Identification of a mitochondrial form of kynurenine aminotransferase/glutamine transaminase K from rat brain

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Abstract A soluble aminotransferase with kynurenine aminotransferase (KAT) activity has been recently isolated from rat brain. This enzyme corresponds to a cytosolic form of glutamine transaminase K (GTK). In addition to the cytosolic enzyme, a mitochondrial-associated form of this KAT/GTK also exists. In the present work we have isolated a rat brain cDNA clone encoding a KAT/GTK enzyme identical to the soluble form but carrying an additional stretch of 32 amino acids at its NH_2-terminus. Several structural features of this sequence resemble those of leader peptides for mitochondrial import. Evidence that the isolated cDNA encoded for mitochondrial KAT/GTK was obtained after transfection of HEK-293 cells with the cDNA coding for this new KAT/GTK isoenzyme. In fact, a significant enrichment of both KAT and GTK enzymatic activities was found in the crude mitochondrial fraction of the transfected cells.

Key words: Kynurenine aminotransferase; Glutamine transaminase K; Cysteine-*S*-conjugate β-lyase; Signal peptide; Kynurenic acid; Rat brain

1. Introduction

Kynurenine aminotransferase (KAT; EC 2.6.1.7) catalyses the irreversible transamination of the L-tryptophan metabolite L-kynurenine to form kynurenic acid. Due to the postulated role of kynurenic acid as a putative endogenous modulator of glutamatergic neurotransmission (see [1] for review), particular attention has recently been devoted to the presence of KAT isoenzymes in cerebral tissues [2]. Kynurenic acid is, in fact, an antagonist at the glycine site of *N*-methyl-D-aspartate receptors, and increased levels of kynurenic acid may exert a neuroprotective action in some pathological conditions [3]. Since several pyridoxal-5′-phosphate (PLP)-dependent aminotransferases with broad substrate specificities are apparently able to catalyse this reaction, the identification of the isoenzymes responsible for the biosynthesis of cerebral kynurenic acid is of importance for claryfying the role of this metabolic pathway in pathophysiology. A cDNA clone from rat brain encoding a soluble enzyme with KAT activity has been recently isolated [4] and it has been found that this protein corresponds to a cytosolic form of rat kidney glutamine transaminase K (GTK; EC 2.6.1.14) [5,6]. This aminotransferase form displays substantial cysteine-*S*-conjugate β-lyase activity [5,7] and has also been referred to under this nomenclature. Due to its β-lyasic activity, this enzyme may be involved in the renal and cerebral toxicity of several halogenated xenobiotics [8]. The human form of kidney cytosolic cysteine-*S*-conjugate β-lyase/GTK has been recently cloned and shown to have 82% amino acid similarity to the rat form [9].

In addition to the soluble cytosolic enzyme (S-KAT/GTK), the existence of a mitochondrial KAT/GTK located in the matrix of mitochondria [10] has also been reported. Interestingly, mitochondrial GTK activity appears to be predominantly in rat brain, whereas the opposite is true for peripheral organs such as kidney [11]. In the present work, we report the isolation of a rat brain cDNA clone encoding a mitochondrial-associated form of KAT/GTK (M-KAT/GTK).

2. Materials and methods

2.1. Preparation of poly(A)$^+$ RNA

Total RNA was extracted from rat cerebellum, and rat primary cortical neuronal and astrocytic cultures by the guanidinium isothiocyanate/cesium chloride method [12]. Poly(A)$^+$ RNA was obtained by two purification cycles on oligo(dT)-cellulose spun columns (Pharmacia Biotech).

