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Complete amino acid sequence of pig kidney fructose-1,6-bisphosphatase

(regulatory enzyme/gluconeogenesis/reactive sites)

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ABSTRACT The covalent structure of the pig kidney fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) subunit has been determined. Placement of the 335 amino acid residues in the polypeptide chain was based largely on automated Edman degradation of eight purified cyanogen bromide fragments generated from the S-carboxymethylated protein. The determination of the amino acid sequence of the largest cyanogen bromide fragment (154 residues) required additional analysis of subfragments obtained by tryptic cleavage at arginyl residues and by mild acid cleavage of an Asp-Pro peptide bond. Alignment of the cyanogen bromide fragments was accomplished by analysis of a product of limited proteolysis by an endogenous protease and by characterization of the tryptic peptides isolated from S-[^{14}C]carboxymethylated fructose-1,6-bisphosphatase. This sequence information has permitted the identification of several reactive sites of functional and structural significance in pig kidney fructose-1,6-bisphosphatase.

Fructose-1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, a reaction that is absolutely essential for gluconeogenesis. The enzyme isolated from gluconeogenic tissues (e.g., liver and kidney cortex) has been studied extensively (for reviews, see refs. 1–3), but structural information required to define structure–function relationships in this important regulatory enzyme is still fragmentary. Horecker and co-workers have determined the sequences of several fragments of rabbit liver fructose-1,6-bisphosphatase that have placed the NH_2-terminal 78 residues (4, 5) and the COOH-terminal 88 residues (6) in the subunit chain. In addition, the selective modification of this enzyme by pyridoxal 5′-phosphate (7, 8) has permitted the identification of sequences presumably at the active and AMP-allosteric sites. Structural studies have also been carried out on sheep liver fructose-1,6-bisphosphatase. Fisher and Thompson (9) have published the sequence of residues 1–60 in this enzyme.

Our sequence analysis has been carried out on the fructose-1,6-bisphosphatase from pig kidney cortex. We have recently described the amino acid sequence of the 60-residue NH_2-terminal peptide produced by digestion of this enzyme with subtilisin (10) as well as the sequence of an internal 63-residue cyanogen bromide fragment containing a phosphorylatable serine (11). In this communication, the complete sequence of the 335 amino acids of the subunit of pig kidney fructose-1,6-bisphosphatase is presented and discussed. The experimental details of the sequence determination will be published elsewhere.

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METHODS

Unless otherwise stated, pig kidney fructose-1,6-bisphosphatase was prepared as described by Colombo and Marcus (12), with only a minor modification (10). The protein was carboxymethylated as described (10) with iodoacetic acid (Sigma) or its ^{14}C-labeled form (Amersham). The experimental procedures used for cleavage with cyanogen bromide (3 mg/mg of protein), citraconylation of amino groups, and cleavage with trypsin were those described by Marcus *et al.* (11). Limited proteolysis with subtilisin and separation of the fragments was carried out as described (10). Heating for 30 min at 100°C in 0.015 M HCl was used to obtain preferential cleavage of Asp-Pro bonds.

Peptides were purified by gel filtration on Sephadex G-75, G-50, and G-15 columns (2.5 × 200 cm or 1.5 × 195 cm) equilibrated and eluted with either 30% acetic acid or 50 mM NH_4HCO_3 (pH 8.5). For the purification of some peptides, an additional step of ion exchange chromatography was required. This step was carried out on a column (0.9 × 26 cm) of DE-52 cellulose (Whatman) equilibrated with 50 mM NH_4HCO_3 (pH 8.5). The column was eluted with a linear gradient of NH_4HCO_3 (pH 8.5; 50–500 mM in 160 ml).

Peptides in column effluents were detected by alkaline hydrolysis and reaction with fluorescamine (13). Tyrosine fluorescence was measured directly by its emission at 305 nm after excitation at 280 nm. Fluorescence measurements were made in an Aminco spectrophotofluorometer equipped with a fixed-voltage J10-222A photomultiplier microphotometer using 10-mm-light-path cuvettes. Radioactivity was measured in a Packard Tri-Carb scintillation spectrometer using 4 ml of scintillation fluid (Budget-Solve from RPI).

Amino acid compositions were obtained by automated ion exchange chromatography on a single column according to the general procedure of Spackman *et al.* (14) with a Dionex 502 analyzer or a Glenco Scientific (Houston, TX) custom modular amino acid analyzer. Analyses were carried out on samples hydrolyzed in 6 M HCl for 24 hr at 112°C at reduced pressure. The quantitation obtained in these analyses served as a reference for the precise determination of polypeptide concentrations in samples subjected to automated Edman degradation.

