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PURIFICATION OF CARNOSINE SYNTHETASE FROM AVIAN MUSCLE BY AFFINITY CHROMATOGRAPHY AND DETERMINATION OF ITS SUBUNIT STRUCTURE

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An extract of chick pectoral muscle was prepared in which the level of carnosine synthetase (L-histidine; β-alanine ligase (AMP-forming), EC 6.3.2.11) activity was approx. 10-times that of previous preparations. In affinity chromatography studies, this material was applied to a Cibracon blue-agarose column, and elution of carnosine synthetase by carnosine was attempted. Results indicated that the elution was not specific as the eluate contained large amounts of myosin. An $(NH_4)_2SO_4$ fraction (21–30% satn.) of the crude extract was prepared which, in comparison to the crude extract, had a higher specific activity, was more stable on storage at 4°C and had much lower myosin content. On affinity chromatography of this fraction, apparently homogeneous carnosine synthetase was eluted with carnosine, and the specific activity of the preparation was 1700-times that of the fresh crude extract. Amino acid analysis of the preparation indicated that it had a very high histidine content (141 per 1000 residues). On analysis of the preparation by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), a polypeptide of M_r 119 000 was observed, whereas gel permeation chromatography of the native enzyme indicated an M_r of 250 000, suggesting that the native enzyme is a dimer.

Introduction

Carnosine (β-alanyl-L-histidine) is a dipeptide that has been found in a wide variety of animal tissue [1], and in particular is present at relatively high concentrations in striated muscles [2] and certain nerve cells [3]. Although the precise physiological function of carnosine is still unknown, a variety of proposals for its biological role have been made, based on its distribution, the variation of its concentration in response to stimuli, and its ability to ligand divalent cations. These proposals have included its possible function in muscle as a physiological buffer [2], an effector of myosin ATPase [4], an activator of fructose-1,6-bisphosphatase [5], as a metal chelator [6,7], and in nerve cells, as a neurotransmitter [8].

Because of interest in the function of carnosine, considerable attention has been focused on its biosynthesis in terms of tissue specificity, turnover rates and possible regulatory features. The principal biosynthetic route for this compound is from β-alanine and L-histidine in an ATP-dependent reaction catalyzed by carnosine synthetase (L-histidine: β-alanine ligase (AMP-forming), EC 6.3.2.11):

L-histidine + β-alanine + ATP

→ β-alanyl-L-histidine + AMP + PP_i
(carnosine)

As carnosine synthetase catalyzes a reaction that is relatively unique in metabolism and that is far removed

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from equilibrium in vivo, the enzyme-catalyzed reaction is probably an important rate-limiting step [9] in the biosynthesis of carnosine and other related histidine-containing dipeptides such as anserine, homocarnosine and ophidine [1]. Earlier work demonstrated that avian muscle contained substantially higher levels of carnosine synthetase activity than mammalian muscles [10] and it was found that the enzyme, which is a soluble sarcoplasmic component, could be extracted in a crude but unstable form [11]. Preliminary kinetic studies on such preparations have provided evidence for a reaction mechanism [11,12] and for possible physiologically important inhibitors [13]. In addition to these studies on the muscle enzyme, recent work has also been performed on the enzyme present in mammalian olfactory bulb, and although a purification procedure for the enzyme was reported [14], the product obtained was still heterogeneous with respect to its polypeptide composition.

In view of the interest in carnosine synthetase, it seemed important to obtain a more purified form of the enzyme than had previously been avialable so that its chemical and biological properties might be investigated in more detail. In this report, we describe a purification procedure for the enzyme using affinity chromatography which yields a preparation of apparent electrophoretic homogeneity, and it is proposed that the native enzyme is a dimer of approximate M_r = 250 000.

Materials and Methods

Materials. Reactive blue 2-agarose (containing immobilized Cibracon blue F3G-A) was purchased from Sigma Chemical Co., Ultrogel AcA-34 was obtained from LKB Instruments and β-[1-^{14}C]alanine with a specific activity of 13.87 mCi/mol was bought from ICN Pharmaceuticals. All other chemicals and reagents were of the highest grade available commercially. White Hyline chicks were maintained on a stock chick diet until 14–18-days-old and were then killed by decapitation and the pectoral muscles excised.