2.2. RT-PCR and Southern blot

Reverse transcription (RT) reactions were carried out in 10 mM Tric-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 mM dTTP, 20 units of RNase inhibitor, 15 pmol of antisense primer (PCR II; 5′-ATAGCTG AGGCCTGTGTTCGA-GGTG-3′), 1 μg of poly(A)$^+$ RNA, and 50 units of cloned Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer) in a final volume of 20 μl at 42°C for 60 min. PCR was performed in a final volume of 100 μl containing 20 μl of RT reaction, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 15 pmol of sense primer (PCR I; 5′-CTA-TTGGACGGAGCAGCGCAATTGT-3′), 1 unit of perfect match Polymerase Enhancer (Stratagene), and 2.5 units of Ampli Taq DNA polymerase (Perkin-Elmer). Cycling was performed at 95°C (1 min), 60°C (1 min), and 72°C (1 min) for 35 cycles. The final cycle had an extension time of 10 min. Ten percent of the PCR products were electrophoresed in 1% agarose gel and alkali-blotted onto zeta membrane (Bio-Rad). The membrane was hybridized with 5′-end ^{32}P-labeled probe (5′-AGGAGCAGGGCTCGGCTTCAGAGTCACGAA-3′). The membranes were rinsed twice, for 1 h each time, with 2 × SSC (300 mM NaCl, 30 mM Na citrate), containing 0.1% SDS, at 50°C.

2.3. Cloning and sequencing of the RT-PCR products

The final PCR product was extracted with phenol/chloroform and then precipitated with ethanol. PCR products were electrophoresed on 1% low-melting agarose gel, and the major band was cut from the gel and subcloned into the blunt-ended *Bam*HI site of the expression vector pBC/CMV. Double-stranded sequencing was performed using primers derived from rat β-lyase cDNA sequence and the Δth DNA polymerase sequencing kit from Toyobo (Japan). All oligonucleotide primers were synthesized by Genosys Biotechnologies Inc.

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Abbreviations: KAT, kynurenine aminotransferase; GTK, glutamine transaminase K; RT, reverse transcription; PCR, polymerase chain reaction.

The nucleotide sequence of KAT/GTK has been deposited into the EMBL data bank under accession no. Z49696.

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2.4. Expression of recombinant KAT/GTK in HEK-293 cells

The cDNA inserts encoding both forms of KAT/GTK were subcloned into the blunt-ended *Bam*HI site of the expression vector pBC/CMV [13], placing transcription of the cDNA under control of the strong immediate early promoter of human cytomegalovirus. Human embryonic kidney fibroblast cells (HEK-293 cell line, ATCC CRL 1573) were transfected as described previously [14]. Two days after transfection, the cells were harvested and washed twice with phosphate-buffered saline and stored at −80°C until analysis.

Subcellular fractionation of the transfected cells was performed by differential centrifugation. The transfected cells were homogenised in 0.28 M sucrose buffered to pH 8.0 with 10 mM Tris-acetate buffer (containing 10 mM 2-mercaptoethanol, 0.5 mM phenylmethansulfonyl fluoride, 50 μM PLP) by means of a glass–teflon homogeniser. Cell nuclei were sedimented by centrifuging the cell lysate at 800 × *g* for 20 min at 4°C and a crude mitochondrial fraction was obtained after centrifugation of the post-nuclear supernatant at 10,000 × *g*. The resulting supernatant was then centrifuged at 100,000 × *g* for 45 min at 4°C to separate microsomes from the cell soluble fraction. The pellets from the various centrifugation steps were resuspended in homogenisation buffer (without sucrose) by means of a Polytron (Kinematica AG) homogeniser.

2.5. Determination of KAT and GTK activity

KAT activity was assayed as previously described [4] by incubating (1 h at 37°C) aliquots of the cell homogenates and crude subcellular fractions in the presence of 5 mM L-kynurenine and 2 mM pyruvate in a final volume of 200 μl of 150 mM Tris-acetate buffer, pH 8.0, containing 70 μM PLP. Kynurenic acid formed was quantified by HPLC with spectrophotometric detection at 330 nm [4].

GTK activity was determined as previously described [15] with minor modifications. Briefly, aliquots of the enzyme preparations from the transfected cells were incubated with 10 mM L-phenylalanine and 5 mM of pyruvate in a final volume of 100 μl of 150 mM Tris-acetate buffer, pH 8.0. After incubation at 37°C for 20 min, 0.9 ml of 3.3 M NaOH was added and phenylpyruvate formed was measured spectrophotometrically at 322 nm.