Automated Edman degradation was carried out in a Beckman protein-peptide sequencer (model 890C). Many runs were made using peptide program 102974 of the manufacturer, characterized by a volatile coupling buffer containing *N,N*-dimethylallylamine. Program 021879 using dimethylallylamine and program 121078 with 0.1 M Quadrol were used for selected samples. The phenylthiohydantoin derivatives liberated after each cycle were identified and quantitated as such or as trimethylsilyl derivatives by gas chromatography (15) on a Beckman GC-65 unit. Alternatively, the residues liberated were converted back to the parent amino acid or derivative thereof by

hydrolysis in HI (16), and the resultant amino acids were identified on the amino acid analyzer. More recently, we have identified the phenylthiohydantoin derivatives by HPLC (17).

RESULTS AND DISCUSSION

The complete amino acid sequence of the 335 residues of the pig kidney fructose-1,6-bisphosphatase subunit is shown in Fig. 1. The amino acid composition from the sequence is Asp_{19}-$Asn_{16}Thr_{21}Ser_{18}Glu_{20}Gln_{7}Pro_{15}Gly_{26}Ala_{30}Cys_{6}Val_{29}Met_{9}Ile_{24}$-$Leu_{28}Tyr_{15}Phe_{10}His_{4}Lys_{25}Arg_{13}$. On this basis, the molecular weight of the subunit (including the NH_2-terminal acetyl group) is 36,534, giving a total value of 146,136 for the native tetramer. Our work on the determination of the amino acid sequence of pig kidney fructose-1,6-bisphosphatase was initiated by study of the limited proteolysis of fructose-1,6-bisphosphatase with subtilisin (for reviews, see refs. 1 and 2). The main site of subtilisin cleavage is the peptide bond between alanine-60 and glycine-61, and the major products of subtilisin action on fructose-1,6-bisphosphatases are known as the "S-peptide" and the "S-subunit" (4). The amino acid sequence of the 60-residue NH_2-terminal S-peptide has been determined for fructose-1,6-bisphosphatases from rabbit liver (4), sheep liver (9), and pig kidney (10).

The cleavage of *S*-carboxymethylated enzyme with cyanogen bromide and the isolation of these fragments provided the main tool for the continuation of the structural studies with pig kidney fructose-1,6-bisphosphatase. The cyanogen bromide cleavage products were separated on a Sephadex G-75 column (2.5 × 200 cm) developed in 30% acetic acid. The elution pattern (Fig. 2) shows the presence of five well-resolved peaks (CN I–CN V, Fig. 2) and a sixth region (fractions 205–226, Fig. 2) containing more than one component. Each peak was analyzed for amino acid composition and the purity of each was assessed by auto-

