Preliminary Studies on Horse Muscle Acylphosphatase Structure. Evidence of a Mixed Disulfide with Glutathione*

Giampietro Ramponi,† Gianni Cappugi, Cristina Treves, and Paolo Nassi

ABSTRACT: N-terminal and C-terminal amino acids of native and carboxymethylated horse muscle acylphosphatase have been determined. Histidine and glutamic acid are N-terminal residues of native acylphosphatase, while in the carboxymethylated protein only histidine is present. As regards C-terminal amino acids, tyrosine and glycine are the terminal groups of native protein; only tyrosine is the C-terminal of CM-protein. This difference was explained by demonstrating that a peptide with low molecular weight is SS bound to native protein. The migration of this peptide by high-voltage electrophoresis and its amino acid composition have shown its identity with glutathione.

Horse muscle acylphosphatase (EC 3.6.1.7) was purified in our laboratory by Ramponi *et al.* (1969). This enzyme presents a molecular weight of about 10,000 (Ramponi *et al.*, 1967, 1969) and appears therefore to be one of the smallest enzymes on record. The determination of the isoelectric point gave a value of 11.4 (Ramponi *et al.*, 1967), indicating that muscle acylphosphatase is a basic protein. As regards the amino acid composition, this protein consists, approximately, of 80 amino acids residues (Ramponi *et al.*, 1969). Measurements of optical rotatory dispersion, performed on native acylphosphatase, have revealed a low per cent of α helix (G. Ramponi, 1970, unpublished data).

In the present investigation we report some studies concerning the amino and carboxy end of native and carboxymethylated protein.

Furthermore, a low molecular weight thiol, bound to the protein by disulfide link, was isolated as the sulfonic acid derivative by high-voltage electrophoresis. This compound has been recognized to be glutathione.

Materials and Methods

Horse muscle acylphosphatase was prepared by the method of Ramponi *et al.* (1969). Carboxypeptidase A was purchased from Worthington Biochemical Corp.; glutathione from E. Merck AG, Germany; tritiated water from NEN Chemical GmbH; Liquifluor 25X from Nuclear-Chicago. All reagents were pure chemicals.

Reduction and Carboxymethylation. This procedure was performed according to Crestfield *et al.* (1963). Lyophilized acylphosphatase (30 mg) was dissolved in 7.5 ml of 0.6 M Tris-HCl buffer (pH 8.6) containing 8 M deionized urea and 0.2% EDTA and maintained under nitrogen barrier; after the addition of 0.1 ml of mercaptoethanol, the reaction mixture was made up to 12 ml using a solution 8 M in deionized urea and 0.2% in EDTA. After 4–5 hr at room temperature, a freshly prepared solution of 268 mg of iodoacetic acid (four-times crystallized) in 1.0 ml of 1 N NaOH was added. All the operations following the addition of iodoacetate were carried out in the dark. Fifteen minutes after the addition of iodoacetate the sample was purified from reagents using a 2.5 × 90 cm column of Sephadex G-25 equilibrated with 0.2 N acetic acid and wrapped with an aluminum foil. Fractions of 5 ml were collected at a flow rate of 15 ml/hr, using a LKB peristaltic pump. Fractions containing CM-acylphosphatase were pooled and lyophilized.

Amino Acid Analysis. Analysis was performed according to the procedure of Spackman *et al.* (1958) in a Beckman Unichrom amino acid analyzer equipped with a microcuvet. Protein samples were frozen in 1 ml of 6 N HCl, sealed under vacuum, and hydrolyzed at 110° for 22 or 70 hr. Values for serine, threonine, and tyrosine were corrected by extrapolating to zero time the 22- or 70-hr hydrolysis data.

N-Terminal Analysis. The NH_2-terminal residues of acylphosphatase and CM-acylphosphatase were determined by the dansyl procedure reported by Gray (1967). DNS-amino acids were identified by thin-layer chromatography according to the method of Nedkov and Genov (1966), using internal and external standards.

