simplified and used in a mini-batch fashion with much higher recoveries (Table IV). Membranes were subsequently submitted to repeated NaOH extractions to solubilize the particulate creatine kinase. In spite of the purification achieved, specific activities were low. This may result from the extraction procedures used; exposure of soluble creatine kinase purified from total electrocyte to NaOH resulted in more than 50% loss of activity. Treatment with 10 mM *N*-ethylmaleimide resulted in complete inactivation of both soluble and membrane-associated enzymes. This treatment inhibits the extractability of the membrane-bound creatine kinase (15, 18).

When the membrane-associated enzyme purified by pCMB-agarose was passed through the HPLC column, the BB type of elution profile was also observed. Analysis of the eluted

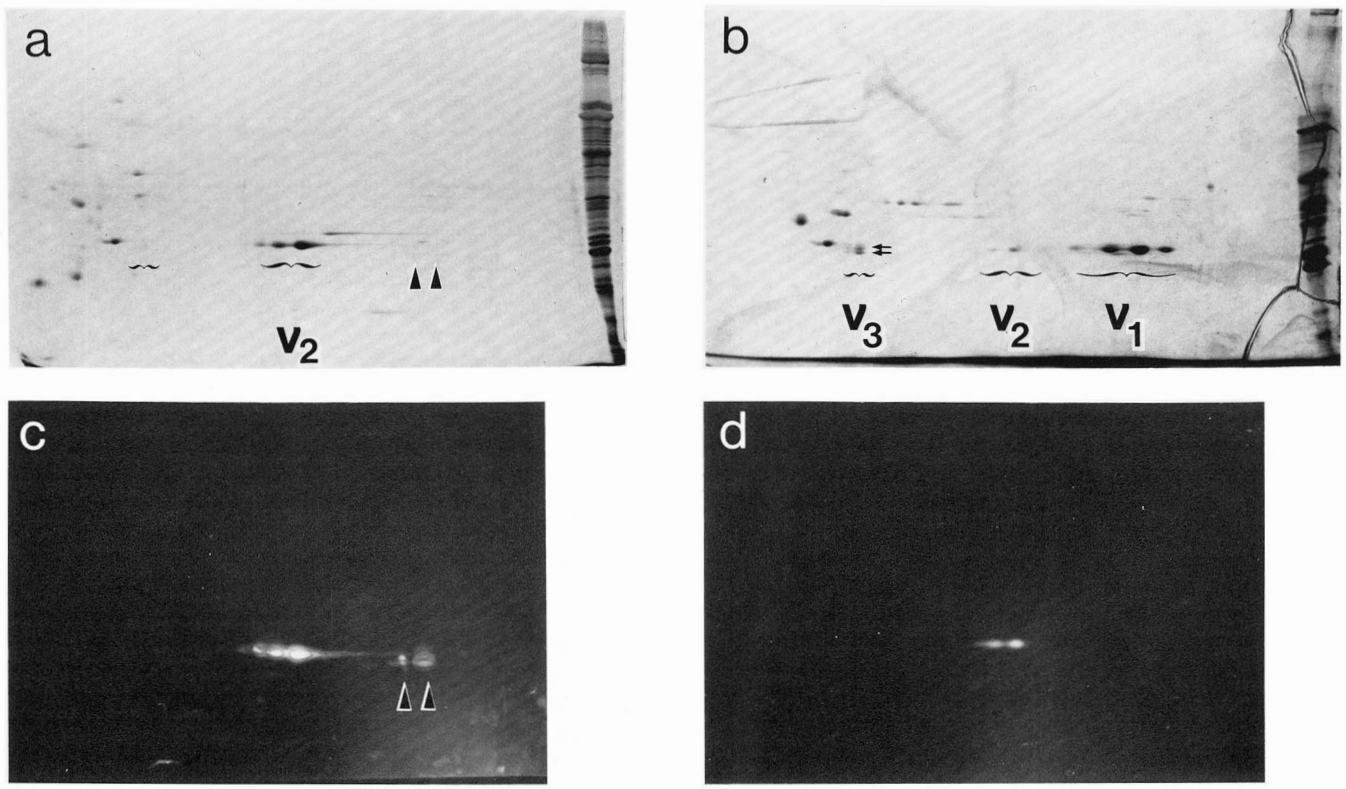

FIG. 7. **Microheterogeneity of the total electrocyte BB and MM creatine kinase and of the AChR membrane-associated BB type of creatine kinase.** *a*, SDS two-dimensional gel of total electrocyte extract. The majority ν_2 isoelectric species is indicated. Its corresponding nitrocellulose paper replica after immunoblotting with anti (chicken)-BB and -MM creatine kinase rabbit antibodies and fluorescein-labeled goat anti-rabbit IgG is shown in *c*. *Arrowheads* indicate the position of anti-MM reaction ahead of the so-called ν_1-proteins. *b*, two-dimensional SDS-PAGE of alkaline extract from AChR membranes. The three isoelectric groups of ν-proteins (ν_1, ν_2, and ν_3) are indicated. The *double arrow* points to a ν_3 doublet. *d*, nitrocellulose blot of *b*; rabbit antibodies against chicken BB creatine kinase followed with fluorescein-labeled goat anti-rabbit IgG. No immune reaction is observed with anti-MM creatine kinase on the AChR membranes.

TABLE III

Amino acid composition of creatine kinases purified from T. marmorata electric tissue and from Torpedo dorsal muscle

Average of two determinations.

Amino acid	mol/100 mol of amino acid	
	Electric tissue creatine kinase	Dorsal muscle creatine kinase
Asx	12.2	10.7
Thr	5.1	5.0
Ser	6.2	5.4
Glx	11.9	10.9
Pro	4.9	5.8
Gly	10.4	9.5
Ala	6.4	5.5
Val	5.9	6.4
Met	2.5	3.0
Ile	3.4	3.8
Leu	9.3	9.1
Tyr	2.1	2.6
Phe	4.0	4.7
Lys	7.8	7.9
His	3.2	4.0
Arg	4.8	5.9

material by two-dimensional PAGE showed two major and a minor polypeptide of $M_r \sim 40{,}000$–$42{,}000$ and a mean pI value of 6.5 (6.3–6.8) (Fig. 8). *Torpedo* dorsal muscle-purified creatine kinase showed a clearly different 2-D pattern (Fig. 8). The denatured membrane-derived enzyme reacted with antibodies against (chicken) BB type of creatine kinase, but not against MM antigens, as was the case with the membrane-bound form (Fig. 7).