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                                             10                                                  20
AcThr-Asp-Gln-Ala-Ala-Phe-Asp-Thr-Asn-Ile-Val-Thr-Leu-Thr-Arg-Phe-Val-Met-Glu-Gln-
                                             30                                                  40
Gly-Arg-Lys-Ala-Arg-Gly-Thr-Gly-Glu-Met-Thr-Gln-Leu-Leu-Asn-Ser-Leu-Cys-Thr-Ala-
                                             50                                                  60
Val-Lys-Ala-Ile-Ser-Thr-Ala-Val-Arg-Lys-Ala-Gly-Ile-Ala-His-Leu-Tyr-Gly-Ile-Ala-
                                             70                                                  80
Gly-Ser-Thr-Asn-Val-Thr-Gly-Asp-Gln-Val-Lys-Lys-Leu-Asp-Val-Leu-Ser-Asn-Asp-Leu-
                                             90                                                 100
Val-Ile-Asn-Val-Leu-Lys-Ser-Ser-Phe-Ala-Thr-Cys-Val-Leu-Val-Thr-Glu-Glu-Asp-Lys-
                                            110                                                 120
Asn-Ala-Ile-Ile-Val-Glu-Pro-Glu-Lys-Arg-Gly-Lys-Tyr-Val-Val-Cys-Phe-Asp-Pro-Leu-
                                            130                                                 140
Asp-Gly-Ser-Ser-Asn-Ile-Asp-Cys-Leu-Val-Ser-Ile-Gly-Thr-Ile-Phe-Gly-Ile-Tyr-Arg-
                                            150                                                 160
Lys-Asn-Ser-Thr-Asp-Glu-Pro-Ser-Glu-Lys-Asp-Ala-Leu-Gln-Pro-Glu-Arg-Asn-Leu-Val-
                                            170                                                 180
Ala-Ala-Gly-Tyr-Ala-Leu-Tyr-Gly-Ser-Ala-Thr-Met-Leu-Val-Leu-Ala-Met-Val-Asn-Gly-
                                            190                                                 200
Val-Asn-Cys-Phe-Met-Leu-Asp-Pro-Ala-Ile-Gly-Glu-Phe-Ile-Leu-Val-Asp-Arg-Asn-Val-
                                            210                                                 220
Lys-Ile-Lys-Lys-Lys-Gly-Ser-Ile-Tyr-Ser-Ile-Asn-Glu-Gly-Tyr-Ala-Lys-Glu-Phe-Asp-
                                            230                                                 240
Pro-Ala-Ile-Thr-Glu-Tyr-Ile-Glu-Arg-Lys-Lys-Phe-Pro-Pro-Asp-Asn-Ser-Ala-Pro-Tyr-
                                            250                                                 260
Gly-Ala-Arg-Tyr-Val-Gly-Ser-Met-Val-Ala-Asp-Val-His-Arg-Thr-Leu-Val-Tyr-Gly-Gly-
                                            270                                                 280
Ile-Phe-Met-Tyr-Pro-Ala-Asn-Lys-Lys-Ser-Pro-Lys-Gly-Lys-Leu-Arg-Leu-Leu-Tyr-Glu-
                                            290                                                 300
Cys-Asn-Pro-Met-Ala-Tyr-Val-Met-Glu-Lys-Ala-Gly-Gly-Leu-Ala-Thr-Thr-Gly-Lys-Glu-
                                            310                                                 320
Ala-Val-Leu-Asp-Ile-Val-Pro-Thr-Asp-Ile-His-Gln-Arg-Ala-Pro-Ile-Ile-Leu-Gly-Ser-
                                            330                     335
Pro-Glu-Asp-Val-Thr-Glu-Leu-Leu-Glu-Ile-Tyr-Gln-Lys-His-Ala
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FIG. 1. Amino acid sequence of the subunit of pig kidney fructose-1,6-bisphosphatase tetramer.

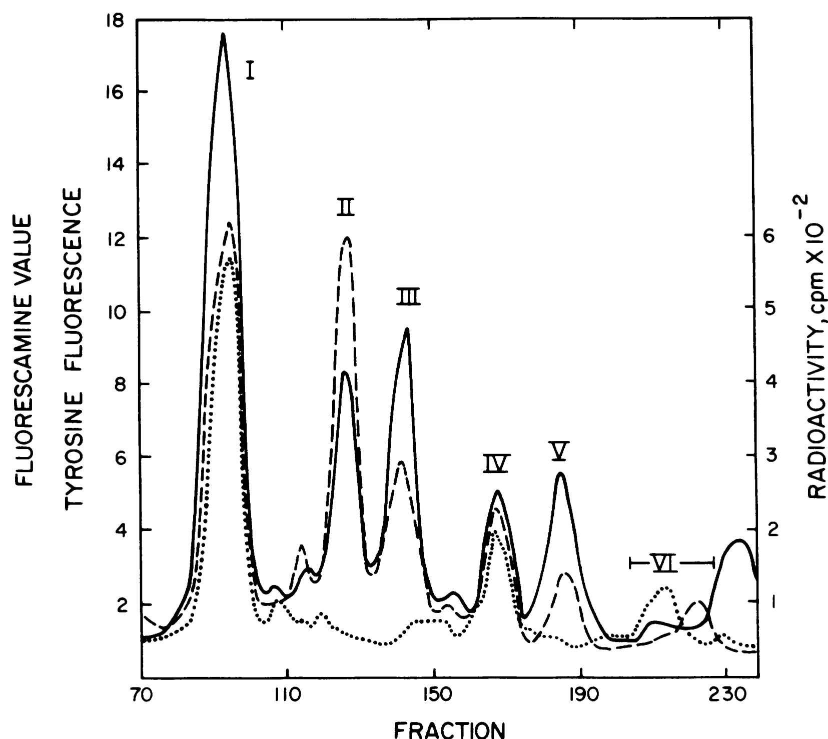

FIG. 2. Gel filtration of the fragments produced by cyanogen bromide cleavage of S-[^{14}C]carboxymethylated pig kidney fructose-1,6-bisphosphatase. The sample (650 nmol in 7 ml of 30% acetic acid/7 M urea) was applied to a column (2.5 × 200 cm) of Sephadex G-75 equilibrated and eluted with 30% acetic acid. Aliquots were subjected to alkaline hydrolysis and analyzed with fluorescamine (——). Other aliquots were used to assay for radioactivity (⋯⋯) and for tyrosine fluorescence measurements (– – –). Various peaks in the chromatogram are designated by Roman numerals based on their order of elution. Peaks I–V and region VI (indicated by a bar) were pooled and lyophilized. Urea eluted as the final fluorescamine-positive peak (fractions 226–248).

