Volume 317, number 1,2, 152–156 FEBS 12089 February 1993

Characterisation of wild-type and mutant forms of human monoamine oxidase A and B expressed in a mammalian cell line

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Received 11 December 1992

Monoamine oxidase (MAO)-A and MAO-B are FAD-containing mitochondrial enzymes which catabolize biogenic and xenobiotic amines. The N-terminal regions of both forms of MAO contain an ADP-binding consensus sequence found in several dinucleotide-dependent enzymes, but otherwise show remarkable sequence differences. In order to investigate whether the N-terminal region of MAOs participates in the different catalytic properties and inhibitor specificities exhibited by MAO-A and MAO-B, we constructed chimeric A/B forms and expressed them in a human embryonic kidney cell line (293 cells). The MAO-A chimeric form containing the N-terminus (36 amino acids) of MAO-B and the B chimera having the first 45 amino acid sequence of MAO-A were both catalytically active. Compared to the respective wild-type form, they did not show any significant difference in their catalytic properties (K_m, k_{cat}) towards the substrates tested or in their sensitivity towards inhibitors. This indicates that the N-terminal region of the two isoenzymes is not involved in the different specificities of MAO-A and MAO-B. Substitution of Cys-397 of MAO-B, i.e. the residue covalently anchoring FAD, with an Ala or a His residue resulted in the total loss of enzymatic activity, suggesting that the covalent coupling of FAD to MAO occurs specifically at the -SH group of cysteine.

Monoamine oxidase; FAD-binding domain; Chimeric enzyme; Site-directed mutagenesis; 293 Cell line

1. INTRODUCTION

The mitochondrial monoamine oxidases (MAO, EC 1.4.3.4., flavin-containing), MAO-A and MAO-B, are enzymes involved in the metabolism of several amines including neurotransmitters, trace amines, and xenobiotics. The two isoenzymes are differently localized in various tissues, show different specificity towards substrates, and are selectively blocked by various inhibitors [1]. In humans, the two proteins, encoded by distinct genes [2], exhibit a high degree of sequence identity (about 70%) [3,4]. Comparison of the primary sequences of MAO-A and -B with those of other known proteins revealed little sequence homology except for the N-terminal sequence of both isoenzymes (residues Asp-15 to Glu-43 and Asp-6 to Glu-34 for MAO-A and -B, respectively) which presents an ADP-binding sequence fingerprint found in several NAD- and FAD-dependent enzymes [6]. This region, predicted to fold into a $\beta\alpha\beta$ conformation and which is most often present at the protein N-termini, interacts with the ADP moiety of NAD and FAD. As previously pointed out [7], whereas MAO-A and MAO-B are identical for the amino acids which constitute this dinucleotide-binding site, remarkable sequence differences, with some positions conserved in MAOs of the animal species studied so far, are present in the N-terminal regions of MAO-A and MAO-B. This observation has prompted the hypothesis that these differences in sequence may be at least partly responsible for the specificity of the two isoforms towards various substrates and selective MAO inhibitors [1]. In order to investigate this, we set up an expression system for MAOs in a human cell line and we constructed chimeric A and B forms carrying the N-terminal sequence of the other isoenzyme. In addition, we report on the effect on the enzymatic activity of the substitution of Cys-397 of MAO-B, i.e. the residue covalently anchoring FAD, with an alanine or histidine. These experiments were carried out in order to investigate whether the coupling of FAD in MAO has a strict requirement for cysteine to have enzymatic activity.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{3}H]Ro 41-1049 (spec. act. 30.8 Ci/mmol; 1 Ci = 37 GBq) and [^{3}H]lazabemide ([^{3}H]Ro 19-6327, spec. act. 17 Ci/mmol) were synthesized by Dr. H. Harder (Isotope Synthesis Department, F. Hoffmann-La Roche, Basel). [^{14}C]5-hydroxytryptamine (5-HT, 56 mCi/mmol) and [^{14}C]phenylethylamine (PEA, 50.8 mCi/mmol) were purchased from Amersham (Little Chalfont, UK) and New England Nuclear (Boston, MA, USA), respectively. All other chemicals were of analytical grade and obtained from various sources.