Immunochemical Differences between Total Electrocyte and AChR Membrane-associated Creatine Kinase Species—The major polypeptide constituents of total *Torpedo* electric tissue and alkaline extracts of the AChR membranes correspond to the proteins of $M_r = 40{,}000$–$43{,}000$ spanning the 5.8–8.5 pI range (Fig. 7, *a* and *b*). But whereas those proteins of intermediate pI (6.3–6.8) constitute the predominant components of the total electrocyte proteins, the more basic (pI 7.0–8.5) species predominate in the membrane extracts (Fig. 7*b*).

Immune replicas doubly labeled with anti-BB and -MM creatine kinase antibodies revealed that both BB and MM creatine kinase isoenzymes are present in the electrocyte (Fig. 7*c*). The predominant BB creatine kinase antigens corresponded to at least three polypeptides of intermediate pI value (6.3–6.8), whereas the MM creatine kinase antigens ran in a more basic pI range (7.3–7.8) ahead of the prominent so-called ν_1-proteins (Fig.7*c*). Alkaline extracts of AChR membranes displayed reactivity at the level of two spots (pI 6.5–6.7), (Fig. 7*d*). Thus, although the polypeptide pattern of the $M_r = 40{,}000$–$43{,}000$ polypeptides in AChR membranes is rather complex, the immunoblot of the membrane-associated creatine kinase appeared to be simpler than that of the total electrocyte; two and three cross-reacting polypeptides were observed in each case, respectively.

TABLE IV

Mini-batch purification of creatine kinase activity from AChR membranes of Torpedinidae

	Total activity	Activity	Protein concentration	Specific activity	Recovery from previous step
	munits	*units/ml*	*mg/ml*	*units/mg*	%
Sequential NaOH extractions of creatine kinase activity from *T. marmorata* AChR membranes					
AChR membranes[a]	140	1.4	4.2	0.33	100
Combined NaOH supernatants[b]	120	0.171	0.085	2.01	86
Dialysate of NaOH supernatant	91	0.516	0.28	1.84	76
Sequential NaOH extractions of creatine kinase activity from *D. tschudii* AChR membranes					
AChR membranes	250	0.25	1.25	0.2	100
Combined NaOH supernatants[c]	108	0.18	0.05	3.6	43
pCMB-agarose purification of alkaline extracts of *T. marmorata* AChR membranes					
AChR membranes[c]	143	1.43	4.2	0.34	100
Combined NaOH supernatants[c]	114	0.19	0.15	1.27	80
pCMB-agarose (mini-batch)					
20 mM dithiothreitol eluate	57.8	0.29	0.08	3.62	51

[a] The AChR membranes from *T. marmorata* used had 4.2 mg protein/ml and a specific activity of 3.4 nmol of [^{3}H]α-bungarotoxin/mg protein.

[b] The sequential alkaline extractions were carried out in a Reacti-vial under gentle stirring with 10 mM NaOH, 5.5 mM NaOH, and 1 mM NaOH for 30 min each at 4 °C in buffer F (see "Material and Methods").

[c] The *D. tschudii* AChR membranes had a specific activity of 1.8 nmol α-toxin/mg protein. Two additional NaOH extractions were carried out (6-fold NaOH extraction).

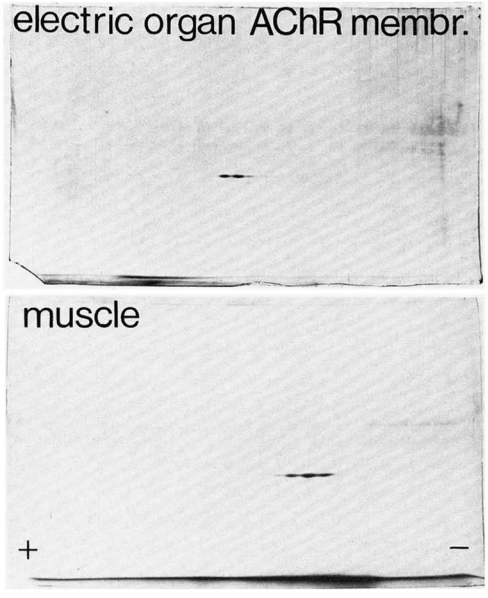

FIG. 8. **Two-dimensional SDS-PAGE patterns of purified creatine kinase.** *Top,* membrane-associated creatine kinase from alkaline extract of AChR membranes (pI 6.3–6.7) and *bottom,* soluble creatine kinase from *Torpedo* dorsal muscle (pI 7.3–7.6) purified by pCMB-agarose as in Table IV and I, respectively.

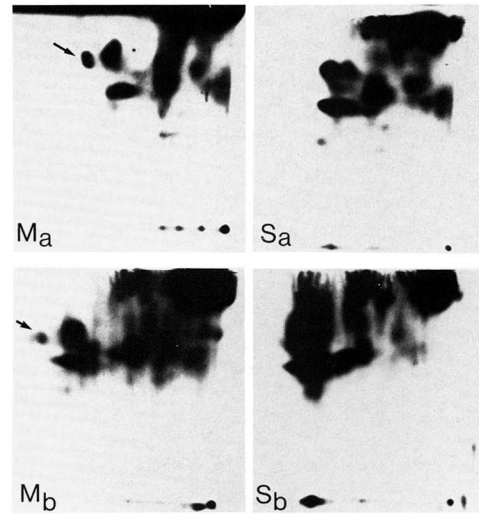

FIG. 9. **Tryptic maps of soluble- and membrane-associated creatine kinase.** Individual subunits of the two forms of creatine kinase obtained from two-dimensional SDS gels of total electric tissue and AChR membranes, respectively, were radioiodinated, trypsin-digested, and processed as given in "Methods." The digests of the more *acidic* subunits of membrane-associated (M_a) and soluble (S_a) creatine kinase are shown on the *left* and *right top panels,* respectively. The corresponding *basic* subunits of membrane (M_b) and soluble (S_b) creatine kinase are shown on the *bottom panels.* Notice the presence of a conspicuous tryptic peptide in the membrane-associated subunits (*arrows*), but not in the cytosol enzyme subunits. Membrane and soluble acidic subunits (*top panel*) were run in parallel; the same applies to the basic subunits.

