A membrane-associated creatine kinase (EC 2.7.3.2) identified as an acidic species of the non-receptor, peripheral ν-proteins in *Torpedo* acetylcholine receptor membranes

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Function of one of the M_r *43000 proteins* *Membrane-associated enzyme*
Acetylcholine receptor-rich membrane *Peripheral membrane protein*
Adenosine 5'-triphosphate:creatine phosphotransferase (EC 2.7.3.2)

1. INTRODUCTION

A group of proteins named the 43K-protein [1,2], the ν-peptide [3], the ν-doublet [4], or the M_r 43000 proteins [5] is probably one of the most abundant non-receptor constituents of acetylcholine receptor (AChR)-rich membranes. Early studies associated the ν-proteins with the site of action of local anaesthetics [6], but it was subsequently shown that their extraction modified neither the binding of these ligands nor the AChR-controlled ion permeability [2,7]. More recently, enhanced freedom of AChR rotational motion [8–10] has been observed after extraction of these (and other) non-receptor peripheral proteins by alkaline stripping. In [4] the involvement of the ν-proteins in processes like synapse formation during ontogenesis, receptor clustering and stabilization of the adult synapse was proposed on the basis of the observed changes in AChR supramolecular organization which follow on from extraction of these proteins. Subsequently, the stabilization of one of the AChR oligomeric species, the 13 S dimer, was found to be coincident with the lack of extraction of the ν-proteins [11]. Antibodies raised against alkaline extracts of *Torpedo* membranes outline the profile of the mammalian neuromuscular junction [12], and purified anti-ν-protein antibodies reveal that their antigenic determinants are predominantly exposed on the inner, cytoplasmic face of the AChR-rich membrane [11]. Lactoperoxidase iodination of holey vesicles confirms this localization [13].

Aside from the heterogeneity apparent in conventional $NaDodSO_4$ electrophoresis [4,14], charge heterogeneity is displayed by the ν-proteins [5,15]. The more basic subspecies, ν_1, remains associated with AChR membranes after extensive purification by affinity partition, whereas the more acidic subspecies, ν_2, and ν_3, are also found in electrocyte soluble extracts [5]. The tightness of the association with the membrane is thus variable for the different ν-proteins, which do not occur exclusively in membrane-bound form. Exogenous cross-linking of AChR membranes has recently been described as being related to the disappearance of ν_1, and appearance of a filamentous network [16], though neither the chemical nature of this network nor its relationship with ν_1 were shown. Thus it does not necessarily follow that the

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modulation of AChR mobility depends on the presence of ν_1 [16].

This work concerns the finding of creatine kinase (EC 2.7.3.2, 'CK'), one of the phosphokinases that catalyzes phosphoryl group transfer to a guanidino function, in AChR-rich membranes from *Torpedo* electric tissue. Enrichment of this enzymatic activity coincides with the purification of the ν-proteins and more specifically, with the subspecies of intermediate p*I*-value (6–6.5). Specific cross-reactivity of the AChR membrane-associated proteins with anti BB (brain) type of CK antibodies is observed. No such cross-reactivity is apparent in AChR membrane-associated ν-proteins with antibodies against the MM (muscle) type of isoenzyme. The kinetic properties of the membrane-associated and soluble (electric tissue) CK do not differ; the apparent Michaelis constants for MgADP and creatine phosphate are also similar to those found in the CK isoenzyme (MM type) present in *Torpedo* skeletal muscle.

2. MATERIALS AND METHODS

2.1. *Membrane purification and extraction procedures*

Native or *N*-ethylmaleimide alkylated AChR membranes from *Torpedo marmorata* were isolated by the procedures reported in [17]. Membrane fractions of relatively low spec. act. were chosen for most of the purification steps in order to maximize the contribution of non-receptor proteins. The immune blotting experiments were performed on both low and high spec. act. ([^{3}H]α-neurotoxin) membrane fractions. Alkaline extraction of non-receptor proteins was done essentially as in [2] with the modifications given in [11]. Alternatively, lithium di-iodo-salicylate extraction of the membranes was carried out [7].