Preparation and preliminary fractionations of the crude muslce extract. The extraction procedure used was a modification of the procedure of Kalayankar and Meister [15]. Fresh chilled or thawed pre-frozen pectoral muscle was homogenized in a Waring Blendor at 4°C for 2 min in 8 vol. buffer 1 (0.65 M NaCl/0.04 M $KHCO_3$/0.5 mM dithiothreitol/0.1 mM phenylmethanesulfonyl fluoride, pH 7.8). The homogenate was then centrifuged at 30 000 × *g* for 1 h and the supernatant retained (approx. 20 mg protein/ml).

The $(NH_4)_2SO_4$ fractionation of the crude extract was performed at 4°C and pH 7.8, and fractions were obtained at 0–20%, 21–30%, 31–40% and 41–50% saturation. The salt was added over a 15-min period, and after a further 10 min, precipitates were collected by centrifugation at 12 000 × *g* for 25 min, resuspended in water and dialyzed against buffer 1 and stored at 4°C.

Affinity chromatography. After exhaustive washing of the Reactive blue 2-agarose with buffer 1 at 4°C, the gel was packed as a 1 × 7 cm column and equilibrated with the same buffer at 5 ml/h. Samples of supernatant or the 21–30% $(NH_4)_2SO_4$ fraction, (approx. 100 mg protein), were applied to the column and non-adsorbed material was washed out of the column with buffer 1 over 24 h at 5 ml/h, with 5-ml fractions collected. Elution of adsorbed material from the column was performed by changing the eluant either to 8 M urea (to elute all adsorbed protein) or to buffer 1 containing 50 mM carnosine (in attempts to specifically elute carnosine synthetase). In both cases, elution was continued for a further 36 h.

Gel permeation chromatography. After washing the Ultrogel AcA-34 with buffer 1 at 4°C, the gel was packed as a 1.5 × 100 cm column, and after further equilibration at 5 ml/h, 5-ml fractions from the affinity column or a mixture of proteins of known molecular weight were applied to the column, and 5-ml fractions were collected at 5 ml/h.

Assay of carnosine synthetase activity. Assays were performed by a modification of the procedure [15] on which conversion of radioactive β-alanine to carnosine is measured. Samples for assay were dialyzed against 10 mM Tris/0.2 mM dithiothreitol, pH 7.5 at 4°C before use, and up to 200 µl of the dialyzed sample was then used in an assay medium (final volume 500 µl) which contained 30 mM β-alanine ($7 \cdot 10^8$ cpm/l)/3 mM L-histidine/3 mM $MgCl_2$/3 mM ATP/50 mM Tris-HCl, pH 7.5. The high concentration of β-alanine was used in the assay because of a report [16] that some carnosine synthe-

tase enzymes have a relatively high K_m (1.8 mM) for this substrate. The assay, which was performed in 1.5 ml capacity polyethylene centrifuge tubes, was started by the addition of enzyme and proceeded for 1 h at 35°C, after which it was terminated by the addition of 250 µl ethanol followed by boiling for 1 min. After cooling, the solution was clarified by centrifugation and 50 µl supernatant together with 2 µl 0.1 M carnosine (used as an internal marker) were fractionated by electrophoresis for 1 h at 3 kV in pH 6.5 pyridine-acetate buffer [17] on Whatman 3 MM paper. The dried electropherogram was stained with ninhydrin solution [18], and the visualized carnosine and neutral amino acid (containing β-alanine) spots were cut out and counted in either a Packard TriCarb Scintillation Spectrometer or a Beckman LS-800 Liquid Scintillation Spectrometer with appropriate corrections made for self-absorption. The ratio of counts incorporated into the carnosine spot to the counts in the neutral (β-alanine) region was then used to calculate the amount of β-alanine converted to carnosine in the assay, based on the known specific activity of the β-alanine used. The specific activity of the enzyme was expressed in units of nmol carnosine synthesized/min per mg protein (nmol · min^{-1} · mg^{-1}) under the conditions of the assay.

