Molecular Identification of Carnosine Synthase as ATP-grasp Domain-containing Protein 1 (ATPGD1)*§

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Jakub Drozak1, Maria Veiga-da-Cunha2, Didier Vertommen3, Vincent Stroobant4, and Emile Van Schaftingen4

From the 1Laboratory of Physiological Chemistry, the 2Hormone and Metabolic Research Unit, and the 4Ludwig Institute for Cancer Research, de Duve Institute, Université Catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium

Carnosine (β-alanyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) are abundant dipeptides in skeletal muscle and brain of most vertebrates and some invertebrates. The formation of both compounds is catalyzed by carnosine synthase, which is thought to convert ATP to AMP and inorganic pyrophosphate, and whose molecular identity is unknown. In the present work, we have purified carnosine synthase from chicken pectoral muscle about 1500-fold until only two major polypeptides of 100 and 90 kDa were present in the preparation. Mass spectrometry analysis of these polypeptides did not yield any meaningful candidate. Carnosine formation catalyzed by the purified enzyme was accompanied by a stoichiometric formation, not of AMP, but of ADP, suggesting that carnosine synthase belongs to the “ATP-grasp family” of ligases. A data base mining approach identified ATPGD1 as a likely candidate. As this protein was absent from chicken protein data bases, we reconstituted its sequence from a PCR-amplified cDNA and found it to fit with the 100-kDa polypeptide of the chicken carnosine synthase preparation. Mouse and human ATPGD1 were expressed in HEK293T cells, purified to homogeneity, and shown to catalyze the formation of carnosine, as confirmed by mass spectrometry, and of homocarnosine. Specificity studies carried out on all three enzymes were in agreement with published data. In particular, they acted with 15–25-fold higher catalytic efficiencies on β-alanine than on γ-aminobutyrate. The identification of the gene encoding carnosine synthase will help for a better understanding of the biological functions of carnosine and related dipeptides, which still remain largely unknown.

Carnosine, a dipeptide consisting of β-alanine and L-histidine, was extracted for the first time from Liebig’s meat extract in 1900 to become the first ever peptide isolated from animal tissue (1). Carnosine is the best known representative of a series of imidazole dipeptides such as homocarnosine (γ-aminobutyryl-L-histidine), anserine (β-alanyl-N-π-methyl-L-histidine), and balenine (β-alanyl-N-τ-methyl-L-histidine), which have long been reported to be present at high concentrations in skeletal muscle and the central nervous system of vertebrates.

The β-alanyl containing peptides are mainly found in skeletal muscle, whereas the γ-aminobutyryl containing peptides are typical of the central nervous system, probably due to the availability of its precursor γ-aminobutyric acid in this tissue. The concentration of carnosine in skeletal muscle of vertebrates varies according to the species from 0.6 mM (mouse) up to 10 and 30 mM (human and horse, respectively) (2). However, the dipeptide is also present at high (1–2 mM) concentrations in the olfactory bulb of the mammalian brain and has been detected in muscles of some invertebrates such as crabs, shrimp, and oysters (Ref. 3, for review see Ref. 4). Homocarnosine is detected at concentration of 0.3–1.5 mM in different regions of the human brain (5). Anserine is abundant in rabbit leg muscle (17 mM) and chicken pectoral muscle (43 mM), whereas balenine is mainly found in muscles of marine mammals such as whales and dolphins (up to 45 mM) (2).

The biological function(s) of carnosine and related dipeptides is still mysterious, although several theories have been proposed. Because of its abundance and its pKa close to the physiological pH, carnosine is thought to act as a buffer, neutralizing lactic acid produced in a working muscle (4). It has also been proposed to be an anti-glycation agent (for review, see Ref. 6) and a blood glucose regulator (7), whereas the dipeptide present in the olfactory system might be either a neurotransmitter or a neuromodulator (for review, see Ref. 8). Carnosine and anserine have been shown to be efficient chelators of copper ions in vitro (9) and all carnosine-related dipeptides have been postulated to be potent endogenous antioxidants (10). However, none of these putative physiological functions has been definitively verified yet.

Similarly, information on the enzyme that catalyzes the formation of carnosine and related dipeptides remains highly deficient. Carnosine is known to be synthesized from β-alanine and L-histidine by an ATP-dependent synthase (EC 6.3.2.11), which has been partially purified from different sources (11–14) and shown to also catalyze the synthesis of homocarnosine. The fate of ATP in the reaction has never been directly demonstrated, but based on indirect arguments, the nucleotide triphosphate is thought to be converted to AMP and inorganic pyrophosphate.

Two proteins encoded by different genes were shown to degrade carnosine in humans and other mammals (15). The first one (CN2, formerly named human tissue carnosinase,

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2 Chercheur Qualifié de la Fonds National de la Recherche Scientifique.

3 Collaborateur Logistique de la Fonds National de la Recherche Scientifique.

4 To whom correspondence should be addressed: UCL and de Duve Institute, Ave. Hippocrate 75, B-1200 Brussels, Belgium. Tel.: 3227647564; Fax: 3227647598; E-mail: emile.vanschaftingen@uclouvain.be.