mated Edman degradation. These cyanogen bromide fragments serve as a basis for discussion of the complete sequence analysis schematized in Fig. 3. The sequence of each purified peptide was established by automated Edman degradation of the fragment itself or of derivative peptides.

Residues 1–172. This region includes both the known (10) S-peptide (residues 1–60) and CN IA (Fig. 3). Analysis of peak I (Fig. 2) showed that it contained two overlapping components, CN IA and CN IB, both terminating at methionine-172. These two chains resulted from cyanogen bromide cleavage of the bond between methionine-18 and glutamate-19 (CN IA) and the incomplete cleavage of that between methionine-30 and threonine-31 (CN IB). This knowledge permitted the treatment of peak I as a single peptide for further fragmentation reactions. The complete structure of CN IA (residues 19–172) was determined by automated Edman degradation of purified tryptic fragments of citraconylated peak I. Five of these tryptic fragments (Glu-19–Arg-22, Lys-23–Arg-25, Gly-26–Met-30, Gly-26–Arg-49, Thr-31–Arg-49) were readily identified as parts of the S-peptide (10). The largest tryptic fragment, which corresponded to the sequence Lys-50–Arg-110, led us deep into the unknown region of CN IA. The ordering of fragments, Gly-111–Arg-140 and Lys-141–Arg-157, was established by sequence analysis of a peptide obtained by mild acid cleavage of the bond between aspartate-118 and proline-119 of CN I (AP-2 in Fig. 3). Determination of the primary structure of CN IA was completed by sequence analysis of the purified homoserine-containing fragment, Asn-158–Met-172.

Residues 173–248. This stretch of sequence included three cyanogen bromide fragments, CN VIA (residues 173–177), CN VIB (residues 178–185), and CN II (residues 186–248). CN II is pure in peak II (Fig. 2) and we have already reported its complete sequence (11). CN VIA and CN VIB were found together with CN VI in the region designated VI in Fig. 2. The three peptides were purified by additional steps of gel filtration on Sephadex G-15 and by ion exchange chromatography on DE-52 cellulose. The alignment of these fragments was provided by analysis of a tryptic peptide from S-[^{14}C]carboxymethylated fructose-1,6-bisphosphatase that comprised residues 158–198. This fragment (T-SH5 in Fig. 3) therefore overlaps CN IA, CN VIA, CN VIB, and CN II.

Residues 249–335. This region of the molecule includes four cyanogen bromide fragments, CN V (residues 249–263), CN IV (residues 264–284), CN VI (residues 285–288), and CN III (residues 289–335). CN V was first identified in peak V (Fig. 2) as a mixture of peptides including fragments from the NH_2-terminal region. Isolation of pure CN V was achieved by cyanogen bromide cleavage of the S-subunit (Gly-61–Ala-335), which lacks the NH_2 terminus of the enzyme.

The alignment CN II–CN V–CN IV was obtained by sequence analysis of a fragment (EP-2 in Fig. 3) obtained by Sephadex G-50 gel filtration of S-carboxymethylated pig kidney fructose-1,6-bisphosphatase prepared as described by Colombo and Marcus (12) but after incubation of the pH 4.5 extract for 18 hr at 4°C. This preparation method yields a "nicked" form of the enzyme that has an alkaline pH optimum in contrast to the native "neutral pH optimum" fructose-1,6-bisphosphatase. The EP-2 fragment (M_r, *ca.* 11,000) is a minor product of proteolytic cleavage of fructose-1,6-bisphosphatase by endogenous proteases present in the kidney extract; it is generated in addition to the major product corresponding to the 60-residue NH_2-terminal peptide.