Differences between Soluble and Membrane-associated Creatine Kinase Isoenzymes and Their Individual Subunits—Soluble and AChR membrane-associated creatine kinase isoenzymes, as well as their individual subunits separated by 2-D SDS-PAGE, were radioiodinated, submitted to tryptic digestion, and further analyzed by mono- and bi-dimensional peptide mapping. Most of the tryptic peptides were common to the two forms of the enzyme, but some prominent fragments were present and absent in each of the species, respectively. This was more easily appreciated when the individual acidic and basic individual subunits of the enzyme were compared (Fig. 9).

Kinetics of the Soluble and Membrane-associated Creatine Kinase Isoenzymes and Creatine-P Concentration—Comparison of the rate of ATP production by the membrane-associated creatine kinase and by the soluble isoenzyme purified from *Torpedo* dorsal muscle showed the expected dependence on the concentration of the two substrates, creatine-P and Mg^{2+}-ADP (Fig. 10). Zero ordinate intercepts of the Lineweaver-Burk plot yielded apparent K_m values for the corresponding substrate, showing little dependence on the other substrate. The apparent K_m values for creatine-P derived from this type of plots (see below) are close to the concentrations of the substrates found in actual measurements: 1.0–2.0 μmol/g tissue for resting electric organ and 10–13 μmol/g in dorsal muscle. Secondary treatment of the reciprocals of V_{max} and [S] according to Florini and Vestling (42) (Fig. 11) yield the apparent equilibrium dissociation constants for the following

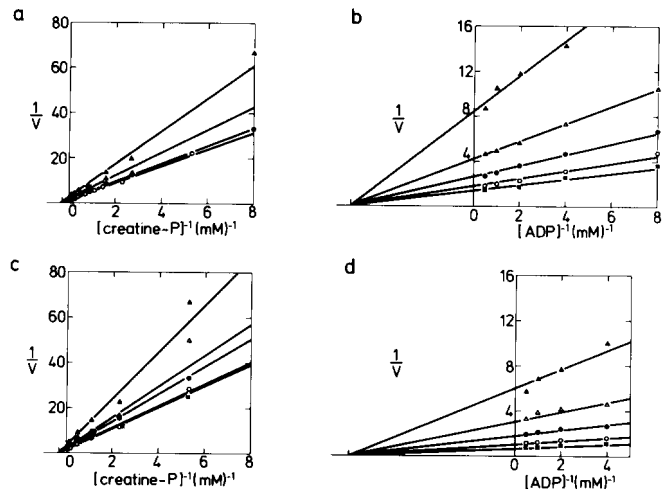

FIG. 10. **Lineweaver-Burk plots of AChR membrane-associated (*a* and *b*) and *Torpedo* dorsal muscle soluble (*c* and *d*) creatine kinase as a function of creatine phosphate (*a* and *c*) and ADP (*b* and *d*) concentrations.** The initial velocities were measured in the incubation mixture given under "Experimental Procedures." The absorbance changes yielding the velocity parameters are derived from measurements carried out at a wavelength of 334 nm and at 25 °C. The substrate concentrations are 0.25 mM (▲——▲), 0.5 mM (○——○), 1 mM (△——△), 2 mM (●——●), and 4 mM (*——*) ADP (*a*) or (*c*); 0.5 mM (△——△), 1 mM (▲——▲), 2 mM (●——●), and 10 mM (*——*) creatine-P (*b*) or (*d*).

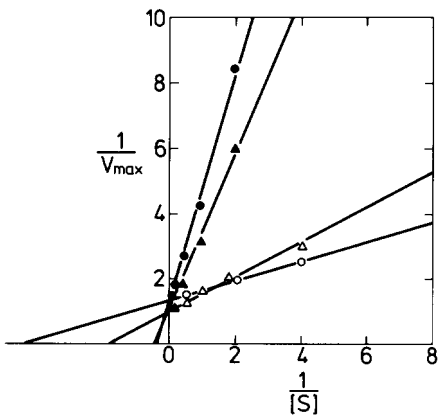

FIG. 11. **Derivative plot of the data of Fig. 10 according to Florini and Vestling (42).** The substrate [*S*] symbols are (○——○) and (●——●) for ADP and creatine-P interacting with the AChR membrane-associated creatine kinase, respectively; (△——△) and (▲——▲) the same for the soluble, *Torpedo* dorsal muscle creatine kinase. The values of the dissociation constants obtained are listed in Table V.

TABLE V

Apparent equilibrium dissociation constants for the AChR membrane-associated and soluble muscle creatine kinases-MgADP-creatine-P complexes as given in Equations 5–8

Apparent K_d	AChR membranes	Torpedo dorsal muscle
	mM	*mM*
K_1	0.17	0.14
K_2	3.1	3.3
K_3[a]	2.5	2.22
K_4[b]	0.22	0.54

[a] Apparent K_m for creatine-P.
[b] Apparent K_m for MgADP.

reaction steps, where CK is creatine kinase and Cr is creatine.

$$\text{CK} + \text{MgADP} \xleftrightarrow{K_1} [\text{CK} - \text{MgADP}] \quad (5)$$

$$\text{CK} + \text{Cr-P} \xleftrightarrow{K_2} [\text{CK} - \text{Cr-P}] \quad (6)$$

$$[\text{CK} - \text{MgADP}] + \text{Cr-P} \xleftrightarrow{K_3} [\text{CK} - \text{MgADP} - \text{Cr-P}] \quad (7)$$

$$[\text{CK} - \text{Cr-P}] + \text{MgADP} \xleftrightarrow{K_4} [\text{CK} - \text{Cr-P} - \text{MgADP}] \quad (8)$$

The apparent K_d values are summarized in Table V.