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EC 3.4.13.18) is a Mn$^{2+}$-dependent cytosolic enzyme ubiquitously expressed in human tissues. This enzyme is now named “cytosol nonspecific dipeptidase,” because it does not degrade homocarnosine and exhibits a rather broad specificity toward various dipeptides. The second one (CN1, EC 3.4.13.20) is a genuine carnosinase (former human serum carnosinase), which breaks down both carnosine and homocarnosine and is found in serum and brain tissue. Interestingly, deficiency of this enzyme leads to hypercarnosinemia and hypercarnosinuria and was associated with neurological symptoms (16, 17).

Further progress on the role of carnosine and homocarnosine would benefit highly from the identification of the enzyme that synthesizes it and this was the purpose of the present work. We chose to purify this enzyme from chicken muscle, a rich source of carnosine synthase, and succeeded to identify it by combining protein purification and mass spectrometry analysis with a data base mining approach.

EXPERIMENTAL PROCEDURES

Materials—Reagents, of analytical grade whenever possible, were from Sigma, Acros (Geel, Belgium), Roche Applied Science, or Merck (Darmstadt, Germany). [$^3$H]β-Alanine and [$^{14}$C]γ-aminobutyric acid were purchased from Moravek Biochemicals (Brea, CA). DEAE-Sepharose, Q-Sepharose, Superdex-200 resins, 1-ml Histag Trap HP (Ni$^{2+}$ form) and PD-10 columns were obtained from GE Healthcare. ATP-Sepharose was a kind gift (to D. V.) from Serenex. AG50W-X4 (100–200 mesh) resin came from Bio-Rad, and Vivaspin-15 centrifugal concentrators were from Sartorius (Stockport, United Kingdom). Enzymes and DNA modifying enzymes as well as the TurboFect transfection reagent were obtained from Fermentas (St-Leon-Rot, Germany). FirstChoice® human brain RNA was from Applied Biosystems (Halle, Belgium).

Assay of Carnosine Synthase Activity—Carnosine synthase activity was determined by measuring the incorporation of either [$^3$H]β-alanine or [$^{14}$C]γ-aminobutyric acid into the corresponding dipeptide. The standard incubation mixture (0.11 ml) contained 50 mM Hepes, pH 7.5, 10 mM KCl, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM MgATP, 5 μM of leupeptin, and 5 μM of antipain) with a Ultra Turrax homogenizer. The homogenate was centrifuged for 30 min at 15,000 × g and the supernatant (550 ml) split into two equal halves. One-half was immediately utilized and the other was frozen at −70 °C and subjected to the same procedure a few days later. The supernatant (250 ml) was diluted with buffer A to 375 ml and applied to a DEAE-Sepharose column (200 ml) equilibrated with the same buffer. The column was washed with 400 ml of buffer A, developed with a NaCl gradient (0–0.5 M in 1000 ml) in buffer A, and fractions (7 ml) were collected. The most active fractions of the two columns were pooled (55 ml), diluted to 334 ml with buffer B (50 mM Tris–HCl, pH 8.0, 10 mM KCl, 1 mM DTT, 1 mM EGTA, 1 mM MgCl$_2$, 5 μM of leupeptin, and 5 μM of antipain), and applied to a Q-Sepharose column (12 ml) equilibrated with buffer B. The column was washed with 36 ml of buffer B containing 35 mM NaCl, and the retained protein was eluted with a NaCl gradient (35–500 mM in 300 ml in buffer B). The most active fractions (23 ml) were pooled, concentrated to 2.3 ml in 2 ml of buffer B, centrifuged at 13,000 × g for 10 min. The clear supernatant was diluted 5 times with 20 mM Hepes, pH 7.5, and 2 ml were applied to AG50W-X4 columns (1 ml, Na$^+$ form), equilibrated with 20 mM Hepes, pH 7.5. The unreacted radiolabeled substrate was removed by washing the columns with 10 ml of the same buffer and carnosine or homocarnosine were eluted with 5 × 2 ml of 20 mM Hepes, pH 7.5, containing 0.5 M NaCl. To elute dipeptides that were more positively charged, the columns were washed with 5 × 2 ml of 1 M NH$_4$OH. In all cases, the samples to be counted were mixed with 5 volumes of scintillation fluid (Ultima Gold, PerkinElmer Life Sciences) and the incorporated radioactivity was analyzed with a Packard Tri-Carb 2300 TR liquid scintillation counter.