2.2. *Construction of chimera between MAO-A and MAO-B and mutagenesis*

Since the MAO-B cDNA sequence has already a *Sma*I restriction

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site at amino acid position Arg-36 [3], a unique restriction site for endonuclease *Sma*I was introduced into the pCMV-MAO-A cDNA sequence at the corresponding amino acid position Arg-45 by site-directed mutagenesis using an in vitro mutagenesis kit system (Amersham). The presence of the introduced restriction site was proven by restriction analysis of isolated plasmid DNA from randomly selected colonies.

The plasmids pCMV-MAO-A-*Sma*I and pCMV-MAO-B were digested by endonuclease *Sma*I. The resulting C-terminal fragments were gel purified and ligated into the *Sma*I-digested and gel-purified pCMV-MAO-B and -MAO-A vectors, respectively. The chimeric MAO constructs, containing the N-terminal part of the other isoenzyme, were as follows: MAO-(A45)B; MAO-(B36)A (see Fig. 1).

For transfection, the native (wild-type) and hybrid constructs were purified by alkaline lysis and cesium chloride density gradient centrifugation.

2.3. *Engineering of MAO-B Cys-FAD mutations*

A 1,670 bp MAO-B *Eco*RI/*Hin*dIII fragment was subcloned into the vector pCMV. Site-directed mutagenesis was performed by PCR (polymerase chain reaction) with overlapping mismatch primers according to the procedure used by Higuchi et al. [8]. The resulting changes of the amino acids were as follows: Cys-397 → His; Cys-397 → Ala.

The mutagenesis was confirmed by DNA sequence analysis of the entire PCR-fragment with appropriate oligonucleotide primers. The mutant DNAs were digested with *Eco*RI/*Hin*dIII and subcloned into the vector pCMV for expression in the mammalian cells. The vector pCMV was composed of an SV40 origin, the human cytomegalo virus promoter, and the M13 origin of replication which can be used for sequencing and mutagenesis.

2.4. *Expression of constructs in 293 cells*

For transfection, 293 cells (human embryonic kidney cells, ATCC CRL 1573) were seeded in a concentration of 2.5×10^5 cells/ml and grown for 24 h at 37°C under 5% CO_2, in MEM+ (Gibco-BRL, Gaithersburg, MD, USA) with the addition of 20 mM HEPES, 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin. The subconfluent cells were then washed with DMEM (Gibco-BRL) and incubated in the same medium with a mixture of 1 μg of expression vector and 5 μg of Transfectam (IBF Biotechnics, Villeneuve-La Garenne, France) per ml, for 2 h at 37°C under 5% CO_2 [9]. The cells were grown for another 48 h in MEM+ and then collected, washed twice with PBS, pH 7.4, and frozen as a cell pellet.

Transfection was performed with the native MAO-A and -B constructs, both in sense and antisense orientation (mock-transfection) to the promoter, and with the chimera or mutated constructs described above.

2.5. *Enzyme activity determination and radioligand binding*

Aliquots of the transfected cells were thawed and gently homogenized in 0.5 ml of 20 mM Tris, pH 8.0, containing 0.5 mM EGTA and 0.5 mM phenylmethanesulfonyl fluoride. Enzymatic activity was assayed radiochemically as previously described [10]. For kinetic analysis, aliquots (5–20 μl) of the homogenates were incubated (10 min at 37°C) in the presence of various concentrations of radiolabeled substrates. Experimental curves were fitted to Michaelis–Menten kinetics using a computer program (ENZFITTER, Elsevier-Biosoft, Amsterdam, The Netherlands). Inhibition experiments were performed by preincubating (15 min at 37°C) the cell homogenates in the presence of various inhibitors. MAO-A and MAO-B activities were then assayed after the addition of 106 μM [^{14}C]5-HT or 1 μM [^{14}C]PEA, respectively, and by incubating for further 10 min at 37°C.

The amount of wild-type MAO-A and MAO-(B36)A mutant active centers was determined by [^{3}H]Ro 41-1049 binding [11]; [^{3}H]lazabemide was used for titration of wild-type MAO-B and its (A45)B chimera [12]. Radioligand binding saturation curves were analyzed using the LIGAND computer program [13].

Protein content was determined by the Pierce BCA Protein Assay (Rockford, IL, USA) according to the manufacturer's instructions.