C-Terminal Analysis. C-terminal analyses were performed by hydrazinolysis, selective tritiation, and digestion with carboxypeptidase A.

Hydrazinolysis was carried out by a modification of the method of Akabori *et al.* (1952). Samples of acylphosphatase and CM-acylphosphatase were introduced into glass tubes and dried. Anhydrous hydrazine (0.5 ml) was added and the tubes were sealed *in vacuo*. After 48 hr at 70° the solutions were cooled and dried over sulfuric acid *in vacuo*. The residue was dissolved in 1 ml of distilled water in a centrifuge tube; 0.5 ml of benzaldehyde was added and the mixture was shaken for 2–3 hr. This treatment was repeated and the combined aqueous layers were dried. Before the analysis on the automatic amino acid analyzer, the material was dissolved in 0.2 N citrate buffer (pH 2.2).

The selective tritiation method (Matsuo *et al.*, 1966) is based on oxazolone formation at carboxy end of proteins or peptides by the action of acetic anhydride. The oxazolones contain an active hydrogen and incorporate tritium when treated with $[^3H]H_2O$ and pyridine. Protein or peptide was dissolved in 0.1 ml (100 mCi) of tritiated water; 0.2 ml of pyridine and 0.05 ml of acetic anhydride were added and the mixture was kept at room temperature for 5 hr. After evapora-

* From the Department of Biochemistry of the University of Florence, Florence, Italy. *Received December 2, 1970.* This work was supported by Grants from the Italian Consiglio Nazionale delle Ricerche and from the Ministero della Pubblica Istruzione. Some of this material was presented at the 39th National Meeting of the Società Italiana di Biologia Sperimentale, Sept 21–24, 1970, Pavia, Italy.

† Present address: Istituto di Chimica biologica dell'Università, Viale Morgagni, Florence, Italy; to whom to address correspondence.

TABLE I: Hydrazinolysis of Native and Carboxymethylated Acylphosphatase (200 nmoles of Protein Was Analyzed).

Protein	n/moles Found	
	Acylphosphatase	CM-acylphosphatase
Amino acid		
Lysine	0	0
Histidine	0	0
Arginine	0	0
Aspartic acid	Trace	Trace
Threonine	Trace	Trace
Serine	10	10
Glutamic acid	Trace	Trace
Proline	0	0
Glycine	150	10
Alanine	Trace	Trace
Half-cystine	0	
CM-cysteine		0
Valine	0	0
Methionine	0	0
Isoleucine	0	0
Leucine	Trace	Trace
Tyrosine	80	60
Phenylalanine	0	0

TABLE II: Selective Tritiation of Native Acylphosphatase, CM-acylphosphatase, and Glutathione.

Protein or Peptide	Counts per Minute[a]		
	Acylphosphatase[b]	CM-acylphosphatase[b]	Glutathione[c]
Amino acid			
Lysine	1,294	3,700	
Histidine	0	0	
Arginine	504	500	
Aspartic acid	1,398	1,290	
Threonine	0	500	
Serine	948	1,600	
Glutamic acid	35,668	1,274	156,240
Proline	0	0	
Glycine	17,786	1,600	97,806
Alanine	0	650	
Cystine	0		200
CM-cysteine		74	
Valine	0	500	
Methionine	0	675	
Isoleucine	0	0	
Leucine	1,170	2,550	
Tyrosine	22,502	22,124	
Phenylalanine	0	400	

[a] Expressed as the sum of counts per minute of all fractions of each amino acid. [b] 100 nmoles was analyzed. [c] 385 nmoles was analyzed.

tion *in vacuo* at 40°, the residue was washed ten times with distilled water and subjected to hydrolysis in 6 N HCl for 24 or 72 hr. The identification of labeled amino acids was carried out by an improved procedure devised in our laboratory, consisting in the use of amino acid analyzer (Cappugi *et al.*, 1971). The exit of flow colorimeter was connected to a fraction collector; 2-ml fractions were collected. A 0.8-ml portion of every fraction was introduced into a counting vial and successively 0.5 ml of distilled water and 10 ml of scintillation solution (one-third parts of Triton X-100 and two-thirds parts of Liquifluor diluted 1:10 with toluene) were added.