Purification of Chicken Carnosine Synthase—Chicken pectoral muscle (250 g) was homogenized with 4 volumes (w/v) of buffer A (50 mM Hepes, pH 7.5, 10 mM KCl, 1 mM DTT, 1 mM EGTA, 1 mM MgCl$_2$, 5 μM of leupeptin, and 5 μM of antipain) with a Ultra Turrax homogenizer. The homogenate was centrifuged for 30 min at 15,000 × g and the supernatant (550 ml) split into two equal halves. One-half was immediately utilized and the other was frozen at −70 °C and subjected to the same procedure a few days later. The supernatant (250 ml) was diluted with buffer A to 375 ml and applied to a DEAE-Sepharose column (200 ml) equilibrated with the same buffer. The column was washed with 400 ml of buffer A, developed with a NaCl gradient (0–0.5 M in 1000 ml) in buffer A, and fractions (7 ml) were collected. The most active fractions of the two columns were pooled (55 ml), diluted to 334 ml with buffer B (50 mM Tris–HCl, pH 8.0, 10 mM KCl, 1 mM DTT, 1 mM EGTA, 1 mM MgCl$_2$, 5 μM of leupeptin, and 5 μM of antipain), and applied to a Q-Sepharose column (12 ml) equilibrated with buffer B. The column was washed with 36 ml of buffer B containing 35 mM NaCl, and the retained protein was eluted with a NaCl gradient (35–500 mM in 300 ml in buffer B). The most active fractions (23 ml) were pooled, concentrated to 2.3 ml in 2 ml of buffer B, centrifuged at 13,000 × g for 10 min. The clear supernatant was diluted 5 times with 20 mM Hepes, pH 7.5, and 2 ml were applied to AG50W-X4 columns (1 ml, Na$^+$ form), equilibrated with 20 mM Hepes, pH 7.5. The unreacted radiolabeled substrate was removed by washing the columns with 10 ml of the same buffer and carnosine or homocarnosine were eluted with 5 × 2 ml of 20 mM Hepes, pH 7.5, containing 0.5 M NaCl. To elute dipeptides that were more positively charged, the columns were washed with 5 × 2 ml of 1 M NH$_4$OH. In all cases, the samples to be counted were mixed with 5 volumes of scintillation fluid (Ultima Gold, PerkinElmer Life Sciences) and the incorporated radioactivity was analyzed with a Packard Tri-Carb 2300 TR liquid scintillation counter.

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The abbreviations used are: DTT, dithiothreitol; ATPGD1, ATP-grasp domain-containing protein 1; AP$_{A}$, P$_{1}P_{3}$-diadenosine 5‘)-pentaphosphate; EST, expressed sequence tag; Hsp90, heat shock protein 90; MS, mass spectrometry; HPLC, high pressure liquid chromatography.
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200-purified chicken carnosine synthase was incubated at 37 °C in the presence of 1 mM β-alanine and 3 mM l-histidine in a reaction mixture (0.33 ml) containing 50 mM Hepes, pH 7.5, 0.2 mg/ml of bovine serum albumin, 10 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 1 mM MgATP, 1 mM Na₃VO₄ (a nonspecific inhibitor of ATPases), and 100 μM diadenosine pentaphosphate (Ap₅A, a potent inhibitor of adenylate kinase (19)). Neither Na₃VO₄ nor Ap₅A affected the formation of carnosine at the concentrations used. To confirm the complete inhibition of adenylate kinase activity by Ap₅A, the enzyme was incubated in the same reaction mixture in which β-alanine and l-histidine had been replaced by 100 mM AMP.

After 0, 60, and 120 min at 37 °C, 0.1 ml of the reaction mixture was removed and mixed with 30 μl of ice-cold 20% (w/v) HClO₄ to stop the reaction. Samples were centrifuged at 13,000 × g for 5 min at 4 °C and the supernatants (0.12 ml) immediately withdrawn and neutralized with 20 μl of 3 M K₂CO₃. The salt precipitate was removed by centrifugation (13,000 × g for 15 min) and the clear supernatants (0.1 ml) were analyzed in an Agilent 1100 HPLC. Separation of adenine nucleotides was achieved by chromatography on a Whatman Partisphere SAX column (4.6 × 125 mm, 5-μm particle size) in a gradient of 0.01–0.5 M NH₄H₂PO₄, pH 3.7, at a flow rate of 2 ml/min (20). The detection of nucleotides was performed with a diode-array detector at λ = 254 nm. Quantification was achieved using external standards of ATP, ADP, and AMP.

Identification of Chicken Carnosine Synthase by Tandem Mass Spectrometry—The bands co-eluting with carnosine synthase activity in the ATP-Sepharose purification step were cut from a 10% polyacrylamide SDS gel and digested with trypsin. In-gel digestions and desalting of the peptides were performed as described in Ref. 21. Peptides were analyzed by LC-tandem mass spectrometry in a LTQ XL ion-trap mass spectrometer (Thermo Scientific) fitted with a microelectrospray probe. The results were analyzed using X-calibur software (Thermo Scientific) and the proteins were identified using Proteome Discoverer (Thermo Scientific) with a False Discovery rate ≤5%, as delivered by a target-decoy data base search. To identify chicken ATPGD1, the downloaded chicken data base of the present work.

The amplified DNA products of the expected size were digested with the appropriate restriction enzymes and cloned into the pEF6/HisB expression vector (Invitrogen), which allows the production of proteins with an N-terminal His₇ tag, and was verified by sequencing. The sequences of mouse brain and muscle Atpgd1 were the same, and only the construct derived from muscle was used in further experiments. For transfections, HEK-293T cells were plated in 85-mm Petri dishes at a cell density of 2.1 × 10⁶ cells/plate in Dulbecco’s minimal essential medium supplemented with 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% (v/v) fetal bovine serum, and grown in a humidified incubator under 95% air and 5% CO₂ atmosphere at 37 °C. After 24 h, each plate was transfected with 6 μg of the appropriate vector using the TurboFect transfection reagent (Fermentas) according to the protocol provided by the manufacturer. After 48 h the culture medium was removed, the cells were washed with 5 ml of phosphate-buffered saline and harvested in 0.5 ml of 50 mM Hepes, pH 7.5, containing 10 mM KCl, 1 mM MgCl₂, 5 μg/ml of leupeptin, and 5 μg/ml of antipain. The cells were lysed by freezing in liquid nitrogen and after thawing and vortexing, the extracts were incubated on ice with 125 units/ml of DNase I (Sigma) for 30 min and then centrifuged at 4 °C (15,000 × g for 30 min) to remove insoluble material.