2.6. *Immunoblot analysis*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on slab gels (12.5% monomer concentration) according to Lämmli [14] and proteins were blotted on nitrocellulose sheets as previously described [15]. Blots were then incubated overnight with specific monoclonal antibodies against MAO-A (MAO A-6C11-5C7, generously provided by Prof. C.W. Abell, University of Texas, Austin, TX, USA) or against MAO-B (MAO B-1C2 [16], hybridoma cell line obtained from ATCC, HB 8176). Immunoreactive bands were then detected, after incubation with peroxidase-linked sheep anti-mouse antibodies, by the Amersham enhanced chemoluminescence (ECL) detection system.

3. RESULTS

3.1. *Expression of wild-type and chimeric MAO forms in 293 cells*

Untransfected 293 cells showed relatively low levels of endogenous MAO activity which was of the A type. The K_m and V_{max} values for 5-HT were 190 ± 35 μM and 4.90 pmol·s^{-1}·mg protein^{-1}, repectively. The amount of endogenous MAO-A, determined by [^{3}H]Ro 41-1049 binding, was 0.43 ± 0.05 pmol/mg protein. Cells mock- transfected with MAO-A and -B antisense constructs did not show differences in enzymatic activity when compared to untransfected cells.

To investigate the properties of the recombinant wild-type MAO-A and -B and their respective forms with the N-terminal sequence of the other isoenzymes, the corresponding cDNAs were transiently expressed in 293 cells. The kinetic constants of the wild-type and chimeric forms were then determined in the cell homogenates using the substrates [^{14}C]5-HT and [^{14}C]PEA, preferred by MAO-A and MAO-B, respectively. Both the wild-type and chimeric MAOs were found to be expressed in a catalytically active form. The observed kinetic parameters are summarized in Tables I and II. As can be seen, the K_m values of the two substrates for recombinant MAO-A and MAO-B were in the range of those reported in the literature for the natural enzymes (see

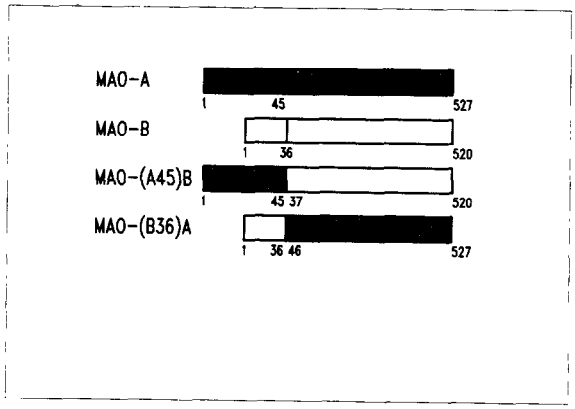

Fig. 1. Diagram of constructs of MAO-A and MAO-B and their A/B chimera. The wild-type and the chimeric forms are schematically summarized: shaded and empty bars corresponds to MAO-A and MAO-B sequences, respectively. The numerals shown below the bars are the position of amino acids in each form.

Table I

Kinetic constants of 5-HT deamination by wild-type MAO-A and by its (B36)A chimer

	K_m (μM)	V_{max} (pmol·s^{-1}·mg prot.)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
MAO-A wild-type	94.0 ± 7.8	52.2 ± 1.8	12.2 ± 0.4	1.3×10^5
MAO-(B36)A	65.2 ± 2.5	52.1 ± 4.5	12.6 ± 1.1	1.9×10^5

Mean ± S.E.M. of 3–4 experiments performed in duplicate.

e.g. [17]). The two A/B chimeric MAO forms did not significantly differ from the respective wild-type enzymes in their affinities for 5-HT and PEA. The same was true for the values k_{cat} (calculated from V_{max}/[MAO], see below) and, consequently, for the apparent second-order rate constants (k_{cat}/K_m), which were virtually identical in the wild-type and the respective chimeric enzymes. In the case of MAO-B and MAO-(A46)B the kinetic properties for 5-HT could not be accurately determined since, after inhibition of endogenous MAO-A with 2 μM Ro 41-1049, the K_m of this substrate for both proteins was >2 mM.