DISCUSSION

Creatine kinases from fish display a wider heterogeneity than the corresponding isoenzymes from higher vertebrates (see *e.g.* Ref. 4). In addition, their purification is reported to involve difficulties associated with their higher lability and propensity to denature in comparison to isoenzymes from other sources (25). This is reflected in variations in the electrophoretic behavior of purified creatine kinase from electric tissue occurring in the course of hours and in the diminution of specific activity upon lengthy storage. The presence of the hybrid MB type and the total absence of the BB and MM isoenzymes in electric organ and muscle of *Electrophorus electricus* have recently been reported (43). Specific activities in the order of 10–30 units/mg were found for the isoenzymes of this freshwater fish. Since the specific activities obtained in fish species are usually lower than those observed in the mammalian isoenzymes (25), activities of the 300–600 units/mg of the creatine kinase purified by pCMB-agarose chromatography from *T. marmorata* (Table I) or *D. tschudii* (Table II) electric tissues compare favorably with those obtained from green sunfish muscle (25), *Electrophorus* electric tissue (43), or *Torpedo californica* (20).

An apparent subunit molecular weight of about 42,000 has been reported for trout creatine kinase (4). This is in the same M_r region as the conspicuous proteins previously referred to as ν-proteins of the electrocyte and the AChR-rich membranes. These proteins display microheterogeneity manifesting itself as a function of the redox state (Fig. 5) or as charge differences (Fig. 7). Monodimensional SDS-PAGE already makes this heterogeneity apparent; skeletal muscle creatine kinase is split into two bands of M_r = 40,000 and 42,000, *i.e.* migrating slower than the α-subunit of the AChR, when electrophoresis under denaturing conditions is carried out in the absence of reducing agent or upon 5,5′-dithiobis-(nitrobenzoic acid) oxidation (Fig. 5). The latter treatment appears to increase the proportion of the slow migrating component of this doublet. Molecular weight heterogeneity of creatine kinase had previously been reported (5, 44). M_r values of 40,200 and 42,700 were determined by ultracentrifugation for the reduced and nonreduced forms of rabbit muscle creatine kinase subunits, respectively (44). This behavior was attributed to the different reactivity of side groups present in inherently asymmetric subunits of the creatine kinase dimer (45).

Isoelectric focusing reveals charge microheterogeneity in both purified creatine kinase and the M_r = 40,000–43,000 proteins (Fig. 7). At least five different species of ν were originally reported (46). Subsequently, they were resolved into three major nonhomologous species: ν_1, ν_2, and ν_3, with various subspecies each (10). Association with the AChR membranes appears to be stronger for the more basic ν_1 species (10), although the nature of this association is not known in this and the other peripheral proteins of similar apparent mass. Although the extractability from the membrane of all species

at low ionic strength/alkaline pH would suggest ionic interactions with some membrane constituent, the lack of extraction of the more basic and intermediate pI protein species in membranes stabilized *ab initio* by *N*-ethylmaleimide alkylation (15, 18) indicates the participation of some other types of forces in which thiol groups may be involved. The definition of "membrane-bound" as well as "cytosol" forms of the M_r = 40,000–43,000 proteins is also operational, depending on the method of membrane purification (affinity partitioning, gradient centrifugation, countercurrent distribution; see Ref. 10). Methodological aspects may also be responsible for the reported variations in the two-dimensional patterns of these proteins (10, 18, 19, 47). Other sources of microheterogeneity and electrophoretic behavior may be their reported susceptibility to phosphorylation (46) and various degrees of amidation occurring in the course of fractionation.

In the present study, we show that creatine kinase is one of the most prominent protein constituents of the *Torpedo* electrocyte. Immunoblots identify it with at least two types of polypeptides having the same apparent molecular weight but different charge (pI 6.3–6.8). Using identical criteria, the MM isoenzyme, representing a minority component of the total electrocyte extracts, is identified with polypeptides migrating slightly ahead of the basic "ν_1"-proteins (pI 7.3–7.6, Fig. 7). These polypeptides can thus be clearly resolved from both the BB isoenzyme and the ν_1-proteins, which also display charge heterogeneity within a wider pI range (7.3–8.5, Fig. 7).

Probably the more substantiated of the effects of the peripheral proteins having in common the apparent mass of about 40,000–42,000 is their influence on the freedom of motion of the AChR protein, both in terms of its rotational and translational mobility. In native AChR membranes, or in those stabilized *ab initio* by *N*-ethylmaleimide alkylation, the AChR appears to lack the characteristic 10–25-μs rotational correlation time of the 9 S, M_r = 250,000 monomer (13) which is observed in membranes depleted of these proteins. In such membranes, the lateral motion of the AChR monomers and dimers appears to lack any lateral translation hindrance (48), this freedom of motion being reflected in their mode of packing (9). Some of these actions have been postulated to result from the participation of the "ν"-proteins, directly or indirectly, in the thiol-dependent receptor aggregational states (15, 16). Whether a creatine kinase-driven energy mechanism is operative or whether other peripheral proteins are involved in these remains to be elucidated.

Other reported effects of the peripheral M_r = 40,000–43,000 proteins concern their influence on the susceptibility of the AChR to thermal denaturation (49) or enzymatic attack (22, 50). Hucho and co-workers (51) also suggested that such proteins protect the AChR from endogenous proteases.

The kinetics of ATP production by the BB type of AChR membrane-associated creatine kinase are similar to those of the soluble MM type of isoenzyme purified from the dorsal muscle of *Torpedo* (Figs. 10 and 11 and Table V). Both follow the same trends as the corresponding isoenzymes from higher vertebrates (52). Our results show that there is a slightly higher affinity for creatine-P after formation of the Mg^{2+}-ADP-enzyme complex (Equations 6 and 7, K_2 and K_3 in Table V) in both membrane-associated and -soluble enzymes. On the other hand, formation of the binary complex with creatine-P diminished the affinity for Mg^{2+}-ADP for both soluble and membrane-associated enzymes (Table V). It follows that there is an asymmetrical interaction between the nucleotide and the guanidine binding sites for both particulate and soluble creatine kinase isoenzymes.