For the purification of mouse and human recombinant ATPGD1, the supernatant (17 ml) was diluted 2-fold with buffer A (50 mM Hepes, pH 7.5, 300 mM NaCl, 10 mM KCl, 1 mM MgCl₂, 5 μg/ml of leupeptin, and 5 μg/ml of antipain) and applied on a HisTrap HP column (1 ml) equilibrated with the same buffer. The column was washed with 30 ml of buffer A and
the retained protein was eluted with a stepwise gradient of imidazole (60–300 mM, 60 ml) in buffer A. Both human and mouse ATPGD1 were eluted with 150–300 mM imidazole in homogeneous form as confirmed by SDS-PAGE (not shown). The enzyme preparations were desalted on PD-10 columns equilibrated with 50 mM Hepes, pH 7.5, 10 mM KCl, 1 mM DTT, 1 mM EGTA, 1 mM MgCl2. The protein concentration was determined spectrophotometrically according to Bradford (18) using bovine γ-globulin as a standard. 0.5 (mouse) or 1 mg (human) of pure recombinant enzyme was obtained from 40 or 70 mg of soluble HEK293T cell protein, respectively. The purified enzymes were supplemented with 2 mg/ml of bovine serum albumin and stored at −70 °C.

Product Analysis—To obtain a sufficient amount of the dipeptide formed in the reaction catalyzed by recombinant mouse ATPGD1 for mass spectrometry analysis, the reaction mixture was scaled up 10-fold. Briefly, 14 μg of mouse ATPGD1 were incubated for 16 h at 37 °C in 1 ml of a reaction mixture containing 50 mM Hepes, pH 7.5, 200 μg of bovine serum albumin derived from the enzyme preparation, 10 mM KCl, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT, 3 mM β-alanine, 3 mM L-histidine, in the absence or presence of 3 mM MgATP. The reaction was stopped by the addition of 0.2 ml of 30% (w/v) HClO4. After neutralization with 3 M K2CO3, the salts were removed by centrifugation (13,000 × g for 15 min) and the clear supernatant was diluted 5 times with 20 mM Hepes, pH 7.5. The sample (6 ml) was applied to AG50W-X4 column (1 ml, Na+ form), the unreacted substrates were removed by washing with 12 ml of the same buffer, and the dipeptide was eluted with 2 ml of 1 M NH4OH. The purified dipeptide was evaporated to dryness, dissolved in 1 ml of 200 mM ammonium bicarbonate, and N-acetylated with 10% acetic anhydride (Ac2O, v/v) for 1 h at room temperature. Carnosine and N-acetylcarnosine were further purified by HPLC on a Hypercarb 2.1 × 150-mm column (ThermoHypersil) in a 5–50% gradient of acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 0.2 ml/min. The column eluent was monitored with a UV detector at λ = 210 nm. All mass spectral analyses were performed on a LCQ Deca XP ion-trap spectrometer equipped with an electrospray source (Thermo Scientific). The sample dissolved in methanol was introduced directly into the source at a flow rate of 4 μl/min. The LCQ was operated in positive mode under manual control in the Tune Plus view with default parameters and active automatic gain control. To confirm the structure of the precursor ions, low-energy collision-induced dissociation (relative collision energy of 25%) was utilized.

Calculations—Vmax and Km for the peptide synthase activity of studied enzymes were calculated with Prism 4.0 (GraphPad software) using a nonlinear regression.

RESULTS

Purification of Chicken Carnosine Synthase—During its purification, carnosine synthase activity was assayed by measuring the conversion of [3H]β-alanine to [3H]carnosine in the presence of L-histidine and ATP. Carnosine synthase was purified from chicken breast muscle about 1500-fold by a procedure involving chromatography on DEAE-Sepharose, Q-Sepharose, Superdex-200, and ATP-Sepharose. The ligase was eluted as a
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**TABLE 1**

| Fraction          | Volume (ml) | Total Protein (mg) | Total Activity (pmol min⁻¹) | Specific Activity (pmol min⁻¹ mg⁻¹) | Purification (fold) | Yield (%) |
|-------------------|-------------|--------------------|-----------------------------|-----------------------------------|---------------------|-----------|
| 15,000 × g supernatant | 750         | 17,240             | 12,710                      | 0.74                              | 1                   | 100       |
| DEAE-Sepharose     | 55.6        | 64.7               | 4,459                       | 68.9                              | 93                  | 35.1      |
| Q-Sepharose        | 23          | 16.7               | 3,775                       | 226                               | 305                 | 29.7      |
| Superdex 200       | 3           | 2.27               | 1,660                       | 731                               | 987                 | 13.0      |
| ATP-Sepharose      | 0.4         | 0.139              | 156                         | 1,119                             | 1,512               | 1.2       |

* The data represent values only for the most purified fraction.