The tritiated selective inhibitors Ro 41-1049 and lazabemide are useful radioligand probes for selectively determining the concentrations of the active centers of MAO-A and MAO-B, respectively [11,12,18]. Analysis of the saturation curve of [^{3}H]Ro 41-1049 binding to MAO-A and MAO-(B36)A and of [^{3}H]lazabemide to MAO-B and MAO-(A45)B did not reveal differences in the apparent K_D values obtained (Table III). In the transfected cells MAO-A and MAO-(B36)A were found to be expressed to a similar extent, whereas the expression of MAO-B was approximately 3 times higher than that of its (A45)B chimera. This accounts for the lower value of V_{max} obtained for PEA deamination by MAO-(A45)B (see Table II).

As shown in Table IV, the potency and selectivity of various compounds in inhibiting the enzymatic activity of the chimeric mutant were not different in comparison to the respective wild-type MAO form.

The expression of the clones was also verified by

Table II

Kinetic constants of PEA deamination by wild-type MAO-B and -A and by their N-terminal chimera

	K_m (μM)	V_{max} (pmol·s^{-1}·mg prot.)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
MAO-B wild type	1.7 ± 1.1	16.6 ± 1.6	2.78 ± 0.27	1.6×10^6
MAO-(A45)B	1.6 ± 0.6	6.7 ± 1.3	3.33 ± 0.40	2.0×10^6
MAO-A wild type	154 ± 12	13.1 ± 1.6	3.07 ± 0.37	2.0×10^5
MAO-(B36)A	115 ± 5	13.3 ± 0.2	3.20 ± 0.23	2.8×10^5

Mean ± S.E.M. of 3–4 experiments performed in duplicate.

Table III

Kinetic parameters of [^{3}H]Ro 41-1049 and [^{3}H]lazabemide binding to 293 cells transfected with wild-type and chimeric MAOs

	[^{3}H]Ro 41-1049		[^{3}H]Lazabemide	
	K_D (nM)	B_{max} (pmol/mg prot.)	K_D (nM)	B_{max} (pmol/mg prot.)
MAO-A wild type	22.8 ± 6.0	4.3 ± 0.2		
MAO-(B36)A	25.7 ± 7.6	4.1 ± 0.2		
MAO-B wild type			17.7 ± 1.3	6.0 ± 0.5
MAO-(A45)B			18.7 ± 2.3	2.0 ± 0.1

Mean ± S.E.M. of 3 experiments in duplicate.

immunoblot analysis. As can be seen in Fig. 2A, the anti-MAO-A monoclonal antibody used recognized, besides the wild-type enzyme, the MAO-(B36)A but not the -(A45)B form, for which the intensity of the immunoreactive band was virtually identical to that observed in untransfected or MAO-B-transfected cells. On the other hand, the anti-MAO-B 1C2 monoclonal antibodies bound to the recombinant wild-type MAO-B, whereas no immunoreactivity was observed in untransfected or MAO-A-transfected cells. Interestingly, the antibody did not recognize the MAO-(A45)B nor the MAO-(B36)A proteins (Fig. 2B). This indicates that the immuno-determinant for the antibody is located at the region of the MAO-B N-terminal sequence where, in the hybrid, the two isoforms were joined together.

3.2. *Site-directed mutagenesis at Cys-397 of MAO-B*

In both MAOs the FAD is covalently attached to the apoproteins through a linkage between a cysteine residue (position 406 and 397 in MAO-A and -B, respectively, and the -CH_3 group in position 8α of FAD [19]. Substitution of Cys-397 of MAO-B with a neutral amino acid, i.e. Ala, resulted in the expression of inactive MAO-B. In several FAD-containing enzymes, the cofactor is covalently attached to the N-3 of a histidine residue (see e.g. [20] and examples cited therein). Therefore, we also investigated the effect of substituting the flavin-modified cysteine of MAO-B with histidine. Also in this case the expressed protein was catalytically inactive. By immunoblotting, both the site-directed mutated enzymes were recognized by the anti-MAO-B monoclonal antibody (Fig. 2B, lanes 6 and 7).

