SEQUENCE OF DODECAPEPTIDE CONTAINING ACTIVE LYSINE FROM CHICKEN ATP: CREATINE PHOSPHOTRANSFERASE

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SUMMARY: The particularly reactive two ϵ -NH_2 groups of lysine in chicken ATP: creatine phosphotransferase were labeled with 1-dimethylaminonaphthalene -5- sulfonylchloride and subsequently the protein was hydrolysed with trypsin. A single peptide composed of 12 residues was isolated and its sequence was established (Leu-Leu-Val-Pro-Asp-Ser-Lys-Leu-Phe-Ser-Val-Arg) by using Edman degradation and also hydrolysis with carboxypeptidases A and B before and after exposure to α chymotrypsin. α chymotrypsin catalyses rupture of the peptide bond between phenylalanine and serine.

ATP: creatine phosphotransferase from various vertebrate species has a molecular weight of about 81,000 and contains two subunits (1). Kuby et. al. (2) established the presence of one active site per protomer and it appears that each one of the two active sites contains one -SH group (3), one lysine and one histidine residue (4,5) as catalytically important components. The sequence about the active -SH group has been determined for rabbit ATP: creatine phosphotransferase (6,7) as Tyr-Val-Leu-Thr-CysH-Pro-Ser-AsN-Leu-Gly-Thr-Gly-Leu-Arg. No knowledge is available, however, as to where in the molecule the histidine or the lysine residue might be located. It was the aim of the present study to pinpoint the position of the responsible lysine. This lysine critical for the catalytic action of ATP: creatine phosphotransferase (isolated from chicken skeletal muscle) was reacted specifically with 1-dimethylaminonaphthalene-5-sulfonylchloride (DNSCl) while the essential -SH groups were blocked (4). Only two moles of DNSCl per mole of enzyme were allowed to react. The masking of the -SH

groups was accomplished by treatment with $K_2S_4O_6$(4).

Experimental Procedure

ATP: creatine phosphotransferase was isolated from the leg muscles of chickens according to the procedure of Kuby et. al. (8) and further purified by passage through a DEAE-cellulose column (9). In order to prevent -SH groups from reacting with DNSCl, they were first treated with $K_2S_4O_6$ (4). To 1.25×10^{-5} moles of protein (dissolved in 95 ml 5×10^{-2} M phosphate buffer, pH 8.1) 1.25×10^{-3} moles $K_2S_4O_2$ (dissolved in 2 ml water) were added. After reaction at 4°C for 5 min., the protein solution was dialysed against the phosphate buffer. 5×10^{-5} moles DNSCl (in 5 ml cold acetone) were introduced into the dialysed solution. After 18 hours at 4°C, the mixture was centrifuged and passed with the phosphate buffer through a Sephadex G25 column. Fractions containing the eluted protein were lyophilized.

The number of moles of DNSCl bound per mole of enzyme were estimated from measurements at 335 mμ using a molar extinction coefficient $\varepsilon_M = 3360$ for the conjugated dye (10) and at 280 mμ using a molar extinction coefficient of 7.1×10^{4} for the enzyme (11).

DNS labeled protein was dissolved in a solution made by mixing 25 ml 0.001 M $CaCl_2$ and 25 ml 0.01 M NaCl. 9×10^{-3} μ moles trypsin (in 1×10^{-3} M cold HCl) per 1.2×10^{-6} moles of protein were added and the mixture was kept 45 hours at 30°C. Nitrogen was passed over the solution and the pH was adjusted at intervals to 8.5 with 0.01N NaOH(12).

For paper chromatography of the tryptic digest we used n-butanol, acetic acid, water (4:1:5, v/v) (16 hrs.) and for high voltage electrophoresis pyridine, acetic acid, water (200:8:2792 v/v) (70 mins.) pH 6.5 (2,000 volts) as solvents. The paper was Whatman 3 MM.