Activity of the mitochondrial marker monoamine oxidase-A was assayed as described in [16]. Protein content was measured by using the Pierce Coomassie Plus assay kit according to the manufacturer's instructions.

3. Results

3.1. Isolation of cDNA encoding membrane-associated form of rat brain kynurenine aminotransferase

In the attempt to obtain a cDNA clone for the rat brain KAT/GTK, oligonucleotide primers (PCR I, PCR II) derived from the rat kidney cysteine-*S*-conjugate β-lyase cDNA [5] (GenBank/EMBL accession number S61960) were used to amplify cDNA from rat total brain Poly(A)$^+$ RNA by PCR. PCR products were subcloned and sequenced, and the majority of the clones were found to be identical to those of rat cytosolic KAT/GTK, as reported in [4]. Among these clones, there were several which generated a larger size fragment when digested with *Eco*RI. One of these larger clones was sequenced completely, and its nucleotide and deduced amino acid sequence (Fig. 1) was found to be identical to that of the rat KAT/GTK mentioned above, except for an insertion of 208 base pairs (bp) in the 5′-untranslated region. This insertion created a new initiation codon (ATG) and encoded a NH_2-terminal stretch of 32 amino acids in frame with the previous open reading frame of rat S-KAT/GTK [4–6]. The amino acid sequence deduced from this longer rat brain cDNA clone consisted of 455 amino acids with a predicted molecular mass of 51,649 Da. Analysis of the inserted NH_2-terminal amino acid sequence revealed that this peptide had the structural features of a putative leader sequence for mitochondrial import [17,18]: (i) positively charged amino

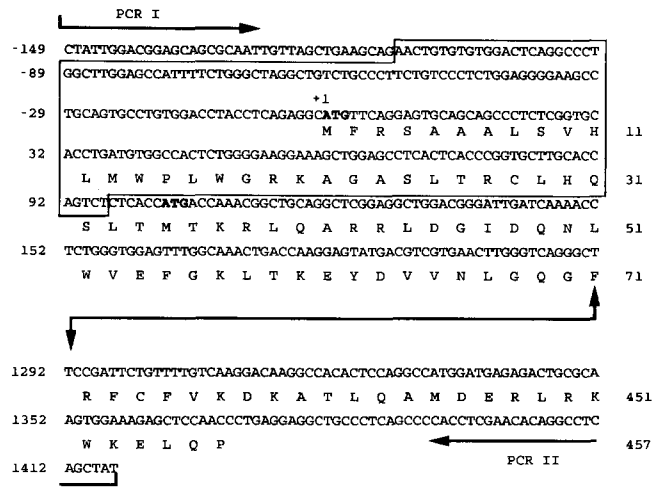

Fig. 1. Nucleotide and deduced amino acid sequences of the M-KAT/GTK cDNA clone. The 208 bp/32 amino acid insertion sequence is boxed. The two in-frame initiation codons (ATG) for M- and S-KAT/GTK are shown in bold characters. The nucleotide sequence is numbered from the codon for initiator methionine (+1) for M-KAT/GTK and indicated at the left of each line. The amino acid numbers are indicated at the right of each line. The two oligonucleotides (PCR I and PCR II) which were used as primers in RT-PCR of rat brain Poly(A)$^+$ RNA are underlined with arrows. The central part of the sequence is not shown in the figure.

acids are distributed along this sequence (predicted isoelectric point, 12.2); (ii) the peptide is enriched in Ala (15.6%), Leu (15.6%), hydroxylated amino acids (Ser+Thr, 15.6%) and Arg (9.3%); (iii) it lacks acidic amino acid residues; and (iv) contains an hydrophobic core. Its NH_2-terminal domain has the potential to fold into a moderately amphiphilic α-helix similar to that of the leader peptide of mitochondrial aspartate aminotransferase from both human and rat. These characteristics strongly suggested that the newly isolated cDNA may encode the mitochondrial-associated form of KAT/GTK (M-KAT/GTK).