4. DISCUSSION

In the present study we have investigated some aspects of the structure-activity relationships of the FAD binding sites of MAOs. At least two regions of the isoenzymes appear to be responsible for the binding of the cofactor: a non-covalent one, corresponding to the ADP-binding $\beta\alpha\beta$ motif located at the N-terminal re-

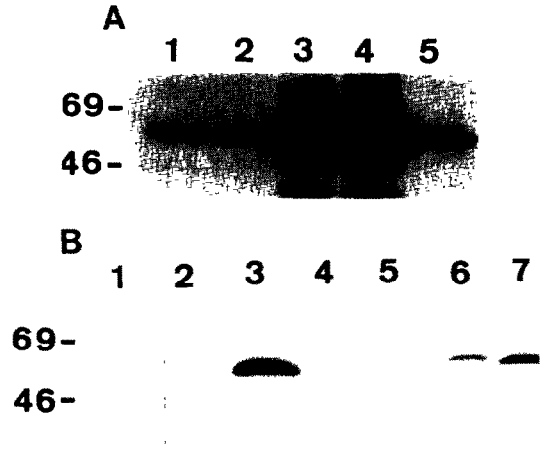

Fig. 2. Immunoblot analysis of 293 cells transfected with the different constructs. (A) Anti-MAO-A antibodies: lane 1, wild-type MAO-B; 2, MAO-(A45)B; 3, wild-type MAO-A; 4, MAO-(B36)A; 5, untransfected cells. (B) Anti-MAO-B antibodies: lane 1, wild-type MAO-A; 2, MAO-(B36)A; 3, wild-type MAO-B; 4, MAO-(A45)B; 5, untransfected cells; 6, MAO-B Cys-397 → Ala; 7, MAO-B Cys-397 → His.

gion [3]; a second, covalent one corresponding to a cysteine residue, to which FAD is bound through a thioether bridge [19], and located towards the C-terminal of the proteins.

The results obtained by constructing N-terminal chimeras of the two proteins indicate that the differences in the amino acid sequences existing within the ADP binding motif of MAO-A and MAO-B do not appear to play a role in the different specificity of the two isoenzymes for substrates and inhibitors. Therefore this domain might mainly play a structural role as a nucleation center [5,21] and for stabilizing the proper enzyme conformation. On the other hand, if amino acids participating in the catalytic mechanism are present, they are likely identical and share the same position within the A and B form. The production of site-directed mutated forms in this region will further help in clarifying the structure-function relationships of the non-covalent dinucleotide-binding domain of MAOs. In addition, our experiments indicate that the 9 amino acid extension at the N-terminus of MAO-A, with respect to MAO-B [3], does not appear to influence the substrate selectivity. Therefore, other sequences have to be searched for, possibly among the less conserved ones, in order to determine the regions of the two enzymes that are responsible for the different topochemistry of their active sites.

Regarding the covalent binding site for FAD, little is known about the coupling mechanism and whether this occurs as a co-translational or post-translational process. Autoflavination has been claimed to be responsible for the covalent attachment of FAD to MAOs [20]. The fact that the MAO-B enzymatic activity is lost upon substitution of cysteine at position 397 with an alanine is clearly an evidence for the requirement for a covalent FAD linkage with the apoprotein. In addition, the requirement for a cysteine is further stressed by the fact that this residue cannot be replaced by a histidine, the residue to which FAD is found to be covalently linked in other flavoproteins [20]. Whether the loss in activity is due to the fact that in MAO-B the FAD cannot be covalently coupled to residues other than cysteine or, alternatively, if this mutant still has covalently bound FAD, but the mutation markedly perturbs the protein conformation, cannot be assessed from the present experiments. Expression of the mutant protein and its purification in high amounts would be in fact mandatory to assess whether covalent FAD is still present.

Acknowledgements: The authors express their gratitude to Prof. C.W. Abell and to Dr. S.W. Kwan (University of Texas, Austin, TX, USA) for the generous donation of antibodies and helpful discussion; Prof. W. Haefely, Dr. J.G. Richards and Dr. P. Schoch (F. Hoffmann-La Roche, Basel) for critical reading of the manuscript; and Mrs. M. Wdonwicki for invaluable secretarial work.

