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# The Complete Amino Acid Sequence of the Low Molecular Weight Cytosolic Acid Phosphatase\*

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This paper presents the complete amino acid sequence of the low molecular weight acid phosphatase from bovine liver. This isoenzyme of the acid phosphatase family is located in the cytosol, is not inhibited by L-(+)-tartrate and fluoride ions, but is inhibited by sulfhydryl reagents. The enzyme consists of 157 amino acid residues, has an acetylated NH<sub>2</sub> terminus, and has arginine as the COOH-terminal residue. All 8 half-cystine residues are in the free thiol form. The molecular weight calculated from the sequence is 17,953. The sequence was determined by characterizing the peptides purified by reverse-phase high performance liquid chromatography from tryptic, thermolytic, peptic, *Staphylococcus aureus* protease, and chymotryptic digests of the carboxymethylated protein. No sequence homologies were found with the two known acylphosphatase isoenzymes or the metalloproteins porcine uteroferrin and purple acid phosphatase from bovine spleen (both of which have acid phosphatase activity). Two half-cystines at or near the active site were identified through the reaction of the enzyme with [<sup>14</sup>C] iodoacetate in the presence or in the absence of a competitive inhibitor (*i.e.* inorganic phosphate).

Ac-AEQVTKSVLFLVCLGNICRSPIAEAVFR  
KLVTQDNISDNVVIDSGAVSDWNVGRSP  
NPRAVSCLRNHGINTAHKARQVTKEDFV  
TFDYILCMDESRLRDLNRKSNQVKNCRA  
KIELLGSYDPQKQLIIEDPPYGGNDADFET  
VYQQCVRCRAFLEKVR-OH

Acid phosphatases (orthophosphoric-monoester phosphohydrolases (acid optimum), EC 3.1.3.2) are ubiquitous in nature and often occur in multiple forms differing in  $M_r$ , substrate specificity, and sensitivity to inhibitors (1-4). In addition, most of these enzymes are glycoproteins and some are also metalloproteins (such as the iron-containing acid phosphatases, porcine uteroferrin and purple acid phosphatase from spleen (5) and bone (6, 7), and the manganese-containing acid phosphatases isolated from some plants (8, 9)). The presence in mammalian tissues of low  $M_r$  isoenzymes was clearly demonstrated by Henrikson (2) who purified the enzyme from bovine liver. Subsequently, De Araujo *et al.* (4) localized the low  $M_r$  acid phosphatase in the cytosol.

Lawrence and Van Etten (10) have recently reinvestigated

the low  $M_r$  acid phosphatase from bovine liver. They reported that they had purified the enzyme to homogeneity and criticized Henrikson's (2) data, particularly with respect to the amino acid composition and specific activity, which they found to be approximately twice as high.

The substrate specificity of the low  $M_r$  acid phosphatases is more restricted than that of the high  $M_r$  acid phosphatases in that the former efficiently hydrolyzes only *p*-nitrophenyl phosphate and riboflavin phosphate (2, 11). In 1980, we reported that the enzyme isolated from bovine liver is catalytically very active with acylphosphates such as carbamoyl phosphate and benzoyl phosphate (12). Taga and Van Etten (11) also found that the low  $M_r$  isoenzyme from human liver has a high activity on acetyl phosphate and suggested a similarity between the low  $M_r$  acid phosphatases and another class of enzymes called acylphosphatases (EC 3.6.1.7). The latter enzymes were extensively studied in our laboratory (13-16): although they have in common a subcellular localization in the cytosol and similar molecular weights, we found that the specificity of the two known isoenzymes of acylphosphatase is limited to acylphosphates and that they do not hydrolyze orthophosphoric-monoesters. In addition, the amino acid sequence was determined for several acylphosphatases from skeletal muscle of vertebrate species (17-23) and for the isoenzyme from human erythrocytes (24).

Recently, Chernoff and Lee (25) demonstrated that the major phosphotyrosyl-protein phosphatase from bovine heart is associated with a low  $M_r$  acid phosphatase. They reported that this enzyme appears to be similar to the low  $M_r$  acid phosphatases from other tissues, including the liver enzyme. In fact, all these enzymes have similar  $M_r$ , pH optima, and  $K_m$  values for *p*-nitrophenyl phosphate, and all are insensitive to inhibition by L-(+)-tartrate and fluoride ions. Furthermore, Boivin and Galand (26) purified two isoenzymes from human red cell cytosol that efficiently dephosphorylate the membrane protein band 3, previously phosphorylated on a specific tyrosine residue by a tyrosine phosphokinase present in the red cell membrane.

## EXPERIMENTAL PROCEDURES AND RESULTS<sup>1</sup>

### DISCUSSION

The complete amino acid sequence of the low molecular weight acid phosphatase (cytosol) from bovine liver is presented in Fig. 1, together with the peptides used to delineate the primary structure. The protein, consisting of 157 amino acid residues, is acetylated at the NH<sub>2</sub> terminus, and has Arg

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<sup>1</sup> Portions of this paper (including "Experimental Procedures," "Results," Figs. 2-11, and Tables I-VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.