2.2. *Thiol-Sepharose chromatography*

The methods usually applied for the purification of mammalian CK were attempted on alkaline or lithium diiodo-salicylate extracts of AChR membranes. Successive ethanol extractions (50–70%) (see [18]) were done at 0°C. After dialysis of the ethanol extract against 50 mM Tris–HCl buffer (pH 7.4) containing 1–2 mM β-mercaptoethanol and concentration by vacuum dialysis, the extracts were chromatographed through *p*-hydroxymercuribenzoate-Agarose CL-6B, or Blue Sepharose CL-6B as described in [19] and [20], respectively. Both procedures have been successfully applied to CK purification, in particular for obtaining the labile fish enzymes [20].

2.3. *Gel electrophoresis and isoelectric focusing*

$NaDodSO_4$–PAGE gel electrophoresis in slab systems followed the procedure in [21], except that samples were not heated for the reasons given in [4]. The 2-dimensional system in [22] was used without modifications. Integration of the Coomassie Blue-stained spots was done after digitalisation of the data acquired with the aid of a high resolution vidicon camera. Computer processing of the digitised optical density profile provided:

(a) a signal-averaged image of a rectangular frame of the stained gel after background subtraction;
(b) integrated area measurements and average positions of the stained spots.

2.4. *Immunological characterization of CK in AChR membranes*

Rabbit antisera against the MM and BB-CK isoenzymes were provided by Dr J.-C. Perriard, ETH (Zürich). Immune replicas of AChR membrane polypeptides were made on nitrocellulose paper as in [11]. After saturation of unspecific sites with 5% horse serum in 50 mM Tris–HCl buffer (pH 7.4) for 2 h, 1:100 dilutions of the first antibody in the same buffer were incubated overnight. Identification of the $NaDodSO_4$-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.

2.4. *Determination of CK activity and enzyme kinetics*

The coupled enzyme assay:

$$\text{creatine-P} + \text{ADP} \xrightleftharpoons{\text{CK}} \text{creatine} + \text{ATP}$$

$$\text{glucose} + \text{ATP} \xrightleftharpoons{\text{hexokinase}} \text{glucose-6-}P + \text{ADP}$$

$$\text{glucose-6-}P + \text{NADP}^+ \xrightleftharpoons{\text{G6P-DH}} \text{gluconate-6-}P + \text{NADPH} + \text{H}^+$$

was used as described [23], except that reduced glutathione was used instead of *N*-acetyl-cysteine. In the case of kinetic experiments, the concentration of creatine phosphate varied between 0.05 and 30 mM, and that of ADP between 0.05 and 4 mM, keeping the other substrate concentration constant.

2.5. *Other analytical procedures*

Protein determination was done as in [24] except for samples containing thiol reagents, in which case the Coomassie Blue procedure (Bio-Rad Laboratories, Richmond) was employed. In both cases the standard was bovine serum albumin. The toxin binding capacity was measured by the DEAE-cellulose filter assay [25].

3. RESULTS

3.1. *CK activity in AChR membranes*

Measurement of CK activity in AChR membranes by the coupled enzyme assay [23] which follows NADPH production yielded values of 0.8 ± 0.5 units/mg protein ($n = 12$). Under similar experimental conditions neither glutathione reductase (EC 1.6.4.2) nor myokinase (EC 2.7.4.3) activities were detected in the membranes. Alkaline stripping, which extracts peripheral, non-receptor proteins and in particular the ν-proteins from AChR membranes, was accompanied by roughly a 2-fold increase in the specific activity of CK recovered in the extract (1.8 ± 0.4 units/mg, $n = 6$). Similar results were obtained with lithium diiodo-salicylate extraction of the AChR membranes.

The AChR membrane-associated CK displayed kinetics similar to those of the enzyme solubilized by alkali stripping procedures. Furthermore, comparison of the membrane-associated enzyme with highly purified CK obtained from the same animal (*Torpedo* dorsal muscle) also showed similar kinetic properties. The apparent Michaelis dissociation constants for creatine-P and ADP were 0.22 mM and 2.5 mM, respectively, for the crude CK found in AChR membranes.