For the method of carboxypeptidase A, lyophilized acylphosphatase was dissolved in 0.1 M phosphate buffer (pH 8.0) containing 0.056 M sodium lauryl sulfate (Guidotti, 1960) and incubated with carboxypeptidase A at 25° with a substrate to enzyme ratio of 15:1. Aliquots were removed periodically and the digestions were terminated by the addition of 0.2 N citrate buffer (pH 2.2); the samples were applied to the amino acid analyzer without further treatment.

Isolation of Protein-Bound Thiol. Native acylphosphatase was oxidized with freshly prepared performic acid to split SS bridge, according to the method of Moore (1963) with slight modification. After oxidation the sample was dried *in vacuo* on solid NaOH. The residue was dissolved in small amount of 0.3 M acetic acid; portions of the solution were applied to Whatman No. 3MM paper (3 × 66 cm) at 27 cm from an edge. Glutathione sulfonic acid separation was performed by high-voltage electrophoresis, in a Savant apparatus, in 0.9 M acetate buffer (pH 2.9) at 5200 V for 30 min (Gross, 1955). The standard was prepared by oxidizing glutathione in the same experimental conditions. One strip was stained with cadmium–ninhydrin reagent. Other strips were not stained and the zone corresponding to sulfonic acid of glutathione was cut out and eluted with distilled water. The solution obtained was lyophilized and the residue was hydrolyzed for amino acid analysis.

Results and Discussion

End Groups. As regards N-terminal amino acids, experiments were performed on native and on carboxymethylated protein. The results have revealed that both histidine and glutamic acid are N-terminal residues of native acylphosphatase, while in the carboxymethylated protein only histidine, as N-terminal amino acid, is present. For the determination of C-terminal amino acids, experiments of hydrazinolysis, selective tritiation, and digestion with carboxypeptidase A were performed.

In Table I the results obtained with hydrazinolysis techniques are summarized. As it appears, the native protein shows two C-terminal residues: tyrosine and glycine; the carboxymethylated protein only tyrosine. A possible explanation of the low yield of tyrosine could be the presence of a peptide bond which is split with a low reaction rate (Bradbury, 1958).

In Table II are reported the results of selective tritiation experiments carried out on native acylphosphatase, CM-acylphosphatase, and glutathione. In the native protein glutamic acid, glycine, and tyrosine are labeled, while in the carboxymethylated protein only tyrosine is labeled. From the above experiments it is evident that the results obtained with the tritiation method are not in agreement with those of hydrazinolysis. In fact with the latter procedure glutamic acid is not detected. In a previous work from this laboratory (Ramponi *et al.*, 1970), we have demonstrated that non-C-terminal glutamic acid can be labeled by tritiation method when the γ-