To investigate the relative abundance of mRNAs encoding the two forms of KAT/GTK in rat brain, PCR was carried out with template cDNAs constructed with Poly(A)$^+$ RNA from rat primary cortical neuronal culture, rat primary astrocytic cultures and rat cerebellum and with PCR I and PCR II primers. The gel-resolved DNA products (Fig. 2A) contained two DNA fragments of 1565 and 1357 bp in length (M-KAT/GTK and S-KAT/GTK), consistent with the two known rat KAT/GTK mRNA species. To demonstrate that the RT-PCR generated DNA fragments corresponded to the two KAT/GTK cDNAs, the gel shown in Fig. 2A was blotted onto a nylon membrane and hybridized with a 5′-end ^{32}P-labeled probe chosen from the internal region (nucleotides 664–693) of the sequence for rat kidney β-lyase reported in [5]. As seen in Fig. 2B, both DNA fragments hybridized with this probe. Judging from the amount of PCR-product from the three tissues examined, the mRNAs encoding both forms appeared to be expressed with approximately equal abundance in rat cerebellum, while the shorter (soluble) form (S-KAT/GTK) was predominant in primary cultures of cortical neurons and astrocytes.

When Poly(A)$^+$ RNAs isolated from rat cerebellum and primary neuronal and astrocytic cultures were subjected to Northern blot analysis using the ^{32}P-labeled nick-translated S-KAT/GTK cDNA (see [4]), a major band of 2.1 kb was detected (not

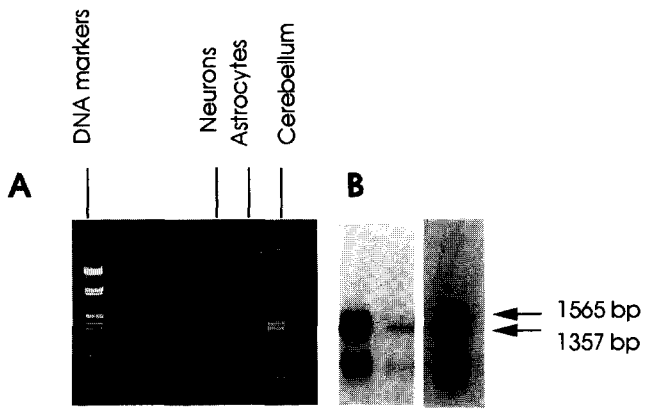

Fig. 2. RT-PCR analysis of Poly(A)$^+$ RNA from rat brain primary cortical neurons and astrocytes and from cerebellum. Poly(A)$^+$ RNA was reverse transcribed, amplified and electrophoresed as described in section 2. The agarose gel was then blotted onto a nylon filter and hybridized with a 5′-end ^{32}P-labeled probe derived from KAT/GTK cDNA [5]. (A) Agarose gel electrophoresis of RT-PCR reactions after ethidium bromide staining. (B) Hybridization with the 5′-end ^{32}P-labeled KAT/GTK probe (nucleotides 664–693 of the sequence of rat kidney cysteine-*S*-conjugate β-lyase; see [5]). The order of the samples is as in (A). The arrows point to the M-KAT/GTK and S-KAT/GTK cDNA fragments containing the rat KAT/GTK mitochondrial and soluble proteins, respectively. DNA markers are *Hin*dIII + *Eco*RI-digested λDNA. The blots for cortical primary astrocytes and neurons were exposed for 45 min and 45 min, cerebellum for 10 min.

shown). The labeling intensity correlated well with the amount of transcript detected by RT-PCR in cerebellum and the two primary cell lines. Unfortunately, the resolution of Northern blot analysis was not sufficient to differentiate between the two mRNA forms.