Another characteristic of the *Torpedo* isoenzymes is their ability to be saturated by relatively low concentrations of ADP and creatine-P. The measured concentrations of the latter high-energy phosphate compound in the resting electric organ, 1–2 μmol/g tissue, indicate that unless a compartmentalization of this substrate occurs, the actual levels in the electrocyte cytosol are also relatively low, most likely close to the calculated apparent K_m of 2.5 mM (K_3, Table V). Analogously, the concentrations of free ADP could also lie within the range of the apparent K_m (0.22 mM), such that small increases resulting from energetic processes exert a regulatory effect on creatine kinase, which would thus work at near-maximal velocity conditions. Such activatory effects of ADP on creatine kinase have been proposed for other cells (53).

The raison d'être of the association of BB creatine kinase with the AChR membrane is not immediately apparent. We have previously discussed the possibility of a trivial adsorption of the enzyme to the membrane (19), but the direct observation of BB creatine kinase on the intact electrocyte by immunocytochemistry, showing its predominant location on the ventral face where the AChR is also present (Fig. 2), strongly favors the possibility that the BB creatine kinase isoenzyme is indeed associated with this membrane *in situ*. Most recently, creatine kinase has been found in synaptic membranes in association with other enzymes involved in energy metabolism (57). The different peptide maps of soluble and membrane-bound creatine kinase also help to dismiss the possibility of a trivial contamination of the membrane preparations with cytosol enzyme. Such differences might also bear relationship to the ability of BB creatine kinase to associate with membranes, a point which will require further investigation. In this context, the presence of a peptide with a high density of positive charges in the membrane-associated isoenzyme subunits and its absence in the soluble enzyme subunits (Fig. 9) are interesting in view of the reported participation of ϵ-amino groups in the association of the ν-proteins with the AChR membranes as proposed by Eriksson *et al.* (58). Although only a 2-fold higher affinity for ADP appears to derive from this association with the membrane, its topographical proximity to the AChR cannot be neglected as an inherently potential advantage (19). Thus, particular significance is acquired by the presence of a rapid and efficient energy-generating system in close contact with the endogenous protein kinase recently localized at the AChR membrane (54) and with its substrate, the AChR (55, 56).

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ISOLATION AND CHARACTERIZATION OF ACETYLCHOLINE RECEPTOR MEMBRANE-ASSOCIATED (NONRECEPTOR ν_2-PROTEIN) AND SOLUBLE ELECTROCYTE CREATINE KINASES (EC 2.7.3.2)

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EXPERIMENTAL PROCEDURES

Materials

Two Torpedinidae species were used, from the North and South Atlantic respectively. Torpedo marmorata electric fish were obtained from the Marine Station at Arcachon, France and specimens of Discopyge tschudii were provided by fishermen near Mar del Plata, Argentina. CK from rabbit muscle (MM type) and all other reagents for the enzyme assays were purchased from Sigma Chemie, Munich, or Boehringer, Mannheim, F.R.G. All other chemicals came from Merck, Darmstadt, F.R.G.

Methods

Preparation of AChR Membranes- Membrane fragments enriched in the AChR protein were isolated from T. marmorata electric organ by the procedures previously reported (16). Minor modifications (A. Braceras and F.J. Barrantes, in preparation) were introduced to obtain membranes from D. tschudii electric tissue.

Protein extraction from AChR Membranes- Alkaline extraction of non-receptor proteins was done essentially as in (7) with the modifications of (15). The double alkaline extraction procedure was further modified as follows: 200 µl of 1 mM NaOH were added to 100 µl of the stock suspension of AChR membranes (4-8 mg protein / ml) in a Reacti-vial and the pH maintained at 11.0. After 10 min stirring at 4° C, the sample was centrifuged at 30,000 rpm for 15 min. The supernatant was carefully withdrawn and immediately adjusted with stock buffer F (40 mM Tris-HCl buffer, pH 8.0, containing 2 mM $MgCl_2$, 1 mM EDTA and 0.2 mM EGTA) to pH 8.0, and the whole procedure repeated five times. Lithium di-iodosalicylate (Li-S) extraction was applied to AChR membranes as in (21). The soluble extracts were dialyzed against buffer F. High salt extraction of the AChR membranes was performed using 0.5 M ClK in buffer F for two 30 min periods, followed by a 15 min centrifugation as above. Sodium cholate, deoxycholate, CHAPS and Triton X-100 (all in a 1% solution) extraction of the AChR membranes was also undertaken in buffer F for 30 min. A brief centrifugation was subsequently used to separate the detergent-soluble CK material and the enzyme activity still present in the membrane remnants. Trypsin digestion of the intact membranes and of the detergent-solubilized material was carried out as in (22).

Extraction of the kinase and precipitation of the Mg^{2+}-enzyme complex- Total Torpedo electric tissue or dorsal muscle homogenates were prepared in 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM KCl, 10 mM 2-EtSH, 1 mM EDTA, 0.2 mM EGTA and 0.1 mM PMSF (buffer E) for 10 s using a Teflon / glass homogenizer at full speed. Extraction was subsequently allowed to proceed by stirring the crude extract at 4° C for 20 min. The crude homogenates were then centrifuged at 13,000 rpm (4° C) in a Beckmann JA 20 rotor for 30 min. The supernatants or the extracts of purified AChR membranes were then submitted to successive 50% and 70% ethanol extractions basically as in (23). Solid NH_4Cl was added to a final concentration of 100 mM and the pH adjusted to 9.0 (4° C) with 5 M NH_4OH. The solution was cooled down to -1° C, to give a final concentration of 50% (v / v). After 30 min the samples were centrifuged at 13,000 rpm at the same temperature for 20 min in a Beckman JA 20 rotor.

The Mg^{2+}-enzyme complex was precipitated from the supernatant by dropwise addition of 2 M $NgSO_4$ to a final concentration of 20 mM, followed by ethanol addition at - 10° C to give a final ethanol concentration of 70% (v/v). The precipitate was collected by centrifugation at -10°C as above. The pellet was resuspended in buffer E at 4°C and centrifuged at 30,000 rpm in a Ti 70.1 Beckman rotor for 30 min. The partially purified enzyme was then dialyzed and concentrated against the appropriate buffer under mild negative pressure using Schleicher and Schüll dialysis bags in the presence of 1-2 mM 2-EtSH.

