CARNOSINE-ANSERINE SYNTHETASE OF MUSCLE

I. PREPARATION AND PROPERTIES OF A SOLUBLE ENZYME FROM CHICK MUSCLE

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SUMMARY

An enzyme prepared from chick pectoral muscle was found to promote the synthesis of carnosine and anserine from their constituent amino acids. Assay involved incubation of the enzyme with either carboxyl-^{14}C histidine or 1-methylhistidine (plus β-alanine), and subsequent destruction of excess free labeled amino acid with ninhydrin. Residual radioactivity represented incorporation into peptides. The latter were characterized by paper chromatography, by hydrolysis with carnosinase, and by butanol extraction following diazotization (in the case of carnosine). The optimum pH for both carnosine and anserine formation was about 7.5, and both Mg^{++} and ATP were required in each case. Experiments with ^{14}C-β-alanine and D- and L-histidine showed that only the natural form of this latter amino acid was effective for the synthetic process.

INTRODUCTION

In vivo investigations[1-4] with isotopic techniques have provided considerable information on the metabolism of carnosine and anserine, including evidence that both dipeptides are formed by direct condensation of their constituent amino acids. Recently such studies have been extended to *in vitro* experiments with muscle strips[5,6]. However, no information has been obtained of the enzymic processes associated with the formation of carnosine and anserine.

Apart from its value in the general problem of peptide bond synthesis, the elucidation of the path of biosynthesis of β-alanyl peptides is of particular interest, in view of the considerable knowledge now available of the mechanism of formation of γ-glutamylcysteine and the condensation of the latter with glycine to form glutathione, with partially purified enzymes of liver[7], yeast[8], and wheat[9].

The present study is concerned with the preparation of an enzyme, carnosine–anserine synthetase (CAS), from chick muscle, and with certain of its characteristics and requirements.

MATERIALS AND METHODS

Amino acids, peptides and enzymes

DL-1-Methylhistidine, DL-anserine, and L-carnosine were obtained from California

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Foundation for Biochemical Research, Los Angeles. Carnosinase was purchased from Mann Research Laboratories, New York.

Radioactive compounds

β-Alanine-1-^{14}C (0.1 mC/mmole) was synthesized, starting with $Na^{14}CN$[10]. DL-histidine-1-^{14}C (0.15 mC/mmole) and DL-1-methylhistidine-1-^{14}C (0.08 mC/mmole) were both synthesized by the method of SAKAMI AND WILSON[11], via glycine-1-^{14}C[2].

Preparation of muscle extracts

2–3 week old chicks (150–250 g) were used. Pectoral muscle (5–10 g/bird) was rapidly excised, chilled to 0°, minced, and then homogenized in a moderately loose fitting Potter glass homogenizer (with Teflon piston), using 4 ml buffer per g muscle. The homogenizer was chilled by an ice bath during this process. The buffer consisted of 3 parts 0.02 *M* $NaHCO_3$ and 1 part 0.04 *M* pH 7.4 tris (tris(hydroxymethyl)-aminomethane), and also contained 10^{-4} *M* versene and 1 mg Tween/liter. The homogenate was centrifuged at 0° for 2 min at about 12,000 *g*. The supernatant (containing CAS) was decanted, and either assayed or else fractionated at once with methanol.

Preparation of enzyme powder

100 ml of the above extract were immersed in a —10° bath, and 105 ml methanol (0°) were added with stirring during 15–20 min.* The resulting precipitate was sedimented by centrifugation for 1 min at 12,000 *g*, and the supernatant retained. To the latter at —5° was added an additional 55 ml of cold methanol during 15 min. A second fraction was collected by centrifuging 2 min at 12,000 *g*. This precipitate was drained free of liquid, transfered with minimum water to a flask and lyophilized. The resulting light pink powder weighed about 300 mg, and contained more than 95% protein (biuret analysis). It was stored at —20°, and was completely soluble in H_2O.