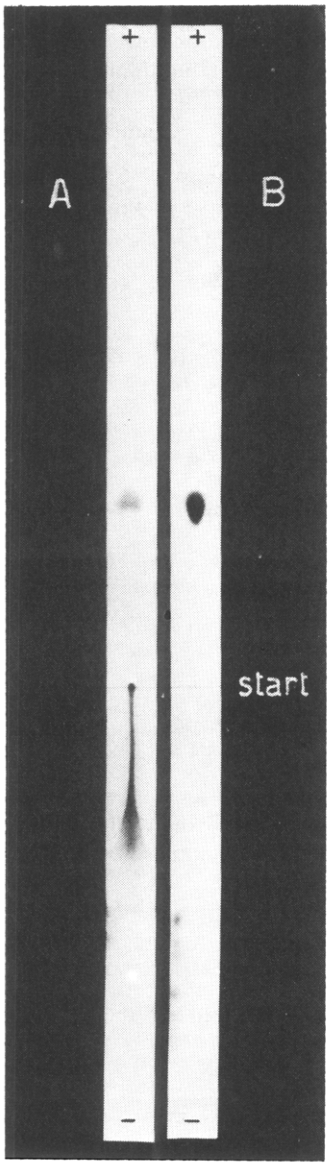
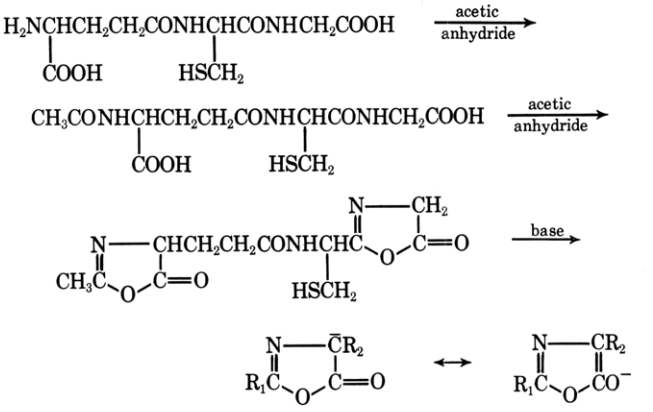


FIGURE 1: High-voltage electrophoresis. (A) Acylphosphatase oxidized with performic acid: the spot which migrates to cathode is oxidized acylphosphatase; the other spot which moves toward the anode is the polypeptide bound to acylphosphatase, which migrates like the standard. (B) Sulfonic acid of glutathione, used as standard.

SCHEME I



glutamyl peptide linkage occurs. In fact in the glutathione experiment also glutamic acid, which is not the C-terminal amino acid, is labeled. In this case glutamic acid can form an oxazolone at amino end, as shown in Scheme I.

Carboxypeptidase A digestion was carried out only on native acylphosphatase. In our experimental conditions only tyrosine is released.

Table III summarizes all the data obtained from the end-group analyses.

Mixed Disulfide from Glutathione and Native Acylphosphatase. In Table IV amino acid analyses of carboxymethylated and native acylphosphatase are reported. It can be observed that, between the two analyses, there is a difference of three residues; in the modified protein, in fact, one glutamic acid residue, one glycine residue and one CM-cysteine residue are not present. This observation, together with the evidence that the two proteins show different end groups, has suggested the

TABLE III: Summarized Data of End-Group Analyses Performed on Native and CM-acylphosphatase.

End Group	Acylphosphatase	CM-acyl-phosphatase
C terminal		
Hydrazinolysis	Glycine Tyrosine	Tyrosine
Selective tritiation	Glycine Tyrosine Glutamic acid	Tyrosine
Carboxypeptidase A	Tyrosine	
N terminal		
Dansyl procedure	Histidine Glutamic acid	Histidine

TABLE IV: Amino Acid Composition of CM-acylphosphatase in Comparison to that of Native Protein.

Amino Acid	CM-acylphosphatase[a]	Acylphosphatase[a]	Acylphosphatase[b]
Lysine	7.66	8.04	8
Histidine	0.52	0.52	1
Arginine	5.00	5.00	5
Aspartic acid	5.92	6.38	6
Threonine	4.82	5.03	5
Serine	9.69	9.85	10
Glutamic acid	8.19	9.22	9
Proline	2.64	3.28	3
Glycine	6.14	6.89	7
Alanine	3.07	3.39	3
Half-cystine		1.65	2
CM-cysteine	0.67		
Valine	7.68	7.61	8
Methionine	1.58	1.57	2
Isoleucine	2.30	2.23	2
Leucine	2.74	3.06	3
Tyrosine	2.60	2.69	3
Phenylalanine	2.62	3.03	3

[a] Residues per molecule of protein. [b] Ramponi *et al.* (1969).