Thiol Group-Sepcific Chromatography- The chromatographic procedure using p-hydroxymercuribenzoate (PCMB)-Agarose CL-6B (24) followed the partial purification by ethanol precipitations (see above) as described in Tables I and II. PCMB-Agarose chromatography was also applied to soluble extracts of AChR membranes in a mini-batch fashion. NaOH or Li-S membrane extracts (0.6 ml) were allowed to react overnight with 0.6 ml gel in a Reacti-vial at 4° C by slowly shaking the suspension in a thermostated bath. Unbound proteins were removed from the gel by repeated washings and successive centrifugations of the gel at 2,000 rpm for 15 min. The supernatant was then assayed for CK activity. In the case of some activity remaining in soluble form, fresh PCMB-Agarose was added and made to react for 30 min. The sedimented PCMB-Agarose was washed three times with 150 µl buffer F containing 100 mM NaCl, followed by one 10 min wash with buffer F. After the four centrifugation steps buffer F containing 20 mM dithiothreitol (DTT) was allowed to desorb the bound protein under gentle stirring for 10 min. The operation was repeated twice, and the eluates collected and dialyzed against buffer F with 1 mM DTT:glycerol (1:1 v/v).

Blue-Sepharose CL-6B Affinity Chromatography- The resin was prepared, stored and recycled as reported (25). Samples were applied in 50 mM Tris-HCl, pH 5.2, containing 5 mM $MgSO_4$, 0.4 mM EDTA, 5.5 mM 2-EtSH and 40 mM NaCl and the column eluted with several column volumes of the same buffer. A linear (0-500 mM) NaCl gradient in the same buffer but at pH 7.9 was used to elute CK activity. Alternatively, a single pulse of 250 mM NaCl was employed.

Enzyme Activity- was assayed following the recommended "optimized procedure" of the German Society for Clinical Chemistry, as described in (26). The coupled reaction:

$$\text{creatine-P} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP} \quad (2)$$

$$\text{glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP} \quad (3)$$

$$\text{glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+ \quad (4)$$

was used as described, except that N-acetylcysteine was replaced by GSH.

On-line, Simultaneous Anion Exchange High Pressure Liquid Chromatography/CK Activity Detection- (Fig. 1). A Varian series 5000 liquid chromatography apparatus (Varian Instr., Palo Alto, Calif.) was used. A 150 x 4 mm (ID) Synchropak AX-300 column (Synchrom, Linden, Ind.) was mounted in series with a Synchrom 400 x 5 mm postcolumn reactor. The column was equilibrated with 0.02 M sodium phosphate buffer, pH 7.4 (buffer A). Samples were prepared in buffer F and injected through a 10 µl loop. Elution with a 0 - 0.5 M NaCl linear gradient in 0.02 M sodium phosphate buffer, pH 7.8, started simultaneously with sample injection, progressing at a rate of 3% buffer B per min. The flow rate was either 0.5 or 1.0 ml/min. The system was operated at pressures of 35 - 60 atm. The column was held at a temperature of 20 - 25° C. The eluate from the ion exchange column was continuously monitored at 280 nm with a Varian UV-50 Vari-Chrom detector in parallel with the recording of the pressure. The effluent of the ion exchange column was mixed with the reaction mixture composed of the enzymes and substrates given in Equation 2-4 (Kit from Boehringer Mannheim GmbH, F.R.G.) in the reactor thermostated to 37°C. The eluate of the post-column reactor was monitored with a Fluorichrom (Varian Instr.) fluorescence detector. The activity detected by the system was calculated according to the formula $y = 3.851 \times -9.535$ where x is the enzyme activity applied to the column and y is the measured activity (41). The limit of sensitivity of the system was 1 unit/l. The data were recorded before and after post-column reaction with two CDS-111 Chromatographic Data Systems (Varian). Retention times and peak areas were calculated with the same instruments.

Enzyme Kinetics- Determination of the kinetic parameters of the membrane associated and soluble CK was carried out as in (27) with the following modifications: a) the buffer employed was 50 mM morpholinopropane sulfonic acid (MOPS); b) 5 mM dithioerythrol and 4 mM AMP were used throughout; c) the assay mixture was supplemented with 1 mM ouabain to prevent splitting of ATP by residual ATPase activity. When the enzyme kinetics were studied as a function of substrate concentration, the concentration of creatine-P was varied between 0.05 and 30 mM and that of ADP between 0.05 and 5 mM, keeping the other substrate concentration constant.

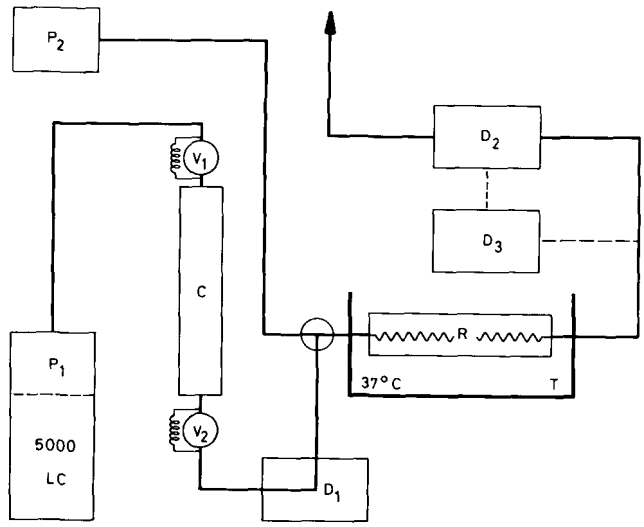

Figure 1. Schematic Diagram of the High Performance Ion-exchange Liquid Chromatography Instrumentation. LC: Varian 5000 liquid chromatographer; P_1, binary solvent system pump; P_2, pump delivering coupled enzyme reactants; V_1, precolumn injection valve, with 10 µl loop (Rheodyne model 7120); C, AX-300 anion-exchange column (Synchrom, Linden); V_2, postcolumn injection valve, used to check column transit times; D_1, UV column detector, R, postcolumn reactor, in thermostated bath T; D_3, postreactor UV detector; D_2, postreactor fluorescence detector.