The sole DNS-labeled peptide, located under U.V. light, was extracted from the paper with water and subjected to the same chromatography-solvent two additional times (24 hours and 28 hours respectively) and after a

third extraction to n-butanol, acetic acid, water in the ratio 3:1:1, (v/v) followed by high voltage electrophoresis in pyridine, acetic acid, water (6.6:66:2927 v/v) pH 3.5. Purity of the DNS-labeled peptide was established by two-dimensional paper chromatography employing n-butanol, acetic acid, water (4:1:1, v/v) and tert., butanol, 88% formic acid, water (70:15:15, v/v) as solvent systems.

For treatment with carboxypeptidase 1 μmole peptide was digested simultaneously with 0.1 mg of carboxypeptidase A and B each in 1 ml 0.1 M NH_4HCO_3 (pH 8.3) at 37-40°C. (The concentrations of peptides were estimated by the method of Waddell (13)). Aliquots (.05-.1 μmole) were removed at intervals, 2 drops acetic acid were added and the mixture was lyophilized. A sample from each aliquot was analysed by descending chromatography employing n-butanol, acetic acid, water (200:30:75, v/v) as solvent or was applied to the amino acid analyser.

For digestion with chrymotrypsin 1 μmole peptide in 1 ml 1% NH_4HCO_3 (pH 8.0) was incubated at 30°C with 0.15 mg of 3 times cyrstallized α-chymotrypsin (dissolved in 0.1 ml cold .001N HCl) for 24 hours. Enzyme action was terminated by addition of 100 μl acetic acid and the mixture was lyophilized. For separation of peptides by paper chromatography, we employed n-butanol, acetic acid, water (4:1:5, v/v) (16 hours) as solvent. Purity was checked by paper chromatography using the same solvent in the ratio 4:1:1 (v/v) followed by high voltage electrophoresis with the buffer of pH 6.5.

RESULTS AND DISCUSSION

Spectrophotometrically, it was observed that, under the conditions employed in the present study, 2.6 moles of DNSCl were bound per mole of ATP: creatine phosphotransferase. Paper chromatography (solvent: n-butanol, acetic acid, water, 4:1:5, v/v) of the tryptic digest of

DNS-ATP: creatine phosphotransferase yielded one fluorescent spot (R_f 0.29). Its mobility during high voltage electrophoresis at pH 6.5 proceeded slightly towards the anode. The purified peptide did not show any absorption at 278 mμ and gave no positive reaction with Ehrlich's reagent (14) indicating absence of tyrosine and tryptophan. An aliquot of peptide hydrolysed in 6 N HCl for 20 hours at 110°C in a sealed tube and chromatographed in n-butanol, acetic acid, water (4:1:5 v/v) yielded a marked fluorescent spot (that was also ninhydrin positive) with an R_f value of 0.70 and which cochromatographed properly with synthetic ε-DNSlysine. (A faint ninhydrin positive spot having the same R_f value as free lysine appeared with the spots representing the other amino acids contained in the peptide). The presence of ε-DNS-lysine in the hydrolysate of the peptide was further ascertained by two-dimensional TLC on Eastman Chromagram Sheet, Type K301R (Silica Gel) using the solvent systems: water, 90% formic acid (200:3 v/v) and n-butanol, heptane, acetic acid (6:6:2 v/v) (15).

TABLE I

Composition of DNS-Peptide

Amino Acid	Ratio of amount of amino acid present to phenylalanine present in peptide
Asp	0.96
Ser	2.02
Val	1.95
Leu	3.30
Phe	1.0
Arg	1.11
DNS-lys	Could not be integrated adequately
Pro	

Upon analysis of the hydrolysate of this peptide on the amino acid analyser the following amino acids were detected (Table 1):

Digestion of this DNS-peptide obtained from the trypsin-treated protein with carboxypeptidases A and B released arginine at 10 min., arginine and valine at 30 min., arginine, valine and serine at 90 min., arginine, valine, serine and phenylalanine at 150 min., and arginine, valine, serine, phenylalanine and leucine at 180 min., suggesting the sequence of amino acids from the carboxyl-terminus as Arg-Val-Ser-Phe-Leu.