Determination of protein concentration. The Bio-Rad protein assay reagent was used, with a standard curve constructed from bovine γ-globulin [19].

Gel electrophoresis. Samples were analyzed by the procedure of Weber and Osborn [20] with an 8% gel on a vertical flat-bed apparatus [21]. After staining with Coomassie blue R250, photographic negatives on Kodak Plus-X film were made of gels and individual tracks in a negative were scanned on a Joyce Loebl Microdensitometer.

Amino acid analysis. Samples were dialyzed exhaustively against water at 4°C, and the lyophilized material was then hydrolyzed in constant boiling HCl for 24 h at 110°C in vacuo, and after removal of HCl, analysis was performed on a Beckman 119 CL amino acid analyzer equipped with a Spectraphysics System I Computing Integrator.

Results

Activity and stability of the crude muscle extract. Crude extracts were prepared from both fresh, chilled and thawed, pre-frozen muscle, and the enzyme activities of these fractions were measured and compared to the specific activities of crude extracts obtained previously [10,11]. The specific activity in the non-frozen muscle (0.200 ± 0.023 nmol · min^{-1} · mg^{-1}) was about 10-fold of those previously reported, and it was found that freezing of the muscle (for up to 3 months at −20°C) did not drastically reduce the specific activity (0.183 ± 0.023 nmol · min^{-1} · mg^{-1}) of the crude extract. However, storage of the crude extract at 4°C for up to 15 days resulted in a loss of activity of up to 90% of the original specific activity (Fig. 1), and for this reason, further experiments were performed on crude extracts which had been freshly prepared.

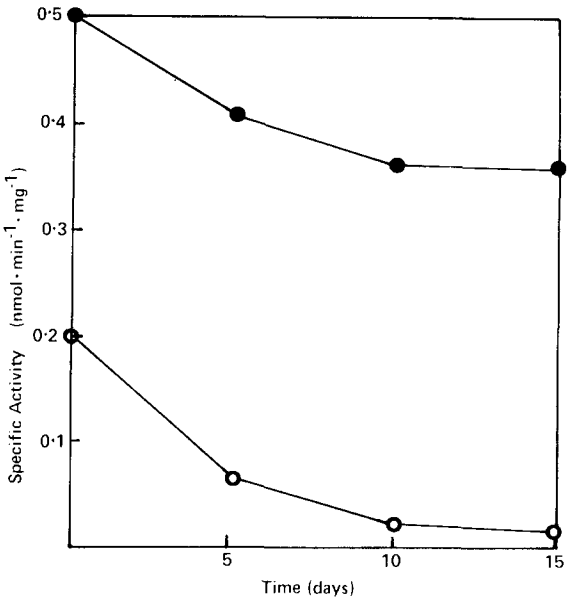

Fig. 1. Temporal stability of carnosine synthetase activity in fractions prepared from chick pectoral muscle. Assays of the carnosine synthetase activity of the crude muscle extract (○———○) and the 21–30% $(NH_4)_2SO_4$ fraction of the crude muscle extract (●———●) were performed on preparations which were stored at 4°C for up to 15 days.

Affinity chromatography of the crude muscle extract. When the crude extract was applied to the Reactive blue 2-agarose column, about 30% of the protein was retained on the column after washing the column with buffer 1, based on absorbance readings at 280 nm of the unabsorbed fraction and the material washed off the column with 8 M urea. When

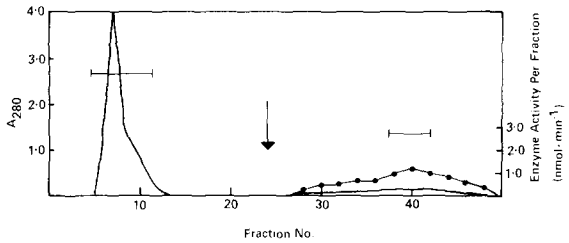

Fig. 2. Affinity chromatography of the crude muscle extract on Reactive blue 2-agarose. Crude muscle extract (100 mg protein) was applied to the affinity column and non-adsorbed protein was washed out with buffer 1, after which elution was continued (at the position indicated by the arrow) by buffer 1 containing 50 mM carnosine. Fractions contained within the bar lines (⊢——⊣) were pooled and used for gel electrophoresis (see Fig. 3). ——, A_{280} and •——•, enzyme activity per fraction.

the 50 mM carnosine solution was used to elute adsorbed material from the column (Fig. 2), less than 5% of the applied protein was eluted, and assays for carnosine synthetase activity showed that the specific activity of the eluted material was maximally about 80-fold that found in the crude extract.