Assay for dipeptide synthetic activity

Measurements were performed on either muscle extract, or on CAS solution prepared by dissolving 8 mg powder per ml of 0.04 *M* pH 7.3 tris buffer (containing 10^{-4} *M* versene). 0.5-ml portions of test solution were added to centrifuge tubes containing specified compounds in a volume of 0.20 ml. These included 1.5 μmoles ATP, 1.2 μmoles Mg^{++}, 2 μmoles unlabeled β-alanine, and 0.5 μmole ^{14}C-DL-histidine or ^{14}C-DL-methylhistidine, unless otherwise stated. An incubation time of 1 h at 37° was generally employed. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA). After centrifugation, 1 ml of the supernatant was measured into a glass-stoppered tube, 40 mg ninhydrin were added, and the tube was heated for 13 min in boiling H_2O, to decarboxylate excess labeled histidine or methyl-histidine. After cooling, the solution was extracted 3 times with 0.5 ml *n*-butanol, to remove ninhydrin and TCA. The aqueous phase was then transfered quantitatively to a rimmed steel planchet, with the aid of rinsing with 1 ml 0.2% Bacto-agar. The latter aided the formation of a uniform layer when drying the planchets with mild heat. The residual ^{14}C on the planchet (representing incorporation into peptide) was

* The temperature of the solution fell from 0 to —5° during this time.

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measured in a windowless flow-gas Geiger counter. The counts/min were converted into μmoles of peptide by reference to suitable standards of radioactive histidine or methylhistidine. The values were then corrected to account for synthesis in the entire original enzyme system. The unit of enzyme activity is defined as that quantity which synthesizes 1 μmole of labeled peptide/h under the above conditions*.

Assay by paper chromatography

This method was employed in experiments with β-alanine-1-^{14}C and non-isotopic histidine. The above assay procedure was modified as follows: The incubation was terminated by adding 2 ml ethanol, and the tube heated briefly at 90° to coagulate the protein. After centrifugation, the supernatant was evaporated to dryness, reconstituted in 0.1 ml H_2O, and 0.03 ml aliquots analysed chromatographically as previously described[6].

RESULTS

Synthetase activity of muscle extracts

Chicken pectoral muscle, which is rich in dipeptides[12], was the best of several tissue sources tested (Table I). An older chicken had somewhat less activity in breast, and very low activity in leg muscle. The other species tested all had quite low enzyme activity in muscle.

TABLE I

ANSERINE SYNTHETASE ACTIVITY OF DIFFERENT MUSCLE SOURCES

Muscle extracts were prepared and assayed as described in the experimental section, with β-alanine and 1-methylhistidine-l-^{14}C as the labeled substrate. ATP and Mg^{++} were not added, since adequate concentrations were available in the extracts.

Animal species	*Type of muscle*	*Enzyme units per g original muscle*
2-week chick (100 g)	pectoral	0.13
Young chicken (800 g)	pectoral	0.07
	leg	less than 0.01
Young adult pigeon	breast	less than 0.01
Young rat	gasrocnemius	less than 0.01
Young rabbit	psoas	less than 0.01

The enzyme units per mg protein were generally in the range of 0.001–0.002 with the chick pectoral extracts, as compared to values of 0.015, 0.13, and 0.68 for glutathione synthetase in extract of wheat[9], liver[7], and yeast[8] (units similarly defined, but with higher substrate concentrations). It may be mentioned that muscle dipeptides have a relatively slow turnover rate ($t_{\frac{1}{2}}$ = 29 days)[3], while the half life of glutathione in liver is a matter of hours. It is not yet known whether CAS has an intrinsically lower activity, or whether this enzyme is simply present in relatively low concentration in muscle.

The extracts of pectoral muscle were not stable, and reaction rates were linear for less than an hour, so that assays were only semi-quantitative. There is also indication (discussed latter) that the considerable concentrations of peptides present

* The amounts of enzyme used were in the region of proportionality of reaction rate to enzyme concentration.

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may have inhibited the synthetic reactions. However, under the assay conditions employed, the incorporation of ^{14}C into dipeptide was about 50 % greater with labeled methylhistidine than with histidine.