3.2. *Expression and subcellular localization of the two KAT/GTK isoforms*

To investigate the enzymatic properties of the two KAT/GTK isoforms, cDNAs for both the putative mitochondrial-associated and the soluble isoenzymes, were subcloned into the eukaryotic expression vector pBC/CMV, which initiates transcription from the CMV promoter [13]. These plasmids (named pBC/CMV-M-KAT/GTK, pBC/CMV-S-KAT/GTK) were transiently transfected into the HEK-293 cells, a cell line which does not show any detectable endogenous KAT and GTK activity. Functional expression of the two KAT/GTK isoforms was initially verified by KAT and GTK activity measurements in the soluble and membrane fractions from cell homogenates. After transfection with the M-KAT/GTK construct, most of the enzymatic activity was recovered in the cell membrane fraction, whereas, in cells transfected with the S-KAT/GTK construct, activity was found in the soluble fraction.

To obtain more information regarding the subcellular distribution of the two KAT/GTK forms expressed in HEK-293 cells, homogenates from cells transfected with the two plasmids (pBC/CMV-M-KAT and pBC/CMV-S-KAT) were subjected to differential centrifugation in order to obtain crude subcellular fractions of the transfected cells. As shown in Fig. 3A, a substantial enrichment in both KAT and GTK specific activity was observed in the crude mitochondrial fraction of cells transfected with the M-KAT/GTK construct. The pattern of monoamine oxidase-A activity (a mitochondrial enzyme) observed in the various cell fractions was essentially similar (not shown), supporting a predominant mitochondrial localisation for M-KAT/GTK. Conversely, cells transfected with the construct encoding the S-form of KAT/GTK exhibited most of the activity in the soluble cell fraction (Fig. 3B).

No major differences were observed in the K_m values of L-kynurenine for either KAT/GTK isoforms when assayed for KAT activity using two different co-substrates. Similar to what has been previosly reported for the cytosolic enzyme [4], the K_m value of L-kynurenine for M-KAT/GTK was lower in the presence of 2-oxoglutarate than with pyruvate, but the V_{max} value was higher in the presence of the latter aminoacceptor (Table 1). However, the ~2.5-fold higher V_{max} value observed for M-KAT/GTK in comparison to the S-form in the presence of 2-oxoglutarate may suggest the existence of some differences in the catalytic properties of the two enzyme forms (see also [10]).

4. Discussion

In the present work we have isolated a rat brain cDNA clone encoding an organelle-associated KAT/GTK (β-lyase) enzyme. Several pieces of evidence suggest that this cDNA clone codes

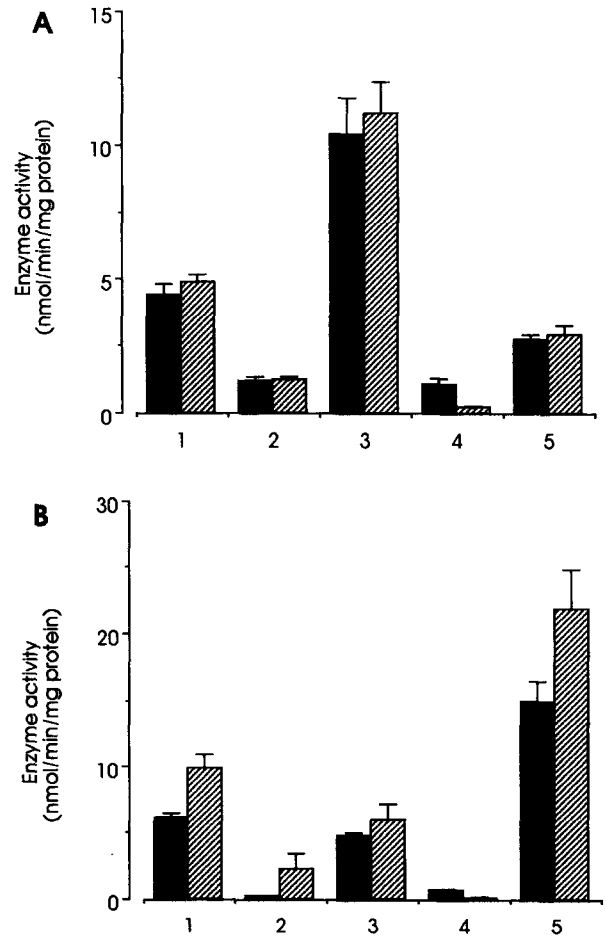

Fig. 3. Determination of KAT (solid bars) and GTK (hatched bars) specific activities in homogenates and subcellular fractions of HEK-293 cells transiently transfected with the pBC/CMV-M-KAT/GTK (panel A) and pBC/CMV-S-KAT/GTK (panel B) constructs. 1 = cell homogenate; 2 = nuclear pellet; 3 = mitochondrial fraction; 4 =microsomes; 5 = soluble fraction.