The fate of ATP during carnosine synthesis indicated that ATP was consumed during the reaction to form carnosine. The reaction mixture was supplemented with 1 mM Na₃VO₄, a nonspecific inhibitor of many ATPases and phosphatases, and 100 μM Ap₅A, to prevent any conversion of ATP to ADP by traces of adenylate kinase that still contaminate the preparation (19). The figure shows that the formation of carnosine was matched by a stoichiometric formation of AMP to ADP by traces of adenylate kinase that still contaminate the preparation (19). The figure shows that the formation of radiolabeled carnosine was determined after chromatographic separation from ATP and ADP. ADP and AMP were determined by HPLC. The figure shows the mean of two experiments performed under similar conditions and yielding similar results (less than 5% variation).

**FIGURE 2. Time course of the changes in carnosine, ADP, and AMP concentration during carnosine synthesis.** A chicken enzyme preparation (11 μg of protein) purified by chromatography on DEAE-Sepharose, Q-Sepharose, and Superdex 200 was incubated for 0, 60, and 120 min with 1 mM MgATP in the absence (empty symbols) or presence (filled symbols) of 3 mM γ- alanine, 1 mM β-alanine, as well as 750 × 10⁴ cpm of [³H]β-alanine. ADP values were calculated by subtracting ADP concentration values in the absence of β-alanine and γ-alanine from the corresponding values in the presence of these substrates. The formation of radiolabeled carnosine was determined after chromatographic separation from β-alanine. ADP and AMP were determined by HPLC. The figure shows the mean of two experiments performed under similar conditions and yielding similar results (less than 5% variation).

The identification of carnosine synthase from chicken pectoral muscle revealed a single peak in each of the purification steps (Fig. 1), indicating the presence of a single enzyme species. The gel filtration step on Superdex-200 disclosed that the size of native carnosine synthase was ~415 kDa (not shown). The overall yield of the purification was only about 1.2% (Table 1), but this low yield was due to the fact that only the most active fractions from one step were used for the next one. For instance, only fraction 27 of the Superdex-200 column (i.e. only about 28% of the recovered activity) was used for the final affinity chromatography.

SDS-PAGE analysis indicated that carnosine synthase coeluted with two major polypeptides of about 100 and 90 kDa in the last purification step (cf. Fig. 1). Both bands were cut from the gel, digested with trypsin, and the resulting peptides were analyzed by tandem mass spectrometry and compared with the chicken, mouse, and human proteomes. Surprisingly, the analysis indicated that both bands contained heat-shock protein 90-α (Hsp90) as well as other proteins, but that none of these appeared to correspond to a putative ATP-dependent ligase. Similar negative results were obtained when the whole ATP-Sepharose-purified fraction was analyzed by tandem MS after trypsin digestion (not shown).

**Fate of ATP during Carnosine Synthesis**—The availability of a substantially purified carnosine synthase preparation enabled us to identify the nucleotide produced from ATP by this enzyme. Fig. 2 shows an experiment in which we followed carnosine synthesis catalyzed by the 1000-fold purified carnosine synthase preparation in the presence of 1 mM γ-Ala and 1 mM 1-His, as well as 750 × 10⁴ cpm of [³H]β-alanine. ADP values were calculated by subtracting ADP concentration values in the absence of β-alanine and γ-alanine from the corresponding values in the presence of these substrates. The formation of radiolabeled carnosine was determined after chromatographic separation from β-alanine. ADP and AMP were determined by HPLC. The figure shows the mean of two experiments performed under similar conditions and yielding similar results (less than 5% variation).

**Data Base Searches for a Carnosine Synthase Candidate**—The finding that carnosine synthase converts ATP to ADP during carnosine synthesis suggested that this enzyme most probably belonged to the ATP-grasp superfamily. The latter includes mostly enzymes catalyzing ATP-dependent ligation of a carboxylate-containing molecule to an amine- or thiol-containing molecule with concomitant formation of ADP and Pi (22). We searched the pfam data base for human or mouse proteins of the ATP-grasp family with unknown function and checked their size and tissue distribution (BioGPS data base and Huge Protein data base (23)). Only one of these proteins, ATPGD1 (also known as KIAA1394), was found to have a size (~900 amino acids) and tissue distribution (mostly expressed in skeletal muscle and brain) similar to that expected for carnosine synthase.

Intriguingly, no chicken ATPGD1 orthologue was present in the chicken protein data base. Furthermore, no atpgd1 gene was detected by performing a BLAST search (24) in the chicken reference genome (as of July 2009). However, four chicken ESTs and several genomic trace sequences were found to encode a chicken protein homologous to mammalian ATPGD1. The complete sequence of atpgd1 was established by sequencing a
full-length protein-coding cDNA obtained by reverse transcription-PCR amplification of chicken breast muscle RNA.