3.2. *Purification of CK activity coincides with enrichment of the ν-proteins*

Literature on CK (see review in [26]) and the ν-proteins [6] indicates abundance of thiol groups in both proteins. The availability of these side chains has been exploited for the purification of human CK [19]. AChR-rich membranes were first submitted to alkaline (one cycle, 1 h at 2°C) and ethanol extractions. Concentrated extracts were chromatographed through the thiol-Sepharose column as shown in fig.1. Non-adsorbed fractions contained mainly a protein identified by 2-dimensional gel electrophoresis as *Torpedo* actin [27] (see also fig.4). Minor amounts of a band migrating with the characteristics of the ν-proteins eluted together with the AChR membrane-associated actin in this fraction. A pulse of 1 mM *p*-hydroxy-mercuribenzoate yielded a main fraction of CK (spec. act. 55–75 units/mg). Further elution with a 2–100 mM β-mercaptoethanol gradient resulted in additional CK activity, but at a lower yield (fig.1). Recovery in these separations was 70% (protein) and 45% (activity). The initial spec. act. of 0.4–1.1 units/mg found in AChR membranes, increased 2-fold upon alkaline stripping, and resulted in about a 70-fold enrichment after thiol-Sepharose purification. The $NaDodSO_4$ pattern of these fractions showed a doublet of bands having electrophoretic mobilities of between M_r 40000 and 43000 (fig.2h). Bulk purification of

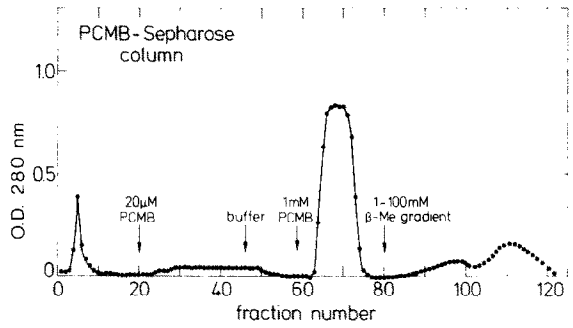


Fig.1. Thiol-Sepharose CL-6B chromatography of alkaline extract of AChR membranes. A 250 μl aliquot (1.5 mg protein) of alkaline extract (one cycle, 1 h at pH 10.9, 4°C) submitted to 50–70% alcohol extractions and concentration against 50 mM Tris–HCl buffer (pH 7.4) was applied to the *p*-hydroxy-mercuribenzoate ('PCMB') column (2.5 ml slurry). Elution of CK activity was accomplished with a pulse of 1 mM PCMB. A 1–100 mM β-mercaptoethanol gradient was subsequently installed, resulting in the elution of additional CK activity. Fractions of 0.8 ml were collected. The corresponding PAGE patterns are shown in fig.2.

CK from total electroplax yielded a similar pattern (fig.2k–m).

3.3. *Immunological characterization of the CK activity in AChR membranes*

Immune blotting techniques were applied to electropherograms of AChR membranes prepared by sucrose gradient centrifugation. Rabbit anti-MM and anti-BB chicken CK antisera at 1:100 dilution were used. No cross-reactivity was observed with the antiserum against the MM isoenzyme, characteristic of skeletal muscle (fig.3d,e). Anti-BB (brain) type CK isoenzyme gave a positive reaction at the level of the ν-proteins (fig.3a,b). Lower antibody dilutions also revealed a much fainter positive reaction at an app. M_r of about 50000 in some membrane preparations (not shown).

3.4. *The AChR membrane-associated CK and ν-protein isoelectric properties*

Alkaline extraction of native AChR membranes

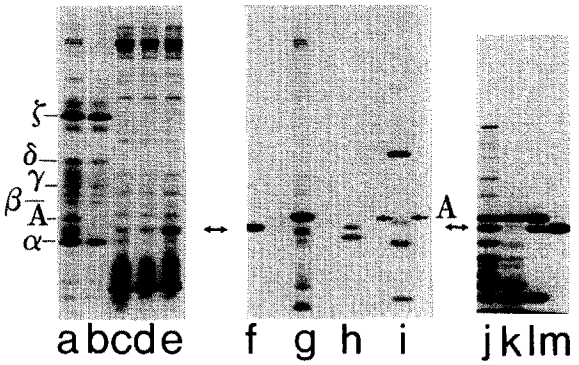
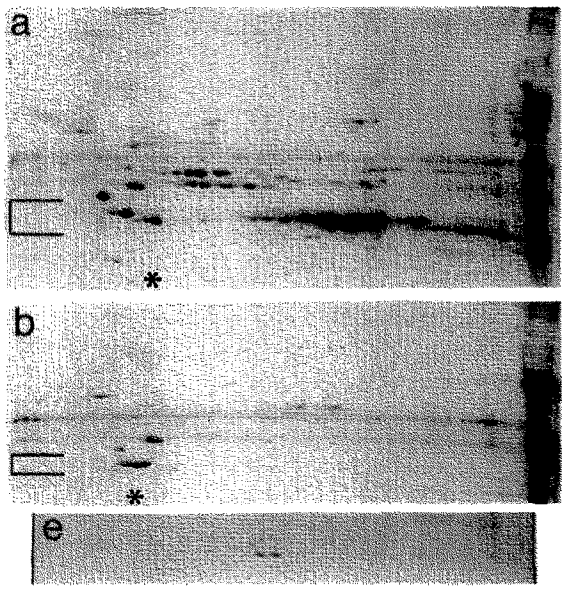