Creatine Phosphate- was assayed using the procedure described in (28). Electric or skeletal muscle tissue from Torpedo was rapidly frozen by immersion in liquid N_2. The frozen tissue was weighed, sandwiched in aluminium foil, and comminuted to a powder under liquid N_2 with a hammer. The operation was completed in a mortar under liquid N_2 and extracted with 4-5 vol. of frozen $HClO_4$ (7% w/v) under continuous mixing with the pestle. The samples were then homogenized in a Virtis 60 homogenizer at 15,000 rpm after thawing. The precipitated protein was thereafter removed by centrifugation in a Sorvall GSA rotor at 10,000 rpm for 20 min. The extract was neutralized with 5 M $KHCO_3$ and clarified by an additional centrifugation in the GSA rotor as above. The pH was finally adjusted to 7.3 and degassed under negative pressure before assaying creatine phosphate using exogenous CK (rabbit muscle).

SDS-Gel Electrophoresis- was done according to Laemmli (29), with the special precautions applying to AChR membranes polypeptides (9). The two-dimensional system of O'Farrell (30) was used when isoelectric focusing preceded the second dimension. Thiol modifications were carried out as reported (31).

Immunological Characterization of CK in AChR Membranes- was carried out essentially as described in (15). Rabbit antisera against the MM and BB-CK isoenzymes were provided by Dr. J.-C. Perriard, ETH (Zurich). Immune replicas of AChR membrane polypeptides were made on nitrocellulose paper (15). After saturation of unspecific sites with 5% horse serum in 50 mM Tris-HCl buffer (pH 7.1) for 2 h, 1:100 dilutions of the first antibody in the same buffer were incubated overnight. Identification of the SDS-denatured antigens was made following reaction with a second (goat) peroxidase, FITC or rhodamine-labelled anti-rabbit IgG (Miles) also at a 1:100 dilution. Silver staining of the nitrocellulose replicas followed Ref. 32.

Indirect Immunofluorescence of CK Isoenzymes in Intact Electric Tissue- Single electrocyte columns were dissected from the electric organ, fixed overnight with 3 % paraformaldehyde in PBS at 4° C, dehydrated through graded series of ethanol (70 % ethanol for 4 h, 80%, 90% and 100% ethanol with two changes each for 1 h), transferred to 100% xylene (two changes for 45 min each), and penetrated with paraffin (Paraplast tissue embedding medium, Lancer, Sherwood Materials Ireland) twice for 45 min at 60° C. The embedded material was solidified at 20°C and stored in the cold. 5 µm sections were cut, floated onto 45°C water and picked up with glass slides previously covered by a thin layer of chrome-alum gelatine (0.1 g ammonium chromate, 1 g gelatine in 100 ml of water). Paraffin was dissolved by two 10 min dips in xylene and the sections rehydrated through a graded series of ethanol (100 %, 90 %, 70 % and 30 % for 10 min each) and washed with PBS (three changes, 15 min each). Sections were incubated in a moist chamber with specific rabbit anti-chicken BB-CK or with anti-chicken MM-CK antibodies diluted to 1-5 µg/ml of specific IgG into PBS-buffer containing 1 mg/ml of bovine serum albumin. The affinity purified antibodies used were specific for the B and M subunits of chicken and crossreacted with Torpedo CK as has been shown previously (18, 19). The antibodies have been extensively characterized (see ref. 33). Preimmune sera or corresponding rabbit IgG were used at the same dilutions as in the controls. After incubation for 30 min at 25°C followed by three washings with PBS, 100-200 µl of 1:100 diluted FITC-conjugated goat anti-rabbit IgG (Cappel) were placed on the same area and the sections incubated for another 30 min. After three washings with PBS and removal of excess PBS, a drop of 50% glycerol in 0.1 M glycine-NaOH at pH 9.0 was placed onto the sections which were then covered by a coverslip, examined with a Zeiss standard-18 epi-fluorescence microscope, and photographed on Ilford HP-5 film.

Amino-acid analyses- The purest fractions of CK obtained from electric organ and dorsal muscle were extensively dialyzed against 10 mM NH_4HCO_3 buffer and lyophilized. Acid hydrolysis was carried out at 110°C for 48 h on 200 µg aliquots.

Peptide Mapping- Two-dimensional peptide mapping of the purified CK or of its individual subunits was carried out after radioiodination of the samples by the method of Bolton and Hunter (34). The ^{125}I-labeled reagent (sp. act. 2200 Ci / mmole, NEN) was allowed to react for 1 h at 0°C and 18 h at 4°C. The samples were otherwise handled according to (35): trypsinolysis was carried out for 24 h. The tryptic digests (1-10 µl) were spotted onto 10 x 10 cm cellulose-coated TLC plates (Eastman Kodak N 13255). Electrophoresis was carried out for 30 min at 1 kV in acetic acid:formic acid:water (15:5:80, by vol.). After drying the plates, the peptides were chromatographed in the second dimension in butanol: pyridine:acetic acid:water (32.5:25:5:20, by vol.). Autoradiography was carried out on Kodak X-AR5 radiographic film using Cronex Hi-Plus (Dupont) intensifier plates.

Other Analytical Procedures- Protein determination was done according to Lowry et al. (36) except for samples containing thiol reagents, in which case the Coomasie Blue procedure (Bio-Rad Laboratories, Richmond) was employed. In both cases the standard was bovine serum albumin. The toxin binding capacity was assayed as in (37).

RESULTS

Partial Purification of Creatine Kinase from Torpedo Electric Organ- The ability of Blue Sepharose CL-6B to retain a variety of enzymes possessing the dinucleotide fold (39) was first exploited. The material used for chromatography was purified by the successive 50%-70% ethanol extraction steps originally described by Kuby et al. (23) for the preparation of muscle creatine kinase, since the Blue-Sepharose chromatography can result in denaturation of the enzyme when too crude tissue extracts are applied, particularly with the reported labile fish creatine kinases (25). In spite of the above precautions, and although attention was paid to the binding capacity of the gel, a significant amount of the enzyme was eluted with the low ionic strength buffer and could not be properly resolved from other proteins present in the extract (Fig. 3).