Automated Edman degradation of the EP-2 fragment showed a sequence Ser-Ala-Pro-Tyr-Gly, indicating that the bond attacked by a lysosomal protease was an asparagine-serine linkage, located between residues 236 and 237 of pig kidney fruc-

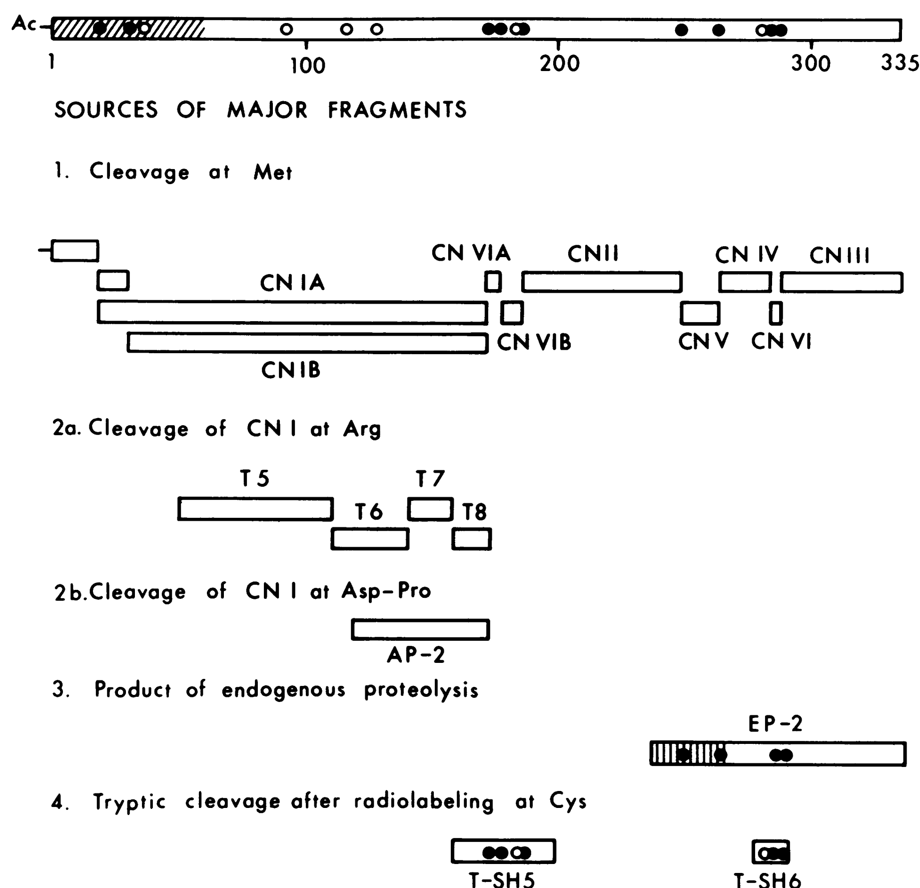

FIG. 3. Diagram showing origin of the fragments of importance in the sequence analysis of pig kidney fructose-1,6-bisphosphatase and their relationship to each other. The length of each bar is proportional to the number of amino acid residues in the peptide. ○ and ●, positions of cysteinyl and methionyl residues, respectively. The hatched section of the top bar designates the known (10) S-peptide fragment (residues 1–60). Cyanogen bromide fragments are identified with the prefix CN and a roman numeral corresponding to their order of elution from a Sephadex G-75 column (cf. Fig. 2). Fragments CN IA and CN IB are two related fragments present in peak I (CN I) of Fig. 2. Fragment CN V was obtained pure in the peak V position (Fig. 2) when generated by cyanogen bromide cleavage of the isolated S-subunit (residues 61–335). Tryptic peptides obtained from citraconylated CN I are identified by the prefix T, but only those corresponding to the region between residues 50 and 172 are shown. Fragment AP-2 was obtained by mild acid cleavage of CN I. Fragment EP-2 was obtained from a sample of fructose-1,6-bisphosphatase overexposed to endogenous proteases during purification (see text). The hatched section of this bar indicates the portion of the sequence determined by automated Edman degradation. Overlapping cysteine-containing tryptic peptides have the prefix T-SH.

tose-1,6-bisphosphatase. Edman degradation of 125 nmol of the EP-2 fragment for 29 cycles confirmed the sequence between serine-237 and proline-265 of Fig. 1 and thus established the complete alignment of CN II–CN V–CN IV shown in Fig. 3. The above results are in accordance with recent work by Xu *et al.* (6) showing that digestion of native rabbit liver fructose-1,6-bisphosphatase with a membrane-bound proteinase from rat liver lysosomes cleaves a peptide bond tentatively located between residues −88 (alanine) and −89 (unknown) from the COOH terminus of rabbit liver fructose-1,6-bisphosphatase.