Fractional precipitation of muscle proteins with methanol

Table II illustrates the results obtained. It is seen that about 80 % of the total activity of the original extract was recovered in the fraction precipitates between 52–63 % methanol concentration, and that this preparation was about 4 times more active than the starting material, per mg protein. This lyophilized fraction (about 300 mg in weight) was used for all subsequent experiments described in the present study. Different batches varied considerably in activity and stability, for reasons not yet understood. These preparations lost much of their activity after 2 weeks, even when stored at —20°.

TABLE II

FRACTIONATION OF CHICK-MUSCLE EXTRACT WITH METHANOL

To 100 ml of extract were added increasing proportions of methanol at —5°, and three successive precipitates were collected and assayed for anserine synthetase.

Methanol concentration	*Total enzyme units*	*Units per mg protein*
Initial extract	2.26	0.0014
52 %	0	
63 %	1.80	0.006
70 %	0.15	

General characteristics of CAS

Fig. 1 shows that the rate of dipeptide synthesis was approximately linear for at least 90 min with labeled histidine, and 60 min with methylhistidine. Because optimal Mg^{++} concentration was not used for the anserine curve, the ratio of anserine/carnosine synthesis at one hour is somewhat smaller than in most subsequent experiments.

The optimum pH for the formation of either carnosine or anserine was approximately 7.5 (Fig. 2). The same value was reported for γ-glutamylcysteine synthesis by liver enzyme[13], while a slightly more alkaline optimum pH of 8–8.5 was found for the second step in glutathione synthesis (involving an α-peptide bond) with enzymes from various sources.

The marked stimulation of carnosine and anserine synthesis by β-alanine is shown in Fig. 3. In both cases the rates approached maxima at about 0.5–0.6 μmole/ml. (The isotopic histidine and methylhistidine were employed at 1.0 μmole/ml.)

In Fig. 4 it is seen that synthesis was most rapid with approximately 0.8–1.0 μmole/ml of radioactive DL-histidine or DL-methylhistidine. This value becomes 0.4–0.5 μmole if only the L-isomer is considered. (β-alanine was present at 2 μmole/ml in these experiments.)

As in the enzymic syntheses of glutathione, glutamine, and pantothenic acid[14], the energy requirement for the formation of the β-alanyl peptides can be derived from ATP (Fig. 5). With both histidine and methylhistidine, maximal stimulation resulted at about 0.004 *M* ATP concentration. Attempts to quantitatively correlate peptide-bond synthesis with liberation of inorganic phosphate were not feasible with the

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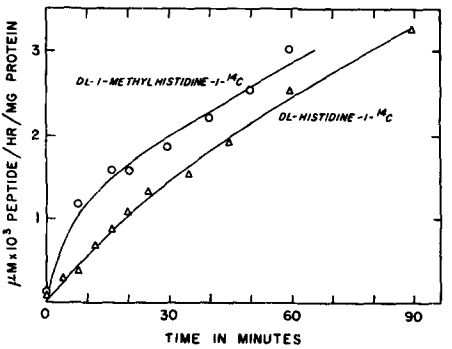

Fig. 1. Rates of carnosine and anserine synthesis by standard CAS preparations. Mg^{++} concentration was 0.002 *M* with labeled histidine, and 0.009 *M* with methylhistidine.

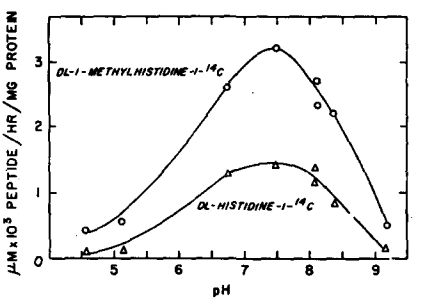

Fig. 2. Influence of pH upon the rates of dipeptide synthesis. Portions of enzyme were dissolved in 0.04 *M* tris buffers, containing sufficient acetic acid to give the required pH values. Mg^{++} was 0.009 *M*.