partial sequences (accounting for about 90% of the entire molecules) with the sequence of the low  $M_r$  acid phosphatase from bovine liver shows virtually no sequence homologies. Furthermore, the two proteins (uteroferrin consists of a single polypeptide of 35 kDa, whereas purple acid phosphatase consists of two polypeptide chains of 20 kDa and 15 kDa) have a low cysteine content: uteroferrin contains 2 cysteine residues per molecule, whereas the 20-kDa purple acid phosphatase chain contains only 1, and the 15-kDa purple acid phosphatase chain contains 2. In contrast, the low  $M_r$  acid phosphatase contains 8 cysteines per molecule ( $M_r = 17,953$ ). The sequences around cysteine residues in uteroferrin and purple acid phosphatase are different from those around the 8 cysteines of the low  $M_r$  acid phosphatase. Uteroferrin and the two chains of purple acid phosphatase have free  $\alpha$ -NH<sub>2</sub> groups at the NH<sub>2</sub> termini, whereas the low  $M_r$  acid phosphatase has an  $\alpha$ -N-acetylated NH<sub>2</sub>-terminal residue. Acetylation at the NH<sub>2</sub> terminus has been postulated to be characteristic of proteins synthesized on free polysomes in the cytosol (27). Thus, our results agree with the data of De Araujo *et al.* (4) on the cytosolic localization of the low  $M_r$  acid phosphatase. We found that iodoacetate causes the inactivation of the enzyme and that the competitive inhibitor P<sub>i</sub> protects the low  $M_r$  acid phosphatase against inactivation (Fig. 10). In agreement with the data of Lawrence and Van Etten (10), our results indicate that at least 1 half-cystine residue is present at or near the active site because 85% inactivation of the enzyme occurred with the carboxymethylation of 0.9 residue of half-cystine per molecule of enzyme (Table I); furthermore, the competitive inhibitor P<sub>i</sub> reduced the rate of inactivation by preventing the iodoacetate reaction with active site sulfhydryl group(s). Because of the difference in inactivation of the enzyme by iodoacetate in the presence and absence of P<sub>i</sub> (Fig. 10), differential modification by [<sup>14</sup>C]iodoacetate (28) was used to distinguish essential active site half-cystines from others that might be modified at the same time at other sites of the molecule. We found that Cys-12 and Cys-17, both labeled by [<sup>14</sup>C]iodoacetate, are protected to the same extent by P<sub>i</sub>, so that these two half-cystine residues are at or near the active site of the enzyme. Nevertheless, Cys-12 reacts with iodoacetate 3.5 times faster than Cys-17 (see the specific radioactivity of Cys-12- and Cys-17-containing peptides in the Miniprint). Thus, Cys-12 contributes most to enzyme's inactivation. In this paper, we present the first complete amino acid sequence for an acid phosphatase.

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#### REFERENCES

- DiPietro, D. L., and Zengerle, F. S. (1967) *J. Biol. Chem.* **242**, 3391–3396
- Heinrikson, R. L. (1969) *J. Biol. Chem.* **244**, 299–307
- Hollander, P. V. (1971) in *The Enzymes* (Boyer, P. D., ed) Vol. 4, pp. 449–490, Academic Press, Orlando, FL
- De Araujo, P. S., Mies, V., and Miranda, O. (1976) *Biochim. Biophys. Acta* **452**, 121–130
- Hunt, D. F., Yates III, J. R., Shabanowitz, J., Zhu, N. Z., Zirino, T., Averill, B. A., Daurat-Larroque, S. T., Shewale, J. G., Roberts, R. M., and Brew, K. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1154–1160
- Kato, T., Hara, A., Nakayama, T., Sawada, H., Hamatake, M., and Matsumoto, Y. (1986) *Comp. Biochem. Physiol.* **83B**, 813–817
- Anderson, T. R., and Toverud, S. U. (1986) *Arch. Biochem. Biophys.* **247**, 131–139
- Uehara, K., Fujimoto, S., and Taniguchi, T. (1974) *J. Biochem. (Tokyo)* **75**, 627–638
- Fujimoto, S., Nakagowa, T., and Ohara, A. (1979) *Chem. Pharm. Bull.* **27**, 545–548
- Lawrence, G. L., and Van Etten, R. L. (1981) *Arch. Biochem. Biophys.* **206**, 122–131
- Taga, E. M., and Van Etten, R. L. (1982) *Arch. Biochem. Biophys.* **214**, 505–515
- Ramponi, G., Cappugi, G., Manao, G., and Camici, G. (1980) *Proceedings of the 26th National Congress Società Italiana di Biochimica, Bologna, September 24–26*, p. 167
- Ramponi, G. (1975) *Methods Enzymol.* **42**, 409–426
- Manao, G., Camici, G., Stefani, M., Berti, A., Cappugi, G., Liguri, G., Nassi, P., and Ramponi, G. (1983) *Arch. Biochem. Biophys.* **226**, 414–424
- Liguri, G., Nassi, P., Camici, G., Manao, G., Cappugi, G., Stefani, M., Berti, A., and Ramponi, G. (1984) *Biochem. J.* **217**, 499–505
- Berti, A., Stefani, M., Camici, G., Manao, G., Cappugi, G., Degl'Innocenti, D., and Ramponi, G. (1986) *Int. J. Peptide Protein Res.* **28**, 15–21
- Cappugi, G., Manao, G., Camici, G., and Ramponi, G. (1980) *J. Biol. Chem.* **255**, 6868–6874
- Camici, G., Manao, G., Cappugi, G., Berti, A., Stefani, M., Liguri, G., and Ramponi, G. (1983) *Eur. J. Biochem.* **137**, 269–277
- Manao, G., Camici, G., Modesti, A., Liguri, G., Berti, A., Stefani, M., Cappugi, G., and Ramponi, G. (1984) *Mol. Biol. Med.* **2**, 369–378
- Manao, G., Camici, G., Cappugi, G., Stefani, M., Liguri, G., Berti, A., and Ramponi, G. (1985) *Arch. Biochem. Biophys.* **241**, 418–424
- Stefani, M., Modesti, A., Camici, G., Manao, G., Cappugi, G., Berti, A., and Ramponi, G. (1986) *J. Protein Chem.* **5**, 307–321
- Camici, G., Manao, G., Modesti, A., Stefani, M., Berti, A., Cappugi, G., and Ramponi, G. (1986) *Ital. J. Biochem. (Engl. Ed.)* **35**, 1–15
- Mizuno, Y., Yamazaki, M., Takasawa, T., Kizaki, T., and Shiokawa, H. (1985) *J. Biochem. (Tokyo)* **97**, 1135–1142
- Liguri, G., Camici, G., Manao, G., Cappugi, G., Nassi, P., Modesti, A., and Ramponi, G. (1986) *Biochemistry* **25**, 8089–8094
- Chernoff, J., and Lee, H. C. (1985) *Arch. Biochem. Biophys.* **240**, 135–145
- Boivin, P., and Galand, C. (1986) *Biochem. Biophys. Res. Commun.* **134**, 557–564
- Wold, F. (1984) *Trends Biochem. Sci.* **9**, 256–257
- Singer, S. J. (1967) *Adv. Protein Chem.* **22**, 1–54
- Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
- Ramponi, G., Cappugi, G., Treves, C., and Nassi, P. (1971) *Biochemistry* **10**, 2082–2085
- Penke, B., Ferenczi, R., and Kovacs, K. (1974) *Anal. Biochem.* **60**, 45–50
- Tarr, G. E. (1977) *Methods Enzymol.* **47**, 335–357
- Bhowan, A. S., Mole, J. E., and Bennett, J. C. (1981) *Anal. Biochem.* **110**, 355–359
- Hermanson, M. A. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., ed) pp. 313–324, Humana Press, Clifton, NJ
- Kasper, C. B. (1975) in *Protein Sequence Determination* (Needleman, S. B., ed) pp. 114–161, Springer Verlag, Berlin