Table 1
Comparison of KAT kinetic properties of the M- and S-form of KAT/GTK recombinantly expressed in HEK-293 cells

Co-substrate	M-KAT/GTK		S-KAT/GTK[a]	
	K_m	V_{max}	K_m	V_{max}
Pyruvate	1630 ± 210	10.8 ± 0.58	1200 ± 300	11.4 ± 0.9
2-oxoglutarate	96.2 ± 26.1	0.39 ± 0.05	86.3 ± 3.4	0.147 ± 0.03

KAT activity of M-KAT/GTK and S-KAT/GTK was measured in the crude mitochondrial and soluble fractions, respectively, of the transfected cells. Nine different concentrations of L-kynurenine were used. The co-substrate concentration was 2 mM. Kinetic constants were calculated by fitting experimental data to Michaelis-Menten equation using the Ultrafit (Biosoft) computer program. K_m values are in μM, V_{max} in nmol/min/mg protein.
[a] Data taken from [4].

for the mitochondrial form of the enzyme. Experiments using crude subcellular fractions of HEK-293 cells transfected with the construct containing the longer KAT/GTK cDNA (pBC/CMV-M-KAT), showed that the majority of the KAT/GTK activity was recovered in the mitochondrial fractions. The mitochondrial enzyme responsible for rat kidney cysteine-*S*-conjugate β-lyase/GTK activity in the mitochondria has been shown to located in the organelle matrix [10], similar to other aminotransferases such as aspartate aminotransferase and serine-pyruvate aminotransferase. Since the purified mitochondrial protein has been reported to have a molecular mass very close to that of its cytosolic counterpart [10], it is likely that the NH_2-terminus of M-KAT/GTK represents a leader sequence which is cleaved after or during import of the protein into the mitochondria. Although mitochondrial targeting peptides do not present highly conserved sequences [17], the NH_2-terminal sequence encoded by the isolated M-KAT/GTK cDNA clone has several features in common with others leader peptides for mitochondrial import.

No information is presently available regarding the putative cleavage site in M-KAT/GTK. Whereas most cleavable mitochondrial leader peptides are removed in a single step by the mitochondrial processing peptidase (MPP) [19], others are cleaved in two sequential steps by two different matrix proteases ([20], and citations therein). If the signal peptide of M-KAT/GTK is removed in a single step, we predict that cleavage may occur at residue Cys^{28}, thus placing an Arg residue at position −2, as is often observed for MPP cleavage sites [20]. In the case of a two-step processing, MPP may instead cleave the leader peptide at Lys^{20}. Formation of the mature protein might then occur after removal of the octapeptide Ala^{21}–Cys^{28} of M-KAT/GTK by mitochondrial intermediate peptidase (MIP). This octapeptide does, in fact, contain the requirements for MIP processing, i.e. a hydrophobic residue at position −8 (Ala) relative to the NH_2-terminus of the mature protein, and serine at position −5 [20]. These hypotheses, however, await experimental evidence.

Regarding the origins of the mitochondrial and cytosolic forms of KAT/GTK, it has been reported that the KAT/GTK gene is unique and no evidence was obtained for a related DNA sequence coding for the mitochondrial form [5]. The two forms may, therefore, derive from initiation of transcription at distinct initiation codons or after alternative splicing of precursor RNA. The first hypothesis seems rather unlikely since two distinct mRNA species were detected by RT-PCR. Therefore, as proposed in [5], alternative splicing of a single gene product may be responsible for the production of the M- and S-form of KAT/GTK. A second in-frame ATG codon is present at position 37 (Fig. 1). Whether this may represent an alternative initiation site remains to be elucidated.