Examination by gel electrophoresis (Fig. 3) of the crude extract, non-adsorbed material and the materials eluted by 8 M urea and 50 mM carnosine buffer revealed that the carnosine eluate was relatively complex in terms of its polypeptide composition, and in particular it contained a large proportion of a polypeptide that co-electrophoresed with rabbit muscle myosin heavy chain (approx. M_r = 200 000). These studies indicate that specific elution of carnosine synthetase from the variety of proteins adsorbed to the affinity column had probably not occurred, and it was decided to attempt preliminary fractionation of the crude extract so that the sample applied to the affinity column would be less heterogeneous, thereby favoring a more specific elution of carnosine synthetase.

$(NH_4)_2SO_4$ fractionation of the crude muscle extract. Previous studies [14] on carnosine synthetase from mouse olfactory bulb had demonstrated the utility of this procedure, and when this approach was used with the crude muscle extract, 74% of the total activity was recovered in the 21–30% $(NH_4)_2SO_4$ fraction and the specific activity of this fraction was approx. 3-fold that of the crude extract. As analysis

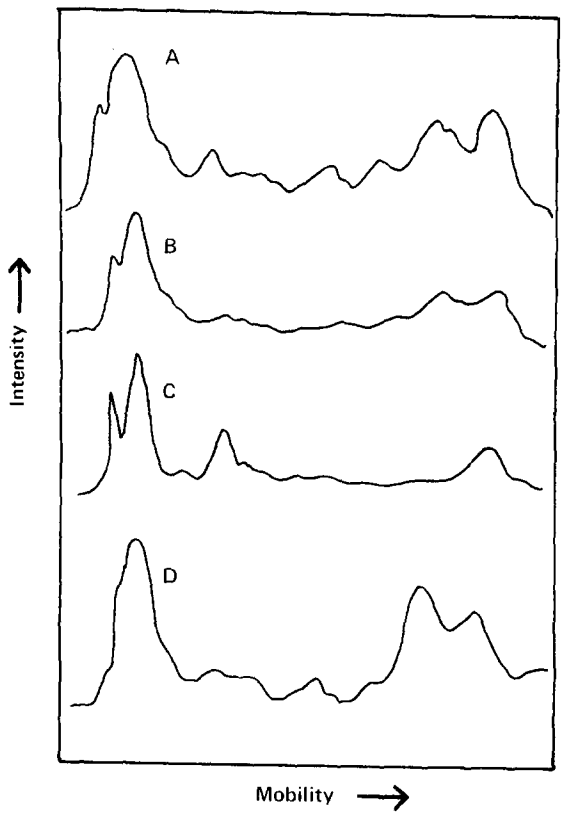

Fig. 3. Gel electrophoretic analyses of fractions obtained from affinity chromatography of the crude muscle extract. Samples from collected fractions were analyzed by gel electrophoresis, and the densitometer scans show higher molecular weight polypeptides at the left, and lower molecular weight polypeptides at the right. Samples: A, crude muscle extract; B, material that was not adsorbed by the affinity column; C, material eluted from the affinity column with the 50 mM carnosine buffer; D, material eluted from the affinity column with 8 M urea.

of this fraction by gel electrophoresis (Fig. 5A) showed that its content of the 200 000 dalton polypeptide was dramatically reduced in comparison to that of the crude extract, it seemed likely that this fraction might be suitable for affinity chromatography. As shown in Fig. 1, the activity in this fraction was somewhat labile during storage at 4°C, and for this reason, prolonged storage of this fraction was avoided prior to the chromatography studies.