## Supplementary Material to:

"The Complete Amino Acid Sequence of the Low Molecular Weight Cytosolic Acid Phosphatase" by Guido Camici, Giampaolo Manao, Alessandra Modesti, Massimo Stefani and Giampietro Ramponi

## EXPERIMENTAL PROCEDURES

**Materials.** Low M<sub>w</sub> acid phosphatase was prepared essentially as described by Lawrence and Van Etten (10). With their technique however, we were not able to obtain a pure protein; a small quantity of a contaminating protein (about 14 KDa) always remained. Therefore we purified the enzyme to homogeneity by means of HPLC on an Aquapore RP 300 (Brownlee Labs. Inc.) column with a TFA/acetonitrile gradient system and checked its purity by SDS-PAGE or by PAGE in urea-containing gels, as described by Manao et al. (14).

Diphenylcarbamylchloride-treated trypsin, pepsinogen, carboxypeptidase Y, diisopropylfluorophosphate-treated carboxypeptidase B and chymotrypsin were obtained from Sigma. *S. aureus* V8 protease was obtained from Miles. ThermoLysin was purchased from Merck (Darmstadt). Reagents and solvents (sequential grade) for sequence determination by the Edman degradation technique were obtained from Fluka A.G. Iodo [2-<sup>14</sup>C]acetic acid was from Amersham Int., with a specific radioactivity of 56 mCi/mmol. All other reagents used were of the highest purity commercially available.

**Determination of free sulfhydryl groups.** Total sulfhydryl group content of acid phosphatase was determined both by spectrophotometric titration with 5,5'-dithiobis(2-nitrobenzoate), Ellman's reagent (29), and by carboxymethylation of the enzyme with iodoacetate in the presence of 6 M guanidinium chloride but in the absence of reducing agents. The spectrophotometric titration was carried out by dissolving the protein in 1 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.01 M EDTA and 6 M guanidinium chloride; the mixture was incubated at room temperature for 20 min prior to addition of Ellman's reagent.  $\epsilon_{412} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$  was used for calculation. Protein concentration was determined by amino acid analysis. The carboxymethylation of the enzyme in the absence of reducing agents was carried out as follows: the enzyme (29 nmol) was dissolved in 40  $\mu$ l of 0.1 M Tris-HCl buffer, pH 8.50, containing 6 M guanidinium chloride and the reaction vessel was flushed with nitrogen. After 20 min incubation at room temperature, 40  $\mu$ l of 64.5 M iodoacetic acid solution (adjusted to pH 8.70 with Tris base) was added. The reaction was carried out under nitrogen atmosphere for 15 min and the carboxymethylated protein was immediately isolated from reagents by HPLC on Aquapore RP 300 (4.6 x 250 mm, 7  $\mu$ m) using a TFA / acetonitrile based solvent system. The protein was hydrolyzed in 6 N HCl and the amino acid composition was determined.

**Reduction and carboxymethylation.** This process was carried out on 0.35  $\mu$ mol of the low M<sub>w</sub> acid phosphatase by the technique previously described (30) except that the carboxymethylated protein was purified from urea and reagents by gel filtration on a column (2 x 40 cm) of Sephadex G 25 superfine equilibrated with 0.2 M ammonia solution.

**Amino acid analysis.** Amino acid analyses were carried out on 3-10<sup>-5</sup> mmol of peptides or protein by means of a Carlo Erba 3024 amino acid analyzer with an SP 4200 computing integrator (30). Cysteine was determined as methionine and tryptophan was assayed by the method of Penke et al. (31). Alternatively, amino acid analysis of peptides (100-500 pmol) was performed by analyzing the phenylthiocarbonyl-derivatives of amino acids by HPLC on a Waters Pico-Tag amino acid analysis column (3.3 x 150 cm, 4  $\mu$ m), using an acetonitrile gradient.

**Enzymic hydrolysis.** Before the various enzymatic digestions, the carboxymethylated acid phosphatase solution in a screw-cap sealed vial was immersed for 5 min in a boiling water bath and then chilled in ice.

Tryptic, peptic, *S. aureus* protease, and thermolytic digestions were carried out as previously described (24). Chymotryptic digestion of C<sub>60</sub>-acid phosphatase (40 nmol) was carried out with a 2.5% (w/v) chymotrypsin in 0.2 M ammonium bicarbonate buffer, pH 8.50, for 4 h at 37°C. With regard to the digestion with carboxypeptidases, C<sub>60</sub>-acid phosphatase (20 nmol) was dissolved in 0.1 M N-ethyl-morpholine-acetate buffer, pH 8.50, and mixed with carboxypeptidase B at a carboxypeptidase/substrate ratio of 0.02 units per nmol. For the digestion with carboxypeptidase Y, the peptide (6 nmol) was dissolved in 0.1 M pyridine-acetate buffer, pH 6.60, and mixed with carboxypeptidase Y at a carboxypeptidase/substrate molar ratio of 1/25. The mixtures were incubated at 30°C; aliquots were withdrawn at different time intervals, and added to 0.2 M sodium salicylate acid, then centrifuged and submitted to amino acid analysis.

**Fractionation of peptides.** Tryptic, thermolytic, peptic, *S. aureus* protease, and chymotryptic peptides were purified by reverse phase HPLC on an Aquapore RP 300 column (4.6 x 250 mm, 10  $\mu$ m); guard column: 4.6 x 30 mm, 10  $\mu$ m) with TFA/acetonitrile gradients. All peaks, except the large initial one, were rechromatographed on the same column but using 0.02 M ammonium bicarbonate/acetonitrile gradients.