Regarding the distribution of the two forms in the rat brain, KAT immunoreactivity has been detected mainly in astrocytes but also in neurons [21]; our preliminary results (see Fig. 2) indicate that the mRNA for S-KAT/GTK is more abundant in rat cortical neurons and astroglial cells, whereas in cerebellum the two transcripts appear to be present in equal amounts. Detailed studies on the regulation of the expression of the two enzyme forms and on their different regional and cellular distribution in brain will be instrumental for clarifying their role in the neuropathophysiology of the kynurenine metabolic pathway.

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References

[1] Stone, T.W. (1993) Pharmacol. Rev. 45, 309–379.
[2] Okuno, E., Schmidt, W., Parks, D.A., Nakamura, M. and Schwarcz, R. (1991) J. Neurochem. 57, 533–540.
[3] Carpenedo, R., Chiarugi, A., Russi, P., Lombardi, G., Carlà, V., Pellicciari, R., Mattoli, L. and Moroni, F. (1994) Neuroscience 61, 237–244.
[4] Alberati-Giani, D., Malherbe, P., Köhler, C., Lang, G., Kiefer, V. and Cesura, A.M. (1995) J. Neurochem. 64, 1448–1455.
[5] Perry, S.J., Schofield, M.A., MacFarlane, M., Lock, E.A., King, L.J., Gibson, G. and Goldfarb, P.S. (1993) Mol. Pharmacol. 43, 660–665.
[6] Mosca, M., Cozzi, L., Breton, J., Speciale, C., Okuno, E., Schwarcz, R. and Benatti, L. (1994) FEBS Lett. 353, 21–24.
[7] Stevens, J.L., Robbins, J.D. and Byrd, R.A. (1986) J. Biol. Chem. 261, 15529–15537.
[8] Cooper, A.J.L. (1994) Adv. Pharmacol. 27, 71–113.
[9] Perry, S., Harries, H., Scholfield, C., Lock, T., King, L., Gibsom, G. and Goldfarb, P. (1995) FEBS Lett. 360, 277–280.
[10] Stevens, J.L., Ayoubi, N. and Robbins, J.D. (1988) J Biol. Chem. 263, 3395–3401.
[11] Cooper, A.J., Abraham, D.G., Gelbard, A.S., Lai J.C.K. and Petito, C.K. (1993) J. Neurochem. 61, 1731–1741.
[12] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[13] Bertocci, B., Miggiano, V., Da Prada, M., Dembic, Z., Lahm, H.-W. and Malherbe, P. (1991) Proc. Natl. Acad. Sci. USA 88, 1416–1420.
[14] Malherbe, P., Köhler, C., Da Prada, M., Lang, G., Kiefer, V., Schwarcz, R., Lahm, H.-W and Cesura, A.M. (1994) J. Biol. Chem. 269, 13792–13797.
[15] Cooper, A.J.L. and Meister, A. (1985) Methods Enzymol. 113, 344–349.
[16] Gottowik, J., Cesura, A.M., Malherbe, P., Lang, G. and Da Prada, M. (1993) FEBS Lett. 317, 152–156.
[17] von Heijne, G., Steppuhn, J. and Herrmann, G (1989) Eur. J. Biochem. 180, 535–545.
[18] Hartl., F.-U., Pfanner, N., Nicholson, D.W. and Neupert, W. (1989) Biochem. Biophys. Acta 988, 1–45.
[19] Arretz, M., Scheider, H., Guiard, B., Brunner, M. and Neupert, W. (1994) J. Biol. Chem. 269, 4959–4967.
[20] Isaya, G., Klousek, F., Fenton, W.A. and Rosemberg, L.E. (1991) J. Cell Biol. 113, 65–76.
[21] Du, F., Schmidt, W., Okuno, E., Kido, R., Köhler, C. and Schwarcz, R. (1992) J. Comp. Neurol. 321, 1–11.