Affinity chromatography of the 21–30% $(NH_4)_2SO_4$ fraction. When this fraction was applied to the affinity column, it was found that approx. 60% of the applied material was bound to the column. When

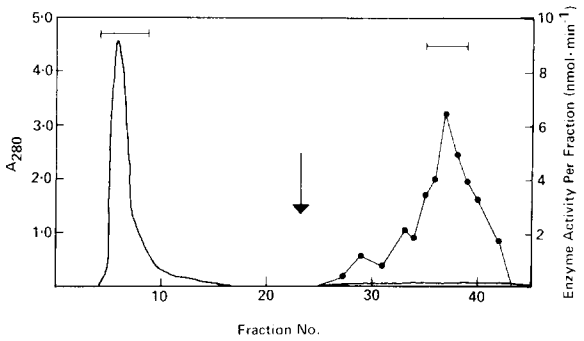

Fig. 4. Affinity chromatography of the 21–30% $(NH_4)_2SO_4$ fraction on Reactive blue 2-agarose. 100 mg protein of this fraction were applied to the affinity column and enzyme assays were performed on the fractions. The position indicated by the arrow shows the point at which the eluting buffer was changed to the buffer containing 50 mM carnosine. Fractions contained within the bar lines (⊢——⊣) were pooled and used in later studies (see Figs. 5 and 6). ————, A_{280} and •——•, enzyme activity per fraction.

the 50 mM carnosine buffer was used as an eluant (Fig. 4), a peak of carnosine synthetase activity was eluted which had an average specific activity across the peak tubes of 343 nmol · min^{-1} · mg^{-1}. This level of specific activity was about 700-fold that in the $(NH_4)_2SO_4$ fraction, and about 1700-fold that in the crude extract. The eluted activity was approx. 80% of that applied to the column, and it represented about 60% of the activity present in the crude extract.

Subunit structure of the eluted carnosine synthetase. Gel electrophoresis (Fig. 5) of the pooled active fractions eluted by carnosine showed that, unlike the material eluted when the crude extract was used, a single polypeptide species was observed on staining with Coomassie blue. The molecular weight of this polypeptide was estimated (Fig. 6A) to be 119 000 ± 6000 using proteins of known molecular weight as standards.

When enzyme from the pooled fraction was chromatographed in non-denaturing solvent on an agarose-acrylamide column, it was eluted at a position which corresponded to a native protein molecular weight of 250 000 ± 20 000 as determined by the elution positions of proteins of known molecular weight (Fig. 6B). These results strongly suggest that the native enzyme is a dimer composed of subunits of M_r = 119 000.