hypothesis that a small peptide bound to native protein by disulfide link could occur. This peptide, removed by reduction and alkylation, could be lost since it remained in the internal volume of the Sephadex G-25 column, used for purification of carboxymethylated protein. Besides, considering that with the selective tritiation procedure also non-C-terminal glutamic acid was labeled, and that this amino acid can be tritiated when the γ-glutamyl peptide linkage occurs, we have thought that the peptide bound to native acylphosphatase could be glutathione, which presents a γ-glutamyl peptide linkage. In order to confirm this hypothesis, an experiment of high-voltage electrophoresis was carried out on acylphosphatase oxidized with performic acid.

Figure 1 shows the results obtained with high-voltage electrophoresis: in strip A it can be seen that the oxidized protein migrates toward the cathode, while a polypeptide moves toward the anode exactly like glutathione sulfonic acid, which was used as standard in strip B.

In Table V is reported the analysis of the peptide eluted after high-voltage electrophoresis; it is evident that the molar ratio between cysteic acid, glutamic acid, and glycine, is 1:1:1. Other amino acids are not present.

TABLE V: Amino Acid Composition of Peptide Bound to Native Acylphosphatase.

Amino Acid	Molar Ratio of Residues
Cysteic acid	1
Glutamic acid	0.99
Glycine	0.97

In this paper it is reported, for the first time, that native horse muscle acylphosphatase is constituted by two chains SS bound; one, whose C-terminal amino acid is tyrosine and N-terminal histidine, the other, with low molecular weight, which is glutathione.

Protein–GSH- and protein–cysteine-mixed disulfide have been identified in human plasma albumin (King, 1961), in protein of Ehrlich ascites cells (Révèsz and Modig, 1965; Modig, 1968), in bovine serum albumin (Modig, 1968), in rat spleen, liver, heart, muscle, kidney, Yoshida ascites sarcoma (Jackson *et al.*, 1968), and in normal and cataractous human lenses (Harding, 1970).

Studies are in progress to investigate the role of glutathione on the enzyme function.

Acknowledgment

We thank Professor Vincenzo Baccari for helpful advice and for his criticism in the revision of the manuscript. We are indebted to Mr. Guido Camici for his skillful technical assistance.

References

Akabori, S., Ohno, K., and Narita, K. (1952), *Bull. Chem. Soc. Jap. 25*, 214.
Bradbury, J. H. (1958), *Biochem. J. 68*, 475.
Cappugi, G., Nassi, P., Treves, C., and Ramponi, G. (1971), *Experientia 27*, 237.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Gray, W. R. (1967), *Methods Enzymol. 11*, 139.
Gross, D. (1955), *Nature (London) 176*, 72.
Guidotti, G. (1960), *Biochim. Biophys. Acta 42*, 177.
Harding, J. J. (1970), *Biochem. J. 117*, 957.
Jackson, R. C., Harrap, K. R., and Smith, C. A. (1968), *Biochem. J. 110*, 37p.
King, T. P. (1961), *J. Biol. Chem. 236*, PC 5.
Matsuo, H., Fujimoto, Y., and Tatsuno, T. (1966), *Biochem. Biophys. Res. Commun. 22*, 69.
Modig, H. (1968), *Biochem. Pharmacol. 17*, 177.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Nedkov, P., and Genov, N. (1966), *Biochim. Biophys. Acta. 126*, 544.
Ramponi, G., Cappugi, G., Nassi, P. (1970), *Biochem. Biophys. Res. Commun. 41*, 642.
Ramponi, G., Guerritore, A., Treves, C., Nassi, P., and Baccari, V. (1969), *Arch. Biochem. Biophys. 130*, 362.
Ramponi, G., Treves, C., and Guerritore, A. (1967), *Arch. Biochem. Biophys. 120*, 666.
Révèsz, L., and Modig, H. (1965), *Nature (London) 207*, 430.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.