Fig.2. NaDodSO$_4$–PAGE of the CK purification resulting from the thiol-Sepharose column chromatography (fig.1): (a) Starting material: AChR membranes (0.9 nmol [^{3}H]α-bungarotoxin/mg protein); (b) Alkali-stripped membranes; (c) Alkaline extract; the same, after 50% (d) and 70% (e) ethanol extractions at 0°C [18]; (f) Rabbit muscle CK; (g) and (h) correspond to the peaks eluted with the void volume and 1 mM PCMB, respectively (fig.1); (i) Standards (from top to bottom): bovine serum albumin (M_r 68000), ovoalbumin (M_r 45000), aldolase (M_r 40000) and chymotrypsinogen (M_r 25000); (j) 50% ethanol extract from electroplax soluble fraction; (k) The succeeding 70% ethanol extract; (l) and (m) correspond to the void volume and the 1 mM PCMB fractions as in AChR membrane extracts (g and h); A is actin; the double-sided arrow shows the position of the ν-proteins.

Fig.3. Identification of the subtype of CK species by immune blotting of AChR membranes with anti-chick CK isoenzyme antibodies: (a), (b), (d) and (e) are replicas of the same samples of AChR membranes, transferred simultaneously to 2 nitrocellulose papers; (a) and (b) were incubated with anti-chick BB-CK isoenzyme antiserum (rabbit) and (goat) anti-rabbit IgG labelled with fluorescein isothiocyanate. Fluorescence is observed at the level of the ν-proteins; (c) shows the Coomassie Blue staining pattern; (d) and (e) correspond to the complementary replica incubated with anti-MM CK antibodies and with pre-immune serum, respectively. Spec. act. of the AChR membranes was 2.8 nmol/mg protein. Aliquots of 25 μg protein were applied in each lane.

results in the depletion of all ν-protein species. Two-dimensional gel electrophoresis reveals a variety of ν-protein isoelectric forms (fig.4a) in the soluble extract. In contrast, only a few spots are observed at the level of the ν-protein bands in the alkaline extracts from membranes prepared throughout in *N*-ethylmaleimide (the 'NEM-membranes' in [11]), as shown in fig.4b. These correspond to the more acidic (p*I* 5.9–6.1) ν-protein species (see [5]). Integrated area measurements of the staining intensities indicate that these acidic species released from NEM-membranes constitute less than 5% of the total population of ν-proteins in native membranes (fig.4c,d). It is also clear that NEM-alkylation prevents extraction of more than 90% of the ν-