Table IV

Inhibition of 5-HT and PEA deamination by various inhibitors in 293 cells transfected with wild-type and chimeric MAOs

	IC_{50} (M)			
	5-HT		PEA	
	MAO-A wild type	MAO-(B36)A	MAO-B wild type	MAO-(A45)B
Ro 19-6327	$>10^{-5}$	$>10^{-5}$	$6.9 \pm 2.7 \times 10^{-9}$	$4.7 \pm 2.0 \times 10^{-9}$
Deprenyl	$2.3 \pm 1.0 \times 10^{-6}$	$1.3 \pm 0.5 \times 10^{-6}$	$6.2 \pm 2.1 \times 10^{-9}$	$4.5 \pm 1.7 \times 10^{-9}$
Ro 41-1049	$2.7 \pm 0.4 \times 10^{-8}$	$3.3 \pm 0.3 \times 10^{-8}$	$>10^{-5}$	$>10^{-5}$
Harmaline	$3.7 \pm 1.5 \times 10^{-9}$	$1.5 \pm 0.7 \times 10^{-9}$	$>10^{-5}$	$>10^{-5}$

Cell homogenates were preincubated (15 min at 37°C) in the presence of 9 different concentrations of various inhibitors. MAO-A and MAO-B activities were then determined by incubation (10 min at 37°C) in the presence of 106 μM [^{14}C]5-HT and 1 μM [^{14}C]PEA, respectively. Values are the mean ± S.E.M. of 2–3 experiments performed in duplicate.

REFERENCES

[1] Cesura, A.M. and Pletscher, A. (1992) Prog. Drug Res. 38, 171–297.
[2] Grimsby, J., Chen, K., Wang, L.-J., Lan, N.C. and Shih, J.C. (1991) Proc. Natl. Acad. Sci. USA 88, 3637–3641.
[3] Bach, A.J.W., Lan, N.C., Johnson, D.L., Abell, C.W., Bembenek, M.E., Kwan, S.W., Seeburg, P. and Shih, J.C. (1988) Proc. Natl. Acad. Sci. USA 85, 4934–4938.
[4] Weyler, W., Hsu, Y.-P.P. and Breakefield, X.O. (1990) Pharm. Ther. 47, 391–417.
[5] Wierenga, R.K., Terpstra, P. and Hol, W.G.J. (1986) J. Mol. Biol. 187, 101–107.
[6] Branden, C. and Tooze, J. (1991) Introduction to Protein Structure, Garland, New York, London.
[7] Powell, J.F. (1991) Biochem. Soc. Trans. 19, 199–201.
[8] Higuchi, R., Krummel, B. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[9] Loeffler, J., Barthel, F., Feltu, P., Behr, J.P., Sassone-Corsi, P. and Feltz, A. (1990) J. Neurochem. 54, 1812–1815.
[10] Da Prada, M., Kettler, R., Keller, H.H., Burkard, W.P., Muggli-Maniglio, D. and Haefely, W.E. (1989) J. Pharm. Exp. Ther. 248, 400–414.
[11] Cesura, A.M., Bös, M., Galva, M.D., Imhof, R. and Da Prada, M. (1990) Molec. Pharmacol. 37, 358–366.
[12] Cesura, A.M., Galva, M.D., Imhof, R., Kyburz, E., Picotti, G.B. and Da Prada, M. (1989) Eur. J. Pharmacol. 162, 457–465.
[13] McPherson, G.A. (1985) J. Pharmacol. Methods 14, 213–228.
[14] Lämmli, U.K. (1970) Nature 227, 680–685.
[15] Towbin, H., Staehelin, H. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[16] Denney, R.M., Denney, C.B., Fritz, R.R. and Abell, C.W. (1982) Mol. Pharmacol. 22, 500–508.
[17] Tipton, K.F., O'Carroll, A.-M. and McCrodden, J.M. (1987) J. Neural Transm. (Suppl.) 23, 25–35.
[18] Saura, J., Kettler, R., Da Prada, M. and Richards, J.G. (1992) J. Neurosci. 12, 1977–1999.
[19] Kearney, E.B., Salach, J.I., Walker, W.H., Seng, R.L., Kenney, W., Zeszotek, E. and Singer, T.P. (1971) Eur. J. Biochem. 24, 321–327.
[20] Birch-Machin, M.A., Farnsworth, L., Ackrell, B.A.C., Cochran, B., Jackson, S., Bindoff, L.A., Aitken, A., Diamond, A.G. and Turnbull, D.M. (1992) J. Biol. Chem. 267, 11553–11558.
[21] Schulz, G.E. and Schirmer, R.H. (1979) in: Principle of Protein Structure, pp. 128–129, Springer, New York.
[22] Weyler, W., Titlow, C.C. and Salach, J.I. (1990) Biochem. Biophys. Res. Commun. 173, 1205–1211.