**COOH-terminal analysis.** Carboxypeptidase B digestion was employed for the COOH-terminal analysis (see Enzymic hydrolysis).

**NH<sub>2</sub>-terminal analysis and sequence determination.** Edman degradation: protein (5 nmol) or peptides (1-10 nmol) were submitted to Edman degradation carried out by the manual technique devised by Tarr (32), modified as follows: the conversion from anilinothiazolones to PTH-derivatives was performed by incubating the dried thiazolone extract at 80°C for 10 min with 0.2 ml of 1 N aqueous HCl, containing ethanol (1% v/v); then, most of the PTH-derivatives were extracted twice with 0.5 ml of ethylacetate. The organic and aqueous phases were separately dried and analyzed. PTH-derivatives present in the organic phase were analyzed by HPLC on a Beckman Ultrasphere ODS column (4.6 x 250 mm, 10  $\mu$ m) according to Brown et al. (33). The analyses of PTH-His and PTH-Arg (aqueous phase) were carried out by HPLC on a small column of Aquapore RP 300 (4.6 x 250 mm, 7  $\mu$ m). The elution employed the isocratic mode (90% 0.04 M sodium acetate buffer, pH 4.40, and 10% methanol, at a flow rate of 1 ml/min).

**FAB mass spectrometry.** FAB mass spectra on peptide T1 were obtained with a VG-Analytical 70-70 EQ instrument as described by Camici et al. (18).

**Nomenclature of peptides.** Peptides are indicated by prefixes corresponding to the type of cleavage by which they are produced: T, tryptic; Th, thermolytic; P, peptic; Sp, *S. aureus* protease, and Ch, chymotryptic peptides.

**Chemical modification with iodoacetate.** Before using, the enzyme was precipitated with 75% ammonium sulfate, centrifuged at 10,000g for 10 min, and the precipitate was dissolved in the buffer used for enzyme modification. The specific activity was 110 units/mg of protein (one unit of activity is defined as the amount of enzyme which catalyzes the hydrolysis of 1  $\mu$ mol of p-nitrophenyl phosphate per min at 37°C). Enzyme modification experiments with iodoacetic acid were performed in 0.13 M cacodylate buffer, pH 6.30, in a final volume of 2.37 ml and 25°C. Four incubation mixtures were prepared. One was 8.4 mM in iodoacetate, a second was 8.4 mM either in iodoacetate and Pi, and a third was 8.4 mM in iodoacetate and 42 mM in Pi. The control contained only enzyme and buffer. About 20 nmol of enzyme was used for each mixture. At various time intervals, 5  $\mu$ l aliquots were taken and the activity was measured at pH 5.50 and 37°C with 4 mM p-nitrophenylphosphate as substrate. **Isolation of <sup>14</sup>C-carboxymethylated peptides.** The enzyme (40 nmol) was inactivated as described above but with C-iodoacetate whose specific radioactivity was 0.59 mCi/nmol. After 140 min, the residual activity was about 15% and the enzyme was purified from reagents by Sephadex G 25 chromatography (2 x 30 cm column). The protein fractions were pooled, freeze-dried, and tested for amino acid composition and radioactivity. Subsequently the protein was dissolved in 80  $\mu$ l of 0.1 M Tris-HCl buffer, pH 8.50, containing 6 M guanidinium chloride and then treated with 4  $\mu$ l of 2-mercaptoethanol at 40°C for 16 h. Then 80  $\mu$ l of 0.65 M iodoacetic acid solution, adjusted to pH 8.70 with Tris base, was added. After 15 min at room temperature the completely carboxymethylated protein was purified by HPLC on Aquapore RP 300 with TFA/acetonitrile based solvent system. Tryptic hydrolysis of the <sup>14</sup>C-carboxymethylated protein was carried out as described. The peptides were separated by HPLC as previously described, and all fractions were tested for radioactivity. Only one tryptic peptide (T2) was labelled. Because this peptide contains Cys<sub>2</sub> and Cys<sub>1</sub>, it was digested with thermoLysin. The peptide (6 nmol) was dissolved in 40  $\mu$ l of 0.1 M ammonium bicarbonate buffer, pH 8.0, containing 1 mM CaCl<sub>2</sub> and treated with 4% thermoLysin (w/w) at 37°C for 210 min. The thermolytic subfragments were separated by HPLC on Aquapore RP 300. All fractions were tested for <sup>14</sup>C-radioactivity.

## RESULTS

The low M<sub>w</sub> acid phosphatase used for the study of the primary structure was a pure protein as checked by PAGE and SDS-PAGE. The amino acid composition and the NH<sub>2</sub>- and COOH-terminal analyses of the C<sub>60</sub>-enzyme are presented in Table I. The amino acid composition is similar to that reported by Lawrence and Van Etten for the same enzyme (10) and is in good agreement with the amino acid composition calculated from the sequence which shows 8 half cystines. The titration with 5,5'-dithiobis(2-nitrobenzoic acid) revealed 7.6 equivalents of free sulfhydryl groups per molecule of enzyme. The carboxymethylation of the enzyme in the presence of 6 M guanidinium chloride but in the absence of reducing agents agreed closely to the results obtained with Ellman's reagent (Table II). Both these findings indicate that all eight half cystine residues occur in the form of free thiol side chains. Furthermore, Table I shows that the NH<sub>2</sub>-terminus is not available for the PITC reagent. Arginine was found as the COOH-terminal residue by carboxypeptidase B time-course analysis. The results, expressed as mol ratios, were as follows: 10 min, 0.24 Arg; 30 min, 0.86 Arg; 2 h, 0.89 Arg; 17 h, 1.00 Arg. These results agree with the amino and carboxyl termini found in the reconstructed primary structure of the enzyme (Fig. 1).