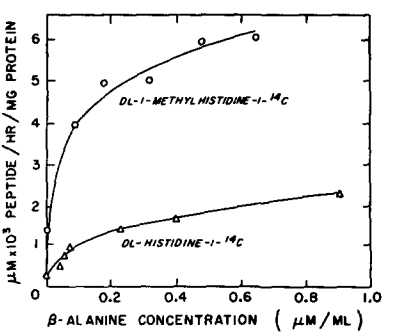

Fig. 3. Effect of varying β-alanine levels on rates of dipeptide synthesis. 0.009 *M* Mg^{++} was employed.

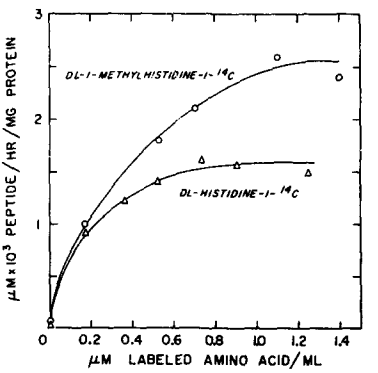

Fig. 4. Influence of increasing concentrations of labeled histidine and of methylhistidine on the rates of dipeptide synthesis. Mg^{++} was 0.009 *M*.

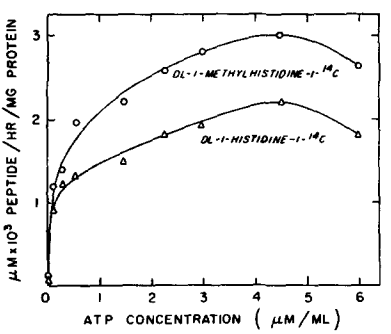

Fig. 5. Dependence of dipeptide synthesis upon adenosine triphosphate. Mg^{++} concentration was 0.009 *M*.

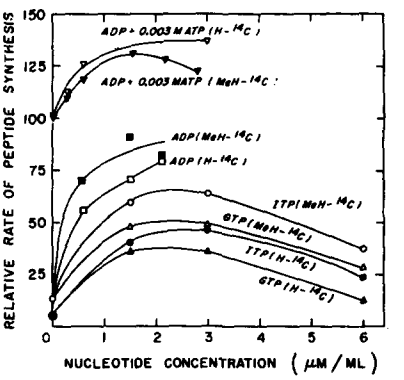

Fig. 6. Effect of various nucleoside di- and triphosphates on carnosine and anserine formation. The values are expressed in terms of the activity obtained with 0.003 *M* ATP (alone), taken as 100.

present crude enzyme preparation, since about 10% breakdown of ATP occured even in the absence of amino acid substrates.

Unlike glutathione synthetases of liver and yeast, CAS was not inhibited by small concentrations of ADP. On the contrary, this nucleotide enchanced significantly the action of ATP (Fig. 6). ADP alone replaced ATP to the extent of 80 % at 0.002 *M* concentration, while somewhat lesser activities were obtained with inosine and guanosine triphosphates. In these experiments the possibility exists of prior transformation of these different nucleotides into ATP by other enzymes in the CAS preparation. It may be noted that ITP was inactive with yeast glutathione synthetase[8].

As in other types of peptide-bond synthesis involving ATP-dependent reactions, magnesium ions were required for the activity of CAS (Fig. 7). With 0.003 *M* ATP present, a comparable concentration of Mg^{++}, about 0.0015–0.002 *M*, was optimal. No significant activity was found in the absence of Mg^{++}, while at high levels of the metal, partial inhibition occured.

CAS was fully active in the absence of K^+, and unaffected by considerable concentrations of this ion. In this respect the present impure preparation differs from the enzymes associated with the two stages of glutathione synthesis (Table III).

Fig. 8 shows that carnosine synthesis was inhibited (about equally) by L-carnosine or DL-anserine, at levels of 0.0015 *M* and higher. Anserine formation was

TABLE III

NON-ESSENTIALLITY OF POTASSIUM IONS FOR DIPEPTIDE SYNTHESIS

In these assays, varying levels of KCl were added to the usual 0.04 *M* tris buffer.