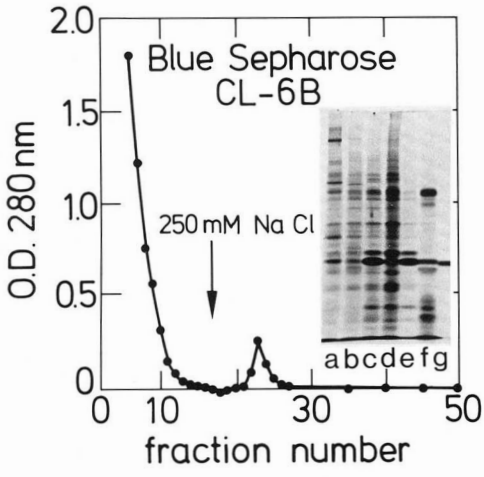

Figure 3. Purification of Creatine Kinase Activity from T. Marmorata Electric Tissue by Affinity Chromatography in Blue-Sepharose CL-6B. About 75% of the material obtained by ethanol extractions of the electric tissue (34 g) homogenate was eluted with the column void volume (3.7 ml) in 50 mM Tris-HCl buffer, pH 8.0 containing 2 mM 2-EtSH. A pulse of 250 mM NaCl in the above buffer resulted in the desorption of a peak, representing 3.8% of the loaded protein, with a specific activity of 155 units/mg protein. Inset: SDS PAGE of the Blue-Sepharose purification. (a) Total homogenate. (b) 50% ethanol extract. (c) 70% ethanol extract Fractions 4 (d), 7 (e) and 23 (f) from the Blue Sepharose column. (g): rabbit muscle creatine kinase.

This was confirmed by SDS gel electrophoresis (Fig. 3, inset) and enzyme activity measurements. A protein of about 43,000 M_r identified as Torpedo α-actin by isoelectric focusing and gel electrophoresis (40) eluted together with the non-adsorbed main creatine kinase polypeptide band (Fig. 3 e). The kinase activity retained by the matrix was subsequently eluted with a pulse of 250 mM NaCl (Fig. 3). The polypeptide pattern of this fraction showed a band having the same electrophoretic mobility as rabbit muscle creatine kinase (Fig. 3g) and a strong component in the region of 80,000-85,000 apparent M_r (Fig. 3f).

Identification of Creatine Kinase Activity by High Performance Liquid Chromatography- HPLC on ion-exchange resins has been successfully applied to the separation of creatine kinase isoenzymes on the basis of their different isoelectric points (41). Anion-exchange HPLC was arranged in series with an enzyme reactor (Fig. 1) fed with the coupled enzyme system specific for creatine kinase (Equations 2-4). The instrumental setup enabled the simultaneous, on-line detection of the protein constituents and their corresponding enzyme activity. Fig. 5 shows the separation obtained with a low speed supernatant of total electric organ homogenate.

Influence of Redox State on Electrophoretic Properties of Creatine Kinase and ν-Proteins- Differences in electrophoretic mobilities of muscle and electric tissue creatine kinase were observed upon PAGE under nondenaturing conditions (Fig. 12 a-c). Such differences can be accounted for in terms of the pI values of the native isoenzymes and their amino acid compositions (Table III). Another source of differences in electrophoretic behavior of both creatine kinase and AChR membrane-associated ν-proteins is related to the redox state of the denatured subunits. Omission of the reducing agent (Fig. 12 d,f) or oxidation of the samples with 5,5'-dithiobis-(nitrobenzoic acid) (Fig. 12 g,i) resulted in the uneven splitting of the rabbit muscle creatine kinase subunits into two apparently different subunits (Fig 12 d and g), a phenomenon not observed with Torpedo isoenzymes. Alkylation with N-ethylmaleimide prevented such splitting (Fig. 12 j,l). Concomitantly, the δ-δ_2 conversion occurred in the membrane-bound AChR subunit.

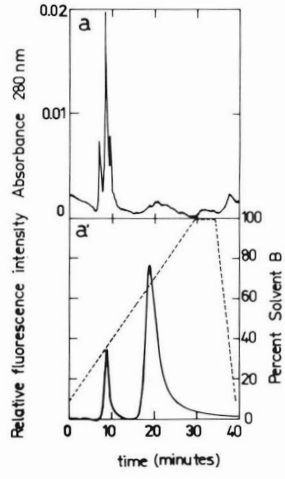

Figure 5. HPLC Separation of CK on Synchropak AX-300 with Simultaneous Enzyme Detection through Coupled Enzyme Reaction. The upper graph (a) corresponds to the protein profile; the lower graph (a') shows the corresponding fluorescence detection of CK activity. The sample corresponds to the low speed supernatant of crude electric organ total homogenate. A ten microliter sample in 20 mM Tris-HCl buffer, pH 7.4, was injected through the loop. The upper graph has been shifted in order to facilitate the comparative visual inspection of the protein and activity profiles. The actual fluorescence patterns of the CK activity eluted 3.48 min later (i.e. the delay involved in passing through the enzyme reactor). Solvent A: 20 mM sodium phosphate buffer, pH 7.8; solvent B: the same, containing 500 mM NaCl. A 250 x 4 mm column was used in this case.

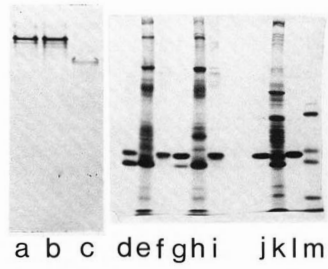

Figure 12. PAGE Patterns of Creatine Kinase and SDS PAGE of AChR Membranes as a Function of Redox Conditions. a), b) and c) correspond to commercial MM creatine kinase (rabbit muscle), Torpedo dorsal muscle creatine kinase and electric tissue creatine kinase respectively, under non-denaturing conditions. Under denaturing conditions (d-m) in the absence of reducing agent rabbit muscle creatine kinase shows two clearly separated bands of M_r 40,000-42,000 (d). Torpedo muscle isoenzyme shows only one band under such conditions (f). Treatment with 5,5'-dithiobis-(nitrobenzoic acid) reveals two bands of uneven intensity with rabbit muscle isoenzyme (g) but not with Torpedo muscle (i). Reduction with dithiothreitol results in the obtention of only one band for both rabbit and Torpedo muscle isoenzymes (j, l) m standards (see Fig. 2).