Of the two cyanogen bromide fragments yet to be placed in sequence, CN III was easily identified as being the COOH-terminal fragment because it lacked homoserine. This places CN VI as the penultimate fragment. Proof for this alignment was obtained by isolation and analysis of a tryptic S-[^{14}C]carboxymethylated peptide (Leu-277–Lys-290) designated T-SH6 in Fig. 3.

The strategies described above permitted elucidation of the complete amino acid sequence of pig fructose-1,6-bisphosphatase shown in Fig. 1. Comparison of this structure with the partial amino acid sequences determined by Horecker and coworkers for rabbit liver fructose-1,6-bisphosphatase shows excellent agreement between the two enzymes up to serine-77 (5). Data that preliminarily extend the sequence information of the rabbit liver enzyme (8) to a methionine designated in that work as residue 163 show areas of strong homology (i.e., with residues 85–120 of Fig. 1), but most of the sequence data are not consistent with our results. No information is available on the rabbit liver enzyme middle portion (*ca.* 85 residues) to allow comparison in that region. Close similarity exists, however, between the two COOH-terminal sequences (compare Fig. 1 of this paper with figure 1 of ref. 6).

The determination of the complete sequence of pig kidney fructose-1,6-bisphosphatase allows us to locate in this structure several of the reactive sites of the enzyme. In addition to the well-established protease-sensitive region around the bond between alanine-60 and glycine-61, we have now identified the peptide bond between asparagine-236 and serine-237 as the second site of cleavage by lysosomal proteases. Earlier, conditions were described for the modification of kidney fructose-1,6-bisphosphatase with pyridoxal 5′-phosphate that result in the selective modification of lysine residues involved in either catalytic activity or AMP inhibition (18, 19). These conditions have been used to modify rabbit liver fructose-1,6-bisphosphatase and the two pyridoxal 5′-phosphate-reactive sites of this enzyme have been identified (6–8). We can now locate these two sites in the established sequence. In the pig kidney enzyme, the sequence surrounding lysine-141 (Tyr-Arg-Lys-Asn-Ser) is

Table 1. Location of reactive sites in pig kidney fructose-1,6-bisphosphatase

Residue or bond	Surrounding sequence	Characteristic
Ala-60 to Gly-61	Gly-Ile-**Ala-Gly**-Ser-Thr	Sensitive peptide bond in area exposed to proteolysis
Asn-236 to Ser-237	Pro-Asp-**Asn-Ser**-Ala-Pro	A second site of cleavage by lysosomal proteases
Lysine-141	Ile-Tyr-Arg-**Lys**-Asn-Ser	Pyridoxal 5′-phosphate-reactive lysine essential for AMP inhibition
Lysine-274	Pro-Lys-Gly-**Lys**-Leu-Arg	Pyridoxal 5′-phosphate-reactive lysine presumably at the active site
Cysteine-116 or -128		Highly reactive SH whose reactivity is increased by AMP
Serine-207	Lys-Lys-Gly-**Ser**-Ile-Tyr	Site of cAMP-dependent phosphorylation in 2 M urea

nearly identical to the sequence Tyr-Arg-Lys-Lys-Ser reported by Suda *et al.* (8) for a sequence presumed to be from the AMP-allosteric site of fructose-1,6-bisphosphatase. The sequence Gly-Lys-Leu-Arg-Leu surrounding a lysine residue from the active site of rabbit liver fructose-1,6-bisphosphatase (7) is identical to the sequence surrounding lysine-274 of the pig kidney enzyme. Fructose-1,6-bisphosphatase contains a highly reactive cysteine residue, the reactivity of which is increased by the presence of the allosteric inhibitor AMP (20). This highly reactive residue, preliminarily identified as cysteine-116 or -128 (unpublished data), is probably the one located between the active and the allosteric sites of fructose-1,6-bisphosphatase (21).

The site of cAMP-dependent phosphorylation of pig kidney fructose-1,6-bisphosphatase exposed in 2 M urea (11) has been identified as serine-207. The locations of the above mentioned reactive sites of pig kidney fructose-1,6-bisphosphatase are summarized in Table 1. It is important to remark, however, that no sequence in the determined primary structure of pig kidney fructose-1,6-bisphosphatase resembles the site of the cAMP-dependent phosphorylation of rat liver fructose-1,6-bisphosphatase (22, 23). This result is in agreement with recent data (24) showing the presence of a unique extension of 30 amino acids at the carboxyl terminus of the rat liver enzyme. This extension coincidentally contains a cAMP-dependent phosphorylatable sequence (22, 23).

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