We reanalyzed the mass spectrometry data obtained with purified chicken carnosine synthase by comparing them with a chicken proteome in which we had introduced the chicken ATPGD1 sequence. This analysis revealed that ATPGD1 was the best hit for the 100-kDa polypeptide (Table 2). Thirty matching peptides (underlined in Fig. 3) were found that covered 497 amino acids, i.e. more than 50% of the predicted sequence. These findings supported the idea that ATPGD1 corresponded to carnosine synthase, as is confirmed below.

**Analysis of the ATPGD1 Sequence**—The sequence of human (GenBank accession number NP_001159694.1) and mouse (GenBank accession number NP_598909.2) ATPGD1 comprised 950 and 957 residues, respectively, and are encoded by genes present on human chromosome 11q13 and mouse chromosome 19. These genes comprise 9 coding exons, with the peculiarity that the first coding exon only encodes the initiator ATG. This splicing is supported by all EST sequences (5 human and 24 murine) corresponding to the 5’-end of the human and mouse cDNAs. Chicken ATPGD1 comprises 930 residues and shares 55% sequence identity with human and mouse ATPGD1. The chicken atpgd1 gene is not available, but a “trace” sequence of genomic DNA also supports the idea that the first coding exon only encodes the initiator methionine. Analysis of the three sequences with TargetP (25) predicts the C-terminal region is the most conserved region (not shown), indicating that similarly to the chicken enzyme it is a homotetramer.

The identity of the product formed from β-alanine and L-histidine by mouse ATPGD1 was verified by purifying this product and submitting it to mass spectrometry. Analysis by electrospray mass spectrometry indicated the presence of a protonated molecular ion with m/z 227, as expected for carnosine (not shown). As shown in Fig. 4A, tandem mass spectrometry analysis of this ion revealed a fragmentation pattern in agreement with the carnosine structure and which was indeed identical with that of commercial carnosine (not shown). The presence of the peak at m/z 156, which corresponded to protonated L-histidine, suggested that the product was β-alanyl-L-histidine rather than its isomer L-histidyl-β-alanine. To verify this conclusion, the ATPGD1 reaction product was N-acetylated by incubation with acetic anhydride. As shown in Fig. 4B, N-acetylation resulted in a characteristic 42-Da mass shift, from m/z 227 to 269. No change in m/z value of the L-histidine fragment ion was detected, confirming that β-alanyl-L-histidine was the product of the activity of recombinant mouse ATPGD1. No carnosine signal was detected in a control reaction that did not contain ATP (not shown).
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Amino acid sequence alignment of human ATPGD1 with its oyster, mouse, and chicken orthologues. Fully conserved residues are highlighted with a black background. The human (GenBank accession number NP_001159694) and mouse sequences (GenBank accession number NP_598909) have been confirmed by PCR amplification of the cDNA and sequencing. The chicken sequence has been determined in the present work (GenBank accession number GU453679). The oyster (C. virginica) sequence was reconstituted from ESTs (GenBank accession number BK007044). The peptides identified by mass spectrometry in the protein purified from chicken pectoral muscle are underlined in the chicken sequence.

FIGURE 3. Amino acid sequence alignment of human ATPGD1 with its oyster, mouse, and chicken orthologues.
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Carnosine Synthase Is an ADP-producing Ligase—Our finding that purified chicken carnosine synthase produces stoichiometric amounts of carnosine and ADP indicates that the reaction catalyzed by this enzyme could only be performed once we realized that carnosine synthase converted ATP to ADP and not to AMP, leading to the hypothesis that carnosine synthase belonged to the ATP-grasp family.

Our identification of carnosine synthase as ATPGD1 rests on two main arguments: the first one is that one of the two major polypeptides that are present in the most highly purified preparation of chicken carnosine synthase corresponds to ATPGD1. The second one is that recombinant mouse and human ATPGD1 catalyze the ATP-dependent synthesis of carnosine and, with a lower affinity, homocarnosine and other related dipeptides. This rather broad specificity with preference for the formation of carnosine is typical of all preparations of carnosine synthase that have been described until now (11–14). Furthermore, we confirmed by mass spectrometry the identity of the product made by the recombinant mouse enzyme as carnosine and not the reverse peptide (L-histidyl-L-α-alanine), which would have a similar elution profile from ion exchange columns.

The purified chicken preparation still contained contaminating proteins, most particularly Hsp90, which was present in similar amounts as ATPGD1. We believe that this contamination is coincidental, being due to the fact that chicken ATPGD1 has similar physicochemical properties as Hsp90, rather than to the two proteins forming a complex. Homogeneously purified human and mouse recombinant ATPGD1 were found to be active as carnosine synthases, indicating that the presence of Hsp90 in the reaction mixture is not required for activity.

The finding that orthologues of atpgd1 are present in other mammals, Xenopus, and chicken, not in certain fishes, but present in certain birds, suggests that ornithine and α-2,4-diaminobutyric acid, most particularly with the mouse enzyme in the latter case. No significant activity was detected with amino compounds without a carboxylic group.