**Purification and characterization of peptides obtained by enzymic hydrolysis.** Fig. 2 shows the preparative HPLC finger print of tryptic peptides. The chromatogram was developed by a gradient of 0.01 M TFA in acetonitrile as indicated in the figure legend, using an Aquapore RP 300 column. All peaks were rechromatographed on the same column but with a solvent system consisting of: a) 0.02 M ammonium bicarbonate, pH 7.0; b) acetonitrile. The gradient program for acetonitrile concentration was the same as that indicated in Fig. 2. The combination of these two HPLC separations permits the purification of most of the tryptic peptides. Some hydrophilic tryptic peptides were eluted together in the large initial peak. The fractions making up this peak were pooled, dried in a vacuum concentrator (300 ml), and then treated with PITC under the conditions used for the coupling step of the Edman degradation. The mixture of phenylthiocarbonyl-tryptic peptides was separated by HPLC on Aquapore RP 300 using a solvent system composed of 0.02 M ammonium bicarbonate, pH 7.0, and acetonitrile (Fig. 3). The fractions corresponding to the peaks were dried in separate mini-reactors and then exposed to an acidic environment for the cleavage step of Edman degradation, and all subsequent operations needed to obtain the PTH-derivatives were carried out. These derivatives were analyzed by HPLC and the Edman degradation was continued on the residual peptides to obtain sequence information. Table III reports the amino acid compositions and yields of all purified tryptic peptides. The sequence information for all tryptic peptides is reported in Fig. 1. Fig. 4 shows the preparative HPLC finger print for the thermolytic peptides. The thermolytic peaks were also rechromatographed under the same conditions described for the tryptic peptides. In addition the big initial peak was treated as described for the tryptic peptides (Fig. 5) reports the HPLC separation of the phenylthiocarbonyl-thermolytic peptides) to obtain sequence information on its hydrophilic peptides. The amino acid compositions and yields of the purified thermolytic peptides are shown in Table III, while the sequence information is given in Fig. 1. Figs. 6-8 report the preparative HPLC finger prints obtained from the peptic, *S. aureus* protease, and chymotryptic hydrolyses, respectively, of the C<sub>60</sub>-low M<sub>w</sub> acid phosphatase. The legends of these figures describe the experimental conditions. All peaks were rechromatographed under the conditions described for the tryptic peptides. With respect to these hydrolyses we did not consider it necessary to characterize the hydrophilic peptides in the

large initial peaks. Tables IV-VI report the amino acid compositions of these last three peptide series, whose sequence information is presented in Fig. 1. Two peptides (Sp<sup>7</sup> and Sp<sup>15</sup>) partially precipitated during the digestion and were separated prior to fractionation of the mixture on the Aquapore RP 300 HPLC column. The precipitate was dissolved in 6 M guanidinium chloride; thus Sp<sup>7</sup> and Sp<sup>15</sup> were purified by HPLC on Aquapore RP 300 using the TFA-acetonitrile solvent system and the same gradient elution used for other Sp peptides. Two anomalous cleavages were observed for P20 (Asp-Pro) bond and P19 (Asp-Ala) bond. The cleavage at the Asp-Pro bond was probably due to the low pH value and the relatively long incubation time at 37°C during peptic digestion. In fact Herendson (34) reported that aspartyl-prolyl bonds are (in some cases) susceptible to cleavage in acidic solutions. As for the partial cleavage at the Asp-Ala bond, it is most likely derived from non-specific digestion, as indicated by its low yield. Kasper (35) has reported that the peptic susceptible bonds in proteins are those formed by the carboxyl group of all L-amino acids except proline.

On the basis of the sequence information from the various peptide series and the amino acid composition of all purified peptides we were able to reconstruct the complete amino acid sequence (Fig. 1).

**Structure of T1.** This peptide represents the NH<sub>2</sub>-blocked (Edman-negative) fragment obtained by tryptic hydrolysis of the C<sub>60</sub>-enzyme. Its structure was obtained by the combination of the following techniques: i) FAB mass spectrometry of the entire peptide; the FAB mass spectrum in Fig. 9 shows a protonated molecular ion at m/z 717, a value which corresponds to a M<sub>r</sub> of 716; ii) digestion of T1 with *S. aureus* protease which generated the NH<sub>2</sub>-acylated T1Sp1 and the T1Sp2 fragments. These peptides were separated by a small column (3 x 30 mm) of ADSOW-X4 (H<sup>+</sup>-form) resin (Bio-Rad). The fragment T1Sp1 was not retained by this column, whereas the fragment T1Sp2 was eluted by 3 M ammonia solution. The amino acid compositions of these peptides were: T1Sp1: Ala(0.9), Glu(1.0), Thr(0.9), Thr(0.9), Glu(1.0), Val(0.9), Lys(1.0). The digestion of the NH<sub>2</sub>-acylated T1Sp1 with carboxypeptidase Y released only Glu, which is assigned to position 2 (this result was expected in view of the specificity of staphylococcal protease). The absence of Ala in the carboxypeptidase Y hydrolysate, which should have appeared in an amount equimolecular to that of Glu, indicates that Ala is the NH<sub>2</sub>-terminal acylated residue.

T1Sp2 was sequenced by Edman degradation and gave the following results: Gln-Val-Thr-Lys. The amino acid composition of T1 (Table II) and the above data indicate the presence of an acetyl group blocking the NH<sub>2</sub>-terminus.

In conclusion the structure of T1 was: Ac-Ala-Glu-Gln-Val-Thr-Lys. Glutamine found at position 3 agrees with the data on the M<sub>r</sub> of T1, calculated from the FAB mass spectrum.

**Active site modification.** Fig. 10 shows the inactivation experiments of the low M<sub>r</sub> acid phosphatase by iodoacetate. The kinetic is pseudo-first order both in the presence and in the absence of Pi, a competitive inhibitor of the enzyme. Inorganic phosphate protects the enzyme against inactivation and these results agree with those reported by Lawrence and Van Etten (10) on the same enzyme, from which we conclude that at least one half cystine residue occurs at the active site of the enzyme. When 85% inactivation had occurred (after about 140 min.) further modification was prevented by quickly separating the enzyme from reagents and salts with gel filtration on Sephadex G 25. Then amino acid analysis was carried out on the modified protein. The results reported in Table I indicate that 85% of enzyme activity loss was related to the carboxymethylation of 0.9 residues of half cystine per molecule of enzyme, while no loss of other amino acid residue was observed. This seems to indicate that one half cystine is involved in the active site of the enzyme. We therefore proceeded to localize the half cystine residue(s) that could be labeled with <sup>125</sup>I-iodoacetate at about 85% inactivation of the enzyme. The labelled enzyme was then