Edman degradation (16) followed by paper chromatography (17) or hydrolysis with 6 N HCl (18) and application to the amino acid analyser allowed identification of the sequence at the amino-terminus as: Leu-Leu-Val-Pro-Asp. Peptide remaining after the fifth step of the degradation was hydrolysed in 6 N HCl and placed on the amino acid analyser (See table II).

Incubation of the DNS-peptide (obtained from the trypsin-treated

TABLE II

Composition of Residue From Fifth Edman Degradation Step of DNS-Peptide

Amino Acid	Ratio of amount of amino acid present to phenylalanine present in peptide
Ser	2.34
Val	0.89
Leu	1.26
Phe	1.0
Lys	0.47
Arg	0.78

protein) with chymotrypsin yielded two peptides: one positive to the Sakaguchi reaction (14) the other fluorescent if examined under U.V. light. The Sakaguchi positive spot, extracted from the paper, hydrolysed with 6N HCl and applied to the amino acid analyser yielded arginine, valine and serine in the ratio 1.0:1.25:1.2. After reaction of the fluorescent peptide (derived from the chymotryptic digestion) with carboxypeptidases A and B amino acids released could be identified as follows: At 10 min., phenylalanine and leucine; at 30 min., phenylalanine, leucine and ϵ DNS-lysine; at 180 min., phenylalanine, leucine, ϵ DNS-lysine and serine, i.e. the sequence here is: Ser-ϵDNS-lys-Leu-Phe.

Thus, the total peptide sequence is: Leu-Leu-Val-Pro-Asp-Ser-Lys-Leu-Phe-Ser-Val-Arg.

αChymyotrypsin catalyses rupture of the peptide bond between phenylalanine and serine.

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REFERENCES

1. Moreland, B., Watts, D.C., and Virden, R., Nature 214, 458 (1967).

2. Kuby, S. A., Mahowald, T. A., and Noltmann, E. A., Biochem. 1, 748 (1962).

3. Mahowald, T. A., Noltmann, E. A., and Kuby, S. A., J. Biol. Chem. 237, 1535 (1962).

4. Kassab, R., Roustan, C., and Pradel, L. A., Biochem. Biophys. Acta 167, 308 (1968).

5. Pradel, L. A., and Kassab, R., Biochem. Biophys. Acta 167, 317 (1968).

6. Thomson, A. R., Eveleigh, J. W., and Miles, B. J., Nature 203, 267 (1964).

7. Mahowald, T. A., Biochem. 4, 732 (1965).

8. Kuby, S. A., Noda, L., and Lardy, H. A., J. Biol. Chem., 209, 191 (1954).

9. Eppenberger, H. M., Dawson, D. M., and Kaplan, N. O., J. Biol. Chem. 242, 204 (1967).

10. Hartley, B. S., and Massey, V., Biochem. Biophys. Acta 21, 58 (1956).

11. Noda, L., Kuby, S. A., and Lardy, H. A., J. Biol. Chem., 209, 203 (1954).

12. Yue, R. H., Palmieri, R. H., Olson, O. E., and Kuby, S. A., Biochem. 6, 3204 (1967).

13. Waddell, W. J., J. Lab. Clin. Med. 48, 311 (1956).

14. Easley, C. W., Biochem. Biophys. Acta 107, 386 (1965).

15. Woods, K. R., and Wang, K. T., Biochem. Biophys., Acta, 133, 369 (1967).

16. Bailey, J. L., Techniques in Protein Chemistry, 2nd edition, (1967). Elsevier Publishing Co.

17. Sjöquist, Acta Chem. Scand., 7, 447 (1953).

18. Van Orden, H. O., and Carpenter, F. H., Biochem. Biophys. Res. Comm. 14, 399 (1964).