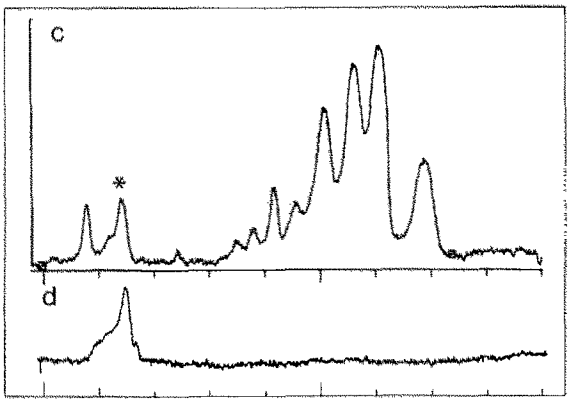


Fig.4. Identification of CK with the acidic subspecies of the ν-proteins by 2-dimensional gel electrophoresis: (a) Alkaline extract of native membranes; (b) Alkaline extract of membranes prepared in *N*-ethylmaleimide throughout [12,18]. The marks on the left indicate the strips scanned for the quantitative analysis shown in c and d; (c) Computer-integrated areas of the strip indicated in (a), including the ν-proteins and actin in alkali extract of native membranes; (d) The same, for NEM membranes (b). Only the more acidic ν-proteins (*) are extracted; (e) CK purified from AChR membranes by thiol-chromatography. The two spots having the electrophoretic mobility of the ν-proteins occur in the 6.4–6.5 pH range; i.e., corresponding to ν-protein subspecies in AChR membranes intermediate between the more basic (p*I* 7–8.5) and the more acidic (p*I* 5.9–6.0, *) species. The isoelectric focusing dimension covers the 4.2 (left)–8.5 (right) pH range.

proteins, a result in agreement with previous reports [11,18].

The CK fractions of highest spec. act. resulting from thiol-Sepharose chromatography (see fig.1) were resolved into two main spots by 2-dimensional gel electrophoresis (fig.4e). This corresponds to the acidic subspecies of ν-proteins, but not to the extremely acidic forms which are extracted from NEM-alkylated membranes by alkaline stripping (fig.4b). The combination of alkylation and the latter treatment therefore enable the separation of different acidic forms of the ν-proteins: The CK-rich fractions remain associated with the membrane, whereas the 5.9–6.1 p*I* subspecies are extracted (fig.4b,d). The ν-protein subspecies displaying CK activity are, however, extracted from native membranes (fig.4a), where they represent less than 5% of the Coomassie Blue staining intensity (fig.4c).

4. DISCUSSION

Creatine kinase has been found in the AChR membranes from *Torpedo marmorata* electric tissue. Purification of this activity is coincident with that of the ν-proteins, conspicuous non-receptor, peripheral membrane proteins. In particular, the purified fractions having the highest CK activity appear to be constituted by subspecies of the acidic forms of the ν-proteins, which also occur in the electrocyte soluble extracts [5]. Their isoelectric values and association with the membranes seem to be intermediate between the more basic ν-protein subspecies (p*I* 7–8) found together with the AChR protein in membranes isolated by affinity partition [5] and the more loosely-bound subspecies found at the acidic end of the p*I*-values (~6). Human MM_2 and X forms of CK also display p*I*-values similar to the ones found here for the AChR membrane-bound form [28].

In mammalian tissues the more acidic forms of CK appear to be associated with the BB-type of isoenzymes [26]. Immune blotting experiments clearly show cross-reactivity of the AChR membrane-associated CK with chicken anti-BB (brain) type of CK. The lack of reactivity of the anti-MM (muscle) type CK antibodies is somewhat surprising, given the embryological similarities between electric tissue and skeletal muscle. The early stages of electrocyte development involve a

myoblast-like period in which spontaneous contractile activity is observed, and the mature electrocyte incorporates nuclei from cells believed to be of myogenic nature throughout its adult life [29]. In adult muscle the MM-type of CK is practically the only one present, but in myogenic cultures the biosynthesis of BB and MB types of CK precede the final installation of the homomeric MM-CK production in the terminally differentiated cells [30,31].

The ν-protein subspecies which we have identified by enzymatic, electrophoretic and immunological criteria as creatine kinase – of the BB type – appears to be a minority constituent of AChR membranes. This may only reflect the loose association of this enzyme with the membrane, as is also evidenced by studies of the ν-proteins and AChR protein by countercurrent distribution and affinity partition techniques [5]. The kinetic properties of the membrane-associated and cytosol CK do not differ, and both appear to share the properties of the MM type of isoenzyme isolated from *Torpedo* muscle.

The present work provides for the first time evidence of a distinct function for one of the ν-proteins. The significance of the presence of an enzymatic function related to the energy metabolism in a membrane-associated subspecies of the ν-proteins remains to be elucidated. One physiologically important process where energy consumption is known to be needed is the internalization step prior to the destruction of ACh receptors (see [32] and references therein). This energy-requiring step mediated by endocytosis is also believed to involve cytoskeletal elements, and is different from the regulation of AChR lysosomal destruction [32]. The close topographical relationship between junctional AChR and ν-proteins, as evidenced by recent cytochemical studies [12], raises the possibility that the differences in turnover rates of junctional and extrajunctional receptors [32] are also reflected at the level of enzymatic functions specifically associated with one of the two types of receptors. Creatine kinase could be one of such AChR-associated entities.