completely carboxymethylated by unlabelled iodoacetate and the C<sub>60</sub>-protein was purified by HPLC as described in the "Experimental Procedure" Section. Then the C<sub>60</sub>-protein was digested with trypsin. Only T2 incorporated <sup>125</sup>I-C<sub>60</sub>-radioactivity. This peptide contains both Cys<sub>12</sub> and Cys<sub>17</sub>. T2 was then digested with thermolysin and the subfragments were separated by HPLC. Figures 11A,B show the chromatogram and the radioactivity, respectively. Only three peaks were C<sub>60</sub>-labelled; these were hydrolyzed and their amino acid composition and sequence revealed that T2Th1 (specific radioactivity = 0.36 nCi/nmol) corresponds to Val-Cys<sub>12</sub>, T2Th2 (specific radioactivity = 0.34 nCi/nmol) corresponds to Phe-Val-Cys<sub>17</sub> and T2Th3 (specific radioactivity = 0.16 nCi/nmol) corresponds to Leu-Gly-Ann-Ile-Cys<sub>17</sub>-Arg. The mean specific radioactivity calculated for Cys<sub>12</sub> was 0.35 nCi/nmol, whereas Cys<sub>17</sub> had a specific radioactivity of 0.10 nCi/nmol, 3.5 times lower than that of Cys<sub>12</sub>. These data demonstrate that the modification of Cys<sub>12</sub> was responsible for the loss of most of the enzymatic activity, although Cys<sub>17</sub> also reacted but to a lower extent. Cys<sub>12</sub> and Cys<sub>17</sub> are close enough to each other so that it appears possible that both were present at or near the active site of the enzyme. To clarify this point we labelled the active site cystine(s) with <sup>125</sup>I-iodoacetate but in the presence of 42 mM Pi. This Pi concentration protects the enzyme almost completely against inactivation by iodoacetate (Fig. 20). The enzyme was processed in the way described above for the modification of the enzyme in the absence of Pi. The thermolytic peptides from T2 were checked for radioactivity and the results, reported in Fig. 11C, clearly demonstrate an efficient protective action by Pi which competes with iodoacetate at the active site of the enzyme. This protection was comparable for all three peaks, since the radioactivity bound to peptides T2Th1, T2Th2, and T2Th3 was 14%, 14%, and 15% respectively, as referred to data obtained in the absence of Pi.

Table I. Amino Acid Composition and Terminal Residues of Bovine Liver Low Molecular Weight C<sub>60</sub>-Acid Phosphatase.

Amino acid <sup>a</sup>	Reduced and carboxymethylated	Carboxymethylated without reduction	Sequence values	Modified <sup>b</sup>	Modified <sup>c</sup> Pi
C <sub>60</sub> -Cysteine	7.9	7.6	(8)	0.9	0.2
Aspartic acid	21.0	23.2	(24)	23.3	22.7
Threonine	5.6	6.2	(6)	5.9	5.7
Serine	9.0	9.8	(10)	9.7	10.3
Glutamic acid	15.8	16.7	(16)	16.8	16.9
Proline	4.9	4.8	(5)	n.d.	n.d.
Glycine	5.5	6.2	(6)	6.4	6.5
Alanine	9.2	9.5	(10)	9.1	9.4
Valine	13.1	14.2	(15)	15.2	14.5
Methionine	0.8	1.0	(1)	1.1	1.1
Isoleucine	7.8	8.5	(9)	8.5	8.2
Leucine	10.7	11.3	(11)	11.0	11.0
Tyrosine	5.1	4.9	(5)	5.1	5.0
Phenylalanine	6.4	6.1	(6)	6.2	5.8
Lysine	9.2	9.1	(9)	9.0	8.8
Histidine	2.0	2.0	(2)	2.1	2.2
Histidine	12.7	12.2	(12)	11.7	11.6
Tryptophan	1.8	n.d.	(2)	n.d.	n.d.

NH<sub>2</sub>-Terminus: Edman<sup>c</sup>: none  
COOH-Terminus: Carboxypeptidase B<sup>d</sup>: Arg

<sup>a</sup>The results are expressed as residues per molecule of enzyme. Values for Ser and Thr were determined by hydrolyses at 110°C for 22 and 70 h in duplicate and extrapolation to zero time. Determined according to (29). <sup>b</sup>The NH<sub>2</sub>-terminal analysis was carried out on 5 nmol of C<sub>60</sub>-acid phosphatase. <sup>c</sup>The carboxypeptidase B digestion was performed on 30 nmol of C<sub>60</sub>-acid phosphatase. <sup>d</sup>85% inactivated by iodoacetate in 140 min. In the presence of 42 mM Pi, 12% inactivated by iodoacetate in 140 min. n.d., not determined.

Table II. Amino Acid Composition of Tryptic Peptides from Bovine Liver Low Molecular Weight C<sub>60</sub>-Acid Phosphatase<sup>a</sup>.

	C <sub>60</sub> -Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position	
T1		3.1		1.8				0.8	0.9						1.0				33	1-6	
T2	1.9	1.2		0.8	0.3			1.0	2.2		1.0	1.8	0.2	1.0				1.0	35	7-18	
T3				0.8	1.0	1.0		1.8	1.0		1.1			1.0				1.0	70	19-27	
T4		6.5	1.1	3.1	1.3			2.4	1.3	4.0		1.8	1.2		1.1			1.2	1.8	28-53	
T5			1.1		0.7			1.8										1.0	57	54-58	
T6	1.2				0.8			0.8	1.0			0.9						1.0	43	59-64	
T7		1.7	1.3					1.0	0.9			1.1			1.0	1.8				56	65-73
T10	1.1	3.8	1.1	1.0	2.0					1.1	0.9	0.8	2.0	1.0	2.0			1.0	39	80-97	
T11			1.8									1.0						1.1	55	98-101	
T14		0.9		0.8	2.0	1.0	1.0	1.0			0.8	2.0	0.9		2.0					12	111-123
T14B		0.9		0.8	2.0	1.0	1.1				0.9	2.0	1.0		1.1					44	113-123
T15	1.1	4.2	1.3		5.9	1.1	1.3	1.0	2.0		1.8	1.1	3.1	1.0				1.0	11	124-147	
T17							1.1	0.8				1.0		1.1	1.2					83	151-155

<sup>a</sup>The digestion was carried out on 50 nmol of C<sub>60</sub>-acid phosphatase. The values are expressed as molar ratios. Values for Ser and Thr were corrected for 15% and 0.5% destruction, respectively. Values of contaminating amino acids at a level of less than 1% are not reported. The hydrophilic peptides T9, T12, T12A, T12B, T13, T14A, T16 and T18 (see Fig. 2) were derivatized with PITC and then separated as phenylthiocarbonyl-derivatives (see Fig. 3), and successively reassayed without determining their amino acid composition. Yield was calculated as moles obtained from moles of protein digested.