DISCUSSION

Molecular Identification of Carnosine Synthase—The two major advances reported in the present work are the molecular identification of carnosine synthase and the fact that this enzyme catalyzes the hydrolysis of ATP to ADP rather than to AMP as previously assumed (11, 12). The two points are linked, because the identification of this enzyme could only be confirmed once we realized that carnosine synthase converted ATP to ADP and not to AMP, leading to the hypothesis that carnosine synthase belonged to the ATP-grasp family.

The kinetic properties and specificity of recombinant human and mouse carnosine synthase are compared with those of the chicken enzyme in Tables 3 and 4. Based on the catalytic efficiency ($k_{cat}/K_m$), β-alanine was about 14, 17, and 25 times better as a substrate than γ-aminobutyrate for the chicken, mouse, and human ligase, respectively, indicating that all three enzymes are much better at synthesizing carnosine than homocarnosine. As for the specificity of the α-amino acid involved in this reaction, L-histidine was for all three enzymes the best substrate. The second best substrate was N-α-methyl-L-histidine in the case of the chicken enzyme and L-ornithine for mouse carnosine synthase. L-Lysine was a significant substrate for all three enzymes, the catalytic efficiency being 5–9% of that observed with L-histidine. Some activity was also observed with L-arginine and L-2,4-diaminobutyric acid, most particularly with the mouse enzyme in the latter case. No significant activity was detected with amino compounds without a carboxylic group.

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Identification of Carnosine Synthase

Kinetic properties of mouse, human, and chicken carnosine synthase

Kinetic properties were determined with the use of homogenous recombinant mouse or human ATPGD1 and chicken muscle carnosine synthase purified by chromatography on DEAE-Sepharose, Q-Sepharose, Superdex 200, and ATP-Sepharose.

| Substrate                  | Mouse ATPGD1 | Human ATPGD1 | Chicken carnosine synthase |
|----------------------------|--------------|--------------|---------------------------|
|                            | $V_{\text{max}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $V_{\text{max}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $V_{\text{max}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
|                            | nmol min$^{-1}$ mg$^{-1}$ | mM | s$^{-1}$ | nmol min$^{-1}$ mg$^{-1}$ | mM | s$^{-1}$ | nmol min$^{-1}$ mg$^{-1}$ | mM | s$^{-1}$ |
| $\beta$-Alanine$^a$        | 300 ± 10     | 0.46 ± 0.04  | 1.16          | 653 ± 2.1    | 0.09 ± 0.01 | 1.28          | 119 ± 4       | 0.033 ± 0.005 | 0.63  |
| $\gamma$-Aminobutyrate$^a$| 238 ± 5      | 6.44 ± 0.33  | 0.07          | 54.7 ± 1.2   | 1.84 ± 0.11 | 0.05          | 841 ± 1.6     | 0.35 ± 0.02   | 0.44  |
| ATP-Mg$^b$                 | 0.67 ± 0.02  | 0.57 ± 0.05  | 0.002         | 0.76 ± 0.01  | 0.42 ± 0.03 | 0.003         | 3.63 ± 0.08   | 0.19 ± 0.01   | 0.032 |
| L-Histidine$^b$            | 0.60 ± 0.02  | 0.11 ± 0.01  | 0.0100        | 0.76 ± 0.03  | 0.37 ± 0.05 | 0.004         | 3.30 ± 0.09   | 0.10 ± 0.01   | 0.055 |
| L-Lysine$^b$               | 0.78         | 1.59         | 0.0009        | 0.61         | 4.67       | 0.0002        | 3.43         | 1.42         | 0.0040 |
| L-Ornithine$^b$            | 1.92         | 0.52         | 0.0066        | 0.28         | 7.66       | 0.0001        | 2.54         | 1.62         | 0.0026 |
| N-$\pi$-Methylhistidine$^b$| 0.66         | 4.51         | 0.0003        | 0.62         | 24.7       | 0.0001        | 3.51         | 0.39         | 0.0150 |

$^a$ Determinations for the indicated substrates were performed with enzyme preparations that were incubated for 20 min in the presence of 3 mM ATP, variable concentrations of either $[1^H + 3^H] \beta$-alanine or $[1^C + 1^C] \gamma$-aminobutyrate and 3 mM L-histidine.

$^b$ Measurements were done in the presence of non-saturating concentrations (1 $\mu$M) of $[1^H + 3^H] \beta$-alanine. Values are the means of two or three separate experiments. The S.E. value is given.

The possibility that there would be two types of carnosine synthase, one producing ADP and the other AMP, is unlikely because we observed only one peak of carnosine synthase in all chromatographic steps. Furthermore, the specificity studies performed here indicate that our chicken enzyme preparation has similar properties to those described previously by other investigators. Taken together, all these considerations indicate that there is only one carnosine synthase and that this enzyme is an ADP-forming ligase.