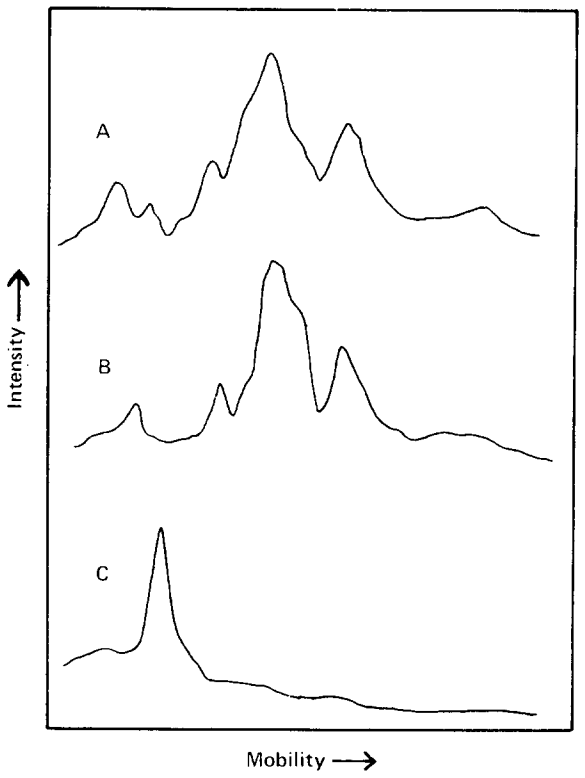

Fig. 5. Gel electrophoretic analyses of fractions obtained from the 21–30% $(NH_4)_2SO_4$ fraction by affinity chromatography. The affinity chromatography of the 21–30% $(NH_4)_2SO_4$ fraction of the crude muscle extract is shown in Fig. 4. Fractions were electrophoresed, and the densitometer scans show high molecular weight polypeptides on the left and low molecular weight polypeptides on the right. Samples: A, 21–30% $(NH_4)_2SO_4$ fraction; B, material from the 21–30% $(NH_4)_2SO_4$ fraction that was not adsorbed by the affinity column; C, material from the 21–30% $(NH_4)_2SO_4$ fraction that was eluted from the affinity column with 50 mM carnosine buffer.

Amino acid analysis of purified carnosine synthetase. Table I shows the amino acid analysis of the enzyme eluted from the affinity column which showed apparent electrophoretic homogeneity. A notable feature of this analysis is the relatively high histidine content. Inspection of chromatograms of the hydrolyzates revealed that no significant amounts of β-alanine were present, indicating that the high histidine content of the enzyme was not caused by binding of carnosine to the enzyme during preparation of the sample for amino acid analysis.

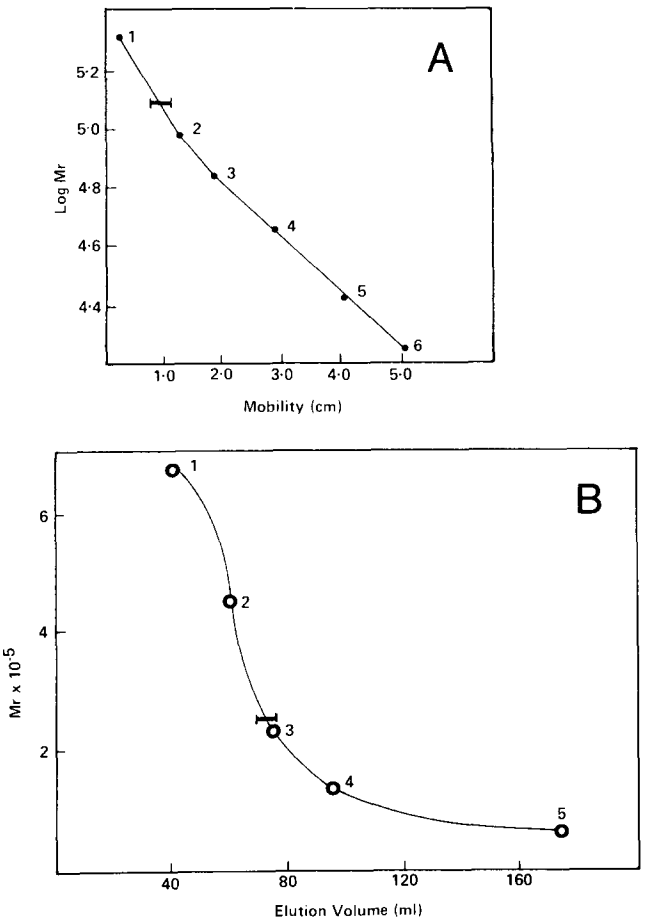

Fig. 6. Molecular weight analysis of denatured and native carnosine synthetase purified by affinity chromatography. The enzyme preparation was the pooled material eluted by 50 mM carnosine buffer during affinity chromatography of the 21–30% $(NH_4)_2SO_4$ fraction of the crude muscle extract (see Fig. 4). A: M_r of the denatured enzyme (⊢——⊣) as determined by gel electrophoresis. Standard proteins are myosin heavy chain (1), phosphorylase (2), albumin (3), ovalbumin (4), chymotrypsinogen (5), and myoglobin (6). B: M_r of the native enzyme (⊢——⊣) as determined by chromatography on AcA-34 gel. Standard proteins are thyroglobulin (1), ferritin (2), catalase (3), lactate dehydrogenase (4) and albumin (5).