Table III: Amino acid composition of thermolytic peptides from bovine liver low molecular weight Ca-acid phosphatase<sup>a</sup>.

	Ca	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position
Th3										1.7		1.5		1.5						35	8-10
Th4	1.2									1.0										22	10-12
Th5		0.3					1.1						1.0							73	13-15
Th6	1.2			1.0	1.0							0.9						1.0		73	16-20
Th8								0.9	0.9						0.9	1.1			1.0	41	24-28
Th9		2.1	1.0		1.0				1.0			1.0								96	29-34
Th10		2.0		1.1								0.9							0.9	50	35-39
Th11		2.7		1.9				1.0	1.0	1.8		0.8							1.0	38	40-50
Th12		1.3		0.9		2.0	1.0	0.9	1.2										2.3	60	51-59
Th14		0.9					1.0					0.9						1.0	1.0	41	63-67
Th18		1.1	1.0		1.1					1.0					0.9	1.1				72	77-82
Th20		1.3											0.9	1.3						74	85-87
Th23		0.9											1.0					1.1		53	96-98
Th24		2.0		1.0	1.0								1.0			1.1		1.0	14	99-105	
Th29	1.2	1.0								0.9							1.2	1.1	24	106-110	
Th26		1.1		1.0	2.1	1.0	4.0					2.0	0.9		1.0				50	115-124	
Th29		4.1	1.0		2.1	1.2	1.1	1.0				1.8	1.0	1.9	1.0				32	125-140	
Th30	1.1				2.1					1.0				0.9						84	141-145
Th31	2.1									1.0									2.1	32	146-151
Th32						1.0							1.0		1.0	1.0				41	152-155

<sup>a</sup>The digestion was performed on 30 nmol of Ca-acid phosphatase. The hydrophilic peptides Th2, Th6, Th7, Th8, Th26 and Th32 (see Fig. 4) were derivatized with PTC and then separated as phenylthiocarbonyl-derivatives (see Fig. 5), and successively sequenced without determining their amino acid composition. Other details are the same as in Table II.

Table IV: Amino Acid Composition of Peptic Peptides from Bovine Liver Low Molecular Weight Ca-Acid Phosphatase<sup>a</sup>.

	Ca	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position	
P2			0.9	1.0					1.9			1.3		1.0						62	4-9	
P4		0.9	1.0					1.3	1.0		1.0	1.0								16	11-16	
P5	1.0		0.3	1.0	1.3	0.9		0.8	0.2		0.8							0.9		38	17-23	
P6								1.0	1.0						1.0					33	24-26	
P7		3.9	0.9	1.1	1.0				1.2		0.9	1.0			1.3		1.1			15	27-38	
P8	1.0		1.0					1.1	1.0	0.9		1.0							3.9	41	39-45	
P10		2.1		1.0		1.9	1.3	1.1	1.0										1.9	1.0	19	49-59
P12		3.2	1.7		2.1		1.0	1.9	1.1		1.0			0.9	2.1	1.8	2.0			14	64-82	
P14													1.0	1.1	1.0					22	86-89	
P15	0.8	2.0		1.2	1.1						0.7	1.2								37	90-96	
P16		1.3		0.9	2.1	1.1	1.0					1.0	1.0		1.0					37	116-124	
P19		3.0		1.0	1.1	1.3						1.5	1.9							12	126-135	
P20		2.9			0.9	1.3	0.9							1.7						24	130-137	
P22			1.0		3.0				1.1					0.8						60	139-144	
P23		1.9								1.0									1.0	47	145-148	
P24	1.1								1.0			1.0	1.0					1.0		39	149-153	
P25						1.1				0.9						1.0		1.1		70	154-157	

<sup>a</sup>The digestion was carried out on 40 nmol of Ca-acid phosphatase. Other details are the same as in Table II.

Table V: Amino acid composition of *S. aureus* V8 protease peptides from bovine liver low molecular weight Ca-acid phosphatase<sup>a</sup>.

	Ca	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	Yield %	Sequence position	
Sp1						1.0			1.0											95	1-2	
Sp2 <sup>b</sup>	1.2	0.9	1.1	1.2	1.3		1.2		2.6			2.0		1.0	0.9					14	3-14	
Sp3	1.0	0.8		0.8	1.0	0.7		1.2			2.2								0.7	21	15-23	
Sp4		3.0	0.9	1.0	1.0			0.9	2.0		0.9	1.0		1.0	1.0				1.0	33	24-37	
Sp5		2.1							1.0	0.9										1.0	33	38-42
Sp6		1.7		2.0			2.0	1.0	2.1										0.9	24	43-52	
Sp6A		1.3		1.9	0.2		1.0	1.0	0.9	0.2										26	43-48	
Sp6B		1.0				1.0	1.0	1.0											0.9	14	48-52	
Sp7	0.8	1.3		2.0		2.1		0.9	1.1			1.0							3.3	11	53-64	
Sp8		2.3	1.2				1.9	0.9			1.0					1.2	2.0			5	65-73	
Sp9			1.0		2.0			1.0	1.2						1.0				1.2	21	74-80	
Sp10		2.0	1.0					1.0						1.9						69	81-86	
Sp11	1.1	1.0			1.0					1.0	1.0	1.0	0.9							37	87-93	
Sp12		2.0			1.0								1.0						1.0	54	94-98	
Sp13	1.2	3.3		0.7	2.2			1.0	1.1		1.0	1.1			3.0		1.7			46	99-114	
Sp14		5.0		1.0	4.0	1.9	2.0	1.2			2.3	3.2	2.7	0.9	0.9					20	115-139	
Sp14A		2.1		0.9	2.8	2.0	1.9				1.7	3.0	2.9		1.0					13	115-133	
Sp14B		2.9			1.2			1.0							1.1					14	134-139	
Sp15 <sup>b</sup>	3.4		1.2		3.0			1.0	1.9			0.9	0.8	0.7					2.1	22	140-154	
Sp16								1.0							1.0		1.1				76	155-157

<sup>a</sup>The digestion was performed on 60 nmol of Ca-acid phosphatase. <sup>b</sup>These peptides partially precipitated during digestion. Other details are the same as in Table II.