Molar concentration of K^+	*μmole* × 10^3 *Peptide/h/mg protein*	
	with DL-*1-histidine-1-*^{14}C	*with* DL-*methylhistidine-1-*^{14}C
0	2.5	6.5
0.002	2.3	6.0
0.011	2.7	6.2
0.027	2.7	5.9

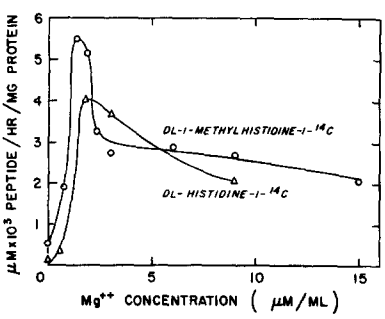

Fig. 7. Essentiality of magnesium ions for carnosine and anserine formation.

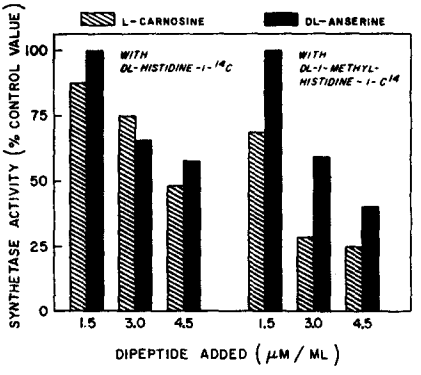

Fig. 8. Inhibition of synthetase activity by carnosine and anserine. The results are expressed in terms of standard assays, without addition of dipeptides.

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similarly depressed, except that DL-anserine appeared to exert a somewhat stronger effect than L-carnosine.

Inorganic phosphate was also found to depress the enzymic formation of dipeptides (Table IV). At 0.01 *M* concentration of phosphate ions the utilization of isotopic histidine was reduced by 55%, and that of methylhistidine by 40%.

TABLE IV

INHIBITORY EFFECT OF PHOSPHATE ON CARNOSINE-ANSERINE SYNTHESIS

Varying quantities of potassium phosphate (pH 7.4) were employed in the assays of CAS activity.

Molar phosphate concentration	*μmole × 10³ Peptide synthesized/h/mg protein*	
	with DL-1-*histidine*-1-^{14}C	*with* DL-1-*methylhistidine*-1-^{14}C
0	3.5	6.8
0.0025	3.1	6.5
0.0050	2.3	5.5
0.0075	1.8	4.7
0.01	1.6	4.1

Solutions of the CAS powder were rather heat-labile, and lost their activity when maintained for a short time at 40° (Fig. 9). At 50°, protein coagulation and inactivation occured in 1–2 minutes.

Characterization of the products of CAS action

The observed relationship between β-alanine concentration and enzyme activity (Fig. 3) implies the formation of β-alanyl peptides. When histidine was the labeled substrate, it was found that the ^{14}C remaining after ninhydrin treatment was largely extracted with butanol, following diazotization (Table V). With labeled methylhistidine, relatively little ^{14}C appeared in butanol extracts, in agreement with the fact that 1-methylated imidazole compounds do not undergo the diazo reaction.

It was also desirable to demonstrate that the enzymically-synthesized peptides could be hydrolyzed by a specific peptidase. When CAS was incubated with its substrates, and carnosinase[15] subsequently added, no appreciable quantities of either carnosine nor anserine could be detected by the usual method (Table VI).

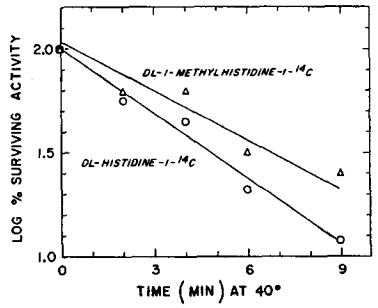

Fig. 9. Thermal inactivation of CAS. The enzyme solution was heated at 40°, and samples removed at varying time intervals for assay of residual activity.

TABLE V

USE OF THE DIAZO REACTION TO DISTINGUISH BETWEEN CARNOSINE AND ANSERINE SYNTHESIS

After assay of CAS powder in the usual manner, the ninhydrin-treated material from several planchets was pooled and reacted with diazotized *p*-chloroaniline[10]. The resulting orange-colored product was extracted with butanol, and the extract evaporated and counted for ^{14}C.