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REFERENCES

[1] Sobel, A., Weber, M. and Changeux, J.P. (1977) Eur. J. Biochem. 80, 215–224.
[2] Neubig, R., Krodel, E.K., Boyd, N.D. and Cohen, J.B. (1979) Proc. Natl. Acad. Sci. USA 76, 690–694.
[3] Hamilton, S.L., McLaughlin, M. and Karlin, A. (1979) Biochemistry 18, 155–163.
[4] Barrantes, F.J., Neugebauer, D.-Ch. and Zingsheim, H.P. (1980) FEBS Lett. 112, 73–78.
[5] Gysin, R., Wirth, M. and Flanagan, S.D. (1981) J. Biol. Chem. 256, 11373–11376.
[6] Sobel, A., Heidmann, T., Hofler, J. and Changeux, J.P. (1978) Proc. Natl. Acad. Sci. USA 75, 510–514.
[7] Elliot, J., Blanchard, S.G., Wu, W., Miller, J., Strader, C.D., Hartig, P., Moore, H.-P. and Raftery, M.A. (1980) Biochem. J. 185, 667–677.
[8] Rousselet, A., Cartaud, J. and Devaux, P.F. (1979) C.R. Hebd. Seances Acad. Sci. Paris 289 D, 461–463.
[9] Lo, M.M.S., Garland, P.B., Lamprecht, J. and Barnard, E.A. (1980) FEBS Lett. 111, 407–412.
[10] Bartholdi, M., Barrantes, F.J. and Jovin, T.M. (1981) Eur. J. Biochem. 120, 389–397.
[11] Barrantes, F.J. (1982) J. Cell Biol. 92, 60–68.
[12] Froehner, S.C., Guldbrandsen, V., Hyman, C., Jeng, A.Y., Neubig, R.R. and Cohen, J.B. (1981) Proc. Natl. Acad. Sci. USA 78, 5230–5234.
[13] Saint-John, Froehner, S.C., Goodenough, A. and Cohen, J.B. (1982) J. Cell Biol. 92, 333–342.
[14] Klymkowsky, M.W., Heuser, J.E. and Stroud, R.M. (1980) J. Cell Biol. 85, 823–838.
[15] Saitoh, T. and Changeux, J.-P. (1980) Eur. J. Biochem. 105, 51–62.
[16] Cartaud, J., Oswald, R., Clement, G. and Changeux, J.-P. (1982) FEBS Lett. 145, 250–257.
[17] Barrantes, F.J. (1982a) in: Neuroreceptors (Hucho, F. ed) pp.315–328, W. de Gruyter, Berlin, New York.

[18] Kuby, S.A., Noda, L. and Lardy, H.A. (1954) J. Biol. Chem. 209, 191–202.
[19] Mandelian, V. and Warren, W.A. (1975) Anal. Biochem. 64, 517–520.
[20] Fisher, S.E. and Whitt, G.S. (1979) Anal. Biochem. 94, 89–95.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[23] Szaz, G., Grauber, W. and Bernt, E. (1976) Clin. Chem. 22, 650–656.
[24] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[25] Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem. 52, 349–353.
[26] Watts, D.C. (1973) in: The Enzymes, vol.8 (Boyer, P.D. ed) pp.383–455, Academic Press, New York.
[27] Zechel, K. and Weber, K. (1978) Eur. J. Biochem. 89, 105–112.
[28] Webers, R.A., Wolters, R.J. and Soons, J.B.J. (1977) Clin. Chim. Acta 78, 271–276.
[29] Richardson, G.P., Krenz, W.D., Kirk, C. and Fox, G.Q. (1981) Neuroscience 6, 1181–1200.
[30] Perriard, J.-C., Caravatti, M., Perriard, E.R. and Eppenberger, H.M. (1978) Arch. Biochem. Biophys. 191, 90–100.
[31] Lough, J. and Bischoff, R. (1977) Dev. Biol. 57, 330–344.
[32] Pamplin, D. and Fambrough, D. (1982) Annu. Rev. Physiol. 44, 319.