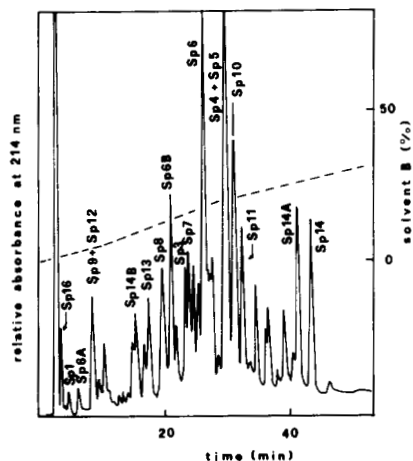


Fig. 7. The HPLC separation of *S. aureus* protease peptides from 60 nmol of the Ca-acid phosphatase. Details are the same as in Fig. 2. The overlapped peptides were rechromatographed at different conditions.

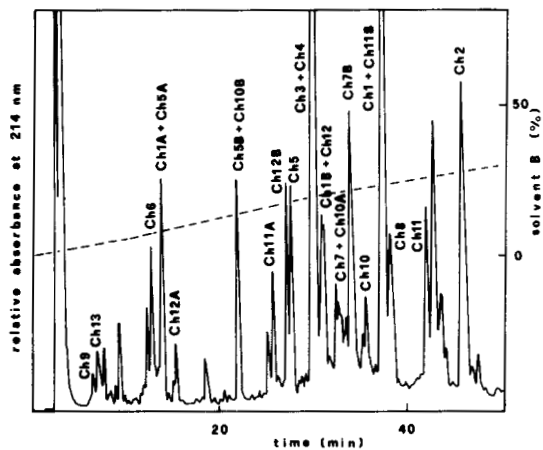


Fig. 8. The HPLC separation of chymotryptic peptides from 40 nmol of the Ca-acid phosphatase. Details are the same as in Fig. 2. The overlapped peptides were rechromatographed at different conditions.

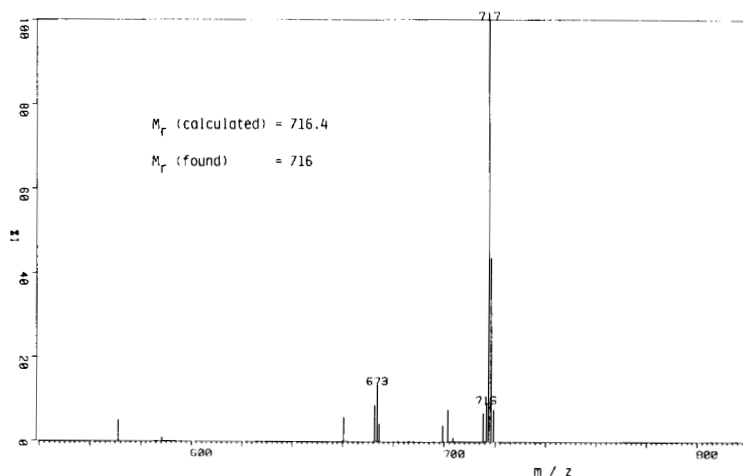


Fig. 9. Positive FAB mass spectrum of T1.

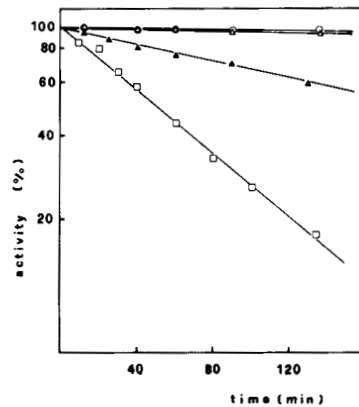


Fig. 10. Inactivation of the low  $M_w$  acid phosphatase by iodoacetate in the presence and in the absence of Pi. Incubations were performed in 0.13 M cacodylate buffer, pH 6.3, containing 1 mM EDTA at 25°C.  $\circ$ — $\circ$ , control;  $\square$ — $\square$ , 8.4 mM iodoacetate;  $\triangle$ — $\triangle$ , 8.4 mM iodoacetate and 8.4 mM Pi;  $\diamond$ — $\diamond$ , 8.4 mM iodoacetate and 40 mM Pi.

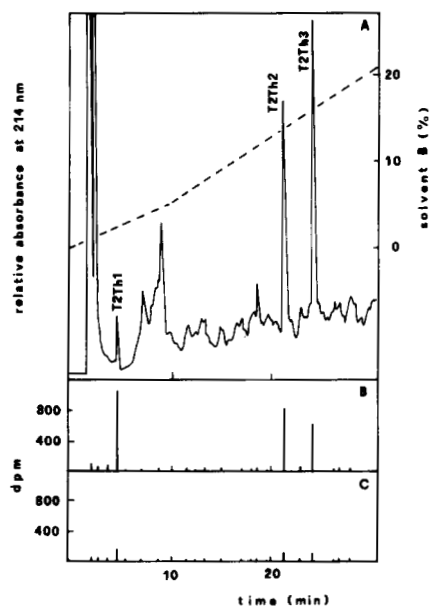


Fig. 11. HPLC separation of the thermolytic subfragments of the peptide T2. A, details are the same as in Fig. 7; B,  $^{14}C$ -radioactivity measured on the chromatographic fractions obtained from the enzyme modified at the active site by  $^{14}C$ -iodoacetate without Pi; C,  $^{125}I$ -radioactivity measured on the chromatographic fractions obtained from the enzyme incubated with  $^{125}I$ -iodoacetate but in the presence of 40 mM Pi.