TABLE I

AMINO ACID ANALYSIS OF PURIFIED CARNOSINE SYNTHETASE

The enzyme was purified by selective elution from a Reactive blue 2-agarose column with 50 mM carnosine buffer of material adsorbed to the column from the 21–30% $(NH_4)_2SO_4$ fraction of the crude muscle extract. The sample was prepared for amino acid analysis as described in Materials and Methods.

Amino acid	Residues per 1 000 Residues [a]
Asx	81.8
Thr	39.6
Ser	50.4
Glx	108
Pro	39.3
Gly	107
Ala	77.5
Cys	14.1
Val	58.1
Met	16.6
Ile	41.5
Leu	65.5
Tyr	14.6
Phe	26.1
His	141
Lys	63.5
Arg	42.6

[a] Excluding the value for Trp, which was not determined.

Discussion

In the present studies, a crude extract of avian muscle has been obtained which has almost 10-times the specific activity of earlier preparations [12,15]. This level of activity was also obtained using thawed pre-frozen muscle, indicating that the activity of the enzyme is not greatly affected by such storage conditions. The major difference between this preparative method and earlier ones is the current inclusion of the reducing agent dithiothreitol and the protease inhibitor phenylmethanesulfonyl fluoride in the extraction buffer. As the latter compound inhibits only the 'active-serine' proteases [22], it is possible that continued proteolysis and subsequent loss of enzyme activity in the preparation is caused by the presence of other types of proteases known to be present in muscle, such as the Ca^{2+}-activated neutral protease [23]. Studies on the stability of the 21–30% $(NH_4)_2SO_4$ fraction of the crude extract show that in comparison to the crude extract, this material has a higher specific activity and is also much more stable during storage. These results suggest that a component such as a protease which was previously responsible for inactivation of the enzyme has been

removed from the 21–30% fraction.

The chromatographic approach to the purification of carnosine synthetase was based on knowledge of the enzyme-catalyzed reaction [11,12], which must involve nucleotide-binding by the enzyme, and on the previous use of immobilized Cibracon F3G-A dye as a ligand for nucleotide-binding enzymes [24]. As the Cibracon group can be expected to bind a wide variety of proteins which have nucleotide-binding sites, a very selective desorption procedure for carnosine synthetase was necessary [25], and it was decided to use a product (carnosine) of the enzyme for desorption on the assumption that relatively few proteins would be present in muscle which had a nucleotide-binding site whose affinity for the Cibracon group would be altered as a result of binding of carnosine to the protein. When this approach was attempted using the crude extract, several different polypeptides were eluted by carnosine, the principal one of which appeared to be myosin. The elution of myosin by carnosine strongly suggests that this protein has a carnosine-binding site and this finding indirectly supports earlier reports that carnosine might be a physiological effector of myosin ATPase activity [4,26,27].

Because of the nonspecific elution obtained with the crude extract, the 21–30% $(NH_4)_2SO_4$ fraction which had a higher specific activity and a lower myosin content was used in succeeding studies. In these studies, a more specific elution of the enzyme by carnosine occurred as is evidenced by the apparent electrophoretic homogeneity of the eluted fractions. The overall yield of enzyme activity recovered was quite high, representing about 60% of the activity present in a fresh crude extract, and its specific activity was approx. 1700-fold higher than that of the crude extract. This level of specific activity (343 $nmol \cdot min^{-1} \cdot mg^{-1}$) is the highest yet attained for any carnosine synthetase preparation, including that obtained from mouse olfactory bulb [14]. The latter preparation had a specific activity of 25 $nmol \cdot min^{-1} \cdot mg^{-1}$ and was electrophoretically heterogeneous.

Studies on the subunit structure of the enzyme preparation strongly suggest that it is composed of two subunits each of $M_r = 119\,000$. It is not yet known if these subunits are chemically dissimilar, and further structural studies will be necessary to clarify this. Regardless of this point, a very interesting feature of the amino acid analysis of the purified enzyme is its surprisingly high histidine content (141 residues per 1000 residues) which is much higher than the average value (approx. 20 per 1000) found in proteins [28]. The significance of this feature is not obvious at this stage, and its explanation will require a detailed structural analysis of the enzyme.

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