The Lack of Specificity of Carnosine Synthase—Previous studies disclosed that carnosine synthase is relatively nonspecific. This conclusion was based on studies with rather impure preparations purified from tissues. Therefore the lack of specificity observed previously could have been due to heterogeneity in the enzymatic preparations. However, both carnosine synthase purified from chicken muscle and the recombinant human and mouse proteins catalyze the synthesis of homocarnosine with catalytic efficiencies that are about 14–26-fold lower than those observed for carnosine synthesis, indicating that a single enzyme is responsible for both activities. Furthermore, our finding that the same sequence was amplified from mouse brain and skeletal muscle cDNA indicates that there is no specific brain isoform that would for instance be specialized in the synthesis of homocarnosine. This conclusion is consistent with previous reports showing the apparent identity of the enzymatic properties of the synthase partially purified from mouse neural tissue or muscle (14) and the high antigenic similarity of carnosine synthase from either rat or rabbit brain, skeletal muscle, and heart (29). From a physiological standpoint, it should be mentioned that the lower catalytic efficiency of carnosine synthase with $\gamma$-aminobutyric acid as a substrate compared with $\beta$-alanine is more than compensated by the higher concentration of $\gamma$-aminobutyric acid compared with $\beta$-alanine in brain (about 1.6–4.6 and 0.06–0.1 mM, respectively, for mouse brain (30)).
As previously reported, carnosine synthase is also not very specific with respect to the amino compound serving as the β-alanine or γ-aminobutyryl acceptor (11, 14). Thus, the chicken enzyme is able to use N-π-methyl-L-histidine, L-arginine, and L-lysine in addition to L-histidine, whereas the mouse and human enzymes use L-ornithine and L-lysine fairly well (cf. Table 3). The β-alanyl-L-lysine or L-ornithine synthesizing activity can now be concluded to be due to an authentic lack of specificity of carnosine synthase rather than to the presence of different isoforms with different specificities. Considering that the concentration of L-lysine in human skeletal muscle is about 1.5-fold higher than that of L-histidine (0.53 and 0.37 mM, respectively (31)) β-alanyl-L-lysine synthesis is expected to proceed in vitro at ~10% of the rate of carnosine synthesis. The concentration of β-alanyl-L-lysine is not known for human muscle, but it is 1000-fold lower than that of carnosine in rabbit muscle (32). This low concentration of the “wrong” peptide is presumably due to the fact that it is degraded by a dipeptidase that is particularly active in muscle and specifically cleaves the β-alanyl or γ-aminobutyryl derivatives of L-lysine, L-arginine, or L-ornithine, but does not act on carnosine or homocarnosine and thus is different from carnosinase CN2 (33). The specific accumulation of carnosine in muscle tissue appears therefore to be due to the existence of two enzymes: one, carnosine synthase, that preferentially makes carnosine, but also synthesizes other dipeptides, and a second enzyme, not yet molecularly identified, that should destroy all the unneeded dipeptides.

Other situations where one enzyme serves to compensate for the lack of specificity of another enzyme have been described. For example, L-2-hydroxyglutarate dehydrogenase serves to degrade L-2-hydroxyglutarate, which is “mistakenly” made by the Krebs cycle enzyme L-malate dehydrogenase (34). Deficiency in the former enzyme causes L-2-hydroxyglutaric aciduria, a neurometabolic disorder. Another example is the ATP-dependent dehydratase that “repairs” the hydrated form of NADH made by glyceraldehyde-3-phosphate dehydrogenase (35). It is likely that other enzymes catalyzing such “metabolite repair reactions” have still to be found.

**Evolution of Carnosine Synthase**—An intriguing aspect in the structure of carnosine synthase is that its sequence has about twice the length expected for a member of the ATP-grasp family. Sequence comparisons indicate that it comprises two ATP-grasp domains, suggesting that the gene encoding carnosine synthase resulted from the fusion of two genes encoding two different ligases. The high conservation of the C-terminal domain indicates that this part of the protein comprises the catalytic site responsible for the ligation of β-alanine to L-histidine and related amino acids. The N-terminal domain is much less conserved and presumably has no catalytic activity in vertebrates. Interestingly, the tripeptide β-alanyl-L-ornithyl-L-ornithine has been described in a bivalve (36). Because carnosine synthases are rather good at synthesizing β-alanyl-L-ornithine, we speculate that this tripeptide is made by a bifunctional enzyme similar to carnosine synthase. Whether the N-terminal domain has now taken another function or is simply a remnant of evolution in present day vertebrate carnosine synthase is unknown.

**Identification of Carnosine Synthase**

The identification of carnosine synthase will allow progress in the understanding of the physiological function of carnosine and homocarnosine. The creation of knock-out models or animals in which carnosine synthase is overexpressed could help define the role of carnosine as a buffer, a radical scavenger in muscle and brain, or as a neurotransmitter in the olfactory system, and the function of homocarnosine as a γ-aminobutyrate reservoir. Knock-out models would tell us whether carnosine and/or homocarnosine deficiency lead to disease. No case of carnosine deficiency has yet been described in humans, but we surmise that this is because carnosine content of muscle and brain is only infrequently measured. We speculate that carnosine synthase deficiency could lead to symptoms like muscle cramp, myopathy, anosmia or hypossmia, seizures, and most probably other neurological problems. As the carnosine synthase gene is in the region of IDDM4 on chromosome 11q13, which is linked with insulin-dependent diabetes (37), it is also possible that carnosine deficiency leads to glucose intolerance. It has indeed been shown that carnosine administration affects the function of pancreatic islets (7). The identification of the gene encoding carnosine/homocarnosine synthase will facilitate the diagnosis of carnosine or homocarnosine deficiency.

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