Labeled amino acid employed	*% Total ^{14}C recovered in butanol extract*
DL-Histidine-1-^{14}C	82
DL-1-Methylhistidine-1-^{14}C	12

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TABLE VI

DESTRUCTION BY CARNOSINASE OF PEPTIDE-^{14}C DERIVED FROM SYNTHETASE ACTION

Following incubation of CAS with usual substrates, the mixture was brought to pH 8, made 0.001 *M* in Zn^{++}, and 1 mg carnosinase added. After an additional hour at 37°, TCA was added, and the measurement of ninhydrin-resistant ^{14}C made by the standard method.

Labeled amino acid used with CAS	*Residual peptide-^{14}C, as % of control (not treated with carnosinase)*
DL-Histidine-1-^{14}C	less than 5
DL-1-Methylhistidine-1-^{14}C	0

TABLE VII

COMPARISON OF STEREOISOMERS OF HISTIDINE IN CARNOSINE SYNTHESIS

CAS was incubated under usual conditions, but with β-alanine-1-^{14}C and an unlabeled form of histidine (both at 1 μmole/ml). Carnosine was subsequently isolated by paper chromatography[6].

Histidine isomer employed	DL	L	D
μmole·10^3 Carnosine/h/mg protein	2.4	2.3	0.3

The use of isotopic β-alanine made possible the separate testing of the L- and D-isomers of histidine, with subsequent chromatographic separation of the synthesized carnosine, from excess ^{14}C-β-alanine (Table VII). It is seen that the carnosine region of the chromatograms was radioactive when either L- or DL-histidine was employed. These gave equal activity, because of the relatively high concentrations employed. However, D-histidine was not significantly active.

DISCUSSION

The apparent absence of carnosinase in muscle tissue[15] has made possible the measurement of net synthesis of small quantities of peptides. While the activity of the CAS preparation was not high, under the usual conditions of assay about 5 % of the isotopic DL-methylhistidine and 2–3 % of the histidine were utilized in an hour. These values may be doubled, assuming that the D-isomers were inactive.

Several lines of evidence including optimum pH, thermal lability, response to metallic ions and nucleoside phosphates, substrate effects, and inhibitions by products, all suggest that a single enzyme catalyzes the synthesis of both carnosine and anserine from their constituent amino acids. However, a more conclusive answer requires further experiments with purified preparations.

Other paths of anserine synthesis such as β-alanyl transfer or the methylation of carnosine cannot be ruled out[2]. It is not yet known whether the methylation of histidine (or carnosine) occurs in muscle tissue or elsewhere, and the mechanism of this process is not understood beyond the fact that methionine can serve as a methyl donor. The inhibitory effects of carnosine and anserine, and of phosphate ions, on the synthetic process suggest a reversible reaction, as in the case of glutathione[8,9]. The lack of inhibition by ADP was perhaps due to its small concentration relative to ATP, and to its transformation into the latter (by myokinase). Subsequent papers will be concerned with the purification of CAS and the detailed mechanism of its action.

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ACKNOWLEDGEMENT

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THE EFFECT OF DL-GLUTAMIC ACID ON THE GROWTH OF *RHODOSPIRILLUM RUBRUM*

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SUMMARY

1. *Rhodospirillum rubrum* failed to grow from small inocula in the presence of DL-glutamic acid although washed suspensions of the organism metabolized both isomers.

2. In the presence of high concentrations of DL-glutamic acid the organism initially failed to divide although cells increased in length. This growth soon ceased and was only resumed after a lag following which the cells acquired a more normal morphology.

3. The only cell constituent observed to be affected by growth on DL-glutamic acid is nucleic acid, and both ribonucleic and deoxyribonucleic acids of the organisms are reduced; the former being affected more than the latter.

INTRODUCTION

Although *Rhodospirillum rubrum* grows readily on a medium containing DL-malic acid and L-glutamic acid as principal carbon and nitrogen sources[1,2] it does not grow

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